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Genotypic characterization of psittacid herpesvirus isolates from Brazil



Marcela Miranda Luppi^{a,*}, Ana Paula Moreira Franco Luiz^a,
Fabiana Magalhães Coelho^a, Roselene Ecco^b, Flávio Guimarães da Fonseca^c,
Mauricio Resende^a

^a Laboratório de Virologia Comparada, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, MG 31270-901, Brazil

^b Laboratório de Patologia, Departamento de Clínica e Cirurgia, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, MG 31270-901, Brazil

^c Laboratório de Virologia Básica e Aplicada, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, MG 31270-901, Brazil

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ABSTRACT

Thirty-six isolates of psittacid herpesvirus (PsHV), obtained from 12 different species of psittacids in Brazil, were genotypically characterized by restriction fragment length polymorphism (RFLP) analysis and PCR amplification. RFLP analysis with the PstI enzyme revealed four distinct restriction patterns (A1, X, W and Y), of which only A1 (corresponding to PsHV-1) had previously been described. To study PCR amplification patterns, six pairs of primers were used. Using this method, six variants were identified, of which, variants 10, 8, and 9 (in this order) were most prevalent, followed by variants 1, 4, and 5. It was not possible to correlate the PCR and RFLP patterns. Twenty-nine of the 36 isolates were shown to contain a 419 bp fragment of the UL16 gene, displaying high similarity to the PsHV-1 sequences available in GenBank. Comparison of the results with the literature data suggests that the 36 Brazilian isolates from this study belong to genotype 1 and serotype 1.

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Introduction

Pacheco's disease was first identified in Brazil in 1930 and described as causing acute hepatitis and death of birds from the Psittacidae family.¹ In 1975, the etiologic agent for this disease was identified to be psittacid herpesvirus (PsHV).² Since

then, PsHV has been related to the death of psittacids in various parts of the world.^{3–6} However, few studies have been conducted in Brazil following the initial discovery and description of the disease.

PsHVs have been classified as members of the genus *Iltovirus* ($\alpha 4$) within the subfamily *Alphaherpesvirinae*, with the scientific name *Psittacid herpesvirus 1*.⁷ The viral genome

* Corresponding author.

E-mail: mmluppi@yahoo.com.br (M.M. Luppi).

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consists of 163,025 base pairs (bp) (GenBank accession no. AY372243), contains 73 open reading frames (ORFs) and has a guanine and cytosine content of 60.95%.⁸

PsHVs have been classified into four genotypes, with distinct biological characteristics. They are thought to be latent in their host species; however, parrots infected with PsHV of a genotype to which they are not adapted have been discovered to contract an acute fatal disease.⁹

Several classification strategies have been proposed for PsHVs, based on their genetic and antigenic differences. Antigenic studies using cross-neutralization tests identified three serotypes of PsHVs and suggested the existence of two others.¹⁰ However, when genotypic differences are considered, viral polymorphisms are more evident, as demonstrated by a study based on PCR amplification patterns, where 10 different variants of PsHVs were identified.¹¹ Another technique that has been used is restriction fragment length polymorphism (RFLP) analysis, which was able to identify up to 14 different patterns when the *Pst*I restriction enzyme was used to digest the viral genome.^{12,13}

The objective of this study was to characterize 36 isolates of PsHV-1, previously obtained from captive parrots from Belo Horizonte, MG, Brazil,¹⁴ by PCR amplification and RFLP analysis using the *Pst*I enzyme.

Materials and methods

Virus isolates

The PsHV isolates used in this study (Table 1) were obtained from chicken embryo fibroblasts (CEF) inoculated with spleen, liver, or kidney tissue fragments, cloacal swabs, or leukocytes isolated from 36 psittacine birds. The birds belonging to 12 different species were held in captivity in institutions located in the city of Belo Horizonte, MG, Brazil.¹⁴ The original samples were collected prior to this study over a course of eight months, between September 2007 and April 2008.

The leukocytes and cloacal swab samples were collected from nine live birds, which displayed no clinical signs of disease at the time of sampling. The kidney, spleen and liver samples were collected from dead birds, of which, 26 had shown anorexia, apathy, and sudden death (no antemortem individual information was available) and one (BH 89), which had been examined before death, showed no clinical changes, but presented sudden death.

A reference virus (KS 144/79) was kindly provided by Prof. Dr. Erhard F. Kaleta from Klinik für Vögel, Reptilien, Amphibien und Fische, Justus-Liebig-Universität, Gießen.

