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The small hydrophobic (SH) gene of North American turkey AMPV-C does not attenuate nor modify host tropism in recombinant European duck AMPV-C

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ABSTRACT

Subgroup C Avian Metapneumoviruses (AMPV-C) has two lineages, one mostly in turkeys and one mostly in ducks. To investigate the molecular basis of AMPV-C host tropism, a reverse genetics system for a duck AMPV-C virus was developed. A recombinant copy and a recombinant virus in which the SH protein had been exchanged for that of a turkey AMPV-C were rescued. No change in cytopathogenic effect or replication profile in vitro were observed for either virus compared to the wild type. In SPF Muscovy ducks the wild type and its recombinant copy were equally pathogenic. Exchanging the SH in the recombinant copy produced the same results.

In SPF turkeys, neither recombinant virus was pathogenic, although both showed a low level of replication. Thus, from the current model, it appears that AMPV-C SH proteins derived from the different species are compatible and that turkey SH does not affect duck AMPV-C pathogenicity.

1. Introduction

Avian Metapneumoviruses (AMPV) have been responsible for major economic losses to the poultry industry and following their discovery in the late 1970's in South Africa they are now detected in most parts of the world (Jones and Rautenschlein, 2013). AMPV infection in the field causes respiratory disease in birds with high morbidity (up to 100% of a flock) and variable mortality (0–50%) depending on the severity of bacterial secondary infections. Respiratory disease after AMPV infection of breeding birds is often less pronounced which has been suggested to be a result of better management conditions (Eterradossi et al., 2015) however, in these birds drops in egg production and shell quality often follow the respiratory signs.

AMPVs belong to the order *Mononegavirales* family *Pneumoviridae* genus *Metapneumovirus* (Afonso et al., 2016) and exist as four different subgroups (A to D) based on their genetic and antigenic properties (Bayon-Auboyer et al., 1999; Brown et al., 2014; Eterradossi et al., 1995; Juhasz and Easton, 1994; Seal, 1998). The subgroup C viruses also have two distinct lineages; a US lineage and a Euro / Asian lineage (Toquin et al., 2006b). In general, literature shows turkeys and chickens to be the conventional hosts of subgroups A and B, turkeys to be the conventional host of subgroup D and US subgroup C lineage, and wild or domestic ducks to be the conventional hosts of US or Euro/Asian subgroup C viruses, respectively. However, until recently this was not confirmed under similar experimental conditions. A recent experimental study was performed where one representative isolate from each subgroup and each AMPV-C lineage was tested in specific pathogen free (SPF) turkeys, chickens and Muscovy ducks (Brown et al., 2018). This study confirmed the previous literature however, sequencing of AMPV-C reisolated from the three bird species also suggested that genome sequence modifications in or around the small hydrophobic (SH) protein open reading frame (ORF) could be linked with host preference (unpublished data). This protein together with the fusion (F) and attachment glycoprotein (G) is located on the surface of the virus, however unlike F and G that have been well characterized (Biacchesi et al., 2004; Brown et al., 2014; de Graaf et al., 2009; Toquin et al., 2003), its function is not well understood. Several studies on viruses belonging to the *Pneumoviridae* family using reverse genetics (RG) have shown however, that deletion of SH results in some level of attenuation (Bukreyev et al., 1997; Ling et al., 2008) and thus it clearly has a role

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across viruses of the pneumoviridae family for virus pathogenicity. For subgroup A AMPVs, deletion of this protein has also been shown to alter cytopathic effect (CPE) in vitro (Ling et al., 2008; Naylor et al., 2004), which could be restored to a typical form using SH protein derived from subgroup B AMPV but not from duck subgroup C virus (Ling et al., 2008). Albeit these data were produced in vitro they do demonstrate compatibility of SH proteins from different subgroups of galliform AMPVs and possibly a lack of compatibility of SH proteins of AMPVs from galliforms and palmipeds. This data also supports the hypothesis that SH has some role in species adaptation.

In order to assess whether the AMPV-C SH protein indeed influences the viruses capability to replicate in a "non conventional host" or affect pathogenicity, a first reverse genetics system based on an AMPV-C isolate (Fr-AMPV-C) belonging to the Euro / Asian duck lineage was developed. This system was used to rescue recombinant viruses encoding either the duck AMPV-C complete backbone with its natural SH ORF or with an SH ORF derived from a turkey AMPV-C virus (AMPV-C Colorado, isolate 193ADV9802). SPF Muscovy ducks and turkeys were inoculated with these viruses and assessed for seroconversion, clinical signs, virus replication, and excretion of live virus over a three-week period.

2. Material and methods

2.1. Construction of Fr-AMPV-C reverse genetics plasmids

Schematic representations of plasmid constructs described in this section are shown in Fig. 1. Cycling conditions and sequences for the different primer pairs are available upon request. All kits and reagents were used according to manufacturer's instructions unless otherwise indicated.

2.1.1. Full length Fr-AMPV-C plasmid

An exact, full length DNA plasmid copy of the Fr-AMPV-C wild type ($_{wt}$Fr-AMPV-C) viral genome (accession no. HG934338) was constructed and named $_{p}$Fr-AMPV-C. Construction was achieved in a series of three reverse transcription reactions, eight PCRs and eight ligation steps similar to that described for the construction of the AMPV-A full length clone (Naylor et al., 2004). However, the current study used a blunt end cloning strategy, as opposed to the sticky end cloning strategy employed by Naylor et al. (2004).

Briefly, viral RNA was extracted from Fr-AMPV-C infected Vero cells using Qiamp Viral RNA mini kit (QIAGEN). Three cDNA fragments were generated from extracted RNA in RT reactions using Superscript II Reverse Transcriptase (Invitrogen). Eight blunt ended PCR fragments covering the entire genome were then generated from the cDNA using *Pfu turbo* DNA polymerase (Agilent) and sequentially ligated into pSMART LC Amp plasmid (Lucigen) using T4 DNA ligase (invitrogen).

The pSMART LC Amp plasmid vector had been previously modified so that inserts were flanked at the 5′ end by the following elements: cytomegalovirus promoter (pCMV)- T7 promoter - hammer head ribozyme (HHr) sequence and at the 3′ end by hepatitis delta virus ribozyme (HDVr) - Bovine Growth Hormone polyA sequence (BGHpA) – T7 terminator sequence.

