



Molecular Biological Characterization of Avian Poxvirus Strains Isolated from Different Avian Species

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1 **MOLECULAR BIOLOGICAL CHARACTERIZATION OF AVIAN**
2 **POXVIRUS STRAINS ISOLATED FROM DIFFERENT AVIAN SPECIES**

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14 ABSTRACT

15 Fifteen strains of Avipoxvirus from different avian species were isolated
16 and molecular-biologically characterized. Most strains did not produce
17 evident pocks on the chorioallantoic membranes of commercial and
18 specific-pathogen free embryonated chicken eggs where, on the contrary,
19 microscopic signs of viral growth were always detected. Polymerase chain
20 reaction of highly conserved P4b gene was positive for all cases confirming
21 to be a reliable diagnostic method for Avipoxvirus. Sequencing of these
22 amplicons confirmed most strains clustered either with Fowlpox virus or
23 with Canarypox virus whereas a possible new clade could be hypothesized
24 for one strain from Japanese quail. Classification of Avipoxvirus strains by
25 amplification of the newly identified locus fpv140 revealed major
26 limitations as only five samples were positive. These results underline the
27 importance to undertake similar studies on higher numbers of Avipoxvirus
28 isolates and on wider genomic regions of this large viral group.

29

30 Keywords: Avipoxvirus, virus isolation, PCR, sequencing

31

32

33 INTRODUCTION

34 The poxviruses belong to the Poxviridae family, whose members are large,
 35 oval to brick-shaped enveloped DNA viruses. Their genome consists of
 36 double-stranded DNA ranging from 130000-375000 nucleotides in linear
 37 configuration. They replicate within the cytoplasm of the cells they infect
 38 (Gubser et al., 2004; Jarmin et al., 2006). The poxvirus family is subdivided
 39 into the Entomopoxvirinae and Chordopoxvirinae subfamilies, which infect
 40 insects and chordates, respectively (Moss, 2001 cited by Gusber et al., 2004).
 41 Within the Chordopoxvirinae subfamily, Avipoxvirus (APV) is the only
 42 characterized genus that can infect non-mammalian hosts (Moss, 2001 cited
 43 by Gusber et al., 2004), including more than 230 of the known 9000 avian
 44 species with a worldwide distribution (Bolte et al., 1999, Tulman et al., 2004).
 45 Nevertheless, APV includes only the following species: Canarypox virus,
 46 Fowlpox virus, Juncopox virus, Mynahpox virus, Pigeonpox virus,
 47 Psittacinepox virus, Quailpox virus, Sparrowpox virus, Starlingpox virus and
 48 Turkeypox virus. Three more species - Peacockpox virus, Penguinpox virus,
 49 Crowpox virus - are still referred to as tentative by the International Committee
 50 on Taxonomy of Viruses (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm).
 51 APVs cause diseases in avian species that are usually referred to as pox and
 52 are characterized by two main forms: the cutaneous form and the diphtheritic
 53 form. In poultry, this disease can cause significant economic losses associated
 54 with decreased egg production, reduced growth, and increased mortality
 55 (Tripathy and Reed, 2003). Pox in canary is usually associated with extremely
 56 high mortality rates due to severe pulmonary damage. The possibility for

57 APVs to threaten the sustainability of populations of endangered birds has
58 been also described (Tripathy et al., 2000).

59 The conventional laboratory diagnosis of APVs is carried out by
60 histopathologic examination, electron microscopy, virus isolation on
61 Chorioallantoic Membrane (CAM) of embryonated chicken eggs or cell
62 cultures, and serologic methods (Tripathy and Reed, 1998). Recently,
63 molecular biological methods have proven to be the most sensitive techniques
64 for the routine diagnosis. In particular, Polymerase Chain Reaction (PCR)
65 based on the amplification of a 578-base pair (bp) region of the highly
66 conserved P4b gene of APVs had been increasingly used for diagnosis in the
67 last few years (Lüschow et al., 2004). Live vaccines against fowlpox made
68 with chicken strains are commercially available to protect chickens and
69 turkeys as well. Additionally, vaccines against canarypox, pigeon pox and
70 quail pox exist, each made with virus strains originating from the specific
71 avian species. In the last few years, the request for new vaccines against APV
72 infections have been increasing to protect a wider range of avian species,
73 particularly endangered species such as some birds of prey. Little is known
74 about the host range of APVs, although it is generally assumed to be limited
75 (Bolte et al., 1999; Jarmin et al., 2006). Moreover, the number of the species
76 and tentative species listed in the genus Avipoxvirus
77 (www.virustaxonomyonline.com) is much smaller than the number of bird
78 species naturally infected by pox and, consequently, the attribution of an APV
79 strain to a novel species within the genus APVs requires biologic, antigenic
80 and genetic characterization (Kim et al., 2003). Although poxvirus genome
81 organization, replication, host range and pathogenesis have been studied

