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Quasispecies Nature of an Unusual Avian Paramyxovirus Type-1 Isolated from Pigeons

CYRIL BARBEZANGE[1,2] & VÉRONIQUE JESTIN[1,*]

[1]*AFSSA (French Agency for Food Safety), Avian and Rabbit Virology Immunology and Parasitology Unit, BP 53, 22440, Ploufragan cedex, France*

[2]*Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 OES, UK*

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Abstract. An avian paramyxovirus type-1 (APMV-1) was classified as virulent according to its Intra Cerebral Pathogenicity Index (ICPI), but as avirulent according to the motif of its F protein cleavage site. Although this atypical APMV-1 was isolated from sick, unvaccinated pigeons, it was not grouped with pigeon variants regarding its antigenic and genetic characterisation. We analysed its quasispecies nature by cloning and sequencing parts of the genome in three different genes to evaluate if heterogeneity might explain the difference observed between the ICPI and the F protein cleavage site motif. Two distinct sub-populations were detected in the phosphoprotein gene. In the fusion protein gene, two clones were found to be related to typical pigeon variants in the hypervariable domain.

Key words: avian Paramyxovirus, Newcastle disease, quasispecies, pigeon

Introduction

Pigeon paramyxovirosis is a form of Newcastle disease (ND), which was first described in the Middle East in 1978 [1]. The disease reached Europe in 1981 and remains enzootic in pigeons in many countries [2]. Typical clinical signs include torticollis, paralysis and the excretion of large volumes of green, watery diarrhoea. The viruses involved are antigenic variants of avian paramyxovirus type-1 (APMV-1 or Newcastle Disease virus or NDV), and so represent a group within APMV-1 called pigeon paramyxovirus type-1 (pPMV-1). The pPMV-1 isolates were responsible for ND natural outbreaks in chicken broilers, breeders and layers in the United-Kingdom in 1984 [3].

*Author for correspondence:
E-mail: v.jestin@ploufragan.afssa.fr

The APMV-1 viruses belong to the newly created genus *Avulavirus* within the viral family *Paramyxoviridae* (accepted proposal available on ICTV website at http://www.danforthcenter.org/iltab/ICTVnet). Their non-segmented single-stranded 15 kb RNA genome of negative polarity contains six genes in the 3′-to-5′ direction: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and RNA-directed RNA polymerase (L) [4]. Each gene codes for a unique structural protein, except gene P, which is polycistronic and codes for two additional non-structural proteins (V and W). APMV-1 strains are internationally recognised as pathogenic if their Intra Cerebral Pathogenicity Index (ICPI) is above 0.7 [5].

RNA viruses are believed to exist within the same individual as a population of closely related sequences known as quasispecies [6]. The possible heterogeneity of APMV-1 strains was

demonstrated by the plaque assay test performed in various cell culture systems [7]. Quasispecies might have an important role in the survival and evolution of RNA viruses as well as in the pathogenesis of a disease. Such a hypothesis was made by Kissi and Lomiczi in 1988 [8,9] regarding the potential risk of pPMV-1 for poultry. Although pPMV-1 may be a heterogeneous population in pigeons, it might become more homogeneous following several passages in chickens with the possibility of selection of virulent sub-populations. Similarly, APMV-1 could become more heterogeneous after serial passages in pigeons.

In a previous study, we characterised an interesting APMV-1 strain (99143) at the molecular level by direct sequencing of amplification products of the six genes [10]. Phylogenetic analyses had grouped this isolate with the vaccine reference strains Hichner B1 and La Sota, irrespective of the gene or protein. The motif of its F protein cleavage site was also found to be typical of avirulent APMV-1 strains. The 99143 isolate could then be considered as an unusual 'classical' (therefore not a pigeon variant) avian paramyxovirus type-1 strain (aPMV-1, to differentiate it from the APMV-1 strains, which include all groups of avian paramyxovirus type-1). Indeed, although 99143 had been characterised as an avirulent strain by antigenic and genetic means, it had been isolated from sick, unvaccinated pigeons and was exhibiting a mesogenic ICPI.

