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The Potential Risk of Pigeon Paramyxovirus-1 Infections in Chickens – Implications for Diagnosis and Control.

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1. Summary

In recent years, several cases of pigeon paramyxovirus-1 (PPMV-1) outbreaks in feral pigeons were described in Switzerland. The potential of PPMV-1 to induce the notifiable Newcastle Disease in chickens is discussed. In order to study this issue and to gain sample material for diagnostic investigations as well as information on epidemiological relevant parameters chickens were experimentally infected with a Swiss PPMV-1 isolate.
The infectivity of the PPMV-1 isolate for chickens was demonstrated by virus shedding after experimental inoculation. Our data suggest that long-lasting shedding can occur in chickens infected with PPMV-1 for up to 60 days. To our knowledge, there are no other studies of chickens which were conducted over such a long period of time. The pathogenicity of the used strain seems to be low for chicken. Different (q)RT-PCR assays were evaluated with Swiss PPMV-1 isolates and various samples were analysed with respect to their suitability to detect viral RNA. At 14 days post infection virus genome was mainly detected in spleen, caecal tonsils, heart, cloacal swabs, liver, proventriculus, duodenum and kidney tissue samples. The level of virus replication was shown to be low. Important implications on disease diagnosis and control have been made in this study. Not all routinely used assays were able to detect viral genome from the tested isolates, therefore molecular based methods for the detection of viral RNA as well as the material should be adapted.

2. Introduction

Newcastle disease virus (NDV), also known as avian paramyxovirus-1 (APMV-1), is a highly contagious agent, causing severe loss in poultry and other bird species. APMV-1 is an enveloped, negative sense, single stranded RNA virus belonging the species avian avulavirus-1, genus *Avulavirus* within the family of *Paramyxoviridae*, of the order *Mononegavirales*. The genus *Avulavirus* consists of 13 serogroups (Avian Avulavirus-1-13) [1, 2]. Newcastle disease was first discovered in 1926 in Indonesia and in Great Britain on a poultry farm near Newcastle-on-Tyne, where the disease received its name from [3]. Currently, two different systems of classification of APMV-1 are used based on the sequence analysis of the gene coding for the fusion protein (F). Aldous suggested to group NDV into six lineages with sublineages [4]. Further sublineages have been proposed since then [5, 6]. A second classification system divides AMPV-1 into two major groups as class I and class II, and subdivides class I into nine genotypes and class II into fifteen genotypes and several sub-genotypes within them [7-10]. The most predominant virulent ND viruses circulating in wild and domestic birds in Europe are genotype V, VI, VII and XIII [1, 11, 12].
The genome of NDV consists of about 15`186-15`198 nucleotides and encodes the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the hemagglutinin-neuraminidase protein (HN), the large polymerase protein (L), the fusion protein (F) and two additional non-structural proteins V and W, which are generated by RNA editing during P gene transcription. The gene order of NDV is 3'-NP-P-M-F-HN-L-5' [9, 13, 14]. NDV is known to be able to infect over 200 bird species, but the initiated disease varies between the host species and virus strains. Isolates can be categorized into lentogen, mesogen and velogen depending on their pathogenicity. Mesogen and velogen isolates usually inherit a multiple basic amino acid sequence at the cleavage site of the fusion protein and a phenylanin at residue 117. The pathogenicity is normally assessed by *in vivo* pathogenicity tests or nowadays by sequencing of the fusion protein cleavage site [15]. High virulent isolates have a mortality up to 100 % in chickens with a fast distribution between flocks and thus enormous economic losses can be caused [16]. In Switzerland, infections of commercial poultry with APMV-1 are notifiable. Infected flocks have to be stamped out and vaccination is strictly forbidden. In many countries infections with NDV is endemic, especially in Asian and African regions despite vaccination [16]. Current vaccination is not able to completely protect animals and prevent from viral shedding and transmission [17].

The pigeon paramyxovirus-1 (PPMV-1) represents a variant of APMV-1. PPMV-1 isolates are generally classified into class II genotype VIb or lineage 4b [18, 19]. The first cases identified as PPMV-1 were originating from countries of the Middle East in the late 1970s [20]. In Europe, PPMV-1 first appeared in birds in the early 1980s and then spread across Europe [21-24]. Since then, PPMV-1 is considered to be endemic in pigeons in many countries [1, 12, 25]. Outbreaks of PPMV-1 in poultry in several countries including Sweden, Estonia, France, Germany and UK have demonstrated that the presence of PPMV-1 in pigeons and other wild birds pose a potential risk for the poultry industry [1, 26-28].

Incubation period usually is seven to 14 days, but longer periods up to four weeks are not unusual [29]. Transmission occurs directly and indirectly by contamination of feed or material. Birds shed virus in both faeces and respiratory secretions. However, it seems that APMV-1 strains which fail to induce respiratory signs, such as PPMV-1, are primarily transmitted by the faecal-oral route of infection [28, 30]. In pigeons, clinical signs of infection include neurological and intestinal signs [31]. Morbidity ranges from 30 to 70 %, whereas mortality varies from 10 to 70 % [31-33]. The spread of PPMV-1 to domestic poultry as well as the potential of PPMV-1 to induce Newcastle Disease in poultry is described in the literature [18, 28, 34, 35]. The clinical manifestation of PPMV-1 infections in poultry varies greatly. Although PPMV-1 isolates usually encode for a virulent amino acid motif at the fusion protein cleavage site, they are often of moderate or low pathogenicity for chickens [36]. The virulence of PPMV-1 isolates appears to escape definitions by standard tests (intracerebral pathogenicity index and amino acid sequence at the fusion protein cleavage site) concerning infections of chickens [28, 30]. Natural infections in chickens, respectively partridges with symptoms as diarrhoea, loss of condition, decline in egg production, neurological symptoms and increased mortality have been reported [28, 34]. The symptoms caused by experimental infections of chickens were variable ranging from asymptomatic to disease with depression, lethargy, inactivity, respiratory signs, nervous signs, conjunctivitis and even sudden death [30, 32, 35, 37-40].

