A single polymerase (L) mutation in avian metapneumovirus increased virulence and partially maintained virus viability at an elevated temperature

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Previously, a virulent avian metapneumovirus, farm isolate Italy 309/04, was shown to have been derived from a live vaccine. Virulence due to the five nucleotide mutations associated with the reversion to virulence was investigated by their addition to the genome of the vaccine strain using reverse genetics. Virulence of these recombinant viruses was determined by infection of 1-day-old turkeys. Disease levels resulting from the combined two matrix mutations was indistinguishable from that produced by the recombinant vaccine, whereas the combined three L gene mutations increased disease to a level (P<0.0001) that was indistinguishable from that caused by the revertant Italy 309/04 virus. Testing of the L mutations individually showed that two mutations did not increase virulence, while the third mutation, corresponding to an asparagine to aspartic acid substitution, produced virulence indistinguishable from that caused by Italy 309/04. In contrast to the vaccine, the virulent mutant also showed increased viability at temperatures typical of turkey core tissues. The notion that increased viral virulence resulted from enhanced ability to replicate in tissues away from the cool respiratory tract, cannot be discounted.

INTRODUCTION

Infection of domestic poultry with avian metapneumovirus (AMPV) is responsible for disease on a worldwide basis. In farmed turkeys high levels of mortality are found (Buys & Du Prees, 1980; Stuart, 1989), while the less severe disease generally encountered in chickens nonetheless results in severe economic losses. Virus replication occurs principally in the respiratory tract and the presence of other pathogens frequently leads to exacerbation of the disease (Cook, 2000).

AMPV is a member of the family Paramyxoviridae and, in common with other members of the family, is a pleomorphic enveloped virus with a genome of negative-sense, ssRNA. It is classified in the subfamily Pneumovirinae, due to presence of an M2 gene, the nature of the P gene and in not following the ‘rule of six’ (Easton et al., 2004). However, the gene order more closely resembles rubuloviruses such as mumps virus than the otherwise more closely related pneumoviruses such as respiratory syncytial virus (RSV). For this reason a new genus Metapneumovirus was established (Pringle, 1998). A second member of the genus, human metapneumovirus, was identified in 2001 (van den Hoogen et al., 2001) and currently these two viruses are its only members. The genome possesses eight discrete transcription units that are generally assumed to code for nine proteins, but the absence of exhaustive studies leaves the possibility that other available ORFs may be expressed. There are four known AMPV subtypes worldwide (A, B, C and D) (Bayon-Auboyer et al., 2000; Juhasz & Easton, 1994; Seal, 2000); however, cases in Western Europe have been mainly of subtypes A and B.

Disease is predominantly controlled by vaccination with attenuated live AMPV (Buys et al., 1989; Cook et al., 1989a, b, 1995, 1996; Patnayak & Goyal, 2006; Williams et al., 1991a, b). However, back passage of such vaccines in birds has been shown to lead to a return to virulence in experimental conditions (Naylor & Jones, 1994) as well as on farms. In a previous study, a vaccine derived from the UK field strain #8544 (GenBank accession no. DQ666911) was found to have reverted to virulence in field conditions. Isolated virus AMPV Italy 309/04 contained just five nucleotide substitutions compared to the vaccine and those were located solely in the matrix (M) and L genes (Catelli et al., 2006). One M mutation at genome position 2879 was
silent and occurred immediately before the stop codon, while the second was non-coding and occurred downstream prior to the transcription stop signal (genome position 2891, M gene coding nt 775). All three L mutations occurred in the coding region with one silent (genome position, 11431, L gene coding nt 2868, no coding change), one conservative (genome position 10109, L gene coding nt 4191, coding D to E) and the other introducing a negatively charged residue (genome position 11442, L gene coding nt 4200, coding N to D). None of these mutations represented back mutations to its progenitor sequence in the vaccine.

The current study precisely determines the contributions made by each of these five mutations in the establishment of virulence in Italy 309/04 by using the same established challenge system (Naylor et al., 1992). Viruses were investigated further to determine whether growth or gene transcription changes were detectable.

**RESULTS**

**Plasmids and recombinant viruses**

Cloned viral sequences were found to be correct and all produced recombinant viruses in the reverse genetics system as demonstrated by the presence of cytopathic effect (CPE) in Vero cells. Full sequencing of resultant viruses showed that no mutations had arisen during the virus rescue procedure. Each recombinant virus had the correct complete sequence.