All plasmids were used to transform *Escherichia coli* MAX Efficiency™ Stbl2™ Competent Cells (Invitrogen). At each step in the sequential cloning process, plasmids were extracted from colonies using NucleoSpin® Plasmid kit (Macherey Nagel) followed by restriction digest mapping and Sanger sequencing using Big Dye Terminator v3.1 cycle sequencing kit (ThermoFisher). The final plasmid $_{p}$Fr-AMPV-C was extracted using Nucleobond® Xtra Maxi Plus (Macherey Nagel) and was confirmed to have the correct sequence by Sanger sequencing as described above.

2.1.2. $_{p}$Fr-AMPV-C with unique engineered BamH1 site

A full length Fr-AMPV-C clone that contained a unique restriction site for the *BamH*I restriction endonuclease (RE) in the F ORF was generated so that rescued viruses could be differentiated simply from $_{wt}$Fr-AMPV-C using BamH1 digestion (see Section 2.5).

This clone was constructed by site directed mutagenesis (SDM) on $_{p}$Fr-AMPV-C using Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific). The F ORF was modified by synonymous mutation to create the site. Cloning procedures, plasmid sequencing and final plasmid preparation were performed as described for $_{p}$Fr-AMPV-C. The final plasmid with the engineered RE site was named $_{p}$Fr-AMPV-C_{BamHI}

2.1.3. $_{p}$Fr-AMPV-C with SH modification

The full length SH ORF from pFr-AMPV-C was exchanged for that of a North American turkey AMPV-C Colorado isolate reference 193ADV9802 (accession no. AJ457967).

Viral RNA was extracted from Vero cells infected with 193ADV9802 using the Qiamp Viral RNA mini kit (Qiagen). cDNA covering the SH ORF of 193ADV9802 was generated using Maxima H Minus Reverse Transcriptase (Thermo scientific). The full length SH PCR product was generated using Phusion Green Hot Start II High-Fidelity DNA Polymerase. Following purification, this PCR product was used as a "megaprimer" for a SDM reaction on pFr-AMPV-C using Phusion Green Hot Start II High-Fidelity DNA Polymerase. This "megaprimer" technique has been explained previously in detail and shown to be successful for modifying large zones of sequence within an AMPV A RG system (Brown et al., 2011). Cloning procedures, plasmid sequencing and final plasmid preparation were performed as described for $_{p}$Fr-AMV-C.

The $_{p}$Fr-AMV-C plasmid containing the full length SH coding sequence from 193ADV9802 was named $_{p}$Fr-AMPV-$C_{SH\text{-}Co}$

2.2. Plasmids expressing the viral replication proteins

PCR products corresponding to the nucleoprotein (N), the

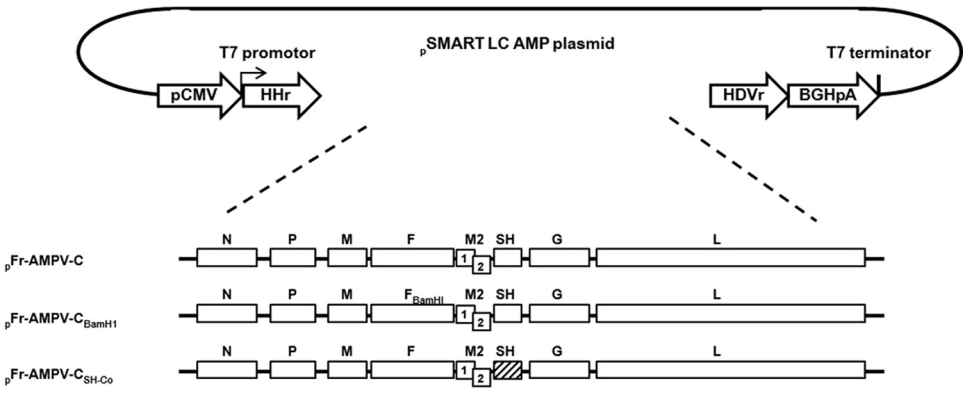

Fig. 1. schematic representation of produced plasmids, $_{p}$Fr-AMPV-C represents the plasmid containing the exact sequence of $_{wt}$Fr-AMPV-C virus. $_{p}$Fr-AMPV-C_{BamHI} corresponds to the plasmid with a synonymous mutation in the F ORF introducing a BamHI RE site. $_{p}$Fr-AMPV-$C_{SH\text{-}Co}$ corresponds to the $_{wt}$Fr-AMPV-C consensus sequence with the SH gene exchanged for that of Colorado North American turkey isolate 193ADV9802. pCMV Cytomegalovirus promotor; HHr Hammerhead ribozyme; HDVr Hepatitis delta viral ribozyme; BGHpA Bovine growth hormone poly A; $_{p}$SMART LC Amp (Lucigen).

phosphoprotein (P) and the full M2 ORFs of Fr-AMPV-C were individually cloned into a $_{p}$CDNA 3.1 vector (Addgene) using Topo cloning technology. The L ORF was cloned in three sequential cloning steps in the same manner and into $_{p}$SMART LC Amp as described in Section 2.1. Cloning procedures, plasmid sequencing and final plasmid preparation were performed as described in 2.1. Final plasmids were named $_{p}$CDNA-N, $_{p}$CDNA-P $_{p}$CDNA-M2 and $_{p}$SMART-L.

2.3. Recovery of recombinant AMPV-C viruses

Three recombinant (rc) viruses were rescued following the protocol described by Zhang et al. (2016). For rescue, 90% confluent BSRT7 (kind gift from Prof. Dr K. Conzelman, Pettenkofer Institute, München, Germany) cells in 6-well plates (Nunclon™ Delta surface, Thermo Scientific) were washed twice with Opti-MEM™ + GlutaMAX™ Reduced Serum Medium (Thermofisher) and were incubated at 37 °C and 5% CO_2 for 30 min with 1.5 ml of the same wash medium. The cells were then transfected using lipofectamine 2000 (Invitrogen) and 2.0 µg of $_{p}$CDNA-N, 2.0 µg of $_{p}$CDNA-P, 0.5 µg of $_{p}$CDNA-M2, 1 µg of $_{p}$SMART-L and 5 µg of either $_{p}$Fr-AMPV-C, $_{p}$Fr-AMPV-C_{BamHI} or $_{p}$Fr-AMPV-$C_{SH\text{-}Co}$. Transfected BSRT7 cells were incubated at 37 °C and 5% CO_2 for four hours, then washed twice with 1 ml of Minimal Essential Medium (MEM, Gibco) and finally cultivated with 2 ml of MEM supplemented with 10% FCS (Eurobio), penicillin, streptomycin and fungizone (200 u/ml, 0.2 mg/ml and 2 µg/ml final concentration respectively) at 37 °C 5% CO_2.