During the last years several outbreaks of PPMV-1 infections in wild pigeons occurred in Switzerland (unpublished data). So far, in Switzerland no case of PPMV-1 infection in poultry has been reported. The infectivity and threat for poultry of currently circulating PPMV-1 isolates is unclear. The prescribed test for APMV-1 diagnosis for international trade implies virus isolation in embryonated eggs or cell culture followed by hemagglutination inhibition assays with NDV-specific antibodies [15]. However, this is a time-consuming method and requires the availability of embryonated eggs.
The quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a specific, sensitive, reproductive and fast method for the laboratory diagnostic of viral infections by detection of viral RNA [41, 42]. Two USDA-validated qRT-PCR assays by Wise et al. are commonly used for APMV-1 detection [43, 44]. However, APMV-1 viruses undergo evolutionary changes leading to a genetic divergence which may produce false-negative APMV-1 RNA results [5, 44, 45]. Therefore, besides these widely used qRT-PCR methods by Wise et al. [43] other assays have been proposed for NDV diagnostics in order to avoid failure of RNA detection [11, 45, 46]. According to the OIE, suitable samples for virus identification from dead birds are oronasal swabs and tissue samples from lung, kidney, intestine with content, caecal tonsils, spleen, brain, liver and heart. From live birds, swabs from trachea or cloaca are suitable [15].
In Switzerland, mainly kidney and brain tissues as well as swabs are used for PPMV-1 diagnostics. The suitability of these sample materials in order to detect PPMV-1 RNA in infected chickens has not been evaluated. Brain, cloacal swabs as well as kidney tissue from birds are commonly known as problematic sample material for RNA extraction and qRT-PCR [47, 48].

Recent PPMV-1 outbreaks in Switzerland in pigeons prompted us to investigate the infectivity and pathogenicity of a currently circulating Swiss PPMV-1 isolate for chickens. In order to investigate these issues chickens were experimentally infected with a Swiss PPMV-1 isolate via the natural route. The aims of our study included the determination of

parameters of PPMV-1 infections in chickens such as the duration of viral shedding as well as the induction of an immune response. Moreover, currently used diagnostic techniques were evaluated for their suitability to diagnose a PPMV-1 infection in chickens. Additionally, RT-PCR and qRT-PCR assays were evaluated for the detection of different Swiss PPMV-1 isolates.

In summary, the current routinely used APMV-1 qRT-PCR assays showed to be not appropriate to detect PPMV-1 infections in chickens due to the genetic divergence of PPMV-1 or the potentially low level of viral RNA in samples. In contrast, two different not-routinely used methods are more suitable for virus genome detection. The chickens experimentally infected with a Swiss PPMV-1 isolate remained healthy. Prolonged viral shedding up to 60 days post infection was noticed. The study demonstrates the need to investigate several sample materials since the common Swiss practise of investigating only kidney and brain tissue can fail to detect viral RNA. Our investigations point out important insights on PPMV-1 diagnostics.

3. Materials and methods

3.1. Virus isolate

A PPMV-1 isolate named T383 originating from an outbreak in feral pigeons in 2014 in Switzerland was used for experimental infection of chickens. In the loft where the disease occurred 45 out of 80 pigeons died within 10 days, showing remarkable greenish diarrhoea. One of the dead pigeon was necropsied at the National Reference Centre for Poultry and Rabbit Diseases in Zurich. The pathological findings coincided with peracute death. Viral RNA was detected by qRT-PCR in kidney material. The isolate was identified as a PPMV-1 strain by sequencing. At the Institute of Virology and Immunology, the virus isolate was propagated in embryonated eggs from kidney tissue.

3.2. Experimental design

White Leghorn chickens raised under optimized hygiene conditions were obtained from the *in-house* breeding facility. The chickens were kept on straw on the floor allowing free movement and contact. From all chickens used in this study, swabs were taken on day 0 before inoculation. In a first experiment, nine 2-week old chickens were inoculated oculonasally with three different doses of virus diluted in PBS (three animals per group). 25 µl inoculum was deposited on each eye angle and on each nostril with a laboratory pipette (full virus dose in a total of 100 µl). The virus stock solution consisted of $10^{8.2}$ TCID50/mL. The infective virus was applicated undiluted, 10^{-2} and 10^{-4}. For the first 24 h after infection, the groups were kept separately in cages. Afterwards they were hold together on the floor. The birds were daily monitored for clinical symptoms and cloacal swabs were taken to assess viral shedding. This trial was terminated 14 days post infection.

In a second experiment, a total of 19 chickens were inoculated with the challenge dosage defined by the first experiment (undiluted). Three different age groups were challenged:
1-week old (n=7), 3-week old (n=7) and 6-week old (n=5).
The animals were monitored daily. Cloacal and oropharyngeal swabs were taken daily during the first two weeks after infection, every second day until day 24, every 2 to 3 days until day 52 and then on day 56, 58, 60, 63, 66 and 70. Swabs from Copan with flexible minitips with 1 ml UTM™ medium were used (COPAN ITALIA spa, Brescia, Italy) for sampling. Blood samples were collected from all chickens of the second experiment from the *V. ulnaris* (under the wing) before inoculation and subsequently on day 4, 7, 14, 21, 28, 35 and 70 post inoculation.

After the termination of the trials, sera were taken from all birds. Additionally, all chickens of the first experiment as well as a few of the second experiment were subjected to necropsy to assess virus tropism.

3.3. Sample preparation and RNA extraction

The swab samples were thoroughly vortexed after sampling and centrifuged. Afterwards, the UTM medium in the swab tubes was pipetted off and used for further proceedings. The RNA extraction was carried out with the NucleoMag® 96 VET Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) MagMax Express 96 processor (Applied Biosystems™ by Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's instructions.

Tissue samples were homogenized diluted 1:10 in DMEM (catalog number: 21063029) with Penicillin-Streptomycin in concentration 400 U/ml penicillin respectively 400 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Gibco™ by Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the TissueLyser II *(*Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA extraction from tissues samples was performed using the standard phenol-chloroform extraction with TRIzol® Reagent (Ambion™ by Thermo Fisher Scientific, Waltham, Massachusetts, USA).

In order to control the efficiency of nucleic acid extraction and to detect potential inhibitory effects an internal control consisting *of in vitro* generated EGFP transcript was added to the sample prior to extraction.

3.4. (q)RT-PCR for the detection of viral RNA

For the detection of viral RNA a previously described assay based on the amplification of a part of the gene coding for the polymerase (L gene) was used [46] in combination with an assay for the detection of the internal control. For qRT-PCR reactions, the Superscript III Platinum One-Step qRT-PCR Kit (Invitrogen by Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. The reaction mixture contained 1 µl of RNA, 6.25 µl 2X Reaction Mix, 2.47 µl of RNAse free H2O, 0.03 µl of ROX Reference Dye (25 µM), 0.25 µl of Magnesium Sulfate (50 mM), 0.25 µl of SuperScript® III/Platinum® Taq Mix, 0.25 µl of each primer for viral RNA and internal control amplification (end concentration 400 nM, respectively 100 nM), 0.5 µl of each probe for the detection of viral RNA (end concentration 200 nM) and 0.25 µl of the probe for the amplification of the internal control (end concentration 100 nM). The thermal cycling protocol was as follows: 48°C for 30 min, 95°C for 2 min, 50 cycles at 95°C for 15 sec, 56°C for 30 sec and 72°C for 30 sec. The 7500 Fast real-time PCR system (Applied Biosystems™ by Thermo Fisher Scientific, Waltham, Massachusetts, USA*)* was used and results were analysed with the software (SDS Software v 1.3.1).