As no sequence differences were noted between the 99143 isolate and vaccine strains in parts of the genome we analysed that would account for the difference in ICPI, we wondered if the quasispecies nature of 99143 might be responsible for the discrepancy between the ICPI test and the F protein cleavage site motif. Here we report the results of a quasispecies analysis we performed on three genes of the 99143 isolate.

Materials and Methods

Virus Strain History

In 1999, a strain of aPMV-1 was isolated in France from unvaccinated racing pigeons with signs of paramyxovirosis including paralysis, anorexia and mortality along with positive serology for NDV by haemagglutination inhibition assay in the pigeons of the same loft. This strain, referred to as 99143, was isolated from a mix of lungs and brains and has been propagated three times in embryonated chicken eggs. An ICPI of 1.4 was determined using one-day-old chicks according to European standards [5]. However, the monoclonal antibody 161/617, which is specific for pPMV-1 variants [11], did not inhibit the haemagglutination property of 99143 and this isolate was sent to the European Community Reference Laboratory in Weybridge (UK) for further characterisation. Following testing using the complete range of monoclonal antibodies described by Alexander et al. [12], the 99143 isolate was assigned to the group E along with avirulent vaccine strains such as the B1-like strains, although the virulent ICPI was confirmed at 1.26.

Polymerase Chain Reaction (PCR), Cloning and Sequencing

To evaluate the quasispecies nature of 99143, the P, F and HN gene PCR products we used to determine the consensus sequences already published [10] were cloned. After purification using a QIAquick® Gel Extraction kit (QIAGEN), each product was cloned using a ZeroBlunt® TOPO® PCR cloning Kit for sequencing (Invitrogen) according to the manufacturer's instructions. Plasmid purifications were performed using a QIAprep® Spin Miniprep kit (QIAGEN), and the presence of insert was checked by digestion with *Eco*RI restriction enzyme. Clones with insert were sequenced in both directions by the dye terminator method (ABI PRISM DyeTerminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) on an automatic DNA sequencer ABI 373XL (Applied Biosystems) with the upper and lower primers used for the amplification and with inner primers designed to carry on with the sequencing. Sequences of each clone were assembled with the CAP program and compared with the consensus sequence of the genes previously described (AJ415881, AJ415880 and AJ415884).

Sequence Analysis

Sequence alignments were performed using the Clustal W program. The PHYLIP package was used for the phylogenetic analyses. For nucleotide

sequence analysis, alignments of homologous sequences were first submitted to the Seqboot program to generate 100 sets of data for bootstrapping. Then, the distance matrix using Kimura 2 parameter model was analysed with the Neighbor-Joining program. Finally, bootstrap values obtained with the Consense program were written onto a representative tree of the Neighbor-Joining results showing branch lengths.

Results

For the HN gene, a sequence of 662 nucleotides (between nt 36 and 697 of the open reading frame, ORF) was analysed for 19 clones. The master or dominant sequence (HN.a) was identical to the consensus sequence, and only five clones showed one mutation with it (Table 1). From these results the viral population could be considered to be homogeneous.

In the P gene of the 99143 isolate, the potential quasispecies nature was shown by analysing a sequence of 644 nucleotides (between nt 38 and 681 of the ORF) for 20 clones. Two distinct subpopulations could be described (Table 1). The sequence of 12 of the 20 clones was similar to the consensus (P.a to P.j). The remaining eight clones (P.k to P.l) all differed from the consensus by a T > C mutation at both positions 137 and 140 of the ORF, leading to a Valine-to-Alanine and a Leucine-to-Proline substitution, respectively.