82 extensively (Moss, 2001 cited by Gusber et al., 2004), less is known about the
83 evolutionary relationships of these viruses. Recently, the number of poxvirus
84 genome sequences submitted to the databases has increased considerably.
85 Much recent interest in the poxviruses has centred on their use as virus vectors
86 (Gusberg et al., 2004). Moreover, the increasing interest for APV is partially
87 due to the possible threat such pathogens may play in limiting the population
88 of endangered birds as recently described by Tripathy et al. (2000).
89 Still little information exists about the phylogenetic relationships among
90 APVs. More details about their evolution could be valuable to better
91 understand their host spectrum, epidemiology and pathogenesis. Recently,
92 phylogenetic studies, focused on the sequencing of P4b gene, have pointed
93 out that the vast majority of APV isolates clustered into the two major
94 Clades of the phylogenetic trees, i.e. Fowlpox virus (Clade A) and
95 Canarypox virus (Clade B) (Lüschow et al., 2004; Weli et al., 2004; Jarmin
96 et al., 2006). Attempts to identify new loci would permit a more robust
97 classification of APVs. Recently, Jarmin et al. (2006), sequenced locus
98 fpv140 of some strains of APVs. Their results suggest that the size of the
99 primary product of the fpv140 locus PCR allows easy distinction between
100 Clades A and B, being 1800 bp and 2400 bp, respectively.
101 This study is aimed at obtaining a more comprehensive phylogenetic
102 framework for the APVs by analysing P4b and fpv140 loci of APV strains
103 isolated from naturally occurring cases of pox infections in different avian
104 species.

105

106

107 MATERIALS AND METHODS

108 Viruses:

109 The study was based on 15 strains collected as fragments of cutaneous or
 110 pulmonary pox lesions during routine diagnostic activity. Two
 111 commercially available vaccine strains against fowlpox and canarypox were
 112 also included. The avian species of origin, month and year of collection are
 113 listed in Table 1.

114

115 Virus isolation: Tissue samples were stored at -20°C until use. Samples
 116 from each clinical case were minced and ground with sterile quartz sand
 117 using a mortar and pestle, suspended in a balanced salt solution containing
 118 50 IU/ml penicillin and 50 µg/ml streptomycin. Following low-speed
 119 centrifugation at 100×g for 10 min, 0.1 ml of the supernatant of the 15
 120 suspensions was inoculated on the CAMs of 11-day-old chicken embryos
 121 from commercial and Specific-Pathogen Free (SPF) (SPAFAS®) line as
 122 well. The inoculated eggs were incubated at 37.5°C for 7 days and then
 123 examined for pocks on the CAM. No further passages were carried out.
 124 Samples of each CAM were collected for histopathology.

125

126 Histopathology

127 Tissue samples collected from each clinical case and from each CAM were
 128 fixed in 10% buffered formalin embedded in paraffin, sectioned at 4 µm and
 129 stained with haematoxylin and eosin.

130

131 DNA extraction and PCR amplification

132 DNA was extracted from 25 mg of the skin or pulmonary lesions of all the
 133 clinical cases and from 25 mg of the two lyophilized vaccines by QIAamp
 134 DNA Mini Kit (Qiagen, Italy, Milan) following the manufacturer's
 135 guidelines. Tissue digestion with proteinase K was performed at
 136 56°C/overnight. The DNA concentration was measured fluorometrically and
 137 DNA samples were stored at -80°C until analysis.
 138 PCR reactions for APV-specific PCR and for fpv140 as well consisted of 25
 139 µl PCR buffer containing 1.5 mM of MgCl₂, 6 pmol of each primer, 200 µM
 140 of each dNTP and 1.25 U Taq polymerase (Invitrogen, Milan, Italy).The
 141 APV-specific PCR was performed using a primer pair described by Huw
 142 Lee & Hwa Lee (1997) based on P4b sequence of Fowlpox virus strain
 143 HP444 (forward primer: 5'-CAGCAGGTGCTAAACAACAA-3': reverse
 144 primer: 5'-CGGTAGCTTAACGCCGAATA-3'). Identical protocol as
 145 described by Lüscho et al. (2004) was used with the exception of clinical
 146 isolate QP-241, whose optimal annealing temperature (50°C) was
 147 established by gradient PCR. Amplification was performed after initial
 148 denaturation for 2 min at 94°C for 35 cycles and consisted of 1 min
 149 denaturation at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C.
 150 A final extension step was performed for 2 min at 72°C. Fpv140 was
 151 amplified by PCR as described by Jarmin et al., (2006) using a forward
 152 primer with slight modification, 5'-GAAGTAGAGTTATCGGTTC-3', and
 153 the same reverse primer M2912, 5'-GGTGATCCATTTCCATTTC-3'.
 154 Amplification was performed after initial denaturation for 5 min at 94°C for
 155 35 cycles, and consisted of 1 min denaturation at 94°C, 1 min annealing at
 156 55°C, and 2 min extension at 72°C. A final extension step was performed

for 15 min at 72°C. Then, five microlitres of the amplified PCR products were separated by 2% (for P4b) and 1.2% (for fpv140) agarose gel electrophoresis and stained with ethidium bromide. PCR products of P4b gene with the specific size were purified by QIAquick PCR purification kit (Qiagen, Milan, Italy), and both strands sequenced by BMR Genomics (Padova, Italy). The sequences were submitted to the GenBank database under accession numbers listed in Table 1.

