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Development of a polymerase chain reaction for the detection of abalone herpesvirus infection based on the DNA polymerase gene

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Abstract:

A 5781-base pair (bp) fragment of genomic DNA from the Taiwanese abalone herpesvirus was obtained and showed 99% (5767/5779) homology in the nucleotide sequence and 99% (1923/1926) in the amino acid sequence with the DNA polymerase gene of the abalone herpesvirus strain Victoria/AUS/2007. Homology of the amino acid sequence with the DNA polymerase of ostreid herpesvirus 1 was 30% (563/1856). In this study, a PCR-based procedure for detecting herpesvirus infection of abalone, *Haliotis diversicolor supertexta*, in Taiwan was developed. The method employed primer sets targeting the viral DNA polymerase gene, and was able to amplify DNA fragments of the expected size from infected samples. Primer sets of 40f and 146r were designed for amplification of an expected PCR product of 606 bp. Combining the new PCR protocol with histopathology, this assay can serve as a reliable diagnostic for herpesvirus infections in abalone.

Highlights

► Sequencing of the abalone herpesvirus from field cases was carried out. ► A PCR was developed from the DNA polymerase of herpesvirus for detecting herpesvirus infections in abalone. ► This test is a useful method for epidemiological studies of abalone herpesvirus in the field.

Keywords: Herpes virus ; Abalone ; *Haliotis diversicolor supertexta* ; DNA polymerase ; Polymerase chain reaction

1. Introduction

Herpesvirus infections have been reported in various marine bivalve molluscs worldwide since the early 1990s. The first description of a virus morphologically similar to members of the Herpesvirales (Davison et al., 2009) in a bivalve mollusk was in the eastern oyster, *Crassostrea virginica* in 1972 (Farley et al., 1972). A wide host range was then reported for herpesvirus infections in bivalves including various species of oysters, clams, and scallops (Renault and Novoa, 2004 ; Renault, 2008 ; Burge et al., 2011). Mortality of abalone (molluscan genus *Haliotis*) associated with herpes-like viral pathogens has been reported in several species and subspecies including *H. discus discus* (Otsu and Sasaki, 1997; Nakatsugawa et al., 1999), *H. discus hannai* (Wang and Li, 1997), *H. diversicolor* Reeve (Wang et al., 2000), *H. diversicolor aquatilis* (Song et al., 2000), and *H. diversicolor supertexta* (Zhang et al., 2001). Recent outbreaks of infection with a herpesvirus, *Haliotis* herpesvirus (AbHV) that induced acute and high mortality were described in cultured abalone, *H. diversicolor supertexta*, in Taiwan (Chang et al., 2005). A herpesvirus identified as *Haliotis* herpesvirus 1 (AbHV-1) also induced mortality in abalone including blacklip abalone *H. rubra*, greenlip abalone *H. laeviga*, and their hybrids in Australia. However, clinical signs of Australian

AbHV infection differed from those of the Taiwanese abalone in that moribund Australian abalone had a swollen mouth and prolapsed odontophore (Hooper et al., 2007), while those in Taiwan diseased abalone exhibited mantle recession and muscle atrophy (Chang et al., 2005), but lacked the oral lesions observed in Australia.

AbHV is a neurotropic virus causing ganglioneuritis; abalone 1 year or older are typically affected (Huang et al., 1999; Chang et al., 2005). Lesions include necrosis of the cerebral ganglia and nerve bundles in the muscle of the foot as well as in the muscular layers beneath the visceral organs (Chang et al., 2005). Similar neurological involvement, also noted as ganglioneuritis was reported in the blacklip abalone and greenlip abalone by Hooper et al., (2007). A herpes-like virus was also reported from abalone causing amyotrophia characterized by the development of tumour-like lesions in the nerve trunk of Japanese black abalone, *H.* (=*Nordotis*) *discus discus* (Nakatsugawa et al., 1999).