The analysed fragment of the F gene consisting of the first 645 nucleotides of the ORF was interesting because we were not able to clearly distinguish two sub-populations among the 27 clones. Twenty-five clones all differed from the consensus sequence by two silent mutations in the hypervariable domain of F (G > A at position 87 and C > T at position 189) and consequently formed the main sub-population (Table 1). However, the two remaining clones (F.p and F.q) were really distant from this main sub-population. Interestingly, out of the 43 differences detected for each of these two clones compared with the consensus, 28 positions were found to be identical to the consensus sequence of two typical pPMV-1 isolates (99299 and 99106) previously characterised [10] (Table 1). Moreover, the F protein cleavage site motif of all 27 clones was identical to the non-pathogenic ^{112}G**R**QG**RL**IG^{119} found for the consensus sequence of 99143.

Distance matrix phylogenetic analyses with the Neighbor-Joining method and bootstrapping were carried out with all the mutant sequences for each gene (Fig. 1). All the mutant sequences grouped with their consensus for P and HN genes. However, the second sub-population described for the P gene was supported by a bootstrap value of 93%. Furthermore, the two distant clones appeared intermediate between the 99143 isolate and the pPMV-1 strains with regards to the F gene.

Discussion

The quasispecies nature of RNA viruses represents an interesting hypothesis to understand their pathogenesis and evolution. Domingo et al. [13] defined quasispecies as a rated spectrum of mutants dominated by a master sequence, which may or may not coincide with the consensus sequence (usually determined by direct sequencing of PCR products). Domingo and colleagues [13–17] suggested that each mutation should be used to define sub-populations even if the mutation was only seen once. However, Smith et al. [18] reported that only the clear distinctions between potential sub-populations should be described, whilst all the other mutations should be considered as punctual. We chose to present all the mutations in Table 1 but to define sub-populations only by obvious positions, therefore when more than one clone presented the same sequence. Thus, two distinct subpopulations could be described when considering the P gene, and, with respect to the F gene, two clones exhibited only 93.3% identity (602 nucleotides/645) with the master sequence. To our knowledge, this is the first time that such a study is carried out and such observations are reported.

As mentioned in the Materials and Methods section, the 99143 isolate was sent to the European Community Reference Laboratory at Weybridge (UK) for further antigenic characterisation according to the European Union member state agreement. The isolate was classified in the vaccine strain group by antigenic means, but the virulent ICPI was also confirmed. Recently the 99143 isolate has been included in a molecular epidemiological study on avian paramyxoviruses type-1

Table 1. Summary of the mutations found in the different clones for each gene when compared to the consensus sequence

clone[a]	nb	nucleotide change[b]	amino acid consequence[c]
P.a	3	/	/
P.b	1	189 A > T	63 Q > H
P.c	1	373 A > G	225 S > G
P.d	1	572 A > T	191 Q > L
P.e	1	86 A > G*	29 K > R*
P.f	1	111 T > C*	/
P.g	1	263 C > T	88 P > L
P.h	1	92 C > A	31 A > E
		373 A > G	225 S > G
P.i	1	459 T > C	/
		491 T > C*	164 L > P*
		503 T > C*	168 V > A
P.j	1	139 C > A	47 L > M
		225 A > G*	/
		308 A > G	103 E > G
P.k	6	**137 T > C***	**46 V > A***
		140 T > C	**47 L > P**
P.l	1	96 G > C	32 E > D
		137 T > C*	**46 V > A***
		140 T > C	**47 L > P**
P.m	1	**137 T > C***	**46 V > A***
		140 T > C	**47 L > P**
		234 A > C	/
HN			
HN.a	14	/	/
HN.b	1	447 A > G	/
HN.c	1	558 C > T*	/
HN.d	1	592 G > A	199 D > N
HN.e	1	625 G > A	209 G > S
HN.f	1	643 G > A	215 A > T
F			
F.a	10	**87 G > A**	/
		189 C > T	/
F.b	1	**87 G > A**	/
		99 T > C*	/
		189 C > T	/
F.c	1	**87 G > A**	/
		189 C > T	/
		452 T > A	151 I > N
F.d	1	**87 G > A**	/
		189 C > T	/
		543 G > A	/
F.e	1	**87 G > A**	/
		189 C > T	/
		609 A > G	/
F.f.	1	**87 G > A**	/
		189 C > T	/
		267 C > T	/
F.g	1	**87 G > A**	/
		189 C > T	/
		310 G > A	104 E > K