After manual editing and excluding primer regions the P4b sequences (504 bp) were aligned (clustalW; Thompson et al., 1994) with APV sequences available from GenBank (Table 2) and possible genetic relationships and phylogenetic grouping of the different APVs were investigated using the neighbour-joining method according to the Jukes and Cantor model. *Mollusum contagiosum* virus (MOCV) was used as an outgroup for P4b. The reliability of the tree topologies was tested by bootstrap analysis (1,000 resampling). Pairwise genetic and amino acid distances of those strains displaying greater variability in the phylogenetic tree were calculated by MEGA version 3.1 (Kumar et al., 2001), applying the default setting, with the exception that all sites with ambiguous codes and gaps were ignored.

175

176 RESULTS

177

178 Virus isolation

179 No appreciable differences between commercial and SPF eggs were
 180 observed out. Macroscopically, pocks were evident for samples PA9554,
 181 PA9678, PA295-93, PA262-06 and PA213-07 respectively from song

182 thrush (the first two), canary, gyrfalcon and turkey. Small and single lesions
 183 were detected for samples QP-241 (Japanese quail) and PA153-05 (common
 184 buzzard). The CAMs inoculated with the other samples appeared
 185 moderately thickened and oedematous or quite normal (Table 3).

186

187 Histopathology

188 The histological evaluation of the cutaneous lesions revealed marked
 189 hyperkeratosis and achantosis associated with multifocal ulcers, cellular
 190 debris and large aggregates of cocci. Most keratinocytes were characterized
 191 by ballooning degeneration with large intracytoplasmic, 15 to 20 mm, pale
 192 eosinophilic inclusions surrounded by a clear halo (Bollinger bodies).
 193 Proliferative bronchitis associated with numerous characteristic poxviral
 194 inclusions were observed in the pulmonary lesions. Evidence of viral
 195 replication varying in severity was observed in the CAMs collected from all
 196 the inocula and consisted of focal hypertrophy and hyperplasia of the CAM
 197 epithelium associated with ballooning degeneration and Bollinger bodies.
 198 Oedema and fibroplasia of varying severity and heterophilic infiltration
 199 were also detectable in all samples.

200

201 PCR and sequencing

202 APV-specific DNA was detected in the vaccine samples and in the lesion
 203 samples from all the clinical cases. The size of all the amplified products of
 204 the P4b gene (about 580 bp including primers) was in agreement with the
 205 size of the published nucleotide sequences (Figure 1). The derived
 206 phylogenetic trees are shown in Figure 2. All the strains of this study except

207 that from quail (QP-241) can be included into Clade A (Fowlpox virus) or
 208 Clade B (Canarypox virus) according to the phylogenetic classification by
 209 Jarmin et al. (2006). One strain from grey partridge (PA4078), one from
 210 canary (PA1-09-92), one of the two strains from pheasant (PA9922), and the
 211 isolate from turkeys (PA213-07) clustered in subclade A1, whereas the
 212 second strain from grey partridge (PA7761), strain PA295-93 from canary,
 213 strains PA153-05 from common buzzard, and strain PA262-06 from
 214 gyrfalcon belonged to subclade A2. The isolates from pigeon (PA303-94),
 215 dunnoch (PA248-07), hooded crow (PA147-07), song thrush (PA9554 and
 216 PA9678), and one strain from canary (PA7746) belonged to subclade B1.
 217 Mean distance percentage of 27.4% and 28.7% was evaluated between
 218 nucleotide sequences of QP-241 and Clade A and B respectively, of 23.7%
 219 between PA1-09-92 and subclade B1, 8.3% between PA4078 and PA7761,
 220 3.4% between PA147-07 and subclade B1 and 2.7% between PA9678 and
 221 subclade B1. Mean distance percentage of 18% and 23.8% was evaluated
 222 between derived amino acid sequences of QP-241 and Clade A and B
 223 respectively. Amplification of the gene fpv140 gave PCR positive results for
 224 7 strains including the two vaccines (Figure 3). Amplicons from canarypox
 225 vaccine, and from the canary samples 7746 and PA1-09-92 were 2400 bp
 226 whereas amplicons from fowlpox vaccine and from PA9922, PA295-93, and
 227 PA 153-05 (pheasant, canary and common buzzard respectively) were 1800
 228 bp. The other isolates were all negative.