Cell culture and virus production

Primary CEF (chicken embryo fibroblast) monolayers were prepared as described previously¹⁵ and grown in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Gibco, Life Technologies, Grand Island, NE, USA) and 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA).

Each isolate was inoculated into a culture flask containing a confluent CEF monolayer. Following observation of a

cytopathic effect, the cells and medium were harvested. Viral precipitation was carried out with saturated ammonium sulfate solution, as described previously.¹⁶

Extraction of viral DNA

Total DNA was extracted using a proteinase K/phenol-chloroform-isoamyl alcohol protocol as described previously.¹⁷

The purified DNA was eluted with water and stored at 4 °C for at least 24 h before use. The total DNA was analyzed and quantified using a NanoDrop ND-1000 spectrophotometer at 260–280 nm (Thermo Fisher Scientific).

RFLP analysis

The DNA samples extracted from the PsHV isolates and from the reference virus (KS 144/79) were analyzed by RFLP for genotypic classification using the restriction enzyme *Pst*I (20,000 U/mL, New England Biolabs, Inc., Ipswich, MA, USA), as recommended by the manufacturer.

The resulting DNA fragments were separated by electrophoresis on a 0.5% agarose gel in 1× Tris-acetate-EDTA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 5 µL of ethidium bromide (10 mg/mL) (Life Technologies, Grand Island, NE, USA) at room temperature under constant voltage of 20 V for about 15 h. The BenchTop 1 kb DNA Ladder (Promega, Madison, WI, USA) was used as a DNA size marker. The gels were observed at 254 nm using a MacroVue UV transilluminator (GE Healthcare, Cleveland, OH, USA) and photographed.

Restriction profile analysis

The fragments were visually identified on the gel photographs. Based on the DNA marker, an approximate molecular size of each fragment was calculated. Thus, it was possible to prepare a visual graphic picture of the restriction patterns obtained.

To prepare a dendrogram, the RFLP patterns were converted to binary matrices by assigning "1" for presence and "0" for absence of each fragment generated. Cluster analysis based on similarity was performed by the neighbor-joining method¹⁸ using the Sorensen-Dice coefficient with the program NTSYS-pc (Exeter Software, Setauket, NY, USA),¹⁹ as described in previous research studies.

PCR amplification patterns

A 200 µL aliquot of a cell culture suspension was used for DNA extraction using sodium iodide and silica according to the method described by Boom et al.²⁰ The extracted total DNA was analyzed and quantified by measuring absorbance at 260 nm on a NanoDrop ND-1000 spectrophotometer.

Each sample was amplified using six different pairs of primers described in the literature^{9,11} and capable of producing up to 10 different patterns by standard amplification (Table 2). The PCR reaction conditions were as described by Tomaszewski et al.,^{9,11} with modified annealing temperatures (T_m) for each pair of primers (optimized by gradient PCR), as specified in Table 2.

Table 1 – List of isolates and their respective sources of isolation.

Specie	Common name	Sample	Isolate
<i>Pyrrhura cruentata</i>	Blue-throated Parakeet	Leukocyte	BH 117
<i>Trichoglossus haematodus</i>	Rainbow Lorikeet	Leukocyte	BH 137
<i>Amazona xanithops</i>	Yellow-faced Amazon	Leukocyte	BH 145
<i>Pionites leucogaster</i>	White-bellied Parrot	Cloacal swab	BH 167
<i>Psittacula krameri</i>	Ring-necked Parakeet	Cloacal swab	BH 168, BH 169, BH 170, BH 171, BH 172
<i>Amazona aestiva</i>	Blue-fronted Amazon	Liver	BH 78, BH 80, BH 100, BH 102, BH 103, BH 104, BH 107, BH 284, BH 285, BH 316, BH 318, BH 341
<i>Anodorhynchus hyacinthinus</i>	Hyacinth Macaw	Liver	BH 89
<i>Aratinga leucophthalmus</i>	White-eyed Conure	Liver	BH 955
<i>Eupsittula aurea</i>	Peach-fronted Parakeet	Liver	BH 106, BH 1430, BH 1431
<i>Forpus xanthopterygius</i>	Blue-winged Parrotlet	Liver	BH 108, BH 958
<i>Pionus sp.</i>	Pionus	Liver	BH 342
<i>Psittacara leucophthalmus</i>	White-eyed Conure	Liver	BH 101
<i>Amazona aestiva</i>	Blue-fronted Amazon	Spleen	BH 1362, BH 1407, BH 1411
<i>Amazona amazonica</i>	Orange-winged Amazon	Spleen	BH 1408
<i>Psittacara leucophthalmus</i>	White-eyed Conure	Spleen	BH 1096
<i>Amazona aestiva</i>	Blue-fronted Amazon	Kidney	BH 343
Positive control			KS144/79

Table 2 – The used primers with respective Tm and possible amplification patterns as reported earlier.