Light microscopy has been used to diagnose both AbHV and AbHV-1 infections due to the prominent neurological lesions induced by these viruses in Taiwan and Australia, respectively (Chang et al., 2005; Hooper et al., 2007). Additional techniques, such as transmission electron microscopy, can be used

77 to confirm the diagnosis. Both microscopic techniques are time consuming and
78 inadequate for epidemiological studies because herpesviruses may persist in
79 clinically healthy hosts (carrier or latent-state infection) and recur under
80 stressful conditions (Whitley, 1996). Thus rapid, high-throughput molecular
81 diagnostic methods are needed to aid in diagnosing AbHV.
82 The genome sequencing of one bivalve herpesvirus, ostreid herpesvirus
83 (OsHV)-1, allowed the development of DNA-based diagnostic techniques.
84 Polymerase chain reaction (PCR)-based methods for detecting OsHV-1 DNA
85 isolated from fresh, frozen and paraffin-embedded samples were previously
86 published (Renault et al., 2000 ; Barbosa-Solomieu et al., 2004 ; Batista et al.,
87 2007 ; Martenot et al., 2010 ; Segarra et al., 2010). Different OsHV-1-specific
88 primer sets (n=20) were used on abalone samples from Taiwan, but failed to
89 amplify DNA from AbHV-infected abalone (Chang and Renault, unpubl. data).
90 A TaqMan® PCR-based technique specific for gene containing motifs V and VI,
91 characteristic of SF2 helicase was available for detection of herpesvirus
92 infections in abalone (OIE 2009; Corbeil et al., 2010).
93 Although the DNA polymerase gene seems to be highly conserved, some
94 differences did exist between virus isolates from Taiwan and Australia. Thus,
95 this virus gene appears of interest for generic diagnosis purpose to detect most

of virus isolates in the field. In the present study a conventional PCR technique targeting the AbHV DNA polymerase gene was developed and tested on abalone samples from field locations (farms).

2. Materials and methods

2.1. Biological materials

AbHV-infected abalone, *H. diversicolor supertexta*, collected from a grow-out farm that suffered high mortalities in 2004 in northern Taiwan were used as the material source. Abalone collected from farms in southern Taiwan with no history of AbHV infection were used as negative controls. Nerve tissues of 300 moribund abalones collected from one batch from a AbHV-affected farm were excised and held at -80°C until DNA extraction. Nerve tissues of 300 control abalones were also excised and similarly stored.

2.2. Viral purification, negative staining and DNA Extraction

AbHV was purified using methods modified from Le Deuff and Renault (1999) and Tan et al., (2008). Briefly, 2 g of pleuropedal ganglia, pedal nerve cords, head and epipodial tissue from AbHV-infected abalone was used to purify virus particles. Cells were lysed using a combination of homogenization and sonication. Viral particles were separated from abalone nervous tissues by a

115 discontinuous sucrose gradient composed of five fractions, of 60%, 50%, 40%,
116 30%, and 10% sucrose (w/v), prepared in seawater followed by
117 ultracentrifugation at 112,398 x*g* (25,000 rpm using an SW28 rotor, Beckman,
118 Brea, California, USA) for 1 h at 4 °C. Fractions (1~2 ml) were collected at
119 each interface, and each corresponding fractions were pooled from different
120 gradient tubes. Fractions were diluted four times by adding seawater in a
121 drop-wise manner, and the virus was pelleted at 300,000 x*g* for 90 min (Le
122 Deuff and Renault, 1999). DNA from the virus pellets was extracted using a
123 QIAamp Stool Mini Kit (QIAgen, Hilden, Germany) following the manufacturer's
124 instructions. A portion of the purified virus pellets (100 µL) was centrifuged at
125 100,000 x*g* for 10 min, negatively stained with 2% phosphotungstic acid and
126 examined with electron microscopy (Hitachi, HF-3300, Tokyo, Japan) (Bozzola
127 and Russell, 1992).