229

230

231 **DISCUSSION**

232 Fifteen strains of APVs from clinical cases affecting different avian species
233 were isolated and characterized by molecular biological methods to increase
234 the knowledge about the complex relationships between these viruses and
235 their hosts. All cases occurred in Northern Italy and were diagnosed during
236 routine diagnostic activity. Some of the APV strains infected avian species
237 where APVs are not or rarely reported (song thrush, grey partridge,
238 dunnock, hooded crow) (Bolte et al., 1999). Interestingly, the onset of some
239 episodes was registered in wintertime (Table 1) when APV infections are
240 considered to be uncommon. Although only one passage was carried out,
241 virus isolation gave some interesting results. Commercial eggs were used
242 first. Seven strains produced evident pocks on the CAMs whereas such
243 gross lesions were not observed with the other strains (Table 3). Whether
244 pocks were visible or not, evidence of viral growth was detected
245 microscopically in all the CAMs (Table 3). The multifocal distribution of
246 such findings excluded the site of injection as the limited area of viral
247 replication. It is well known that some APV strains, especially from wild
248 avian species, have no or poor growth in embryonated eggs or cell cultures
249 (Tripathy & Reed, 1998; Krone et al., 2004). Our histopathological results
250 pointed out all the 15 strains did grow in embryonated eggs after just one
251 passage thus suggesting their possible ability to adapt to the embryos. This
252 will be investigated in future studies. The possible role of maternal
253 antibodies against fowlpox in the unapparent and limited growth of most
254 strains on eggs from commercial breeders (Rampin et al., 2007) was ruled

255 out, inasmuch as the same inocula were subsequently injected into SPF eggs
 256 with strictly comparable results.

257 As for the molecular biological analysis, gene P4b amplification products of
 258 the expected size were obtained for all the strains of this study, thus
 259 confirming that PCR is an extremely valuable diagnostic method for APV
 260 infections. Sample QP-241 required gradient PCR to assess optimal
 261 annealing temperature, as the protocol published by Lüschoff et al. (2004)
 262 gave no result. Sequencing of these amplicons revealed that all of our
 263 isolates but QP-241 cluster in the two major Clades of APVs: A (Fowlpox
 264 virus) and B (Canarypox virus) (according to Jarmin et al., 2006) (Figure 2).
 265 Strain QP-241 from Japanese quail was known to have peculiar
 266 characteristics among APVs (Rinaldi et al., 1972). Sequencing of its P4b
 267 gene revealed mean distance percentages of 27.4% and 28.7% with Clade A
 268 and B, respectively, corresponding to 18% and 23.8% mean amino acid
 269 distance. As the mean distance percentage between nucleic acid sequences
 270 of Clade A and B is 25%, a possible new Clade of belonging for strain QP-
 271 241 could be proposed. This is supported by previous reports which showed
 272 DNA trees with a third major clade consisting of APVs from psittacines
 273 (Lüschoff et al., 2004; Jarmin et al., 2006). Psittacines viruses display
 274 peculiar nucleic acids changes and this is in accord with in vivo cross-
 275 protection studies (Jarmin et al., 2006). Similarly, QP-241 from Japanese
 276 quail was proven to have unique biological characteristics. Nevertheless, its
 277 genetic divergence needs to be ascertained through study of larger genomic
 278 regions. Interestingly, only another sequence of P4b gene from Japanese
 279 quail ([Table 1-DQ873809](#)) was found in the GenBank database, but it

280 phylogenetically belongs to Clade A2. As for the other strains, two strains
 281 from canary (PA1-09-92 and PA295-93) clustered in Clade A, precisely in
 282 subclade A1 (Fowlpox virus) and not in Clade B (Canarypox virus).
 283 Moreover, strains from the same avian species can display great nucleic acid
 284 divergence: APVs from grey partridge cluster in two different subclades
 285 (A1 and A2) with mean distance percentage between these two strains of
 286 8.3%. As for the strains from song thrush (PA9554 and PA9678), both were
 287 considered members of subclade B1 although the mean distance between
 288 the two strains was 2.7%, versus 0.7% as the mean distance within subclade
 289 B1 members. Similarly, PA147-07 from hooded crow is placed in subclade
 290 B1 with nucleic acid divergence of 3.4%. These results further sustain what
 291 is reported for some APV strains from sparrows, falcons and pigeons
 292 (Lüscho et al., 2004; Jarmin et al., 2006) and confirms that sequences of
 293 isolates taken from the same species can be found in different sub-clades or
 294 even different clades. This supports the evolutionary taxonomy of the host
 295 does not appear to have a major role in driving APV evolution as reported in
 296 previous reports (Jarmin et al., 2006). Alternatively, it is not possible to
 297 exclude P4b gene is inappropriate for a correct evaluation of evolutionary
 298 taxonomy. The possible consequences of the ancient and empirical use of
 299 APV strains of different origin (chicken and pigeon) for immunization of
 300 other avian species should also be considered. In this context, another report
 301 (Adam et al., 2005) described identical P4b sequences in six different
 302 genera of free-ranging birds in Virginia (USA). Our results cannot sustain
 303 such finding as all the obtained P4b sequences display a certain degree of
 304 nucleic acid divergence. It should be considered the fifteen clinical isolates

305 of this study come all from Northern Italy, but they were collected in a very
306 long time span and in different periods. Nonetheless, the ability of APV
307 specific strains to naturally infect multiple avian species requires further
308 investigations.