Table 1. Continued.

clone[a]	nb	nucleotide change[b]	amino acid consequence[c]
F.h	1	**87 G > A**	/
		189 C > T	/
		415 G > A	139 A > T
F.i	1	**87 G > A**	/
		189 C > T	/
		379 C > T	127 L > F
F.j	2	**87 G > A**	/
		189 C > T	/
		191 A > G	64 K > R
		290 A > G	97 D > G
F.k	1	**87 G > A**	/
		189 C > T	/
		607 G > A	203 A > T
F.l	1	**87 G > A**	/
		189 C > T	/
		201 G > A	/
		627 G > C	209 E > D
F.m	1	**87 G > A**	/
		104 G > A	35 R > K
		189 C > T	/
		421 C > G	141 L > V
		642 A > T	/
F.n	1	10 A > G	4 R > G
		17 C > T	6 S > F
		87 G > A	/
		189 C > T	/
		548 A > C	183 Q > P
F.o	1	**87 G > A**	/
		189 C > T	/
		477 C > T*	/
		490 G > A*	/
		501 G > A	/
		507 T > C	/
		509 A > G	170 D > G
		523 C > T	/
F.q	1	Ditto F.p, but	
		132 T > A	/
		543 G > C	181 K > N
F.p	1	11 G > A*	4 R > K*
		18 T > C*	/
		23 A > G*	8 K > R*
		25 A > T*	9 N > I*
		31 G > A	11 A > I
		32 C > T	11 A > I
		37 A > C*	13 M > L*
		41 T > C*	14 M > T
		42 G > A	/
		47 C > T*	16 T > I*
		48 T > C*	/
		50 T > C*	17 I > T*
		54 G > A*	/

Table 1. Continued.

clone[a]	nb	nucleotide change[b]	amino acid consequence[c]
		55 G > A*	19 V > T
		56 T > C	19 V > T
		58 G > A*	20 A > I
		59 C > T	20 A > I
		60 G > A	20 A > I
		64 G > A	22 V > I
		67 C > T	/
		72 T > C*	/
		81 T > C*	/
		85 G > A*	29 A > T*
		89 A > G*	30 N > S
		93 C > T*	/
		94 A > C*	32 I > L*
		99 T > C*	/
		120 A > G*	/
		123 A > G*	/
		129 G > C	/
		132 T > G	/
		141 C > T*	/
		153 C > T*	/
		165 A > T	/
		193 C > T*	/
		195 C > G*	/
		205 C > A*	69 L > I
		207 G > A	69 L > I
		216 T > C*	/
		231 G > A	/
		240 C > G*	/
		243 G > A*	/
		246 T > A	82 D > E*

[a]Each clone was assigned a code including the gene name and a lowercase letter to differentiate mutants.

[b]Observed mutations in a clone when compared to the consensus sequence of each gene (access Number AJ 4158854, AJ 415881, AJ 415880 for HN, P and F, respectively). Each mutation is expressed as the position in the open reading frame of the nucleotide concerned, followed by the base found in the consensus, > for 'mutated into', and the base found for the clone. The / symbol was used when no differences were found with the consensus. The *symbol indicates that the base found for the clone is identical to that found in the pPMVI sequences. Position in bold are used to define sub-populations.

[c]Consequences of the mutations on the amino acid sequences. The / symbol was used when the mutation is synonymous. For all non synonymous mutations, the affected position in the protein is followed by the amino acid found in the consensus (using the one letter codification of the amino acids), > for 'changed into', and the amino acid found for the clone. The * symbol indicates that the amino acid found for the clone is identical to that found in the pPMVI sequences.

[19], which confirmed our previously published sequence showing that 99143 is genetically close to known vaccine strains [10]. However, Aldous et al. [19] did not mention the discrepancy of the ICPI test result, nor discuss it in relationship with the fact that 99143 was isolated from sick, unvaccinated pigeons.