128 *2.3. DNA Sequencing*

129 Approximately 5 µg of genomic DNA from ganglia and nerve cords was
130 sheared by nebulization, and DNA sequencing was performed following
131 protocols for the Genome Sequencer GS FLX Titanium System (Roche,
132 Branford, Connecticut, USA). Reads generated by the GS FLX sequencer
133 were trimmed of low-quality sequences and were assembled by a GS de novo

134 Assembler. These sequences were compared using the NCBI blastx program
135 (http://blast.ncbi.nlm.nih.gov). A gene homologous to the DNA polymerase of a
136 herpesvirus was chosen in this study. Analyses were performed and
137 sequences aligned using Phylogenetic Inference with the Automatic Likelihood
138 Model (PALM) (Braithwaite and Ito, 1993; Chen et al., 2009).
139 *2.4. PCR for the detection of Haliotis herpesvirus*
140 PCR primers were designed from the above sequences using the program
141 Primer3. Primers of 40f (5'-TCCATCGAGATTCCCAGTTC-3') and 146r
142 (5'-ACGCCACCCTGTATAACGAG-3') were expected to yield a 606 bp PCR
143 product. PCR amplification was performed as described by Renault et al.,
144 (2000). A 50-µl reaction mixture was prepared with 5 µl of 10x PCR buffer (10
145 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% w/v gelatin), 400 µM
146 dNTPs, 5 µM tetramethylammonium chloride, 40 pmoles of each primer, 2 µl
147 purified DNA, and 2 U of Taq polymerase, and was brought up to volume with
148 sterile distilled water. PCR amplification was performed in a thermocycler
149 (PTC-100, MJ Research, Alameda, CA , USA) with an initial denaturation step
150 of 94 °C for 2 min, followed by 35 cycles of 1 min of denaturation at 94 °C, 1
151 min of annealing at 52 °C, and a 1-min extension at 72 °C, followed by
152 incubation for 5 min at 72 °C. Control PCRs without a template were used to

153 test for cross-contamination. All reactions were tested in triplicate. Amplified
154 fragments were separated using a 1% agarose gel and visualized with UV
155 illumination after staining with ethidium bromide. The presence of amplifiable
156 DNA was confirmed in all negative samples using 18S ribosomal RNA gene
157 primers kindly supplied by Dr. Mark Crane (CSIRO Livestock Industries,
158 Victoria, Australia). Primer sets of 18S forward
159 (5'-GGCTACCACATCCAAGGAA-3') and 18S reverse
160 (5'-GCTGGAATTACCGCGGCT-3') were used in this study, and the procedure
161 was carried out as described previously (Corbeil et al., 2010).

162 *2.5. Histolopathology and PCR concordance analysis*

163 Batches were selected for the analysis based on mortality and low harvest
164 rates of grow-out farms in an epizootic area. Archived samples collected
165 between 2003 and 2009 from farms that showed persistent low rate mortality
166 and high cumulative mortality associated with undertermined etiologies were
167 inlcuded in this analysis. Samples of visceral organs were fixed in 10%
168 seawater formalin, embedded in paraffin, sectioned at 5 µm, and stained with
169 Mayer's haematoxylin and eosin (H&E) and examined by light microscopy.
170 Results of histolpathology and PCR were recorded.

171

3. Results

3.1. Nucleotide sequencing and sequence alignment

A 5781-bp fragment of AbHV showed 99% (5767/5779) homology with the polymerase region of the abalone herpesvirus strain Victoria/AUS/2007 (AbHV-1). When translated to its predicted amino acid sequence, the selected AbHV fragment also showed high sequence identity (99%; 1923/1926) with that of AbHV-1. AbHV showed moderate amino acid sequence identity (30%; 562/1856) with that of OsHV-1. This fragment of AbHV also showed moderate to low amino acid sequence identities (23%~46%) with the DNA polymerase of other herpesviruses (Table 1). The nucleotide sequence of the AbHV DNA polymerase gene of the virus isolate from Taiwan has been deposited in GenBank under accession number HQ317456.

A dendrogram depicting the relationship of AbHV to other members of the Herpesvirales order illustrated that the abalone herpesvirus forms a polyphyletic group (Fig. 1). Moreover, abalone herpesvirus from Australia, a abalone herpesvirus from Taiwan, and OsHV-1 clustered in the same subgroup based on the DNA polymerase sequence data.