In order to evaluate different molecular based methods, the following (q)RT-PCR assays were additionally applied: the standard USDA-validated qRT-PCR assays by Wise et al. [43], the qRT-PCR assay by Cattoli et al. [45] and a conventional RT-PCR assay published by Stäuber et al. [49]. Primers and probes were used as published. A selection of PPMV-1 positive swab samples, eight PPMV-1 isolates from Switzerland, one PPMV-1 isolate from England and an APMV-1 (Hitchner B1) vaccine strain were used for this intention.

Further procedure information's are available upon request.

3.5. qRT-PCR for the detection of the housekeeping gene RNA

In order to evaluate the collected swabs in respect to the presence of sufficient material, a novel qRT-PCR assay was designed. Primers and probe were designed to specifically amplify mRNA of the housekeeping beta-actin gene (size of 93 bp) based on GenBank accession no. AB980793.1, GI:672889400 (Table 1). The Primer Express Software for Real-Time PCR Version 3.0 from Applied Biosystems was utilized for this approach. Each reaction mix for the beta-actin qRT-PCR assay contained 3.97 µl of MiliQ H2O, 6.25 µl of 2x Reaction Mix, 0.03 µl of ROX Reference Dye (25 µM), 0.25 µl of Beta-Actin_F (20 µM), 0.25 µl of Beta-Actin_R (20 µM), 0.25 µl of Beta-Actin_P (FAM-BHQ1; 5 µM) and 0.25 µl of enzyme mix. The final concentration of forward and reverse primer was 400 nM and 100 nM for the probe. The beta-actin content was determined from the sample swabs taken from the birds of the second experiment 10 and 30 days post infection.

Table 1. Sequences of primers and probes used for the detection of beta-actin mRNA and EGFP in the qRT-PCR assays

Name	Nucleotide sequence 5'-3'
EGFP2_F	GGG CAC AAG CTG GAG TAC AAC
EGFP2_R	CAC CTT GAT GCC GTT CTT CTG
EGFP2_P	YYE - ACA ACA GCC ACA ACG TCT ATA TCA TGG CC - BHQ1
Beta-Actin_F	CAT TGC TGA CAG GAT GCA GAA G
Beta-Actin_R	ACA GAG TAC TTG CGC TCA GGC
Beta-Actin_P	HEX - CAC CCA GCA CAA TGA AAA TCA AGA TCA TTG - BHQ1

3.6. Virus isolation from sample material

From a selection of tissue homogenates and swab samples, virus isolation was attempted to confirm the presence of infectious virus. Adherent Vero cells were infected in vented 12.5 cm^2 tissue culture flask with 1 ml of 1:10 diluted sample material and incubated for 1 h at 37° C,
5 % CO_2. Afterwards sample dilution was removed and 5 ml medium with supplements was added to each flask. The GMEM (Gibco™ by Thermo Fisher Scientific, Waltham, Massachusetts, USA) contained 2 % FCS filtered through 0.8 µM filters (Biochrom GmbH, Berlin, Germany) and Penicillin-Streptomycin in concentration 100 U/ml penicillin, 100 µg/ml streptomycin and 0.625 µg/ml amphotericin B (Gibco™ by Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were observed after 3, 4, 5 and 6 days for cytopathic effect. In the case of unclear cytopathic effect passage was performed as follows: 3-freeze-thaw cycles followed by centrifugation at 250 rcf for 10 min; the cell culture supernatant was then taken for virus isolation as described above.

3.7. Serology

A commercially available enzyme-linked immunosorbent assay (ELISA) kit (ID Screen® Newcastle Disease Competition, ID.Vet innovative Diagnostics, France) was used for the detection of antibodies against PPMV-1 and carried out according to the manufacturer's instructions. Briefly, sera were diluted 1:5 with sample diluent and added onto the plate. Following different incubation and washing steps, the absorbance was measured on a photometer at 450 nm and given as optical density. Based on manufacturer's validation results, samples showing percentage inhibition values >40 % were considered to be positive.

4. Results

4.1. Evaluation of different molecular based methods for the detection of PPMV-1 RNA

Eight Swiss PPMV-1 isolates as well a PPMV-1 isolate from England and a lentogen APMV-1 (Hitchner B1) vaccine strain were tested with four different qRT-PCR assays (Fig. 1a) and with a conventional RT-PCR assay (Fig. 1b). The qRT-PCR assay by Fuller et al. [46] was the only (q)RT-PCR assay, which gave positive virus RNA results for all investigated isolates. The assays by Cattoli et al.[45], Stäuber et al. [49] and the fusion gene (F-gene) assay by Wise et al. [43] all missed one of the eight tested Swiss PPMV-1 isolates. The F-gene assay shows higher Cq values especially for certain isolates such as the T383 and T040 compared with the values obtained by other methods. However, also values obtained by the Cattoli et al. assay seem higher compared to the values obtained by the Fuller et al. assay. The Wise et al. matrix gene (M-gene) assay [43] gave false negative results for 3 of these 8 Swiss PPMV-1 isolates. A positive result for isolate T366 was only received by the Fuller qRT-PCR assay, however with a high Cq value. The band obtained for the P/pigeon/England/617/83 isolate by the conventional Stäuber et al. assay [49] was faint under UV-light indicating a low amount of viral RNA. In contrast, Cq values obtained by the qRT-PCR point out a high content of viral genome.

The same (q)RT-PCR assays were also evaluated with a selection of collected swab samples from the animal trials. The two USDA-validated qRT-PCR assays from Wise et al. were not able to detect viral RNA from the tested swab samples [43]. Also the conventional RT-PCR assay by Stäuber et al. was not able to detect viral RNA, however, part of the samples didn't give any interpretable results. In contrast, the Fuller et al. and the Cattoli et al. qRT-PCR assays [45, 46] stated the same samples of this selection as PPMV-1 RNA positive (data not shown).

Fig. 1(a) Viral RNA detection with various qRT-PCR assays from different isolates. Negative results are illustrated as Cq value 50.