Primers	Clone 9 F	Clone 9 R	Clone 11 F	Clone 11 R	Clone 23 F	P-UL17/16
Fragment amplified size (bp)	489	378	394	310	281	667
Amplified region	UL19	UL21	UL14/UL15	UL9	UL17/UL16	UL17/UL16
Tm	55 °C	52 °C	51 °C	55 °C	58 °C	58 °C
Amplification patterns/variant						
1	+	+	+	+	+	+
2	–	+	+	+	+	+
3	–	–	+	+	+	+
4	–	–	–	+	+	+
5	–	–	–	–	+	+
6	+	–	+	+	+	+
7	–	+	–	+	+	+
8	+	–	–	–	+	+
9	+	+	–	+	+	+
10	+	–	–	+	+	+

In all PCR assays, reagent mixture without a template was used as a negative control, and DNA from isolate KS 144/79 (PsHV-1) was used as a positive control.

The PCR products were separated on a 1% agarose gel in Tris–Borate–EDTA buffer (TBE: 100 mM Tris-base, pH 8.3, 25 mM EDTA, 50 mM boric acid) (Thermo Scientific, Pittsburgh, PA, USA) at room temperature under constant voltage of 100 V and visualized using a UV transilluminator (GE Healthcare, Cleveland, OH, USA). A 100 bp DNA Ladder (Invitrogen, Carlsbad, CA, USA) was used as DNA size marker.

Sequencing and phylogenetic analysis

Twenty-nine of the isolates analyzed in this study carried a previously sequenced fragment of 419 bp, coding for the UL16 ORF. These already known sequences were used to analyze the RFLP and PCR results.

A neighbor-joining phylogenetic tree was constructed by aligning the nucleotide sequences of the UL16 ORF of the Brazilian PsHV isolates and the sequences of PsHV-1, PsHV-2, PsHV-3 and PsHV-4 obtained from the NCBI sequence

database. One thousand bootstrap replicates were used to assess the significance of the tree topology.

Histopathology

In order to determine the cause of death and PsHV infection-related injuries, histopathological examination was performed on samples from 26 of the 27 dead birds. These results have been published previously.¹⁴

Results

Restriction fragment length polymorphism

The PstI digestion of genomic DNA from the 36 isolates of PsHV revealed five different restriction patterns (Figs. 1 and 2). By comparing the restriction profiles obtained in this study, including the reference isolate (KS 144/79), with those described by Schröder-Gravendyck et al.,¹³ we were able to conclude that 28 isolates belonged to group A1 (Table 3).