3.2. PCR for the detection of AbHV, Haliotis herpesvirus

190 The 40f and 146r AbHV primers amplified the expected PCR product of
191 606 bp from DNA of moribund abalone. No amplification was observed when
192 eel herpesvirus DNA, koi herpesvirus DNA, chicken infectious laryngotracheitis
193 virus DNA, or OsHV-1 DNA served as irrelevant controls (Fig. 2).
194 *3.3. Histopathology*
195 Ganglioneuritis lesions were present in ganglia and nerve tracts of various
196 tissues in PCR-positive samples. These lesions were characterized by tissue
197 necrosis accompanied by infiltration of haemocytes; inclusion bodies were rare.
198 Haemocytes were also observed surrounding the neurilemma (Fig. 3a, b).
199 *3.4. Electron microscopy*
200 A negative-contrast electron microscopy demonstrated viral particles at
201 the interface of the 40%~50% layers of the sucrose gradients. Viral particles
202 were hexagonal, 90~100 nm in diameter, and had a single coat (Fig. 4).
203 *3.5. Histolopathology and PCR concordance analysis*
204 Histopathology and PCR data showed 100% concordance. Samples from
205 6 out of 19 batches examined showed both histological evidence of
206 ganglioneuritis and PCR evidence of AbHV DNA presence. All of the
207 remaining 13 batches of samples from which no AbHV DNA was amplified also
208 lacked histopathological evidence of ganglioneuritis (Table 2). No virions were

observed via electron microscopy using direct negative staining of pooled organs including nerve tissues, the hepatopancreas, gonads, gills, and intestines in any animlas from PCR and histology-negative batches. However, these samples have experienced elevated mortality of unknown etiology in the field.

4. Discussion

For the first time, this study provides information on the phylogeny of the Taiwanese herpesvirus infecting the abalone, *H. diversicolor supertexta*. In this study, partial genome sequences of a 5781-bp fragment from the Taiwanese AbHV DNA polymerase gene showed nearly identical DNA and deduced amino acid sequence (99%) homologies with those of the abalone Victoria/AUS/2007 (AbHV-1). In contrast, AbHV showed moderate amino acid sequence identity (30%) with that of OsHV-1, which infects bivalves, and 23%~46% identity with some vertebrate herpesviruses. The phylogenic analysis showed that the AbHV isolate from Taiwan is a member of the Herpesvirales. In this study, AbHV isolate from Taiwan, AbHV-1 and OsHV-1 were clustered in the same subgroup based on DNA polymerase sequence data (Fig. 1). However, analysis of large genome fragments of the AbHV virus

228 isolate from Taiwan using the supermatcher program revealed that the TC04
229 fragment of the Taiwanese isolate (NCBI accession no. JN083851) had 85.7%
230 (10481/12224) identity to the Victoria/AUS/2007 AbHVscaffold_3172-3200
231 fragment. The TC02 (NCBI accession no. JF967012) and TC08 fragments
232 (NCBI accession no. HQ890941) of the Taiwanese isolate corresponded to the
233 Victoria/AUS/2007 AbHVscaffold_3197-3033 isolate, and had 66.7%
234 (26,884/40,281) and 61.2% (14,529/23,756) identities, respectively. These
235 results suggested that the Taiwan virus isolate, Taiwan/2004, is
236 distinguishable from AbHV-1 (Australia isolate, Victoria/AUS/2007).
237 No OsHV-1 and AbHV belong both to Malacoherpesviridae family. A
238 phylogenic analysis of deducted protein sequences from DNA polymerase and
239 terminase genes showed that although OsHV-1 and AbHV appear to belong to
240 the family Malacoherpesviridae, they are in fact distantly related, and the
241 putative amphioxus herpesvirus shares more sequence similarity to mollusk
242 herpesviruses than to sequences from any of the vertebrate herpesviruses
243 analyzed (Savin et al., 2010). To accommodate this abalone virus, Savin et al.,
244 (2010) suggested creating a new genus called *Haliotivirus* within the
245 Malacoherpesviridae family and assigning the virus infecting abalone in
246 Australia as a species under *Haliotivirus* (as *Haliotid* herpesvirus 1). Although