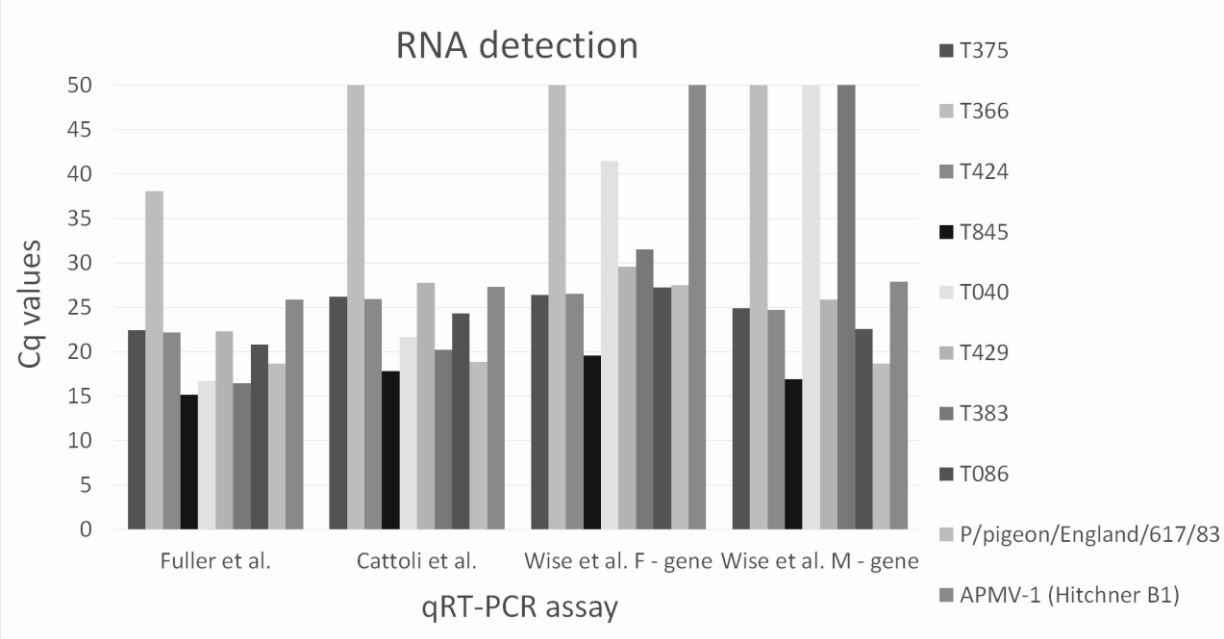
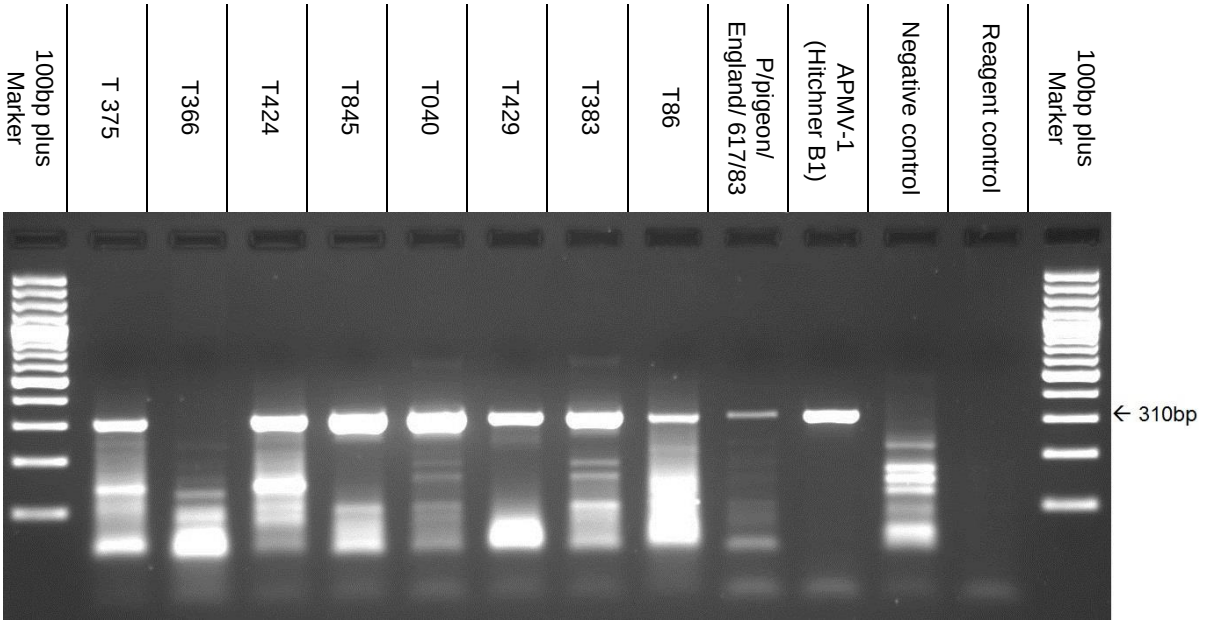


Fig. 1(b) Agarose Gel of the RT-PCR by Stäuber et al. from different isolates



4.2. Clinical manifestation and gross lesions observed in experimentally infected animals

In order to determine the optimal infectious dose for inoculation experiments, a first group of nine 2-week old animals were inoculated oculonasally with three different doses (in total of $10^{7.2}$, $10^{5.2}$ or $10^{3.2}$ TCID50) of infectious virus. All of the infected animals stayed healthy and no obvious clinical signs such as massive diarrhoea or central nervous disorders were observed. In addition, no weight loss was noticed.

Based on this result, the highest dose of infectious virus was used in order to infect chickens of three different age groups in a second experiment.

Interestingly, more liquid cloacal excretions occurred in two animals originating from the 1-week and 3-week old group on day one. In addition, one 1-week old animal showed mild symptoms of diarrhoea/polyuria during three separate days. Similar to the first experiment, none of the animals died or showed massive signs of disease.