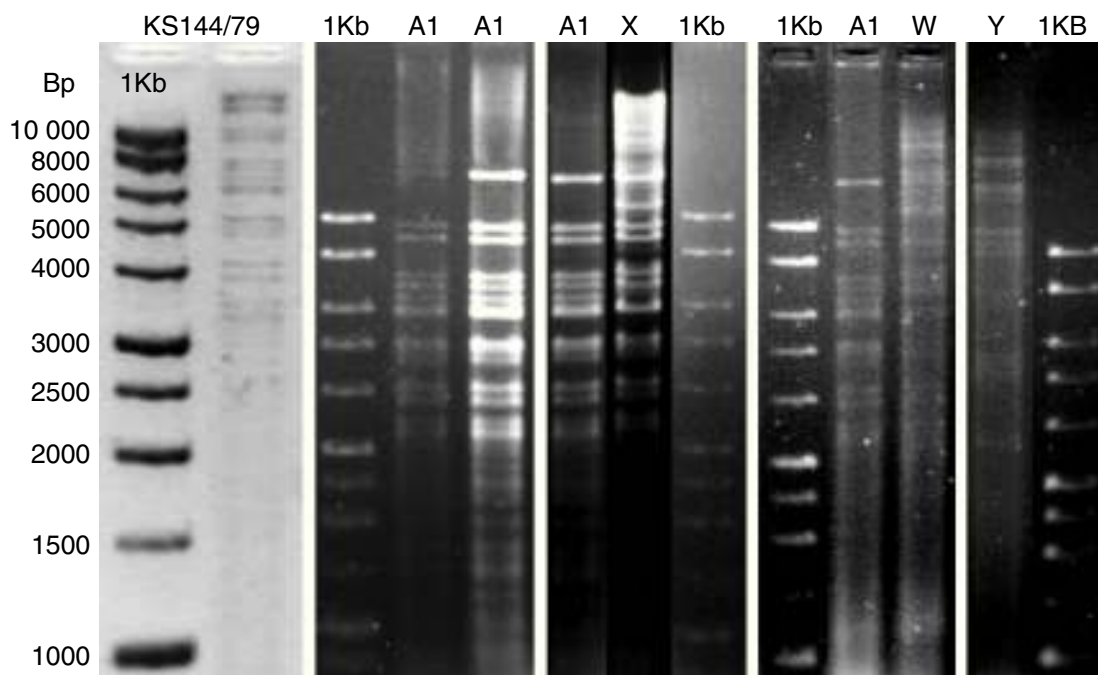


Fig. 1 – Restriction patterns (A1, X, Y and W) obtained by digestion of DNA from seven Brazilian PsHV isolates and reference strain KS 144/79 with *Pst*I, compared with 1 kb DNA Ladder (Promega).

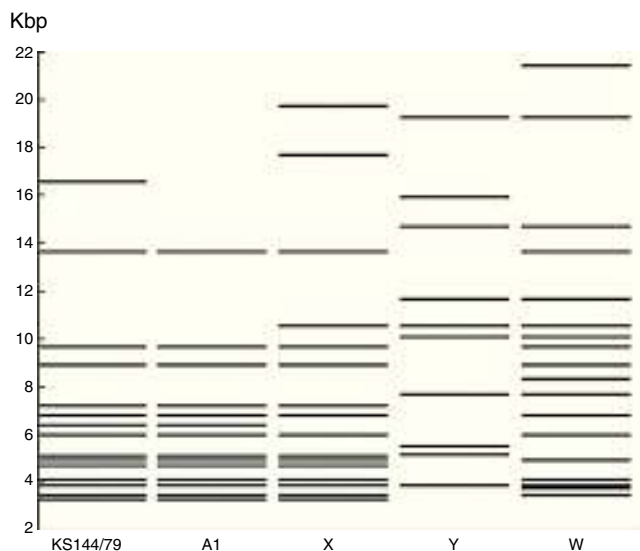


Fig. 2 – A schematic representation of the restriction patterns of PsHV DNA digested with *Pst*I. In The Y-axis represents the molecular weights (kb) based on 1 kb DNA Ladder (Promega).

The digestion profiles of isolates KS 144/79 and BH1411 were different from those of the other 27 isolates in group A1, due to the presence of a fragment of approximately 16,500bp long (Fig. 1). The eight remaining isolates revealed three restriction patterns, X, W, and Y, different from those described in the literature (Table 3). The W pattern was represented by a single isolate, and no visible fragments were

obtained in a repeated experiment. All other patterns were successfully reproduced.

The dendrogram (Fig. 3) shows two distinct groups, one consisting of the isolates that exhibited the Y pattern and the other consisting of the isolates that exhibited the other three patterns (A1, X and W). Similarity was observed between A1 and X patterns.

PCR amplification patterns

Using this technique, it was possible to identify differences (variants) among 27 of the isolates. Among these, 14 isolates were identified as variant 10, nine as variant 8, six as variant 9, two as variant 1, and one of each as variants 4 and 5, according to the classification strategies detailed by Tomaszewski et al.¹¹ The other nine isolates showed amplification patterns incompatible with any of the variants described in the literature (Table 3).

Sequencing and phylogenetic analysis

All sequences obtained showed 99–100% similarity with the PsHV-1 sequences published in GenBank (Table 3).

Phylogenetic analysis showed four distinct clusters. The first cluster grouped the sequences of the Brazilian isolates and the PsHV-1 sequences obtained from GenBank. It was also possible to confirm high sequence identity (99 to 100%) of PsHV-1 and PsHV-4 (Fig. 4).