309 Amplification of the gene fpv140 was carried out as it was proposed as a
310 diagnostic marker to easily distinguish between Clade A and Clade B
311 (Jarmin et al., 2006). Unexpectedly, only 7 out of 17 strains gave positive
312 results (Figure 3) including the two vaccine strains. It was well known this
313 marker fails with Clade C (Psittacinepox virus) and subclade A3, but none
314 of the seventeen strains cluster in those clade and subclade. Consequently,
315 the lack of amplification we observed for most strains reduces the
316 applicability of fpv140 as a diagnostic marker. As for the seven samples
317 with positive results for fpv140 gene, it is noteworthy that strain PA295-93
318 from canary belongs to Clade A (Figure 2) but yielded a 2400-bp product.
319 (Figure 3). This contrasts with previous findings by Jarmin et al. (2006).
320 Although these findings should be confirmed by detailed investigations on
321 larger APV collections, they suggest it is necessary to re-evaluate the role of
322 gene fpv140 as a target for easy distinction between APV clades.
323 Nevertheless, fpv140 gene can still be considered as a potential useful
324 addition to gene P4b in molecular biological characterization of APVs as
325 reported by Jarmin et al. (2006) and Rampin et al. (2007), though there is
326 still a need for other genome markers.

327

328

329 CONCLUSION

330 The virological and molecular biological characterization of the strains
 331 included in this study further detail the wide host range of these viruses.
 332 These analyses of scarcely or previously uncharacterised APV strains
 333 allowed the submission to GeneBank of new sequences of P4b gene now
 334 available for further epidemiological investigations. The unexpected
 335 limitations to the use of fpv140 gene for molecular biological
 336 characterization of APVs underline the importance to undertake similar
 337 studies on wider genomic regions aimed at identifying new pan-genus
 338 markers, such as the P4b locus, that would permit more insight into this
 339 large viral group.

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345 REFERENCES

- 346 Bolte, A.L., Meurer, J. & Kaleta, E.F., 1999. Avian host spectrum of
 347 avipoxviruses. Avian Pathol. 28, 415–432.
- 348 Gubser, C., Hué, S., Kellam, P., Smith, G.L., 2004. Poxvirus genomes: a
 349 phylogenetic analysis. J. Gen. Virol. 85, 105-17.
- 350 Huw Lee, L. & Hwa Lee, K. 1997. Application of the polymerase chain
 351 reaction for the diagnosis of fowl poxvirus infection. J. Virol.
 352 Methods 63, 113–119.
- 353 Jarmin, S., Manvell, R., Gough, R.E., Laidlaw, S.M., Skinner, M.A., 2006.
 354 Avipoxvirus phylogenetics: identification of a PCR length
 355 polymorphism that discriminates between the two major clades. J.
 356 Gen. Virol. 87, 2191-2201.

- Kim, T.J., Schnitzlein, W.M., McAloose, D., Pessier, A.P. & Tripathy, D.N., 2003. Characterization of an avianpox virus isolated from an Andean condor (*Vultur gryphus*). Vet. Microbiol. 96, 237–246.
- Krone, O., Essbauer, S., Wibbelt, G., Isa, G., Rudolph, M., Gough, R.E., 2004. Avipoxvirus infection in peregrine falcons (*Falco peregrinus*) from a reintroduction programme in Germany. Vet. Rec. 154, 110–113.
- Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics. 17, 1244–5.
- Lüschow, D., Hoffmann, T., Hafez, H.M., 2004. Differentiation of avian poxvirus strains on the basis of nucleotide sequences of 4b gene fragment. Avian Dis. 48, 453–62.
- Rampin, T., Pisoni, G., Manarolla, G., Gallazzi, D., Sironi, G., 2007. Epornitic of avian pox in common buzzards (*Buteo buteo*): virus isolation and molecular biological characterization. Avian Pathol. 36, 161–5.
- Rinaldi, A., Mahnel, H., Nardelli, L., Mandelli, G.C., Cervio, G., Valeri, A., 1972. Charakterisierung eines Wachtelpockenvirus. Zentralbl. Veterinarmed. B. 19, 199–212.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–80.
- Tripathy, D.N., Reed, W.M., 1998. Pox. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed W.M (Eds.), Isolation and Identification of Avian Pathogens, 4th edn., New Bolton Center, PA: American Association of Avian Pathologists, pp. 137–140.
- Tripathy, D.N., Schnitzlein, W.M., Morris, P.J., Janssen, D.L., Zuba, J.K., Massey, G., Atkinson, C.T., 2000. Characterization of poxviruses from forest birds in Hawaii. J. Wildl. Dis., 36, 225–230.
- Tripathy, D.N., and Reed W.M., 2003. Pox. In: Saif, Y.M., Barnes, H.J., Fadly, A.M., Glisson, J.R., McDougald, L.R. and Swayne, D.E. (Eds.), Diseases of Poultry (cd version). 11th edn, Iowa State Press , Ames, pp. 253–269.

- 391 Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G. F. & Rock, D.L.,
392 2004. The genome of canarypox virus. *J. Virol.* 78, 353–366.
- 393 Weli, S.C., Traavik, T., Tryland, M., Coucheron, D.H., Nilssen, O., 2004.
394 Analysis and comparison of the 4b core protein gene of
395 avipoxviruses from wild birds: evidence for interspecies spatial
396 phylogenetic variation. *Arch Virol.* 149, 2035-46.
- 397
- 398

399 **Figure captions**

400

401 **Figure 1.** PCR amplification of the P4b gene from the the APV isolates of
402 this study. Lane M, DNA size marker (100-bp DNA ladder); CTRL-,
403 negative reagent control. See Table 1 for virus abbreviation.