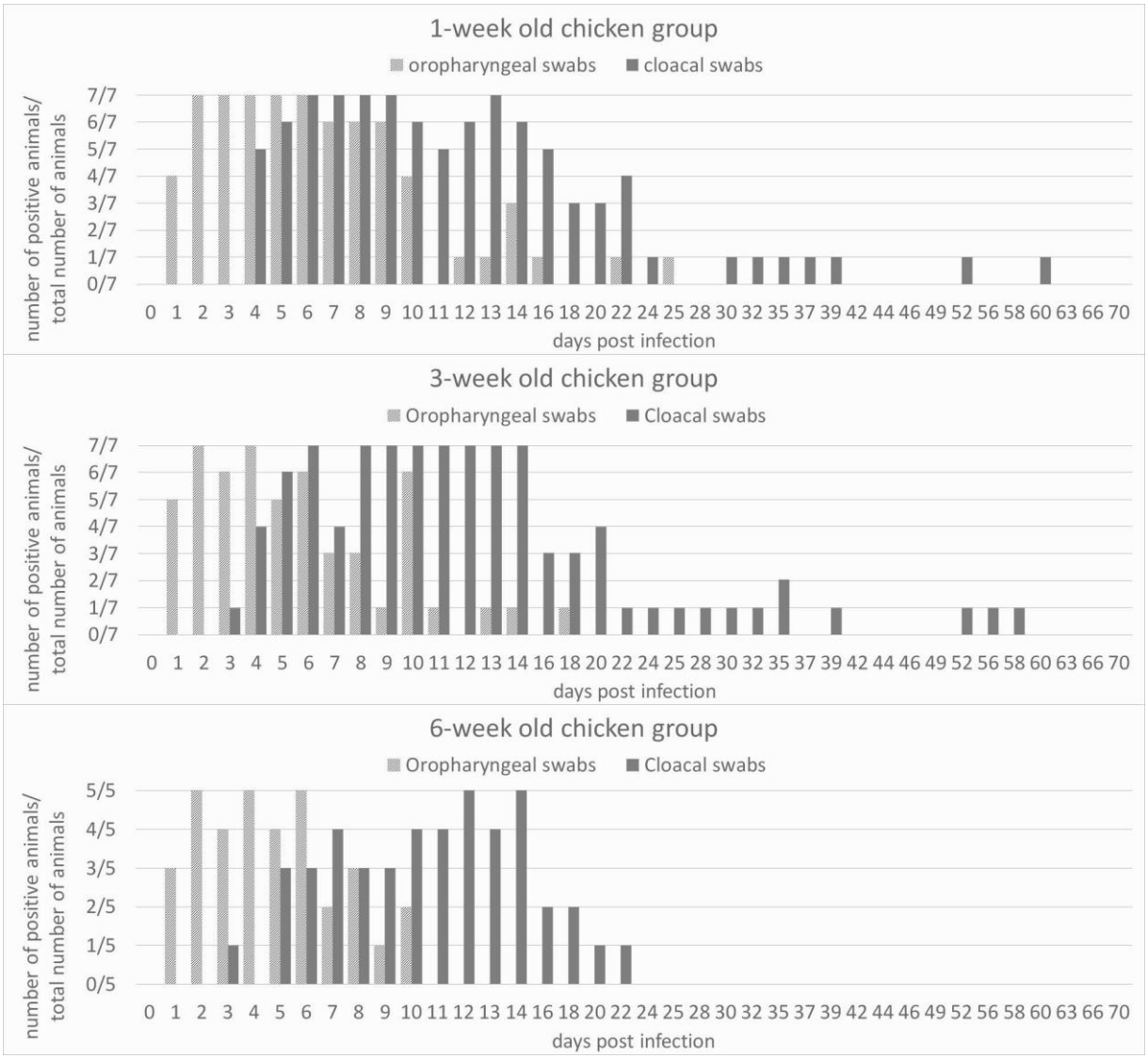
All animals of the first experiment as well as four chickens from the second experiment were necropsied. No obvious macroscopic lesions have been found.

4.3. Viral RNA detection in swab samples

Viral shedding determined by the detection of viral RNA by qRT-PCR was demonstrated in all infected animals in both experiments. However, from one chicken infected with the highest dilution of inoculated virus in the first experiment only one swab sample was positive for PPMV-1 RNA during the sampling period of two weeks, at 14 days post infection (dpi).
In the second experiment, viral shedding was predominantly detected between day 2 and 14 post inoculation. Viral RNA was detected in oropharyngeal swabs only, both types of swabs (oropharyngeal and cloacal) and cloacal swabs only during the first three days, between day 4 and 10 after inoculation and predominantly after 10 days of inoculation, respectively (Fig. 2).
From day 14 post inoculation, viral shedding was less frequently detectable. From that date on, a gradually, scattered viral shedding was observed in a few animals. Viral RNA was successfully detected from swab samples till 60 dpi, 58 dpi, 22 dpi for the 1-week, 3-week, respectively 6-week-old group. In general, PPMV-1 RNA was intermediately detectable in all

inoculated animals.
Quantification cycle (Cq) values obtained by qRT-PCR were low. In the second experiment, the lowest Cq for virus detection was 30.8 with a mean value of 39.54 (1-70 dpi). During the period when both types of swabs were positive (4-10 dpi) the mean Cq was 38.58, whereas afterwards (11-70 dpi) the mean Cq was 40.52.

Fig. 2 Viral RNA detection in swab samples



4.4. Detection of viral RNA in different organ samples

In order to determine the most suitable diagnostic specimens for viral RNA detection, various samples were tested (Table 2). In the first experiment, PPMV-1 RNA was successfully detected in all cloacal swabs, as well as in all spleen and heart tissues collected from the nine inoculated animals. All caecal tonsil samples were virus RNA positive, with the exception of the sample from one animal. The most reliable results for qRT-PCR indicated by the positivity of all triplicates were obtained by the use of caecal tonsils as well as spleen tissues. Viral RNA was detected in the proventriculus and kidney samples of 7 respectively 6 animals, whereas positive results were obtained from liver and trachea in 4 respectively 3

collected samples. In contrast, viral genome was detected only in 2 of 9 collected lung as well as brain samples.

In order to gain more information about the distribution of virus genome over a longer time period after inoculation, one or two animals per age group were necropsied at 70 dpi. Although cloacal swabs have been PPMV-1 RNA negative at this point of time, PPMV-1 RNA was still detectable in tissue samples from all necropsied animals. Viral RNA was detected in ceacal tonsils and proventriculus from all four animals, whereas spleen and duodenum from three animals were RNA positive, in addition heart and kidney samples were positive from two animals. Interestingly, no viral RNA was detected in liver, trachea, lung and brain tissue at this point of time after inoculation.

The mean Cq value of all tested positive tissue samples was 40.5. The Cq values obtained at 14 dpi from the first experiment were lower (mean 39.7) than the ones from the second experiment at 70 dpi (mean 43.3).

Table 2. Virus genome distribution in experimental infected chickens

Sampling time point after infection (in weeks)	Age at infection time point (in weeks)	Virus dose (TCID50 ml^{-1})	Animal number	Liver	Heart	Trachea	Proventriculus	Duodenum	Caecal tonsils	Spleen	Lung	Brain	Kidney	Cloacal swab
2	2	$10^{7.2}$	1	+	++	+	-	+	++	++	+	++	-	+
			2	-	++	-	+	++	++	+	+	+	++	++
			3	+	+	+	+	+	++	++	-	-	-	+
		$10^{5.2}$	4	-	+	-	+	++	++	++	-	-	++	+
			5	+	++	-	+	+	-	+	-	-	+	++
			6	++	+	-	-	-	++	++	-	-	++	++
		$10^{3.2}$	7	-	++	-	++	+	++	++	-	-	++	+
			8	-	++	+	+	-	++	+	-	-	-	+
			9	-	+	-	+	-	++	++	-	-	++	+
10	1	$10^{7.2}$	10	-	-	-	+	+	+	+	-	-	-	-
			14	-	-	-	+	+	+	++	-	-	+	-
	3		17	-	+	-	+	+	+	+	-	-	-	-
	6		22	-	+	-	+	-	++	-	-	-	+	-

++ PPMV-1 RNA positive (all wells of the triplicates)
+ PPMV-1 RNA positive (but not all wells of the triplicates)
- PPMV-1 RNA negative

4.5. Serology

In order to analyse the immune response against PPMV-1 in experimentally infected chickens, serological analysis by ELISA were performed. No antibodies were detected in blood samples before infection as well as on day 4 post infection. Inhibition values obtained by the ELISA at 7 dpi came up to 73 %, 76 % and 70 % in 1-week, 3-week and 6-week old animals respectively indicating an immunological reaction. Subsequently, a strong increase

of antibody titer was observed till 21 dpi, a maximum was reached at 35 dpi and titers remained high till the end of the experiment (a mean value of 93 percent inhibition at 70 dpi) (Fig. 3).

