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Herpesvirus Infection in Tortoises (*Malacochersus tornieri* and *Testudo horsfieldii*)

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Abstract. Large numbers of pancake tortoises (*Malacochersus tornieri*) and Horsfield tortoises (*Testudo horsfieldii*) in three consignments imported into Japan died soon after arrival. Some tortoises in the first consignment were dead on arrival. Postmortem examination of two of the pancake tortoises and four of the Horsfield tortoises revealed necrotizing lesions of the oral mucosa in both species, primarily in the tongue. Eosinophilic to amphophilic inclusion bodies were visible in the nuclei of mucosal epithelial cells in the lesions. Similar inclusion bodies were observed in the liver, spleen, adrenal glands, stomach, lungs, kidneys, small and large intestines, pancreas, and cerebrum of the pancake tortoises and in the liver, spleen, and pancreas of the Horsfield tortoises. Electron microscopic examination of the cells containing inclusion bodies showed herpesvirus-like particles about 100 nm in diameter in the cytoplasm. Nested polymerase chain reaction analysis using a herpesvirus consensus primer method confirmed the presence of a characteristic herpesvirus base sequence in tissue from these lesions.

Key words: Herpesviridae infection; polymerase chain reaction; tortoises (*Malacochersus tornieri* and *Testudo horsfieldii*).