Three days post transfection, 1 ml of supernatants were harvested and stored at −70 °C for later use. Cell sheets were resuspended by pipetting in the remaining 1 ml and then co-cultivated onto pre-established 50% confluent Vero cells in 25 cm^2 flasks (Corning) in MEM supplemented with HEPES (20 mM), 10% FCS, penicillin, streptomycin and fungizone at 200 u/ml, 0.2 mg/ml and 2 µg/ml final concentration respectively. Successful rescue was confirmed by the development of cytopathic effect (CPE) and immunofluorescent staining using turkey anti-AMPV-C polyclonal antibody (see 2.7), RT-PCR, and BamH1 digestion for rescues using $_{p}$Fr-AMPV-C_{BamHI} (see Section 2.5). Supernatants of positive rescue experiments were inoculated onto confluent Vero cells in 150 cm^2 flasks. Cell sheets and supernatants were harvested (at the appearance of CPE 4–6 days after inoculation) and homogenized, then centrifugated at 300 g for 5 min at + 4 °C and stored at −70 °C as viral stocks. The full length genome sequences of rescued virus stocks were confirmed using next generation sequencing (NGS, see Section 2.4)

Rescued viruses were named $_{rc}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C_{BamHI} and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$.

2.4. NGS analysis of $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$

RNA was prepared for NGS by centrifugation of the different virus stocks at 17000 g for 10 min at + 4 °C followed by RNA extraction using TRIzol LS (Life Technologies). Sequencing was performed using an Ion Proton Sequencer (Life Technologies), details of sequencing protocols and sequence analysis are available upon request.

2.5. Confirmation of viral rescue of $_{rc}$Fr-AMPV-C_{BamHI}

RT-PCR on RNA extracted from supernatants was used to generate a 445 bp amplicon spanning the region in the F gene where the RE site was located. This amplicon was digested with *BamHI* Fast Digest (Thermo Scientific) for 30 min at 37 °C. Digested products were revealed by electrophoresis on 2% agarose gel, positive digestion being expected to result in two products, one of 294 bp and another of 151 bp.

To eliminate possible PCR amplification from transfected full length plasmid DNA, supernatants were treated twice with DNase I, RNase free (Qiagen). The absence of plasmid DNA was confirmed by performing PCRs without prior RT.

2.6. Virus replication kinetics

$_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C_{BamHI} and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were inoculated onto Vero cells at a m.o.i of 0.05. Briefly, Vero cells at 90% confluence in 96-well plates were inoculated with 50 µl of virus per well, then incubated at 37 °C 1%CO_2. After 1 h of contact, the inoculum was removed, and cells were washed twice with PBS. 250 µl of MEM supplemented with HEPES (20 mM), 2% FCS, penicillin, streptomycin and fungizone at 200 u/ml, 0.2 mg/ml and 2 µg/ml final concentration respectively was added, then the plates were incubated at 37 °C 1%CO_2. At multiple time points post infection (0 h, 12 h, then at day 1, 2, 3, 4, 5, 6 and 9), supernatants were harvested and stored at −70 °C. Virus titres were determined by microplaque assay using the Reed and Muench method.

2.7. Cytopathogenic effect

Inoculated Vero cells were observed daily for the development of cytopathogenic effect. Cells were observed by white light microscopy and by immunofluorescence after cells had been fixed in 50:50 ethanol / acetone followed by immunostaining using turkey serum raised against Fr-AMPV-C and Fluorescein isothiocyanate (FITC) conjugated anti-turkey antibodies (Rabbit anti turkey IgG H + L, Rockland USA PA).

2.8. Animal experiments

2.8.1. Ethical statement

All animal experiments were performed in agreement with the national regulations on animal welfare from the French Ministry for higher education and research and after approval from the French Agency for Food, Environmental and Occupational Health & Safety's (ANSES) ethical committee and with the permission of the “Haut Commissariat des Biotechnologies” for the use of recombinant viruses.

18-day-old SPF Muscovy ducks and 28-day-old SPF Turkeys (Anses-Ploufragan), housed under BSL2 biosafety conditions, were experimentally infected and then monitored over a three-week period. These ages were chosen as they represent the age when the birds reach a higher sensitivity to respiratory infections. In five separate containment cells, three groups of 15 ducks (D0, D1 and D2) and two groups of 15 SPF turkeys (T1 and T2) were inoculated via the intranasal route with 100 µl of virus at $10^{4.5}$ $TCID_{50}$/ml in MEM supplemented with HEPES, penicillin and streptomycin at 20 mM, 200 u/ml, 0.2 mg/ml final concentration respectively. Three days after inoculation five age- and

a) Groups / Inoculum

Species	Group Name	Virus inoculated
Duck	negative controls	MEMH
	D0	$_{wt}$Fr-AMPV-C
	D1	$_{rc}$Fr-AMPV-C
	D2	$_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$
Turkey	negative controls	MEMH
	T1	$_{rc}$Fr-AMPV-C
	T2	$_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$

b) Experimental procedure

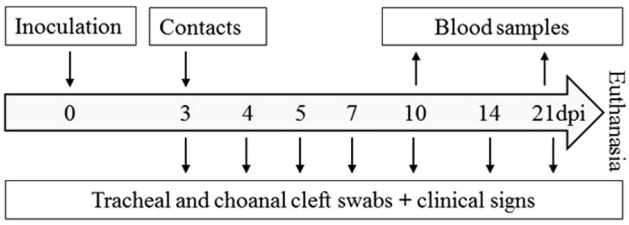

Fig. 2. a) Table showing the inoculum used for each experimental group, **b)** flow diagram of the experimental procedure from the day of inoculation (0) to the end of the trial (21dpi).