Fig. 3 Seroconversion after the experimental infection of chickens

	0 dpi	4 dpi	7 dpi	14 dpi	21 dpi	28 dpi	35 dpi	70 dpi
—1-week old	nd[a]	7%	73%	91%	94%	96%	96%	90%
—3-week old	7%	4%	76%	92%	95%	96%	97%	96%
—6-week old	8%	3%	70%	89%	94%	94%	96%	95%

[a] *nd* not done, no blood collection at this point in time

4.6. Virus isolation

Virus isolation in Vero cells was attempted with a section of viral RNA positive samples (three cloacal swabs, two oropharyngeal swabs and eight tissue samples from the first experiment). All swab samples as well as one kidney sample produced an obvious cytopathic effect in Vero cell monolayer. The observed cytopathic effects included cell death as well as syncytia formation, multinucleated cells originating from the fusion of several cells, appearing between day 3 to 5 post inoculation. All samples used for virus isolation were rejected to passage to proof virus isolation. The same samples produced a cytopathic effect after passaging, however the cytopathic effect was already obvious on day 3 post infection. Before passaging, the positive kidney sample and one of the cloacal samples were not interpretable until day 5 post infection.

4.7. Suitability of swab samples as diagnostic specimens

The amount of sample material is crucial in order to avoid false negative results in (q)RT-PCR assays. Therefore, in order to evaluate swab samples for their cellular content, an additional qRT-PCR for the detection of a house keeping gene was applied. All cloacal and oropharyngeal swabs from day 10 and 30 post infection of the second experiment were tested for the presence of beta-actin mRNA by a novel established qRT-PCR. Analysis of all samples revealed positive results indicating the presence of sufficient cellular sample material.

During cloacal sampling, the attempt was made to coat the swabs optically visible with faeces, however, this was not always successfully achieved. The Cq values of the tested oropharyngeal swabs ranged from 30.4 to 39.7 with a mean value of 33.9. The values obtained for the cloacal swabs ranged from 23.5 to 36.2 with a mean result of 31.1. These

swab samples were also visually assessed for their content of sample material. There was no obvious correlation between the Cq values and the visual indications (as particles in the swab liquid or its discoloration). By centrifugation of the swab liquids, a sample material pellet became visible at the bottom of each swab sample, however in varying sizes.

5. Discussion

5.1. Infectivity and viral shedding

PPMV-1 circulating in pigeons has been reported before to be a potential threat for poultry [1]. The clinical outcome manifestation of PPMV-1 infections in chickens varies greatly [28, 30, 32, 35, 50]. Recent disease outbreaks in urban pigeons due to infection with PPMV-1 prompted us to investigate the infectivity and pathogenicity of a current Swiss PPMV-1 isolate for chickens. All chickens experimentally infected with PPMV-1 T383 isolate shed virus and showed an immune response to the virus. All of the infected birds stayed healthy and no obvious clinical signs were observed, uninfluenced by the different inoculation doses and ages of hosts. Increased unclear liquid cloacal excretions were noticed in three younger animals with no influence on the general health status of the animals. In conclusion, the inoculated isolate seems of low virulence for chickens. However, sequencing of this isolate revealed a virulent amino acid sequence of ^{112}RRQKRF^{117} at the fusion protein cleavage site. These findings confirm that the virulence of PPMV-1 measured by standard tests as the motif at the fusion protein cleavage site may not correspond to the clinical signs in chickens [30].

For quite some time, the effect of passaging PPMV-1 virus in chickens on the pathogenicity for chickens has been discussed. One hypothesis is that virulence increases by passaging through chickens respectively chicken eggs [51, 52]. However, there is not always an effect on the virulence due to passaging, especially concerning the severity of clinical disease [30]. In a recently published study, a passaged PPMV-1 isolate from Pakistan led to high mortality in experimentally infected chickens [35]. The passaging of the Swiss PPMV-1 isolate used for the experimental infection may conceivably have changed the virulence for chickens and could be further examined in a subsequent study.

In our experiments, viral shedding was detectable intermittently. However, the Cq values obtained in the PPMV-1 assay were high and therefore it was not clear whether the viral shedding per se was intermittently or if the amount of the shed viral RNA was intermittently over the detection limit. Viral shedding was detected especially between day 4 and 10 post inoculation. Oropharyngeal swabs were PPMV-1 RNA positive by qRT-PCR mainly at the beginning of this period. Afterwards, for a certain period cloacal as well as oropharyngeal swabs were positive. Finally, viral RNA was detected only from cloacal swabs. The fact that positive data were first received from oropharyngeal swabs could be attributed to the chosen oculonasal inoculation route. Virus isolation was successfully attempted from viral RNA positive swab samples confirming the presence of infectious virus in the swab samples i.e. viral shedding. Viral shedding with medium and high inoculation doses of infectious virus - as applied in the first experiment - seemed comparable, whereas the lowest dose led to less viral shedding. The undiluted virus dose was considered most appropriate for the second experiment as no clinical signs were observed with any of the doses. In the second experiment, viral RNA was more frequently detected in samples from younger chickens compared to those originating from older chickens. Moreover, the viral shedding in younger birds seems to be prolonged. These results, together with the findings of increased liquid cloacal excretions noticed in three younger birds, seem to point out certain differences between the age of infected chickens concerning infectivity and pathogenicity, as already described before [39, 52, 53].
Viral RNA was successfully detected from swab samples over a long time period with the last

detection at 60 dpi. Long-lasting shedding of APMV-1, as observed in our study, has been observed before [54-56], however, this is in contradiction to the statement of Marlier and Vindevogel [31] that pigeons do not stay longer infected by PPMV-1 than six weeks. Our data suggest that long-lasting shedding can occur in chickens infected with PPMV-1. However, to our knowledge there are no other studies of chickens which were conducted over such a long time period and therefore there is not any possibility of comparison.