Histopathology

The histopathology results ranged from no observable changes (Nos. 5, 11, 27 and 35) to hepatitis displaying intranu-

Table 3 – Results of RFLP and PCR amplification patterns compared by sequences genotype.

No.	RFLP results	Accession number	Sequence genotype	Amplification pattern						Variant	Clinical signs	Histopathology
				9F	9R	11F	11R	23F	P-UL16/17			
BH117	A1	JF338864	PsHV-1	+	+	–	+	+	+	9	WCS	–
BH137	Y	JF338865	PsHV-1	+	+	–	–	+	+	–	WCS	–
BH145	A1	UR	UR	+	–	–	+	+	+	10	WCS	–
BH167	A1	JF338867	PsHV-1	+	–	–	–	+	+	8	WCS	–
BH168	A1	JF313339	PsHV-1	+	–	–	–	+	+	8	WCS	–
BH169	Y	JF313340	PsHV-1	–	–	–	–	–	+	–	WCS	–
BH170	X	JF313341	PsHV-1	+	–	–	–	–	+	–	WCS	–
BH171	A1	JF338868	PsHV-1	+	–	–	+	+	+	10	WCS	–
BH172	A1	UR	UR	+	–	–	+	–	+	–	WCS	–
BH78	A1	JF313328	PsHV-1	+	+	–	+	+	+	9	–	Chlamydia
BH80	A1	JF338858	PsHV-1	+	–	–	–	+	+	8	–	SUG L
BH100	A1	JF313329	PsHV-1	+	+	–	+	+	+	9	–	SUG LK
BH102	A1	JF313330	PsHV-1	+	+	+	+	+	+	1	–	SUG LK
BH103	A1	JF313331	PsHV-1	+	–	–	+	+	+	10	–	SUG LK
BH104	A1	JF313332	PsHV-1	+	+	+	+	+	+	1	–	SUG LSK + chlamydia
BH107	A1	JF338862	PsHV-1	+	–	–	+	+	+	10	–	SUG L
BH284	A1	JF313333	PsHV-1	+	–	–	+	+	+	10	–	SUG LK
BH316	A1	JF313335	PsHV-1	+	–	–	–	+	+	8	–	SUG L
BH318	A1	IS	IS	+	–	–	+	+	+	10	–	SUG HS + chlamydia
BH341	A1	UR	UR	+	–	–	+	+	+	10	–	SUG L
BH285	W	JF313334	PsHV-1	+	–	–	+	+	+	10	–	SUG LS + chlamydia
BH89	A1	JF338859	PsHV-1	+	–	–	–	–	+	–	SD	UR
BH955	X	JF338870	PsHV-1	+	+	–	+	+	+	9	–	SUG L
BH106	A1	JF338861	PsHV-1	+	–	–	+	+	+	10	–	SUG LS + chlamydia
BH1430	Y	IS	IS	+	+	–	–	+	+	–	–	Intestinal invagination
BH1431	A1	JF338875	PsHV-1	+	–	–	+	+	+	10	–	SUG L
BH108	A1	JF338863	PsHV-1	+	–	–	–	–	+	–	–	No changes
BH958	Y	UR	UR	–	–	–	–	–	+	–	–	No changes
BH342	A1	JF313337	PsHV-1	–	–	–	–	+	+	5	–	Chlamydia
BH101	A1	JF338860	PsHV-1	–	–	–	+	+	+	4	–	No changes
BH1362	X	JF313326	PsHV-1	+	–	–	+	+	+	10	–	SUG L
BH1407	A1	JF338873	PsHV-1	+	–	–	+	+	+	10	–	SUG L
BH1411	A1	UR	UR	–	+	–	–	–	+	–	–	No changes
BH1408	A1	JF338874	PsHV-1	+	–	–	–	+	+	8	–	SUG LK
BH1096	A1	JF338872	PsHV-1	+	–	–	+	+	+	10	–	Lipidosis
BH343	A1	JF338869	PsHV-1	+	–	–	–	+	+	8	–	SUG L
C+	A1		PsHV-1	+	+	+	+	+	+	1		

UR, unrealized; IS, incomplete sequence; WCS, without clinical signs; SD, sudden death; SUG, suggestive of viral alterations on liver (L), kidney (K) and spleen (S).

clear inclusion bodies, which suggested herpesvirus infection (Nos. 25 and 26) (Table 3). Among the 26 histologically examined birds, 18 had multifocal lymphoplasmacytic hepatitis with random foci of necrosis suggestive of viral infection. Of these, six displayed additional multifocal interstitial lymphocytic nephritis. Four of these birds showed inflammation in the spleen, characterized by marked histiocytic infiltration with concomitantly diagnosed intracytoplasmic bacteria *Chlamydoiphila psittaci*.²¹ The remaining four birds showed changes unrelated to viral infection.