247 AbHV isolate from Taiwan showed some sequence homology with OsHV-1,
248 neither virus is similar enough to be detected using existing primers targeting
249 OsHV-1. Indeed, the primer pairs (C1/C6, C1/C4, C2/C4, C2/C6, C5/C13,
250 C9/C10, A3/A4, A5/A6, B1/B2, B3/B2, B3/B4, Gp1/ Gp2, Gp1/Gp4, Gp1/Gp10,
251 Gp3/Gp4, Gp7/ Gp8, Gp9/Gp10, Gp10/Gp15, IA1/1A2, IA3/IA6) (Arzul et al.,
252 2001a, b, 2002; Renault and Arzul, 2001; Renault, pers. comm.) failed to
253 amplify DNA from AbHV-infected samples from Taiwan (Chang and Renault,
254 pers comm). Thus, AbHV and OsHV-1 may represent two viral species, the
255 former infecting gastropods and the latter infecting bivalves.
256 A conventional PCR for detecting AbHV was developed in the present
257 study targeting the DNA polymerase gene. Indeed, some differences were
258 reported between virus isolates from Taiwan and Australia. The DNA
259 polymerase gene appears of interest for detecting most of virus isolates in the
260 field. A clear advantage of the designed primer set is its application on abalone
261 samples from field locations. Application of the primer set (40f/146r) yielded
262 amplicons of the expected size when DNA samples extracted from infected
263 abalone were used as templates. This amplification was specific, since no
264 fragment was amplified from irrelevant control abalone DNA, or DNA from

265 different herpesviruses, including a OsHV-1, a koi herpesvirus (KHV), an eel
266 herpesvirus, OsHV-1, and an avian herpesvirus.
267 Although major neural tissues appeared to be prominently involved in the
268 disease process, viral DNA was also amplified from the intestines, gills,
269 hepatopancreas, gonads, muscles, and mantle (data not shown). In the field,
270 recurrence of AbHV in abalones on farms following strict disinfection has been
271 noticed in successive years. Severe mortality occurred following low water
272 temperatures suggesting the possible persistence of AbHV on farms and
273 reactivation of the the virus infection by stress (Van Nieuwstadt et al., 2001).
274 However, more studies are needed to verify the relationship between stress
275 and AbHV infection of abalone. Development of diagnostic procedures for
276 detecting early infections of AbHV in abalones would be useful. Results of this
277 study will facilitate epidemiological investigations to ascertain whether other
278 mollusks in this area may be infected. Histopathology or electron microscopy
279 would also be required to confirm AbHV infection (OIE 2009). A retrospective
280 study using this primer set to analyze field samples revealed that 100% (6 of 6
281 batches) of PCR-positive cases had nervous lesions examined by
282 histopathology. Whereas all PCR-negative batches lacked histopathological
283 lesions (Table 2), and no virions were observed by the electron microscopy

using direct negative staining of pooled organs consisting of gonads, the hepatopancreas, intestines, gills, muscles, and mantle. Due to the slow rates of abalone mortality noted in the field, the relationship between these cases and herpesvirus infection needs further study.

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abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. Virology. Journal. 7, 308.

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395

396 Figure captions

397 Fig. 1. Alignment of the deduced amino acid sequences of *Haliotis* herpesvirus
398 (AbHV) with the homologous domains of DNA polymerases from different
399 herpesviruses using a PALM analysis. The percentage of replicate trees in the
400 bootstrap test (100 replicates) is shown next to the branches.

401

402 Fig. 2. The PCR detection of AbHV is specific since all irrelevant controls yield
403 negative results. Lane 1, *Haliotis* herpesvirus (AbHV); lane 2, chicken
404 infectious laryngotracheitis; lane 3, koi herpesvirus; lane 4, eel herpesvirus;
405 lane 5, ostreid herpesvirus (OsHV)-1 DNA; and lane M: 100 bp DNA. The
406 expected fragment size is 606 bp (arrow).

407

408 Fig. 3a. Histology of normal ganglion containing a peripheral layer of glial cells
409 (arrow) of the foot muscle of abalone (M). Scale bar = 25 μm.

410

411 Fig. 3b. *Haliotis diversicolor supertexta*. Histological examination of a ganglion
412 (G) associated with the kidney (K). Diffuse necrosis accompanied by infiltration
413 of hemocytes (arrow) and necrotic cells was prominent in the tissue.
414 Haemocytes surrounding neurilemma was evident. Scale bar = 25 μm.