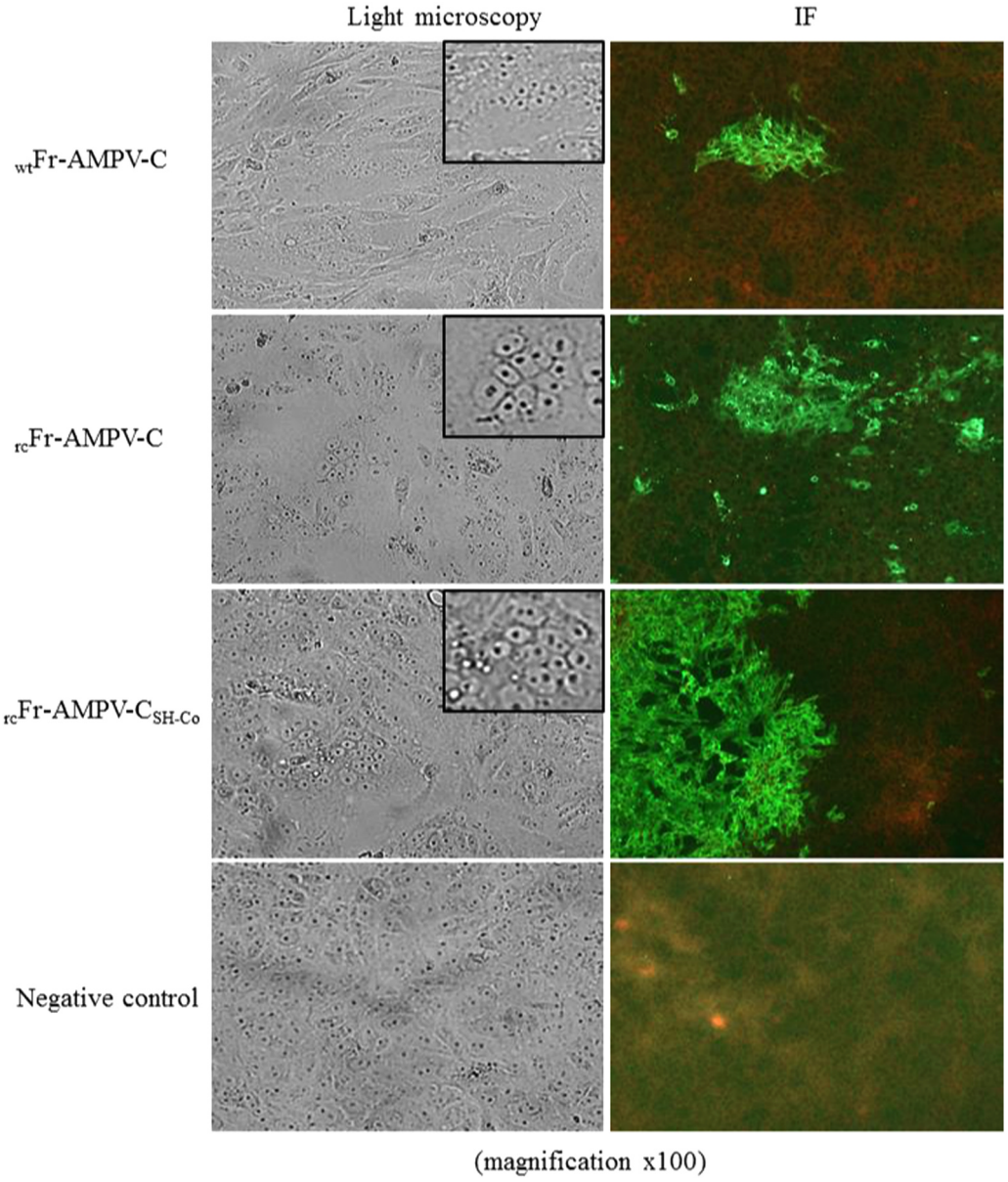

Fig. 3. CPE of $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ by Light microscopy (left panel) and IF (right panel) in Vero cells. Insets show a zoom of multinucleated cells.

species- matched birds were introduced to each group as contacts to monitor horizontal transmission. Details of the specific virus inoculated to a group together with the procedures carried out during the experiment trials are shown in Fig. 2.a. In addition to the procedures shown in Fig. 2.b, two inoculated birds from each group were euthanized at 3, 4, 5, 7 and 10 days post inoculation (dpi), from which trachea, nasal turbinates, lungs, liver, kidneys, testis, bursa of Fabricius, spleen and thymus were collected for future studies concerning tissue distribution that are not discussed in the current paper.

Choanal cleft and tracheal swabs were used to detect AMPV viral RNA loads (see Section 2.9) and to look for excretion of infectious virus by isolation from 5 to 10dpi (see Section 2.10). Blood samples were used to look for the presence of anti-AMPV-C antibodies (see Section 2.11).

A negative control batch composed of five SPF Muscovy ducks and five SPF turkeys were kept under the same conditions as the inoculated subjects. These were inoculated with 100 µl via the intranasal route with the same MEMH used to prepare viral inoculums (see above) as shown in Fig. 2.a. At 5, 10 and 21dpi, clinical signs were observed followed by separate swabbing of the choanal cleft and trachea. At 0, 10 and 21dpi blood samples were collected from all subjects. Samples from control birds were analyzed in the same way as those of inoculated birds. Control birds were also euthanized at 21dpi.

2.9. Detecting viral RNA loads by qRT-PCR

RNA was extracted from tracheal and choanal cleft swabs that had been re-suspended in 1.2 ml of MEMH supplemented with penicillin and streptomycin at 200 u/ml and 0.2 mg/ml final concentrations respectively, or from supernatants of cell cultures, using MagAttract Virus Mini M48 Kit (Qiagen). The levels of AMPV specific RNA in these extracts were then assessed using the recently described Pan MPV SYBR green real time RT-PCR (Lemaitre et al., 2018).