Discussion

The RFLP assays were performed using the restriction enzyme *Pst*I, which had been shown to generate the largest number of PsHV profiles.¹³ These authors observed up to 12 different

restriction patterns while testing 31 isolates of PsHV by *Pst*I-RFLP. In our study, five different patterns were observed among the 36 isolates examined by RFLP analysis with *Pst*I. The difference in the number of patterns between the two studies may be due to the fact that the study by Schroder-Gravendyck et al.¹³ included isolates obtained over a period of 17 years and from different countries, while our isolates were obtained over an eight-month period from the same city/region.

The RFLP pattern generated by digestion of the DNA isolated from strain KS 144/79 showed an unexpected fragment of approximately 16,500 bp in length, not observed by Schroder-Gravendyck et al.¹³ among the products of digestion of DNA from the same isolate with the *Pst*I enzyme. This could be due to an enzyme failure to digest part of the viral DNA or, alternatively, to a mutation in the restriction site that may have occurred during successive passages of the virus in cell culture. This same fragment was also observed among the

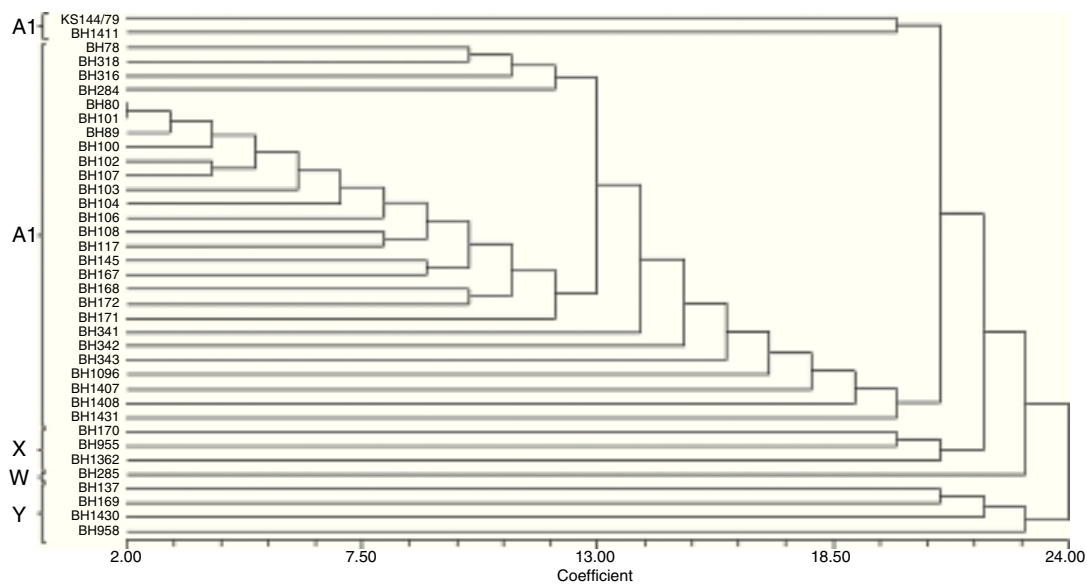


Fig. 3 – Dendrogram generated by NTSYS-pc cluster analysis of the RFLP results, based on the similarity among the profiles produced.

products of digestion of the BH1411 DNA. With the exception of this fragment, the RFLP patterns of these two isolates were identical to those of the other 27 isolates in the same group and corresponded to the previously described A1 pattern.¹³

The patterns other than A1, displayed by eight isolates, had no similarity to the 13 other *Pst*I restriction cleavage patterns described in the literature.^{12,13}

Among the three X-pattern isolates, one was obtained from the cloacal swab of a rose-ringed or ring-necked parakeet (*Psittacula krameri*), a bird naturally found in Africa and Asia, while the other two were obtained from liver and spleen tissue samples of two Brazilian species, a white-eyed Conure (*Psittacara leucophthalmus*) and a blue-fronted Amazon (*Amazona aestiva*), respectively. The W pattern was displayed by a unique isolate (No. 32) obtained from the liver of *A. aestiva*. The four samples that generated the Y pattern were isolated from leukocytes, cloacal swab, and liver tissues of four different bird species, *P. krameri*, *Trichoglossus haematodus*, *Forpus xanthopterygius*, and *Eupsittula aurea*. Therefore, it was not possible to correlate the differences observed in the RFLP patterns of the isolates based on the bird species or source material.