415

416 Fig. 4. TEM examination of a purified Taiwanese abalone herpesvirus

417 presented at the interface of 40%~50% sucrose gradients. Negative staining

418 with PTA. Scale bar = 100 nm.

419

420 Table 1

421 Comparison of DNA polymerase amino acid sequence between abalone

422 herpesvirus from Taiwan 2004 and herpesviruses of other species.

423

Accession no.#	Description	Identity
HQ317456	Abalone herpesvirus Taiwan/2004	
HM631982.2	Abalone herpesvirus Victoria/AUS	99% (1923/1926)
YP_024639.1	Ostreid herpesvirus 1	30% (562/1856)
NP_065512.1	Alcelaphine herpesvirus 1	26% (47/180)
NP_076501.1	Bovine herpesvirus 4	26% (50/193)
NP_040211.1	Saimiriine herpesvirus 2	46% (18/39)
YP_438136.1	Ovine herpesvirus 2	25% (45/180)
YP_002321247.1	Caviid herpesvirus 2	34% (21/62)
NP_570750.1	Macacine herpesvirus 5	25% (47/185)
NP_047983.1	Ateline herpesvirus 3	46% (17/37)
|NP_066916.1	Gallid herpesvirus 3	26% (19/74)
NP_047983.1	Ateline herpesvirus 3	29% (26/91)
YP_004207849.1	Rodent herpesvirus Peru	23% (39/166)
NP_064160.1	Murid herpesvirus 2	29% (25/86)

NP_042605.1	Equid herpesvirus 2	24% (42/174)
NP_042931.1	Human herpesvirus 6	38% (20/52)
NP_612698.1	Panine herpesvirus 2	29% (24/84)
YP_068007.1	Macacine herpesvirus 4	23% (41/181)
NP_733857.1	Callitrichine herpesvirus 3	36% (13/36)
NP_944452.1	Psittacid herpesvirus 1	29% (18/62)
NP_116408.1	Tupaiid herpesvirus 1	34% (21/62)
NP_044863.1	Murid herpesvirus 4	23% (36/159)
NP_077443.1	Cercopithecine herpesvirus 9	33% (12/36)
YP_001096184.1	Cyprinid herpesvirus 3	42% (27/65)
YP_068007.1	Macacine herpesvirus 4	23% (40/177)

424 #: The accession numbers were taken from the NCBI's GenBank.

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428 Table 2

429 Summary of Case histories and results of histopathological and PCR

430 examinations.