5.2. Virus distribution

Eleven different sample materials from inoculated chickens were investigated. At 14 dpi virus genome was mainly detected in spleen, caecal tonsils, heart, cloacal swabs, liver, proventriculus, duodenum and kidney tissue samples. At 70 dpi PPMV-1 RNA was still detected in different tissue samples, especially in caecal tonsils, spleen, duodenum and proventriculus, although no more viral shedding has been detected until this point in time by swabbing. Kidney tissue, which is considered a major organ for NDV detection, was not viral RNA positive in all chickens. Brain tissue, also considered as a major organ for NDV detection, as well as lung tissue were negative in most of the investigated chickens. These findings indicate a rather viscertropic type of virus i.e. with affinity for the internal organs. This is in agreement with the origin of the virus isolate used for the experimental infection, which was isolated from an outbreak in pigeons showing remarkable greenish diarrhoea. Classical PPMV-1 infections in pigeons are usually characterized by a neurological manifestation of the disease and in an initial stage symptoms such as diarrhoea/polyuria may occur. However, atypical digestive forms of PPMV-1 are being seen increasingly frequently and consist of persistent diarrhoea without neurological symptoms [22, 29, 31, 51]. As the tested virus isolate is originating from an outbreak in the field, it is not clear at which stage of infection the outbreak was reported. Therefore, the finding of greenish diarrhoea can either be due to the early stage of infection or due to the atypical form of this isolate. From classical viscerotropic APMV-1 strains it is known that especially lymphoid organs as well as the gastrointestinal tract are affected [57].

In our study, the investigated lung tissues were mostly PPMV-1 RNA negative, although the lung could have been expected to be involved in the infection by the chosen oculonasal experimental infection route. Mainly gastrointestinal and lymphathic tissues were PPMV-1 genome positive, indicating the induction of a natural virus distribution of this viscerotropic virus type due to the applied inoculation route. Part of the infective virus was presumably swallowed after nasal application, which may support a gastrointestinal distribution. Therefore, the inoculation route may affect clinical manifestations [51]. The PPMV-1 RNA distribution in the tissue samples support the common finding of the strain dependent virus tropism of NDV infections [57]. From chickens infected with neurotropic PPMV-1 isolates viral RNA can usually be found in brain tissues, however, not always in kidney tissue [30, 35, 52, 58, 59].

In accordance with other experimental PPMV-1 infections in chickens, lymphatic tissues as in spleen and caecal tonsils as well as in kidney, heart and proventriculus are in general suitable matrices to detect PPMV-1 infection by (q)RT-PCR or virus isolation. But this is neither the case with every isolate [35] nor in every bird of a group. In contrast to this, other studies reported frequent detection of PPMV-1 virus or its RNA in brain and lung tissues in case of PPMV-1 infection in chicken [30, 32, 58-60].

From eight PPMV-1 RNA positive tissue samples, virus isolation was attempted using Vero cells. However, only one of the tissue samples, concretely a kidney sample, produced a cytopathic effect. This is not surprising as Cq values obtained in the qRT-PCR assay were high.

5.3. Virus replication

Cq values obtained by the qRT-PCR assay to detect viral RNA were high for tissue samples as well as for swab samples over the whole sampling period. During the main viral shedding period, the mean Cq values where slightly lower. These low levels of PPMV-1 RNA indicate a low efficiency of replication of this isolate in chickens. Low viral genome replication of PPMV-1 has been reported to be directly associated with low virulence [51, 61]. Dortmans et al. demonstrated that the increase in virulence for chicken of a PPMV-1 isolate by passaging in chicken was the result of mutations in the polymerase complex. These mutations led to an enhanced replication of the virus [61, 62]. In our study, the low PPMV-1 RNA amount in all samples could be associated with the low virulence of the Swiss PPMV-1 isolate used in our case. Thus, passaging in chickens of the used PPMV-1 isolate could have changed the disease outcome and amount of PPMV-1 RNA in sample material due to more efficient replication of the virus.

5.4. Serology

First, the appropriate serological method to test our PPMV-1 sera samples was selected. Hemagglutination inhibition assay [15] as well as ELISA kits were used to detect PPMV-1 antibodies before [35]. Previous studies using ELISA kits to detect PPMV-1 antibodies used a multispecies kit by Swanova, which is no longer available [63, 64].

However, another commercially available ELISA kit designed to detect NDV in domestic and wild species (ID Screen® Newcastle Disease Competition, ID.Vet innovative Diagnostics, France) successfully detected antibodies against PPMV-1 as test results with sera gained from the first animal trial revealed. In order to compare the sensitivity of this ELISA kit and the hemagglutination inhibition assay, a selection of weak positive PPMV-1 sera as well as a serial dilution were used. Both serological methods gave comparable results concerning sensitivity (results not shown). However, only due to our *in-house* use of a questionable region between 25 to 40 percent inhibition for the ELISA kit, it was possible to detect a weak positive serum dilution, which was positive in the hemagglutination inhibition assay. According to the manufacturer's instructions, the questionable region is from 30 to 40 percent inhibition. The ELISA kit was used to assess all sera samples from the animal trial as the procedure is faster, needs less sample material and gives more objective results.

The immune response against PPMV-1 in experimentally infected chickens was analysed. A seroconversion was observed in all birds, starting at 7 dpi. This is in accordance to data previously published [32]. The antibody titer afterwards increased strongly till 21 dpi with a maximum at 35 dpi. Until the end of the trial at 70 dpi, the antibody level stayed high. To our knowledge, there are no other studies in chickens with serological monitoring over such a long period after experimental infection.
High antibody level induced by the inoculation with PPMV-1 reveals an effective immune response [32, 65]. In this study, the chickens infected with the Swiss PPMV-1 isolate showed a high antibody titer, which may have contributed to the observed low virulence of this isolate.

5.5. Detection of PPMV-1 RNA

Concerning PPMV-1 RNA detection, there are two main issues which represent a challenge to diagnostics: the high capacity for mutation in RNA viruses and the potentially low virus replication in chickens.

Two USDA-validated qRT-PCR assays are routinely used in diagnostic laboratories to identify APMV-1 RNA. One of the assays by Wise et al. was designed to detect a conserved region of the M-gene of diverse NDV strains as the other was designed to detect potentially

virulent NDV by targeting a F-gene region. However, it is known that due to the high genetic variability of NDV these commonly used assays fail to detect all NDVs, including PPMV-1 strains, due to primer and probe mismatches [5, 11, 15, 44]. Other qRT-PCR assays have been proposed to detect all NDV strains. One assay was developed based on the Wise et al. M-gene assay, however with a modified probe, whereas another was designed in order to detect a conserved region of the large polymerase gene (L) [45, 46]. In our study, we compared these four qRT-PCR assays and a conventional RT-PCR assay for their ability to detect PPMV-1 RNA. Therefore, eight Swiss PPMV-1 isolates as well as a lentogen APMV-1 (Hitchner B1) vaccine strain, a PPMV-1 strain from England and swab samples from our trials were tested.