404

405 **Figure 2.** Phylogenetic tree of nucleotide sequences of the 4b core protein
406 gene of APV isolated in this study (marked with asterisk *),
407 reference APV sequences and the MOCV orthologue sequence,
408 rooted on MOCV. The tree was obtained by the neighbour-joining
409 method calculated with the Jukes and Cantor model. Bootstrap
410 testing of phylogeny was performed with 1000 replications and
411 values equal to or greater than 70 are indicated on the branches (as a
412 percentage). The length of each bar indicates the amount of
413 evolution along the horizontal branches as measured by substitution
414 per site. APV Clades A to C and subclades are labelled. Reference
415 sequences are available from Jarmin *et al.* (2006).

416

417 **Figure 3.** PCR amplification of the fpv140 gene from the the APV isolates
418 of this study. Lane M, DNA size marker (1000-bp DNA ladder);
419 CTRL-, negative reagent control. See Table 1 for virus abbreviation.

420

Table 1 – Details of APVs used in this study

Code	Avian species of origin	Nature	Date	Origin	Accession numbers
FWPVD	Chicken (<i>Gallus gallus</i>)	Commercial vaccine	--	Europe	*AM050380
Diftosec CT (Merial)					
CNPVV	Canary (<i>Serinus canarius</i>)	Commercial vaccine	--	Europe	--
Duphar-Fort Dodge					
QP-241 §	Japanese quail (<i>Coturnix japonica</i>)	Clinical isolate	1963	Northern Italy	GQ180200
PA4078	Grey Partridge (<i>Perdix perdix</i>)	Clinical isolate	12-1978	Northern Italy	GQ180201
PA7746	Canary (<i>Serinus canarius</i>)	Clinical isolate	12/1985	Northern Italy	GQ180203
PA1-09-92	Canary (<i>Serinus canarius</i>)	Clinical isolate	09/1992	Northern Italy	GQ221269
PA7761	Grey Partridge (<i>Perdix perdix</i>)	Clinical isolate	09-1986	Northern Italy	GQ180204
PA9554	Song Thrush (<i>Turdus philomelos</i>)	Clinical isolate	10-1991	Northern Italy	GQ180205
PA9678	Song Thrush (<i>Turdus philomelos</i>)	Clinical isolate	02-1992	Northern Italy	GQ180206
PA9922	Pheasant (<i>Phasianus colchicus</i>)	Clinical isolate	12-1992	Northern Italy	GQ180207
PA295-93	Canary (<i>Serinus canarius</i>)	Clinical isolate	1993	Northern Italy	GQ180208
PA303-94	Pigeon (<i>Columba livia</i>)	Clinical isolate	09-1994	Northern Italy	GQ180209
PA153-05	Common Buzzard (<i>Buteo buteo</i>)	Clinical isolate	09-2005	Northern Italy	**EF016108
PA262-06	Gyr Falcon (<i>Hierofalco rusticolus</i>)	Clinical isolate	11-2006	Northern Italy	GQ180210
PA147-07	Hooded crow (<i>Corvus corone</i>)	Clinical isolate	06-2007	Northern Italy	GQ180211
PA213-07	Turkey (<i>Meleagris gallopavo</i>)	Clinical isolate	10-2007	Northern Italy	GQ180212
PA248-07	Dunnock (<i>Prunella modularis</i>)	Clinical isolate	10-2007	Northern Italy	GQ180213

Legend: -- not available; § Rinaldi et al., 1972; *Jarmin et al, 2006; **Rampin et al., 2007

Table 2 - Details of poxvirus sequences obtained from GenBank.