In the present study the numerous differences between the two clones of the F gene were only found in the hypervariable domain of F, and the non-pathogenic F protein cleavage site motif was identical to the consensus. Thus, the potential quasispecies nature of the F gene could not give a clear explanation to the high ICPI of the 99143 isolate. Some hypotheses might explain these two clones. They may represent a recombination phenomenon between a vaccine aPMV-1 and a pPMV-1, although this has never been described in avian paramyxoviruses [20]. The 99143 isolate might also be a mix between a vaccine aPMV-1 and a pPMV-1, although we were not able to detect P or HN clones of 99143 which would have shared more similarities with a typical pPMV-1. The possibility of recombination events or co-infection could be tested by using specific primers designed for pPMV-1 strains as these primers might be more specific to the pPMV-1-like sequences. The two clones could also represent hypermutated forms of an original vaccine strain. Such a hypermutated form has been mentioned by Domingo et al. [13] for a wild strain of measles virus (another member of the family *Paramyxoviridae*), for which a hypermutated form was responsible for a specific form of the disease.

We were unable to connect the presence or absence of quasispecies to a particular pathogenicity. The introduction of a proline residue at position 46 of the P protein amino acid sequence in the minor sub-population may have an influence on the function of the P, V and W proteins and also on the fitness of this sub-population, which may be able to survive on its own or may need to coexist with the main sub-population. This mutation in the P gene may have an influence on the pathogenesis in pigeons.

The proportion of mutants we obtained by PCR-cloning-sequencing may not correspond to *in vivo* events. Most published studies on viral quasispecies used classical Taq polymerase and nested PCR to amplify viral genomes, and generate 10–20 clones