2.10. Detecting excretion of infectious Virus

Tracheal and choanal cleft swabs that had been re-suspended as described in Section 2.9 were inoculated directly onto Vero cells for

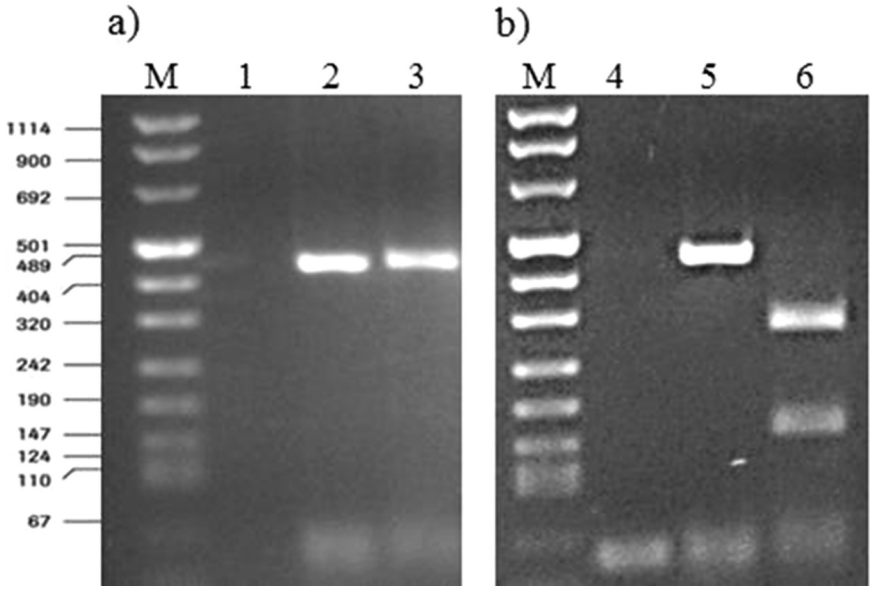

Fig. 4. Electrophoresis gels of PCR products derived from the F gene of $_{wt}$Fr-AMPV-C (a) and $_{rc}$Fr-AMPV-C_{BamHI} (b). M = marker, lanes 1 and 4 represent PCR reactions performed on extracted RNA without prior RT. Lanes 2 and 5 represent undigested RT-PCR products and lanes 3 and 6 represent RT-PCR products digested by BamH1.

virus isolation as previously described (Giraud et al., 1987; Toquin et al., 2006a). Samples were considered negative after three serial passages onto Vero cells

2.11. Detecting anti-AMPV C antibodies by ELISA

Serological testing was performed with an in house ELISA using Fr-AMPV-C antigen as previously described (Toquin et al., 2000)

3. Results

3.1. Recovery of rcAMPVs from cloned cDNAs

$_{rc}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C_{BamHI} and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were successfully rescued in Vero cells as confirmed by partial destruction of the cell sheet and the formation of syncytia following transfection procedures (Fig. 3 left panel) for each recombinant virus. The cell sheets were also positive by IF (Fig. 3 right panel) and their supernatants positive by RT-PCR (data not shown). The rescue of $_{rc}$Fr-AMPV-C_{BamHI} was confirmed as BamH1 digestion of the PCR product amplified from its F ORF generated two fragments of the expected size (294 and 151 bp) (Fig. 4. lane 6). Control reactions without RT were negative (Fig. 4. lanes 1 and 4)

3.2. NGS analysis of $_{wt}$Fr-AMPV-C and recombinant viruses $_{rc}$Fr AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$

$_{rc}$Fr AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were confirmed to have the exact designed genome sequences, corresponding to their parental full length plasmids $_{p}$Fr-AMPV-C and $_{p}$Fr-AMPV-$C_{SH\text{-}Co}$.

3.3. Kinetic analysis (multi-step growth curves)

During the first 24 h post infection, none of the three viruses were detectable by titration assay. 48 h post infection, $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were detected at $10^{3.94}$, $10^{3.55}$ and $10^{3.30}$ $TCID_{50}$/ml respectively. Between 3 and 6dpi all three viruses gave titres equal too or above $10^{3.90}$ with maximum titres of $10^{5.05}$, $10^{4.55}$ and $10^{4.66}$, for $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ respectively. Titres decreased for all viruses at 9dpi. Titres between the three viruses were within $10^{1.15}$ $TCID_{50}$/ml from 2 to 9dpi (Fig. 5).

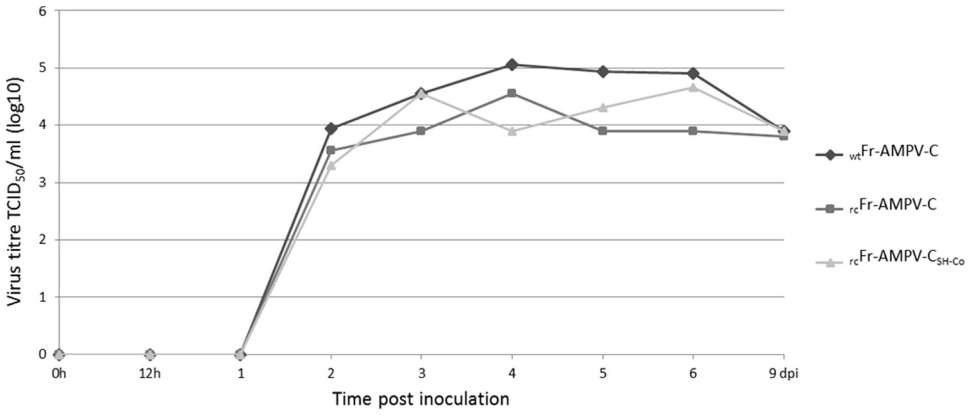

Fig. 5. Multi-step growth curves of wild type and recombinant AMPV viruses inoculated at m.o.i of 0.05 on Vero cells at 0 h. Virus titres were determined by $TCID_{50}$ assay using Reed and Muench method.