Virus name	Abbreviation	Host	GenBank Accession no
Turkeypox 2/11/66	TKPV66	Turkey	AM050387
Turkeypox 10/12/98	TKPV98	Turkey	AM050388
Turkeypox GB 134/01	TKPV134/01	Turkey	AY530304
Sparrowpox 9037 31/5/66	SRPV9037	Sparrow	AM050389
Sparrowpox 9037 31/5/66/23	SRPV23	Sparrow	AM050390
Starlingpox /27	SLPV	Starling	AM050391
Houbarapox 1252/98/42	HOPV1252	Houbara bustard	AM050381
Macawpox 1305/86	MCPV	Macaw	AM050382
Parrotpox 364/89	PRPV	Parrot	AM050383
Falconpox 1381/96	FLPV1381	Falcon	AM050376
Albatrosspox 353/87	ABPV	Black-browed albatross	AM050392
Agapornis APIII	AGPV	Agapornis	AY530311
Canarypox ATCC VR-111	CNPVATCC VR-111	Canary	AY318871
Canarypox GB 724/01-22	CNPV72401	Canary	AY530309
Canarypox 1445/97/33	CNPV1445	Canary	AM050375
Duphar; Fort Dodge	CNPVV	Canary	AM050384
Fowlpox FPV-VR250	FWPVVR250	Chicken	AY453172
Fowlpox HP-B	FWPVHPB	Chicken	AY530302
Fowlpox HP1-444 (FP9)	FWPVFP9	Chicken	AJ581527
Fowlpox Mild (Websters; Fort Dodge)	FWPVM	Chicken	AM050378
Fowlpox Nobilis Variole W (Intervet)	FWPVN	Chicken	AM050379
Fowlpox Diftosec CT (Merial)	FWPVD	Chicken	AM050380
Fowlpox 174/4/04	FWPV174	Chicken	AM050377
Falconpox GB362-02	FLPV36202	Falcon	AY530306
Great titpox GTPV-256	GTPV-256	Great tit	AY453175
Great titpox GTPV-A310	GTPVA310	Great tit	AY453173
Great titpox-A311	GTPVA311	Great tit	AY453174
Ostrichpox GB 724/01-20	OSPV	Ostrich	AY530305
Pigeonpox Peekham 19/11/75	PGPVP	Pigeon	AM050385
Pigeonpox 950 24/3/77	PGPV950	Pigeon	AM050386
Pigeonpox B7	PGPVB7	Pigeon	AY453177
Pigeonpox TP-2	PGPVTP2	Pigeon	AY530303
Sparrowpox DD1258	SRPVDD1258	Sparrow	AY530307
Sparrowpox GB 320/02	SRPV32002	Sparrow	AY530308
Sparrowpox GB 182/98	SCPV18298	Stone curlew	AY530310

Quailpox		Quail	DQ873809
Mourning dove WCV33-03	WCV33-03	Mourning dove	DQ131892
Red-Tailed Hawk WCV1419-03	WCV1419-03	Red-Tailed Hawk	DQ131901

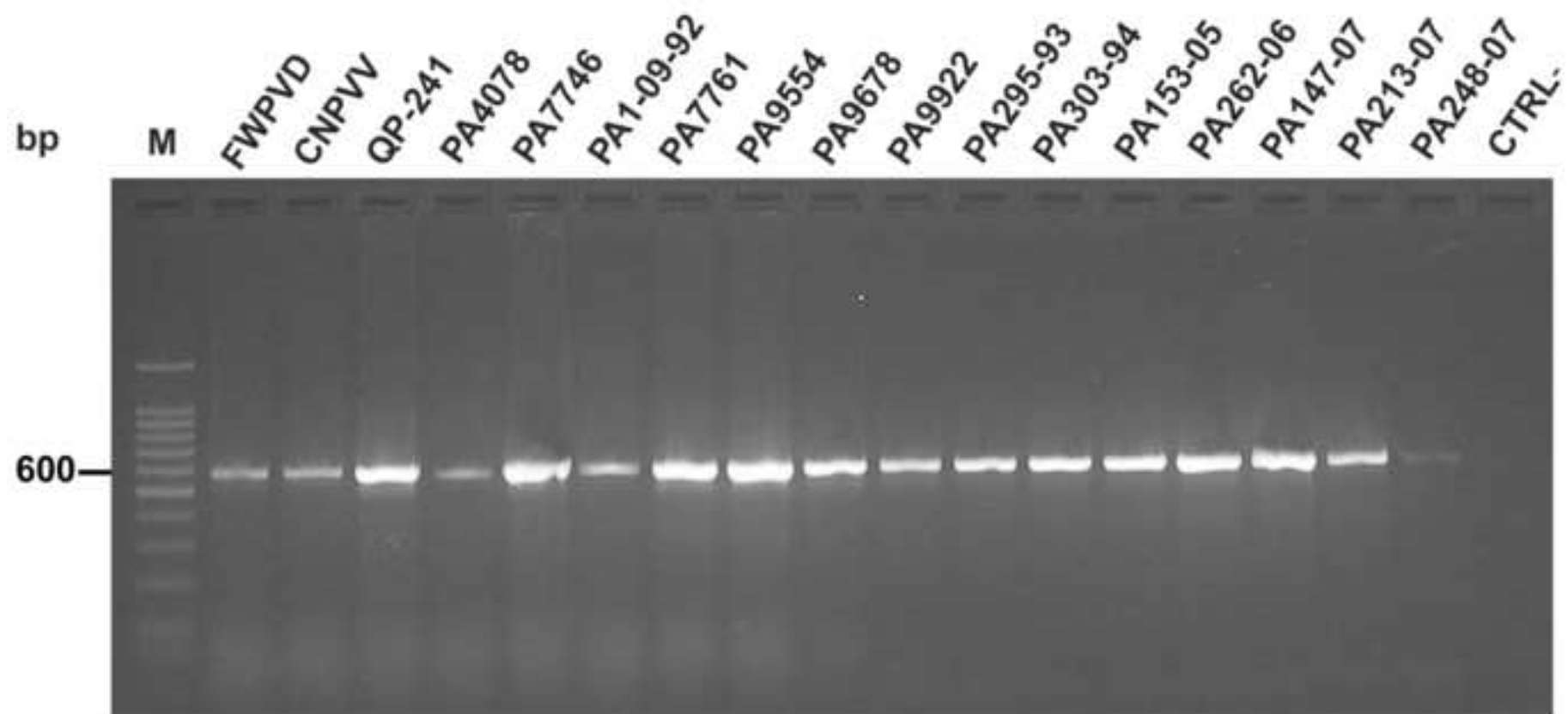
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Table 3 - Results of APV isolation in commercial and SPF embryonated eggs of the clinical isolates used in this study: gross and microscopic findings on CAMs.