**Virulence assessment of recombinant viruses**

Previously, the return to virulence of the isolate Italy 309/04 had been shown to be associated with only five nucleotide substitutions, two grouped in the M gene and three in the L gene (Catelli et al., 2006). The location of these changes occurred in two genome regions so initial investigations focused on whether either or both these regions were important in the observed virulence increase. The subsequent animal study determined the role of individual mutations in virulence increase.

To investigate whether both groups of mutations were involved in virulence, turkeys were infected with either of two recombinants: one containing both the M mutations (rVc-309M12) and the other containing the three L mutations (rVc-309L123). Severity of resultant disease was compared to that seen with the recombinant vaccine (rVc) and the recombinant Italy 309/04 isolate (r-309/04). The daily mean clinical scores for each group are reported graphically in Fig. 1(b). Recombinants rVc-309L123 and rVc produced similar disease levels (cumulative scores 9.5 and 3.5, respectively) to those seen in trial one. Recombinants rVc-309L1 and rVc-309L2 produced disease levels (cumulative scores 3.8 and 3.1, respectively) indistinguishable from the rVc control (P=0.949 and P=0.229, respectively). Recombinant rVc-309L3 produced disease of a similar severity (cumulative score 9.6) to that seen with rVc-309L123 (P=0.934) and this was much more severe than that seen after rVc infection (U=15355; P<0.0001), rVc-309L1 (U=15278; P<0.0001) or rVc-309L2 (U=15792; P<0.0001).

**Growth curves for rVc and rVc-309L3**

For both viruses propagated in Vero and chick embryo fibroblasts (CEF) cells at 37 °C, four very similar 7 day growth curves were produced. When comparing between cell type and virus tested on any given day, titre differences were statistically insignificant. Results can be seen in Fig. 2(a, b). For Vero cells infected with rVc and rVc-309L3, maximum titres were 4.8 and 4.63 log_{10} TCID_{50} ml^{-1}, respectively, while for CEF grown viruses titres were both 4.63 log_{10} TCID_{50} ml^{-1}. When propagated at 40 °C, titres fell for both viruses from 120 h, as seen in Fig. 2(c). For rVc-309L3, titres were generally higher than rVc, between 48 and 72 h (Fig. 4c) amounts of rVc-309L3 were higher than rVc by a factor of five and titre differences at those two times were shown to be significantly different (P=0.039 and P=0.049, respectively).

**Relative gene transcription rates for rVc and rVc-309L3**

When considering like genes in viruses rVc and rVc-309L3 grown at 37 or 40 °C, each specific mRNA was detectable at the same PCR cycle number, thus demonstrating no detectable difference between transcription rates. For this reason only the results for 40 °C are given in Fig. 3.

**CPE in Vero Cells**

The CPE produced by rVc and rVc-309L3 differed with the former producing larger syncytia, as shown in Fig. 4.
DISCUSSION

When an AMPV vaccine reverted to virulence to yield farm isolate Italy 309/04, the changes comprised five nucleotide substitutions of which two occurred in the M gene and three in the L gene. Both M gene mutations and two of the three L gene mutations occurred in close proximity (M 2879 and 2891, L 11431 and 11442) and raised the possibility of their functional connection. One M mutation (2879) was silent and occurred immediately before the stop codon, while the second was non-coding and occurred downstream prior to the transcription stop signal (2891). All three L mutations occurred in the coding region with one silent (11431), one conservative (10109, D to E) and the other introducing a negatively charged residue (11442, N to D) (Catelli et al., 2006).

In this study, the first animal trial showed that introduction of the Italy 309/04 L gene changes to the genome of the vaccine resulted in a virus of similar virulence to the Italy 309/04 isolate. In contrast, the Italy 309/04 M gene changes did not significantly affect virulence, as shown by induced clinical disease being indistinguishable from that caused by the vaccine. This suggests that the M gene changes do not alter virus virulence, but may nonetheless affect an unrelated property. For example in AMPV field disease situations involving vaccine, the vaccine virus has been shown to persist for unexpectedly long periods (Naylor et al., 1997). It may be of value in a further study to ask the question of whether the M gene mutations might be associated with such a change.