3.4. Animal trials

3.4.1. Clinical signs

From 3 to 7dpi, inoculated birds of groups D0, D1 and D2 (ducks inoculated with $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$

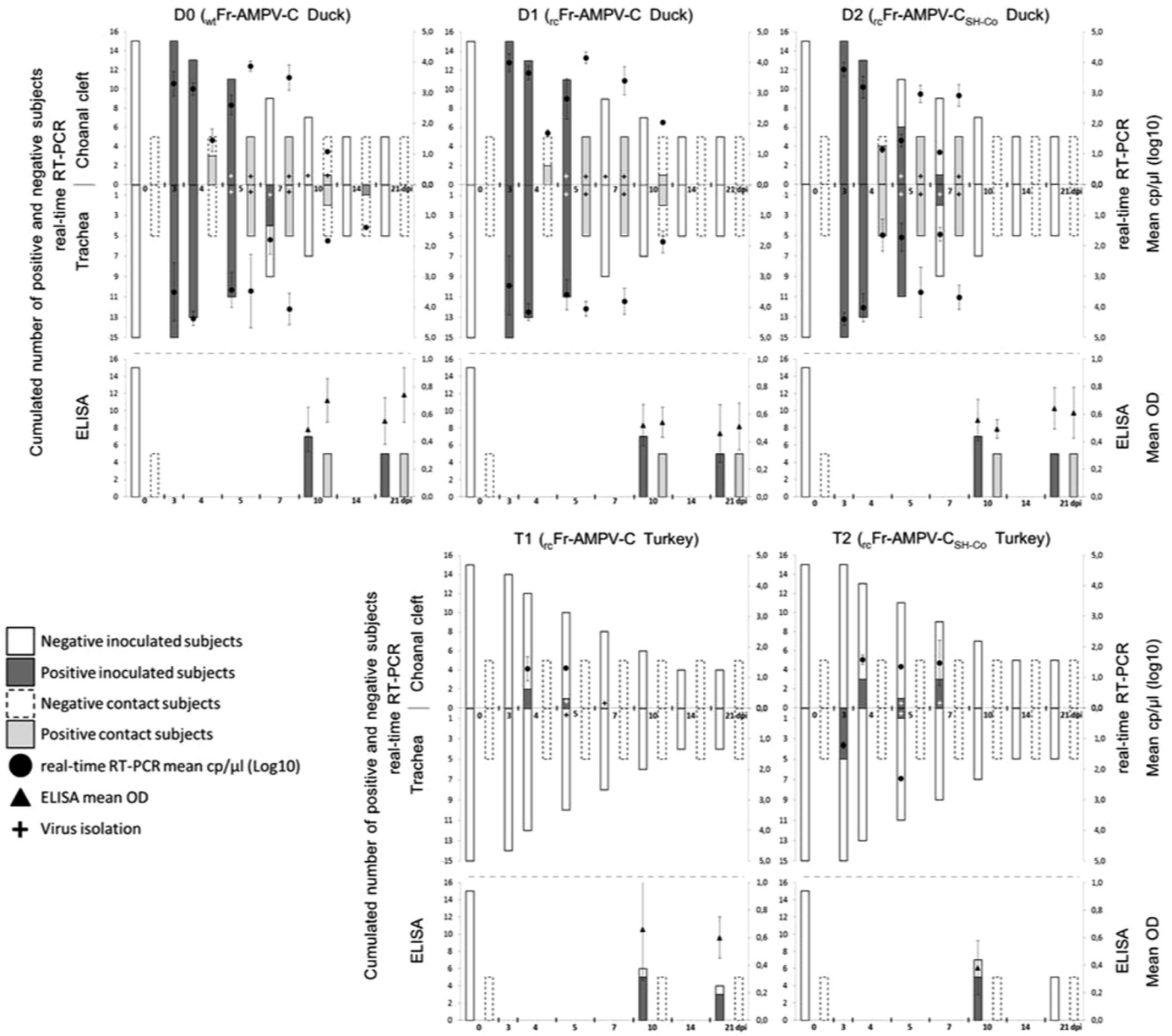

Fig. 6. Representation of the cumulated number of positive and negative subjects by real-time RT-PCR (left Y axis: graphics above dashed lines) and ELISA (left Y axis: graphics below the dashed lines) for duck groups D0, D1 and D2 and turkeys groups T1 and T2 inoculated with their respective viruses. Details of the real-time RT-PCR (mean viral RNA copy number /µl expressed in log10) and ELISA (mean optical density: OD) are shown in the Y axis's to the right in graphics above and below the dashed lines respectively.

respectively) demonstrated typical clinical signs, principally a heavy congestion in the trachea with occasional sneezing and nasal discharge. Contact birds of the same groups demonstrated the same clinical signs from 5 to 10dpi. Inoculated and contact birds had recovered by 10 and 14dpi respectively and remained free of clinical signs for the remainder of the trial. No clinical signs were observed in groups T1 and T2 throughout the duration of the experiment (turkeys inoculated with $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ respectively).

3.4.2. Viral detection and isolation

The following data are shown graphically in Fig. 6

For groups D0, D1 and D2 (Muscovy ducks inoculated with $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ respectively) viral RNA was detected from 3dpi in 100% of inoculated birds in both the choanal cleft and trachea with very similar maximum RNA loads in the choanal cleft of $10^{3.29}$, $10^{4.16}$, $10^{3.69}$ cp/µl and in the trachea of $10^{4.37}$, $10^{4.40}$, $10^{4.40}$ cp/µl respectively. 100% of birds in groups D0 and D1 remained positive in both tissues until 5dpi. After 5dpi no viral RNA was detected in the choanal cleft of groups D0 and D1 or in the trachea of group D1 however, detection continued in the trachea until 7dpi of group D0 yet the percentage of positives had dropped to 44 and the mean viral RNA load to $10^{1.79}$ cp/µl. For group D2, viral RNA was detected in both sampling locations until 7dpi. However, in the choanal cleft the percentage of birds and the viral RNA load had dropped to 55 and $10^{1.43}$cp/µl at 5dpi and to 11 and $10^{1.05}$cp/µl at 7dpi. In the trachea a drop in the percentage of positives and viral RNA load was only observed at 7dpi (22 and $10^{1.62}$ cp/µl respectively). Horizontal transmission of virus from these inoculated birds to their respective contacts occurred in all groups with detection of viral RNA as early as 24 h post contact (4dpi Fig. 6). Detection could be realized in both sampling locations until 7dpc for groups D0 and D1 however, this stopped at 4dpc for group D2. Viral RNA loads where of similar magnitude between contacts of the different groups and

inoculated birds. Concerning the re-isolation of viruses, both inoculated and contact birds of group D0were positive from 5 to 10dpi whereas in groups D1 and D2 these birds were positive at 5 and 7dpi.