When the isolates were classified according to the PCR amplification patterns, the results obtained were also different from those reported in the literature. Using this test, Tomaszewski et al.¹¹ were able to identify 10 different variants of PsHV, with variant 1 having been found most frequently. In our study, only six out of the 10 variants were identified (1, 4, 5, 8, 9, and 10), and variants 10, 8, and 9 (in this order) were most prevalent. In addition, nine isolates displayed patterns that had not been previously described in the literature.

The isolates with the A1 RFLP profile displayed all six PCR patterns found in this study and three others yet to be described. The PCR results of the three isolates with the X RFLP profile (Nos. 29, 30, and 31) showed three different amplification patterns (variants 9 and 10 and unknown).

Isolate No. 32 (W profile) produced a pattern corresponding to variant 10, while all of the Y-profile isolates generated previously unknown amplification patterns. The latter results might suggest that the isolates produced aberrant patterns of amplification.

Due to the variations in clinical signs and histopathological findings it was not possible to propose correlations between pathogenicity of the isolates and the RFLP patterns. For instance, some psittacine isolates with the A1 profile were obtained from healthy birds, while others were obtained from birds which died and whose histopathology showed hepatitis with intranuclear inclusions, suggesting a viral cause.

RFLP analysis is quite useful to readily determine genotypes of isolates and, therefore, is especially helpful to those working in settings where other techniques, such as DNA sequencing, are not immediately available (for instance, in field laboratories). Nevertheless, future studies employing phylogenetics of complete genomes are essential to further characterize new isolates and discover potential correlations between pathogenicity and RFLP patterns.

In addition to the genetic classification of PsHV, previous studies have also focused on classification based on antigenic variations. Serologically, the viruses have been classified into five serotypes based on cross-neutralization testing. Serotypes 1, 2, and 3 have been considered most common, and serotypes 4 and 5 have been represented by single isolates.¹⁰

Previous studies based on sequencing of the 419bp fragment of the UL16 ORF have been able to correlate PsHV genotypes with serotypes. According to these studies, genotype 1 is directly correlated to serotype 1, genotype 2 to serotype 2, and genotype 3 to serotype 3. However, genotype 4 has been correlated to both serotypes 1 and 4. The single isolate representing serotype 5 could not be sequenced, and therefore no correlation with the genotype has been possible.⁹