In the second trial when the three L mutations were added individually to the vaccine sequence, only the 11442 change led to an increase in virulence. Furthermore, the level of disease in this group was indistinguishable from the mutant containing all three mutations, which in turn in trial one had induced disease indistinguishable from disease caused by the recombinant Italy 309/04 itself. This implies that the increase in virulence seen when the vaccine reverted to virulent Italy 309/04 was solely due to the substitution of a negatively charged aspartic acid (D) for a neutral asparagine (N) at a location corresponding to genome position 11442.

Changes in viral properties resulting from single amino acid substitutions are not uncommon and have been

![Fig. 1. Mean daily clinical scores after inoculation of recombinant viruses and mock inoculum in trials one and two (a and b). Eleven turkeys were used per group and a dose of 4 log<sub>10</sub> TCID<sub>50</sub> per turkey was used. Inoculum volumes of 100 μl were divided in two equal parts and dropped onto each eye.](image-url)
reported for other negative-sense RNA viruses such as measles virus (Jiang et al., 2009), Newcastle disease virus (NDV) (Sergel et al., 2000), rinderpest virus (RPV) (Chattopadhyay et al., 2004) and RSV (Cartee et al., 2003; Marriott et al., 1999). Conserved and functional regions of the polymerase have been recognized in many members of the order Mononegavirales (Poch et al., 1990) and in RPV, single amino acid substitutions in D, N or Q of the GDNQ motif have been shown to affect virus phenotype (Chattopadhyay et al., 2004). In RSV, substitution of glycine (G) for serine (S) in the P protein

**Fig. 2.** Growth curves of rVc and rVc-309L₃. Vero cells or CEF monolayers were infected with rVc or rVc-309L₃ at an m.o.i. 0.01 TCID₅₀ per cell. Replicate flasks were frozen every 24 h. After thawing, virus titres at each time point were determined in Vero cells. (a and b) Show growth kinetics at 37 °C of rVc and rVc-309L₃ in Vero cells and CEFs, respectively, and (c) demonstrates growth kinetics of rVc and rVc-309L₃ in Vero cells at 40 °C. Asterisked titres indicate days where rVc and rVc-309L₃ titres were significantly different (P<0.05).

**Fig. 3.** Agarose gel electrophoresis of genomic and mRNA gene transcripts (N to L) amplified from rVc and rVc-309L₃ viruses grown in Vero cells at 40 °C. Samples were removed for analysis at PCR cycles 12, 14, 16, 18, 20 and 22.
conferred thermosensitivity in a minigenome system (Marriott et al., 1999), a D for N substitution in the polymerase was found to affect transcriptional termination (Cartee et al., 2003) and a single L gene mutation resulted in an attenuated temperature-sensitive virus (Juhasz et al., 1997). Again in RSV, a polymerase substitution of tyrosine (Y) to asparagine (N) at residue 1321 was found to be responsible for both increased attenuation and temperature sensitivity (Whitehead et al., 1999). This was in close proximity to the mutation identified in the current AMPV study, though this motif does not occur in one of the known functional or conserved polymerase domains common to many other members of the order Mononegavirales (Poch et al., 1990).

In the current study, five mutations associated with the reversion process have been narrowed down to one causal mutation in the L gene. Previously, virulence decreases seen in producing the vaccine had been shown to be associated with nine mutations, but only two were coding, and both occurred in the F gene (Catelli et al., 2006). In the corresponding reversal of that attenuation reported here, the F gene was unaffected, but another virulent revertant virus arising from the same vaccine, on that occasion generated in experimental conditions, involved F gene mutations (Naylor et al., 2002). This might indicate that virulence in AMPVs arises from any of a range of genome mutations involving several genes, and that the causal L mutation detected in this study forms one of several possibilities.

A study of human RSV showed that the reduced virulence seen in temperature-sensitive mutants was due to mutations in the L gene and furthermore also implicated a mutation in the F gene (Tolley et al., 1996). In NDV, the L gene was found to be associated with a virulence increase and in that case, the mechanism was due to a large increase in replication rates (Rout & Samal, 2008). In the current study, a similar mechanism appears to be involved. Virus rVc-309L3 grown at 40 °C has been shown to replicate more efficiently than rVc and this is probably the cause of the greater disease in birds. While it is common to culture viruses at the accepted nominal normal physiological temperature of 37 °C, the core temperature of avian species approximates to the 40 °C used in the later assays of this study. The L mutation investigated led to the generation of a virus that grew with greater efficiency than the vaccine above 37 °C and which in the bird may be more able than its vaccine progenitor to replicate away from the cooler surface of the respiratory tract. It would be interesting as a follow up study to see whether these two viruses do indeed display different abilities to replicate in warmer tissues of a turkey.