The detection of all tested isolates was only successfully achieved by the assay by Fuller et al. [46] whereas the other assays failed. The assays by Cattoli et al. [45], Stäuber et al. [49] and the F-gene assay by Wise et al. [43] missed all one of the eight tested Swiss PPMV-1 isolates. Additionally, according to expectations the Wise et al. F-gene assay missed the lentogen vaccine strain as this assay is designed to detect virulent NDV. The Wise et al. M-gene assay failed to detect 3 of 8 of these PPMV-1 isolates. This assay is known to be unable to detect certain PPMV-1 isolates due to mismatches at the probe binding site, hence Cattoli et al. developed another probe for this assay.
The Cq values obtained for certain isolates were remarkable higher in the Wise F-gene assay than in the ones obtained by other methods. Already Wise et al.reported that the assay targeting the M gene was substantially more sensitive than the F gene assay [43]. However, also Cq values obtained by the Cattoli et al. assay were rather higher than by Fuller et al. assay. This could be a reason why one isolate was only positive by the Fuller et al. assay as even the Cq value obtained by this method was high.
Conventional RT-PCR assays as the Stäuber et al. [49] are known to be equal or less sensitive compared to qRT-PCR assays [42]. However, one of the reasons for the failure to detect all tested PPMV-1 isolates (or obtained rather faint band as for the P/pigeon/England/617/83 isolate) by this assay were as well primer mismatches, which was confirmed by improved detection of certain isolates with modified primers (data not shown).

From the prior testing of different (q)RT-PCR assays with different isolates, it was not astonishing that the two USDA-validated qRT-PCR assays failed to detect PPMV-1 RNA from the swab samples of the experimental trial. As the virus replication of this isolate was low, the F-gene assay failed to detect any of the testes samples as positive. The M-gene assay failed due to differences in the probe binding site. The samples obtained from the chickens, infected in our animal trial, would have been stated as negative by these assays. The Stäuber et al. assay yielded viral RNA negative as well as non-interpretable results for these swabs (very faint PCR bands and simultaneously many strong unspecific bands). By reamplification of this faint PCR bands or optimisations of the proceeding, such as a higher annealing temperature or prior DNA digestion, viral RNA detection would be possible. Results obtained by the Fuller et al. as well as the Cattoli et al. method for these swab samples gave comparable viral RNA positive results. Although multiplex assays, as the Fuller et al. assay, are considered as reduced sensitive compared to singleplex assays [15].

In routine diagnostics, virus detection from dead pigeons is often attempted from kidney and brain tissue. However, based on our results, it is evident that other organ samples should be taken as well, as kidney and brain tissues can achieve negative results in animals which are positive from other sample materials (mainly caecal tonsils, spleen, heart, cloacal swabs, liver, proventriculus and cloacal/oropharyngeal swabs). It is known that virus tropism is strain dependent and that the detection of PPMV-1 infections can be attempted from a wide range of organs [32, 58].

It has been reported that RNA recovery and amplification by RT-PCR can be interfered by organic material. The success of RNA detection by RT-PCR is therefore dependent on the appropriate RNA extraction method [15, 66]. Inhibitory effects were detected due to our internal EGFP control, especially in proventriculus tissue samples and less frequently also in

cloacal swabs, caecal tonsils, spleen and duodenum sample material. In contrast, brain and kidney tissue did not show inhibitory effects although expected from literature [47, 48]. From such inhibited samples, the RNA extraction was repeated with a 1:10 dilution and re-subjected to RNA detection. Some at first negative PPMV-1 RNA samples proved to be positive afterwards. In particular with samples reaching the detection limit, it is important to mind inhibitory effects.

The amount of cell material gained by swab sampling was determined by the Cq value of the beta-actin mRNA assay. The mean Cq values obtained from the oropharyngeal swabs (33.8) and cloacal swabs (32.1) were rather high compared to the values obtained from tissue homogenates. However, every swab tested for the housekeeping-gene was cell material positive. Interestingly, in preliminary investigations, the same swab type was used to take cloacal and oropharyngeal samples form pigeons and remarkable lower Cq values were obtained in the housekeeping gene assay (a mean value of 21.4 for cloacal respectively 27.4 for oropharyngeal swabs). This means that there may be a bird species dependent difference.
No obvious differences were noticed between swabs coated visibly with faeces and swabs which were optically unremarkable, concerning the Cq values for the cell material content measured by the beta-actin mRNA assay. One reason for this could be that the process of swab sampling was prolongedly conducted with swabs which were not immediately coated with faeces, in order to achieve faecal coated swabs. This may have led to more scratching at the intestinal epithelium and thus higher gain of cell material on the swab. These investigations point out that cloacal as well as oropharyngeal swab samples contain cell material, even if swabs are optically completely unremarkable after sampling and are thus reliable sample materials. These results, together with the findings that PPMV-1 RNA can, among others, mainly be extracted from swabs in the case of infected chickens, support the assertion that swab samples are regarded as suitable sample material from live and dead birds for the detection of PPMV-1 infections [15].

In summary, the current routinely used qRT-PCR assays are not appropriate to detect PPMV-1 infections in chickens due to mismatches at the primer and probe sites and the potentially low amounts of viral RNA in sample materials. In contrast, two different non-routinely used methods showed to be more suitable for virus genome detection for the tested Swiss PPMV-1 isolates and samples with low RNA content [45, 46]. Additionally, in order to detect PPMV-1 infections in chickens, virus genome detection should be attempted from several different sample materials as the same organs are not positive with each isolate and individual animal. Thus, in order to avoid unnoticed PPMV-1 infections in chickens, the diagnostic of PPMV-1 RNA should be adapted concerning the investigated sample materials and the routinely used diagnostic qRT-PCR assays. Nowadays, a wide range of studies and diagnostic laboratory only draw upon the USDA-validated qRT-PCR assays for their NDV diagnostic. Reliable and validated diagnostic methods are crucial for the control and eradication of notifiable diseases.

6. Author statements

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Conflicts of interest

There is no conflict of interest in this paper.

Ethical statement

All animal studies and procedures were carried out in accordance with the Swiss animal protection law and with the national and international animal experimentation guidelines. The animal experiments were approved by the Animal Welfare Committee of the Canton of Berne with the licence number BE58/16.

7. Abbreviations

PPMV-1	pigeon paramyxovirus-1
APMV-1	avian paramyxovirus-1
NDV	Newcastle Disease virus
USDA	United States Department of Agriculture
OIE	World Organisation for Animal Health
dpi	days post infection
TCID50	50 % tissue culture infective dose
ELISA	enzyme-linked immunosorbent assay
PBS	phosphate-buffered saline
RT-PCR	reverse transcription polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
(q)RT-PCR	RT-PCR and qRT-PCR
M-gene	matrix gene
F-gene	fusion gene
Cq	quantification cycle (cycle threshold)
EGFP	enhanced green fluorescent protein

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