APV clinical isolate	Gross lesions on CAMs		Microscopic lesions on
	pocks	thickening	CAMs
QP-241	+	mild/moderate	P
PA4078	-	moderate	P
PA7746	-	moderate	P
PA1-09-92	-	mild/moderate	P
PA7761	-	moderate	P
PA9554	+++	severe	P
PA9678	+++	severe	P
PA9922	-	mild	P
PA295-93	++	mild/moderate	P
PA303-94	-	not observed	P
PA153-05	+	mild	P
PA262-06	+++	severe	P
PA147-07	-	mild/moderate	P
PA213-07	++	moderate/severe	P
PA248-07	-	mild/moderate	P

Legend: +++: > 5 pocks; ++: 3-5 pocks; +: 1-2 pocks; P: present

Figure 1



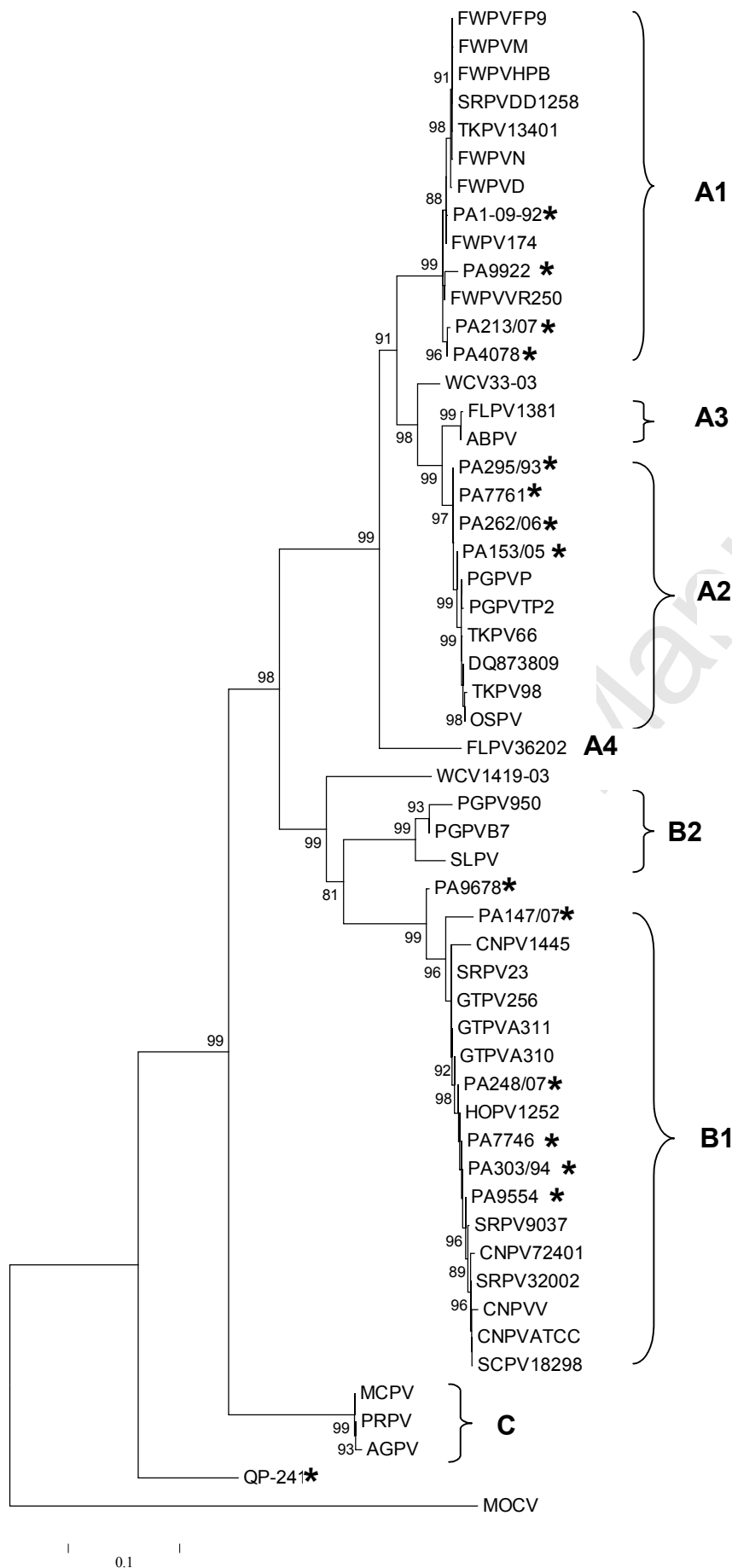


Figure 3