Results from similar viruses have shown that alterations to the viral polymerase can alter its ability to recognize transcriptional signalling sequences. In human RSV (Cartee et al., 2003), an N to D mutation was found to reduce the ability of the polymerase to recognize cis-acting gene end signals, increasing read through and incidence of dicistronic mRNAs. The current study did not reveal any detectable alteration in relative mRNA levels between viruses, or differences in levels of dicistronic mRNAs due to alterations in gene transcriptional stop efficiencies. While the method used to quantify mRNA was incapable of detecting extremely small differences between viruses, results might nonetheless direct follow up mechanistic studies towards investigation of other parameters. As increased thermal stability has been shown to be associated with increased virulence, an obvious initial investigation might be to determine the effect of temperature on the

Fig. 4. CPE in Vero cells 4 days after inoculation of rVc and rVc-309L3 with an m.o.i. of 0.01 at 37 °C.
activity of the viral polymerase. Another line of investigation might be to investigate whether the L3 mutant polymerase is more able to produce a full genome copy at elevated temperature, due to maintained affinity between the ribonucleic complex and the polymerase. Ultimately, it would be likely that determination of a complete mechanism responsible for the increase in virulence would involve a lengthy investigation involving pathogenesis, proteomics and immunology studies.

One observable difference between rVc-309L3 or rVc-309L123 and vaccine related to the CPE they induced Vero cells. Viruses containing the L3 mutation produced a CPE different to that seen in viruses lacking this mutation, as can be seen in Fig. 4. Such phenotypic change in vitro might be explained by the rVc-309L3 polymerase protein altering the balance of mRNAs to levels lower than the limits of detection of the assays used here. However, we have no evidence of such a mechanism, and bearing in mind the current lack of insight into the mechanisms relating CPE and genome alterations in this and similar viruses, it would be unreasonable to attempt to put forward a plausible hypothesis at this stage.

In summary, we have shown that one single L gene mutation led to a virulence increase of similar magnitude to that seen when comparing attenuated vaccine strains to virulent field strains. While the effects of possible further mutations facilitated by the L3 change cannot be excluded; the experimental findings strongly suggest that the L3 change alone was enough to cause the observed virulence. The mutated virus was able to replicate more efficiently at normal avian core temperatures and it appears reasonable to speculate that the increased virulence may have been due to increased stability of the viral polymerase. The identification of this and other virulence determinants may be of value in the attenuation of AMPV, thus helping facilitate the development of improved live AMPV vaccines. In the current case, substitution of a negatively charged amino acid (N to D) at residue 1403 increased virus viability at elevated temperature. Sequencing of a number of field viruses and field strains (Clubbe et al., 2009) has shown this mutation to be within a protein motif that is highly conserved in both A and B subtypes of AMPVs. Furthermore, N is conserved at position 1403 in AMPV subtype C as well as the more distantly related human metapneumovirus. Although this motif does not occur in one of the known functional or conserved polymerase domains common to other members of the order Mononegavirales (Poch et al., 1990), it would appear to be a region of importance within AMPV and it forms a likely target when attempting to rationally generate temperature-sensitive mutants via reverse genetics, as part of a strategy in the development of improved live AMPV vaccines.

**METHODS**

**Cell culture.** Vero cells used were maintained in Dulbecco’s modified Eagles medium (Sigma) supplemented with crystapen (60 μg ml⁻¹), streptomycin (100 μg ml⁻¹), amphotericin (0.5 μg ml⁻¹) and FCS (5%). CEFs were maintained in M199 medium, tryptophosphate broth (10%), crystapen (60 μg ml⁻¹), streptomycin (100 μg ml⁻¹), amphotericin (0.5 μg ml⁻¹) and new born calf serum (5%).