For groups T1 and T2 (turkeys inoculated with $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ respectively) viral RNA was detected in the choanal cleft of a limited number of inoculated birds at 4 and 5dpi for T1 and at 4, 5 and 7dpi for T2. Viral RNA loads were also low at $10^{1.29}$ and $10^{1.31}$ for T1 and $10^{1.57}$, $10^{1.35}$ and $10^{1.47}$ for T2. No viral RNA was detected in the trachea of T1 birds for the duration of the experiment however, in group T2 a limited number of birds were positive at 3 and 5dpi yet similar to the choanal cleft viral RNA loads were low $10^{1.21}$ and $10^{2.30}$ respectively. Despite the low RNA loads virus was re-isolated from the choanal cleft of both groups at 5 and 7dpi and from the trachea of both groups at 5dpi. No horizontal transmission of virus from these inoculated birds to their respective contacts occurred.

3.4.3. Detection of anti-AMPV C humoral antibodies

Sero conversion was observed in inoculated and contact birds of groups D0, D1 and D2 however only the inoculated birds of groups T1 and T2 seroconverted.

4. Discussion

In this study, the first reverse genetics system based on a duck AMPV-C isolate was developed. This system was then used to assess whether a recombinant version of the Fr-AMPV-C wild type virus was equally pathogenic for ducks and if exchanging its SH protein for that of a turkey AMPV-C could contribute to its adaptation to turkeys.

$_{rc}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C_{BamHI} and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were successfully rescued using similar methods as previously described for the first AMPV RG system (Naylor et al., 2004) and protocols applied for the rescue of a turkey AMPV-C (Zhang et al., 2016). The RG system described in the current study and those described previously are all based on the transfection of five individual plasmids, four delivering the RNP proteins and one delivering the viral full-length genome. Although these systems are clearly successful it may be of interest in the future to adopt the recently published technique of Liu et al. (2017) for Newcastle disease virus (NDV). In that study the authors constructed one single plasmid to deliver three NDV RNP proteins and a second to deliver the NDV full-length genome. These authors reported an increase in rescue efficiency when adopting the two-plasmid system as opposed to a conventional four-plasmid system and suggested that this was likely due to the reduced number of plasmids required for complete transfection of a single cell. A two plasmid RG system was not considered as a starting point in the current study as controlling the level of expression of each RNP (critical parameter for AMPV rescue) is easier when having a specific plasmid for each protein.

NGS analysis used to confirm the full-length sequences of all viruses in the study highlighted a single nucleotide position (2932) in wild type viruses where a G or T base could be detected in almost equal proportion in the sequencing reads. These nucleotide changes are non-synonymous, coding for either an arginine or isoleucine amino acid at the last codon in the M ORF respectively. At this position, all sequencing reads from recombinant viruses displayed G, corresponding to their plasmid origin designed on the published consensus sequence G at position 2932 (Brown et al., 2014). This difference in homogeneity provided a means of differentiating $_{rc}$Fr-AMPV-C from wild type virus and at the time highlighted an interesting use of NGS data for discriminating clonal virus populations (designed recombinant viruses) from more heterogeneous viral populations (wild type viruses). Application of this technique could be useful for other RG studies eliminating the need for undesirable tags usually introduced to identify recombinant viruses.

In vitro, differences in viral titres for $_{wt}$Fr-AMPV-C, $_{rc}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were within 10^1 $TCID_{50}$/ml except for one time point between $_{wt}$Fr-AMPV-C and $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ were it was $10^{1.15}$ $TCID_{50}$/ml. As the precision of the Reed and Muench calculation is within 10^1 $TCID_{50}$/ml it is reasonable to assume that all three viruses had the same replication kinetics in Vero cells over the selected time period. Furthermore, all three viruses produced the same CPE in Vero cells. IF for $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ appeared to be more extensive in the current tests, however the same extensive staining by IF can often occur with $_{wt}$Fr-AMPV-C virus, and thus this effect cannot be suggested to be associated with differences in phenotype. In addition, the SH ORF derived from a turkey AMPV-C would appear to be functional and compatible in the duck AMPV-C backbone as previous studies have shown that AMPV-A viruses that do not express SH proteins have an altered phenotype in Vero cells producing “giant syncytia” (Ling et al., 2008; Naylor et al., 2004).

In vivo, the recombinant copy of the wild type virus and the recombinant copy expressing an SH protein derived from turkey AMPV-C were equally pathogenic for Muscovy ducks as the wild type virus. The onset, duration and level of clinical signs, presence of circulating anti-AMPV antibody, RNA loads and duration of its detection, transmission and excretion of infectious virus were almost indistinguishable between the three viruses. Importantly these results showed that the recombinant Muscovy duck virus, on which the SH modified virus was based, maintained the in vivo phenotype of the parental wild type virus. This was critical for assessing the role of the SH swap. Clearly, in the current study, exchanging the duck AMPV-C SH protein for that of a turkey AMPV-C in a duck AMPV-C backbone did not change the viruses pathogenicity in Muscovy ducks. This strongly supported however, a compatibility of the duck and turkey AMPV-C SH proteins at least in the developed $_{rc}$FR-AMPV-C backbone. This is hypothesized as the absence of a functional SH protein would have been expected to result in some level of attenuation, as has been observed for AMPVs and other members of the *Pneumoviridae* family (Biacchesi et al., 2005; Bukreyev et al., 1997; Ling et al., 2008).

The recombinant $_{rc}$Fr-AMPV-C did not cause respiratory disease in turkeys under these experimental conditions. However, the detection of viral RNA and isolation of virus from the respiratory tract of a limited number of inoculated birds would suggest that the virus was able to infect and replicate at a low level. Thus, it would seem reasonable to assume that infection of turkeys by duck AMPV-C could occur in the field, in the absence of respiratory signs.

Swapping the SH protein of this virus for the SH of a turkey virus didn’t change the viral phenotype in the respiratory tract, however, turkeys inoculated with $_{rc}$Fr-AMPV-$C_{SH\text{-}Co}$ produced a shorter duration of detectable, circulating antibodies. Clearly the SH protein swaps undertaken in the current study did not enhance replication of $_{rc}$Fr-AMPV-C in the respiratory tract of turkeys. Whether this modification altered replication elsewhere in the host remains to be determined in the future.