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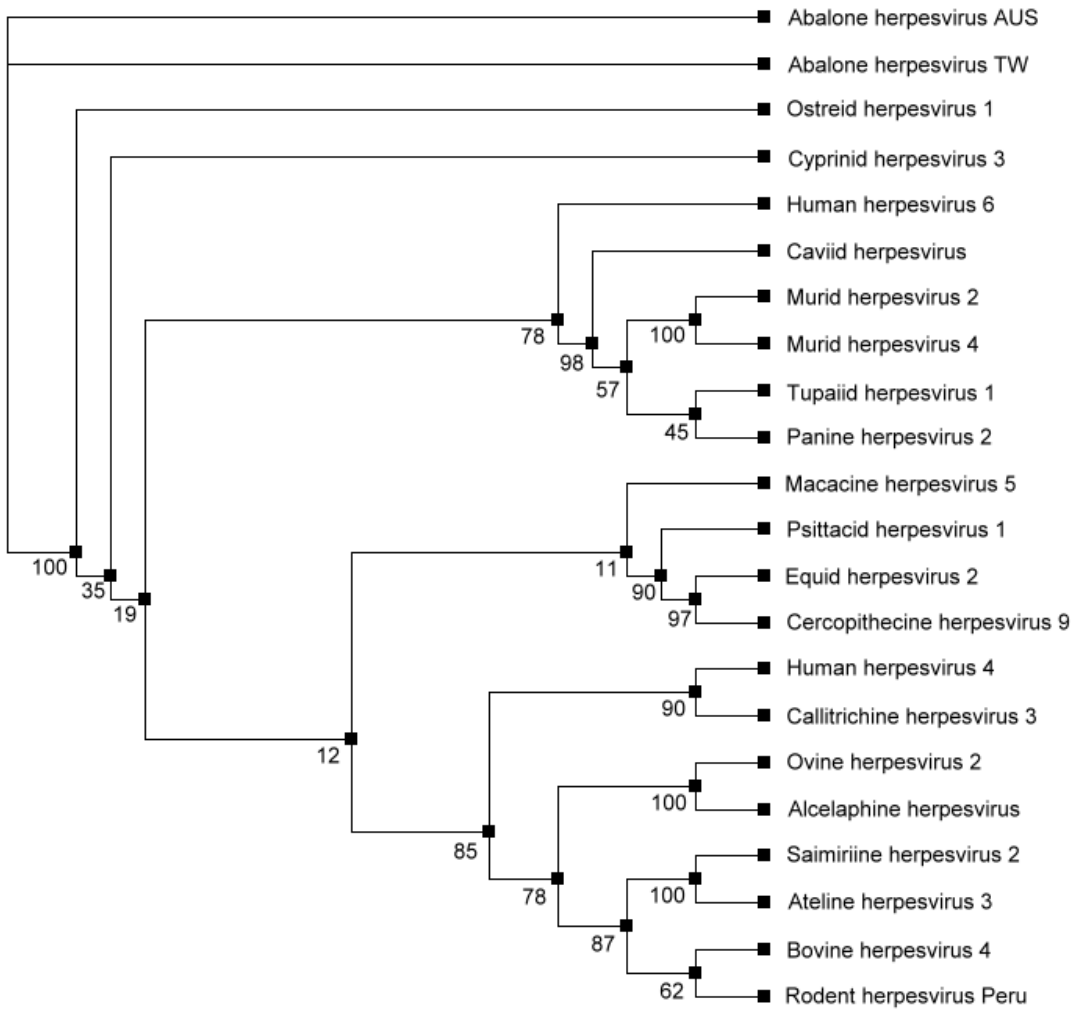
Case no.	Date	Size	History	Histopathology#	PCR
1	2003 Feb.	juvenile	mortality	-	-
2	2003 Feb.	adult	low harvest	+	+
3	2003 Nov.	juvenile	low harvest	-	-
4	2004 Feb.	adult	mortality	+	+
5	2004 Feb.	adult	mortality	-	-
6	2004 Mar.	adult	low harvest	+	+
7	2004 Mar.	adult	low harvest	+	+
8	2004 Nov.	adult	low harvest	-	-
9	2005 Jan.	adult	mortality	+	+
10	2006 July	juvenile	low harvest	+	+
11	2006 Dec.	adult	low harvest	-	-
12	2007 May	juvenile	low harvest	-	-
13	2008 Jan.	adult	low harvest	-	-

14	2008 Jan.	adult	mortality	-	-
15	2008 Oct.	adult	mortality	-	-
16	2008 Oct.	juvenile	low harvest	-	-
17	2008 Oct.	juvenile	low harvest	-	-
18	2009 Aug.	juvenile	low harvest	-	-
19	2009 Aug.	juvenile	low harvest	-	-

#: Results revealed that 32 percent (6 batches/19 batches) of PCR positive cases had nervous lesions under histopathology examination.

435 Fig. 1.



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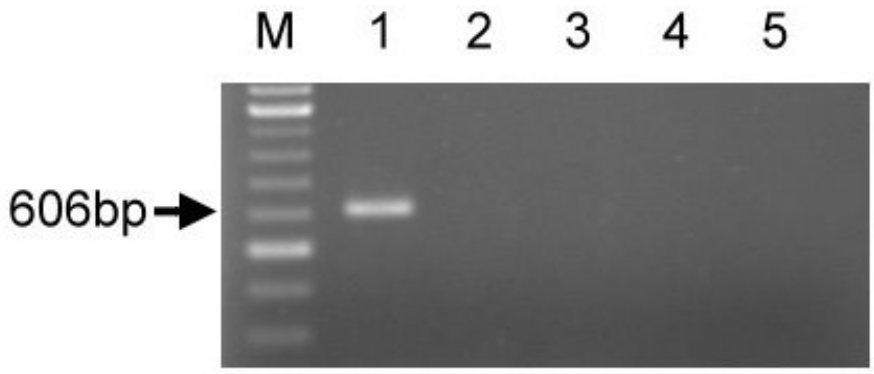
439 Fig. 2.