**Construction of DNA viral copies.** Cloned DNA copies of nine virus variants were constructed by modification of a previous cloned DNA copy of AMPV (Naylor et al., 2004) using DNA copies of the subtype A vaccine under investigation, as outlined in Fig. 5. Primers used in reverse transcription (RT) and PCR are named in Fig. 5 and sequences are reported in Supplementary Table S1 (available in JGV Online). RNA was extracted from subtype A AMPV vaccine strain (RNeasy; Qiagen) and cDNA copies were prepared by RT with superscript II (Invitrogen; 42 °C, 90 min) (Fig. 5a), which were amplified by PCR (Pfu polymerase; Stratagene) to produce two overlapping fragments covering the complete genome (Fig. 5b). These were used in two sequential site-directed mutagenesis (SDM) reactions to generate a plasmid containing an exact copy of the vaccine (pVc) (Fig. 5c). The five mutations needed to convert the plasmid to the Italy 309/04 sequence (p-309/04) were made by SDM using complementary primer pairs and in a similar manner, seven

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**Fig. 5.** Schematic representation of the strategy employed to generate a cloned cDNA copy of the subtype A vaccine sequence. Primers sequences used in RT and PCR are reported in Supplementary Table S1. RNA was extracted from subtype A AMPV vaccine (RNeasy; Qiagen) and cDNA copies were prepared by RT with superscript II (Invitrogen; 42 °C, 90 min) (a) that were amplified by PCR (Pfu polymerase; Stratagene) to produce two overlapping fragments covering the complete genome (b). These were used in two sequential SDM reactions to generate a plasmid containing an exact copy of the vaccine (pVc) (c).
further variants containing combinations of the five mutations were generated. The sequences of complementary primers used in these SDM reactions are shown in Supplementary Table S1. Names and genome sequence details of the seven variants are shown alongside p-309/04 in Fig. 6.

**SDM.** All SDM reactions followed the supplier’s protocol (QuikChange; Stratagene). Where synthesized primer pairs were substituted by blunt-end PCR products, 0.5 µg of sample was treated with exonuclease 1 followed by 80 °C denaturation prior to the SDM reaction.

**Transfection and recovery of recombinant viruses.** Procedures for recovering recombinant viruses followed those described previously (Naylor et al., 2004). In brief, Vero cells infected with an attenuated non-packaging fowlpox recombinant virus (Britton et al., 1996) expressing T7 polymerase were transfected with plasmids containing a cloned virus copy, N gene, P gene, M2 gene and L gene, under the control of the T7 promoter. Names of viruses generated directly relate to their progenitor clones. Rescue of viruses was carried out in a level 2 laboratory for which relevant genetically modified organism (GMO) approval had been obtained from the regulatory authorities.

**Sequencing of cDNA virus copies and rescued virus genomes.** The complete virus sequences in plasmid DNA and reverse transcripts from viral RNA were amplified by PCR and determined as described previously (Naylor et al., 2007). When any cloned virus sequence was found to be incorrect, errors were corrected by SDM. Rescued viruses were sequenced in their entirety. Data were analysed using Chromas software.

**Pathogenicity of recombinant viruses.** Virulence of AMPV recombinants was determined by assessment of clinical signs after inoculation of 1-day-old turkeys in level 2 isolation conditions. Relevant GMO approval had been obtained for the animal studies from the relevant regulatory authorities.

(i) **Viruses and doses inoculated.** Recombinant vaccine or recombinant vaccine with all 309/04 mutations (2879, 2891, 10109, 11431 and 11442), all L mutations (10109, 11431 and 11442), all M mutations (2879 and 2891), single L mutation (10109), single M mutation (11431) and single L mutation (11442) (rVc, r-309/04, rVc-309L123, rVc-309M12, rVc-309L1, rVc-309L2, rVc-309L3 and rVc-309M12, respectively) were titrated in Vero cells and end points were calculated (Reed & Muench, 1938). A dose of 4 log_{10} TCID_{50} was used per turkey.

(ii) **Turkeys.** Unvaccinated commercial turkeys were obtained on two occasions from a commercial hatchery that enforced a high level of biosecurity.