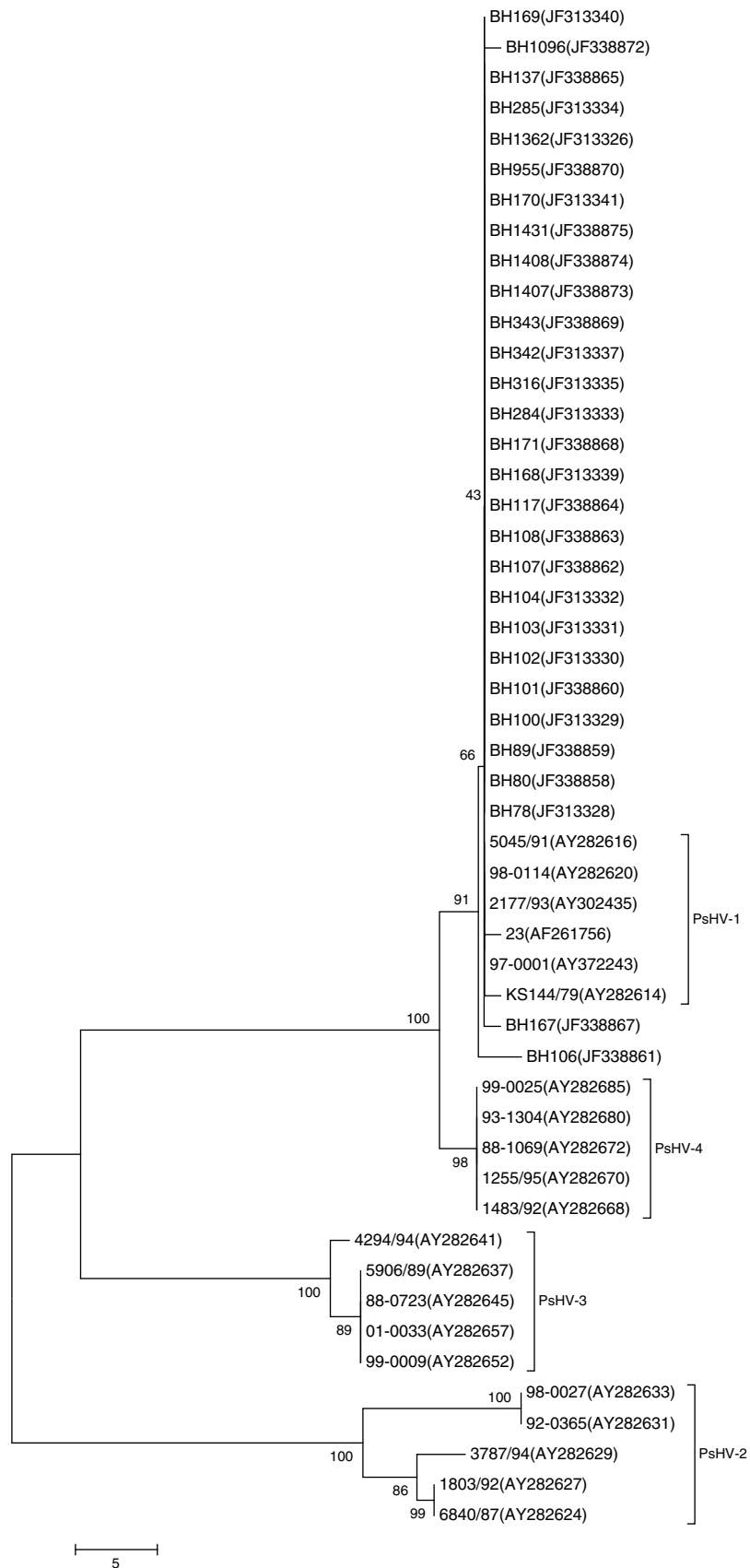


Fig. 4 – Neighbor-joining phylogenetic tree showing four distinct clusters corresponding to viral genotypes PsHV-1, PsHV-2, PsHV-3, and PsHV-4. The isolates from this study were all grouped with PsHV-1. The GenBank accession numbers are given in parentheses.

Schroder-Gravendyck et al.¹³ concluded in their study that the A1 restriction cleavage pattern corresponds to serotype 1. Therefore, we can infer that the 29 isolates from this study that showed the A1 RFLP pattern belonged to serotype 1.

Twenty-nine of the isolates used in this study, including some isolates demonstrating the A1, X, W, and Y patterns, had previously been partially sequenced (GenBank accession nos. JF313326, JF313328, JF313329, JF313330, JF313331, JF313332, JF313333, JF313334, JF313335, JF313337, JF313339, JF313340, JF313341, JF338858, JF338859, JF338860, JF338861, JF338862, JF338863, JF338864, JF338865, JF338867, JF338868, JF338869, JF338870, JF338872, JF338873, JF338874, and JF338875). The DNA fragment sequenced is the 419 bp fragment corresponding to the UL16 ORF that showed 99–100% identity to the sequences of PsHV-1 present in GenBank.¹⁴ Moreover, although the UL16 gene is located in a conserved region within the genome, when the sequences were compared with those of PsHV-2, PsHV-3, and PsHV-4 obtained from GenBank the similarity did not exceed 97%. Therefore, we suggest that all patterns discovered in this study belonged to genotype 1 (PsHV-1) and, probably, to serotype 1.

Phylogenetic analysis showed clustering of the Brazilian isolates with genotype 1 of PsHV. The phylogenetic tree generated indicated the greatest similarity between genotypes 1 and 4 of PsHV. These results are in agreement with the findings of Tomaszewski et al.⁹ who showed that genotype 4 viruses were most similar to genotype 1, and that the differences between those included a single substitution and six deletions.

The present study described three new restriction profiles of PsHV obtained using PstI-RFLP. We report that the most common variants identified by PCR patterns were variants 10, 8, and 9 (in this order). Our data suggest that the 36 Brazilian isolates studied can be assigned to genotype 1 and serotype 1.

Conflicts of interest

The authors declare no conflicts of interest.

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