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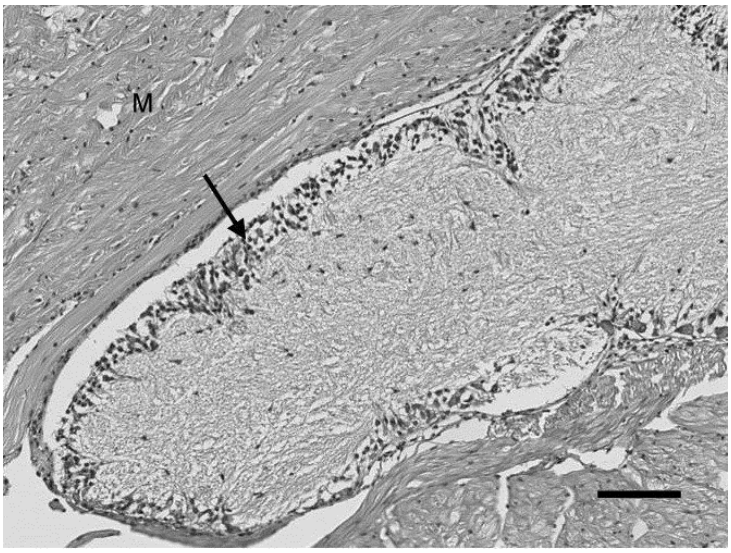
445 Fig. 3a.



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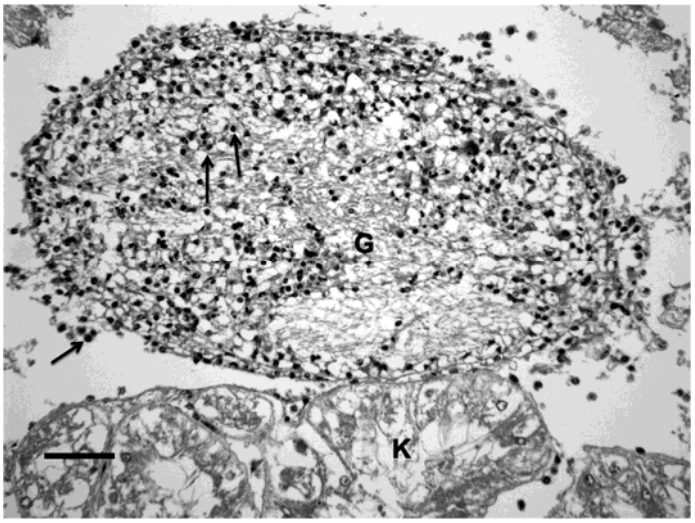
447 Fig. 3b.



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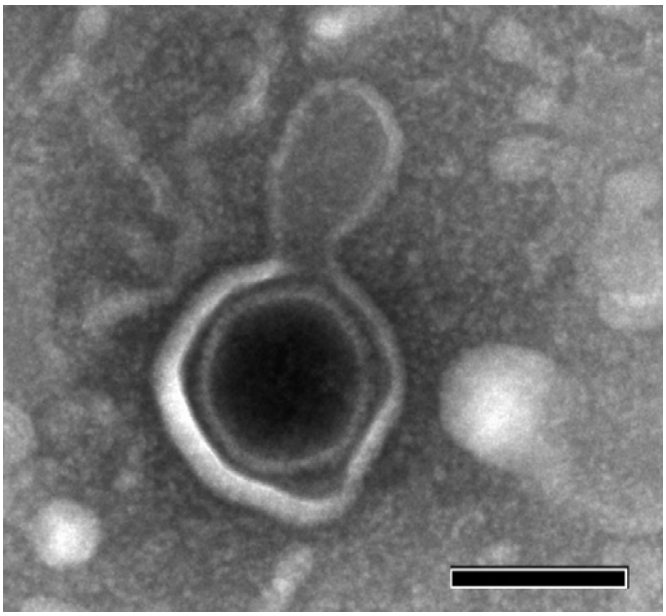
451 Fig. 4.



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