In conclusion a RG system has been developed for the Euro /Asian duck AMPV-C lineage and used to demonstrate that a recombinant virus based on the consensus sequence of Fr-AMPV-C was equally pathogenic for Muscovy ducks under experimental conditions. Furthermore, for the first time this study shows a compatibility between SH proteins of AMPVs from galliforms and palmipeds. To complement the current study, mirror experiments in which recombinant turkey AMPV-Cs modified to have an SH derived from duck AMPV-C should be performed. This would provide a thorough understanding of the AMPV C SH proteins biological role in AMPV C pathogenesis and host range. However, such extensive studies exceeded the scope of the present study and thus will be undertaken within the frame of future collaborations.

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Conflict of interests

The authors declare that they have no competing financial interests

References

Afonso, C.L., Amarasinghe, G.K., Banyai, K., Bao, Y., Basler, C.F., Bavari, S., Kuhn, J.H., 2016. Taxonomy of the order Mononegavirales: update 2016. Arch. Virol. 161, 2351–2360.

Bayon-Auboyer, M.H., Jestin, V., Toquin, D., Cherbonnel, M., Eterradossi, N., 1999. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Arch. Virol. 144, 1091–1109.

Biacchesi, S., Pham, Q.N., Skiadopoulos, M.H., Murphy, B.R., Collins, P.L., Buchholz, U.J., 2005. Infection of nonhuman primates with recombinant human metapneumovirus lacking the SH, G, or M2-2 protein categorizes each as a nonessential accessory protein and identifies vaccine candidates. J. Virol. 79, 12608–12613.

Biacchesi, S., Skiadopoulos, M.H., Tran, K.C., Murphy, B.R., Collins, P.L., Buchholz, U.J., 2004. Recovery of human metapneumovirus from cDNA: optimization of growth in vitro and expression of additional genes. Virology 321, 247–259.

Brown, P.A., Allée, C., Courtillon, C., Szerman, N., Lemaitre, E., Toquin, D., … Eterradossi, N., 2018. Host specificity of avian metapneumoviruses. In: Proceedings of the International Avian Respiratory Disease Conference, Athens, Georgia, p. 38.

Brown, P.A., Lemaitre, E., Briand, F.X., Courtillon, C., Guionie, O., Allee, C., Eterradossi, N., 2014. Molecular comparisons of full length metapneumovirus (MPV) genomes, including newly determined French AMPV-C and -D isolates, further supports possible subclassification within the MPV Genus. PloS One 9, e102740.

Brown, P.A., Lupini, C., Catelli, E., Clubbe, J., Ricchizzi, E., Naylor, C.J., 2011. A single polymerase (L) mutation in avian metapneumovirus increased virulence and partially maintained virus viability at an elevated temperature. J. Gen. Virol. 92, 346–354.

Bukreyev, A., Whitehead, S.S., Murphy, B.R., Collins, P.L., 1997. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. J. Virol. 71, 8973–8982.

de Graaf, M., Schrauwen, E.J., Herfst, S., van Amerongen, G., Osterhaus, A.D., Fouchier, R.A., 2009. Fusion protein is the main determinant of metapneumovirus host tropism. J. Gen. Virol.

Eterradossi, N., Toquin, D., Guittet, M., Bennejean, G., 1995. Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. Vet. Med. 42, 175–186.

Eterradossi, N., Toquin, D., Picault, J.P., Jestin, V., 2015. Métapneumoviroses aviaires. In: Brugère-Picoux, J. (Ed.), Manuel de pathologie aviaire, pp. 156–163.

Giraud, P., Le Gros, F.X., Bouquet, J.F., Toquin, D., Bennejean, G., 1987. Turkey Rhinotracheitis: Isolation of a viral agent and first trials with experimental inactivated or attenuated vaccines. In: Proceedings of the 36th Western Poultry Disease Conference, Davis, California, pp. 94–95.

Jones, R.C., Rautenschlein, S., 2013. Avian Metapneumovirus. In: Swayne, D.E. (Ed.), Diseases of Poultry. Wiley-blackwell, pp. 112–138.

Juhasz, K., Easton, A.J., 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. J. Gen. Virol. 75, 2873–2880.

Lemaitre, E., Allee, C., Vabret, A., Eterradossi, N., Brown, P.A., 2018. Single reaction, real time RT-PCR detection of all known avian and human metapneumoviruses. J. Virol. Methods 251, 61–68.

Ling, R., Sinkovic, S., Toquin, D., Guionie, O., Eterradossi, N., Easton, A.J., 2008. Deletion of the SH gene from avian metapneumovirus has a greater impact on virus production and immunogenicity in turkeys than deletion of the G gene or M2-2 open reading frame. J. Gen. Virol. 89, 525–533.

Liu, H., Albina, E., Gil, P., Minet, C., de Almeida, R.S., 2017. Two-plasmid system to increase the rescue efficiency of paramyxoviruses by reverse genetics: the example of rescuing Newcastle Disease Virus. Virology 509, 42–51.

Naylor, C.J., Brown, P.A., Edworthy, N., Ling, R., Jones, R.C., Savage, C.E., Easton, A.J., 2004. Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. J. Gen. Virol. 85, 3219–3227.

Seal, B.S., 1998. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first U.S. avian pneumovirus isolate is distinct from European strains. Virus Res. 45–52.

Toquin, D., Bayon-Auboyer, M.H., Senne, D.A., Eterradossi, N., 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. Avian Dis. 44, 977–982.

Toquin, D., de Boisseson, C., Beven, V., Senne, D.A., Eterradossi, N., 2003. Subgroup C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. J. Gen. Virol. 84, 2169–2178.

Toquin, D., Guionie, O., Allee, C., Morin, Y., Le CoQ, L., Zwingelstein, F., Eterradossi, N., 2006a. Compared suceptibility of SPF ducklings and SPF turkeys to the infection by avian metapneumoviruses belonging to the four subgroups. In: Heffels-Redman, U., Kaleta, E.F. (Eds.), V. International Symposium on Avian Corona and Pneumoviruses and Complicating Pathogens VVB Laufersweiler Verlag. University of Giessen Rauischholzhausen, Germany, pp. 70–76.

Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C., Eterradossi, N., 2006b. European and American subgroup C isolates of avian metapneumovirus belong to different genetic lineages. Virus Genes 32, 97–103.

Zhang, Y., Sun, J., Wei, Y., Li, J., 2016. A reverse genetics approach for the design of methyltransferase-defective live attenuated avian metapneumovirus vaccines. Methods Mol. Biol. 1404, 103–121.