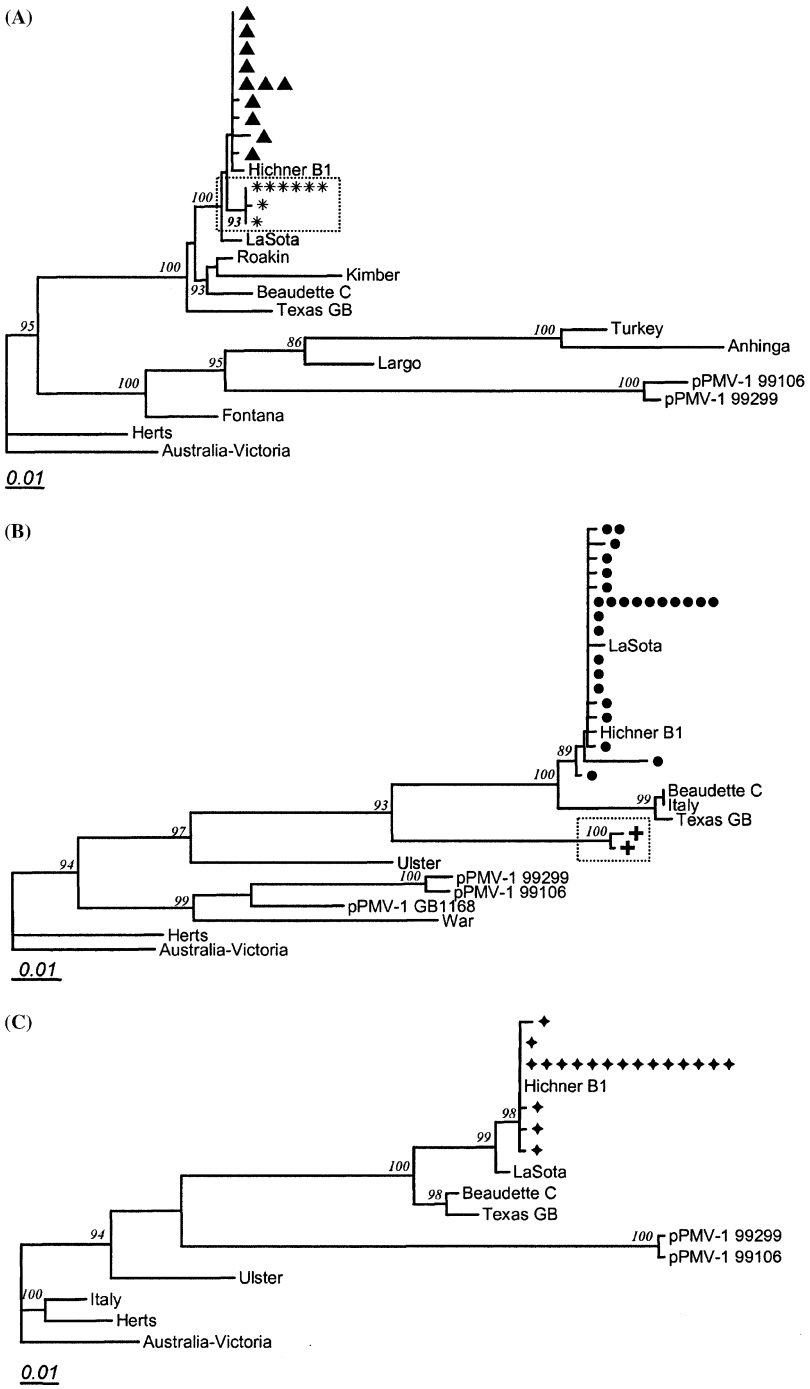

Fig. 1. Phylogenetic analyses performed on nucleotide sequences of the analysed part of the P gene (A), the F gene (B) and the HN gene (C), and including the mutant sequences of 99143 isolate. Unrooted trees were constructed by distance matrix analysis (using Kimura 2 parameter model) with Neighbor-Joining program. The scale bar is representing 0.01 nucleotide substitution per site. Interesting bootstrap values are indicated in italic on the left of each concerned node. The sequences of the APMV-1 reference strains were found in the gene banks and their access numbers are available upon request. Each clone of 99143 was represented by a symbol: ▲ and * were used to distinguish the two described sub-populations of the P gene; ● was used for the main sub-population of the F gene and + for the two distant clones; and finally ✦ was used for the HN gene clones.

for each product. The use of a proofreading polymerase and direct PCRs [10] limited error incorporation during PCR amplifications. Moreover, the cloning-sequencing of PCR products for each gene did not allow us to determine any relationship between the different genes. Further studies could target the three selected genes on individualised viral sub-populations. The selection of heterogeneous sub-populations might be obtained by the plaque assay test [7]. We obtained plaques for the 99143 isolate on chicken embryonated fibroblasts (CEF) without the use of additional trypsin (data not shown). The plaques varied in size or in aspect even though a strain with a non-pathogenic F protein cleavage site motif as in 99143 is not expected to form plaques on CEF without additional trypsin [21]. It would be interesting to further characterise the biological and molecular properties of some selected sub-populations.

Our main research purpose is to better understand the pathogenesis of pPMV-1 in pigeons and chickens. We characterised the quasispecies nature of two isolates to be used as inoculum: a typical pPMV-1 (unpublished) and an unusual aPMV-1 isolated from pigeons (this study). We are interested in following the quasispecies dynamics *in vivo* after serial passages in animals. However, we chose to first focus on a typical pPMV-1 for this kind of *in vivo* study (unpublished), and here we presented only preliminary results on the unusual 99143 isolate. No clinical signs were recorded when the 99143 isolate was oculo-nasally inoculated to four-week-old chickens (data not shown), whereas the original strain was isolated from sick pigeons with signs of pigeon paramyxovirosis. A model for experimental transmission would need to be established in order to reproduce the disease in pigeons. It is also important to note that the pigeons of the outbreak were not vaccinated against pigeon paramyxovirosis and the question arises as to how such a strain might have appeared. Interestingly, a similar aPMV-1 strain was also described in India (UP2–92). This isolate from pigeons was characterised by a mesogenic ICPI but with an F protein cleavage site motif and restriction enzyme patterns typical of apathogenic vaccine strains [22,23]. The isolation of such strains highlights the importance of a better understanding of the virus pathogenesis in pigeons and the role that pigeons might be playing in the appearance or propagation of virulent strains of NDV.

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