(iii) **Experimental design.** Recombinant viruses were tested in two consecutive experimental trials. Birds were tagged, divided into groups of 11 and housed in separate poultry isolators. In trial one, groups were inoculated with r-309/04, rVc, rVc-309L123 or rVc-309M12 and a further group was mock inoculated and kept as the negative control. In trial two, groups were inoculated with rVc, rVc-309L123, rVc-309L1, rVc-309L2 or rVc-309L3 and a further group was mock inoculated and kept as the negative control. In both trials, the inoculum volumes were 100 µl divided in two equal parts and dropped onto each eye. After inoculation, birds were scored for clinical signs daily until signs ceased. At the end of the trials birds were humanely killed and destroyed by incineration.

(iv) **Monitoring clinical signs.** Clinical signs were scored as described previously (Naylor et al., 1992) and outlined as follows: 0 for no clinical signs, 1 for clear nasal exudates, 2 for turbid nasal exudates and 3 for swollen infra-orbital sinuses and/or frothy eyes. Consistent minimal nasal pressure was applied to facilitate extrusion of exudates.

(v) **Statistical analysis.** Distributions of clinical scores within groups were firstly tested using Kruskal–Wallis non-parametric one-way ANOVA. The differences between groups were then tested using the Mann–Whitney test. A P-value <0.05 was considered statistically significant.

**Virus titrations and growth curves.** Titres of freeze-thawed virus infected cultures were determined. T25 flasks of Vero or CEF monolayers were infected with virus rVc or rVc-309L3, at an m.o.i. of 0.01 and incubated at either 37 or 40 °C. For each set of conditions (37 °C Vero cells, 37 °C CEF cells and 40 °C Vero cells), viruses rVc or rVc-309L3 were tested concurrently so that identical flasks were prepared and seven inoculated with each virus. At 24 h intervals, one flask infected with each virus was frozen at −70 °C and stored. Once all 14 flasks had been frozen, they were thawed and the titre of each was determined in 14 concurrently prepared 48-well plates. For each flask, 10-fold serial dilutions were made (10^{-1} to 10^{-7}) and six wells were inoculated with 150 µl aliquots of each dilution. Numbers of wells developing CPE were used to calculate TCID_{50} titres (Reed & Muench, 1938). To determine the statistical significance of differences between rVc and rVc-309L3 geometric mean titres on each day, replicate well-end-points were subjected to Student’s t-test analysis. Because of the equivalence of Vero and CPE titres, Vero cells were predominantly used in later titrations on practical and ethical grounds. For the 40 °C study, replicate titrations were performed using fresh replicate flasks.

**Relative gene transcription in rVc and rVc-309L3.** Total RNA was extracted from 5 days post-infection cell cultures of preparations rVc and rVc-309L3 grown at 40 °C following freeze-thawing, using the QIAamp viral RNA mini kit (Qiagen). Virus rVc-309L3 was diluted 2.5 times prior to extraction to yield identical virus titres prior to extraction. The expected equivalence of the two AMPV genome concentrations was confirmed by RT-PCR from the AMPV 3’ leader into the adjacent N gene using primers APV lead and N2 negative (Supplementary Table S1). In the PCR, reactions were heated to 94 °C for 10 s, 50 °C for 20 s and 72 °C for 1 min, then the cycle was repeated. After 14 cycles, 10 µl aliquots were collected from each reaction at every second cycle. Aliquots were run on agarose gels, visualized using Red Safe (Chembio) and the minimum number of cycles giving band visibility was determined.

**Fig. 6.** Schematic illustration of the AMPV genomes generated in this study. Differences to the vaccine sequence are given for each virus and names specify the gene(s) affected.
Using the above RNA extractions, solely mRNAs in AMPV rVc and rVc-309L3 preparations were amplified using the 3' RACE technique described by Sambrook & Russell (2001). Priming of cDNA copies of mRNAs was done by using dT adapt A/C/G oligo (MWG) that annealed to polyadenylated mRNA tails. PCR amplification used adapt – and gene specific primers (N1 +, P1 +, M8 +, F8 +, M2 mid for, SH 70+, G7 + or L19 +). Primer sequences are given in Supplementary Table S1. As above, aliquots were removed on a two cycle basis during the PCR. Once run and visualized on gels as above, the relative levels of like gene mRNAs in viruses rVc and rVc-309L3 were compared. Sampling every two cycles enabled mRNA concentration differences of four (23) times and greater to be detected. This procedure had been previously shown not to generate amplicons from cDNA genome copies nor genome itself; hence amplicons solely arose from copying and amplification of viral mRNAs.

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