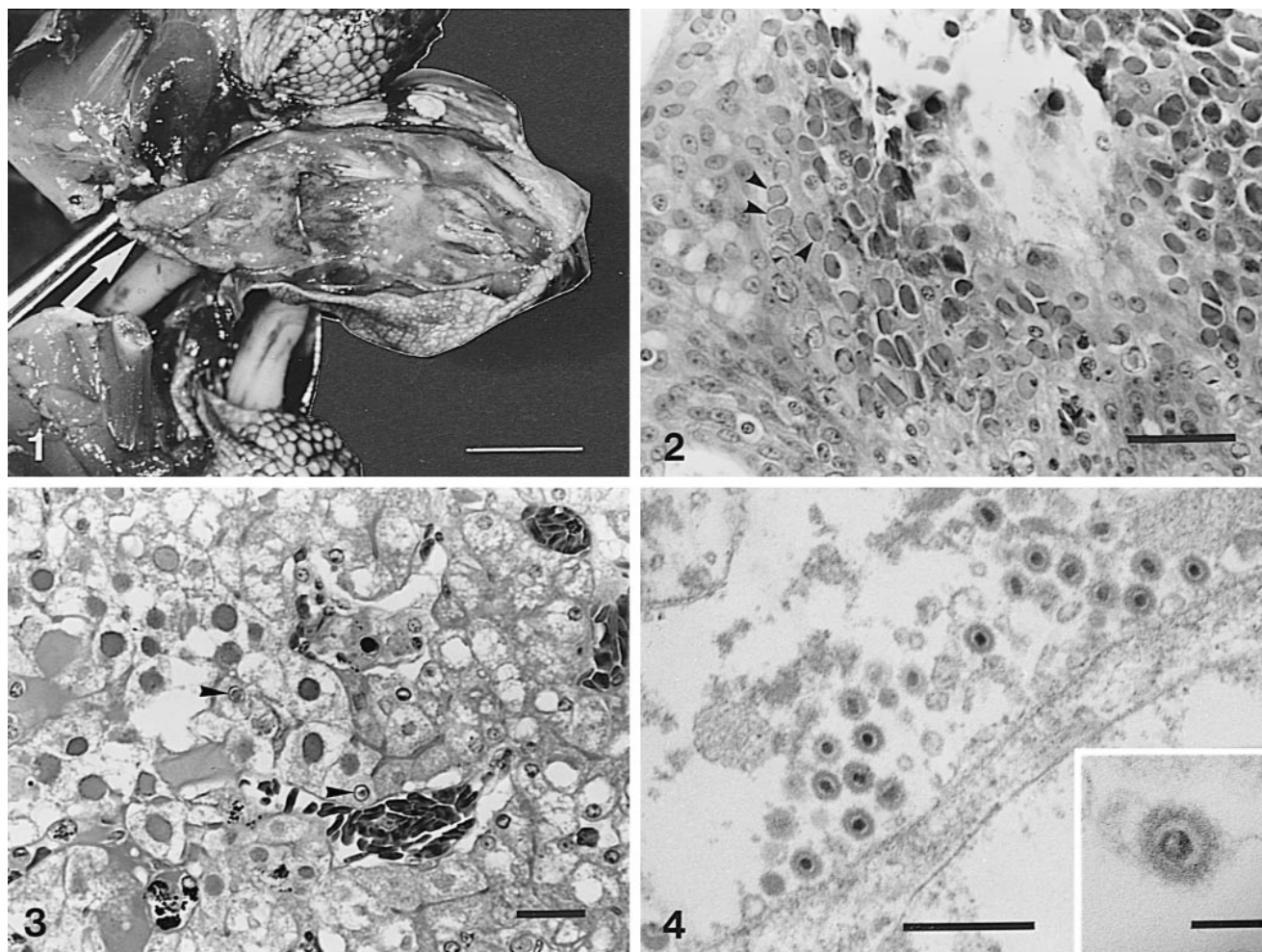


Fig. 1. Opened oral cavity; tortoise No. 3. The tongue (arrow) has a pseudomembrane. Swelling and redness were visible in the oral mucosa. Bar = 1.0 cm.

Fig. 2. Esophageal mucosa; tortoise No. 4. The mucosa is eroded, and inclusion bodies are seen occupying the nucleus of epithelial cells (arrowheads). HE. Bar = 70 μ m.

Fig. 3. Liver; tortoise No. 1. In degenerate and necrotic hepatocytes on the left, the nuclei are fully occupied by inclusion bodies. Other inclusion bodies of the bird's-eye type (arrowhead) are also seen in the nucleus of hepatocytes. HE. Bar = 30 μ m.

Fig. 4. Electron micrograph. Liver; tortoise No. 1. Cytoplasm of a degenerating hepatocyte is seen to contain numerous nonenveloped viral nucleocapsids, most of which contain an electron-dense body. Bar = 500 nm. *Inset:* Higher magnification of a virus particle from the oral lesion of tortoise No. 3. Lead citrate and uranyl acetate. Bar = 100 nm.

Herpesviruses have been detected in a wide range of vertebrates. In chelonians,^{1,11} herpesvirus infections have been identified in at least seven genera (*Gopherus*,⁹ *Chelonia*,¹⁰ *Geochelone*,⁴ *Testudo*,⁶⁻⁸ *Graptemys*,⁵ *Clemmys*,³ *Chrysemys*) and in five types of tortoises: Greek (*Testudo graeca*),^{7,8} Horsfield (*Testudo horsfieldii*),⁶ Hermann's (*Testudo hermanni*),^{6,7} desert (*Gopherus agassizii*),⁹ and Argentine tortoises (*Geochelone chilensis*).⁴ Diagnosis of herpesviral infection is generally based on the presence of intranuclear inclusion bodies, electron microscopic identification of viral particles,^{6,11} isolation of the virus, and/or immunohistochemical confirmation using anti-herpesvirus antibodies. In the present study, tissue samples from two species of tortoise involved in a mass outbreak of suspected herpesviral infec-

tion were subjected to light and electron microscopy. The usefulness of polymerase chain reaction (PCR) analysis using a herpesvirus consensus primer method¹² to diagnose herpesviral infection in tortoises was also assessed.

Of some 3,000 Horsfield tortoises imported into Japan in two consignments, some of those in the first consignment were dead on arrival, and about one-half of the remainder died soon after arrival. Approximately 50 pancake tortoises subsequently imported by another dealer were kept in the same pen that had housed the Horsfield tortoises. Thirty-five of these were quickly sold, but within 60 days of arrival, the remaining 15 tortoises developed signs of loss of appetite, cervical extension, labored breathing through the mouth, respiratory murmur, and oral and nasal discharge. The entire

tongue was covered with a reddish-white fibrinous coating. All 15 tortoises became debilitated and died.

Two of the 15 pancake tortoises (tortoises Nos. 1 and 2) and four Horsfield tortoises (tortoises Nos. 3–6) were submitted for light microscopical study. For electron microscopy, formalin-fixed tissues of tortoises Nos. 1 and 5 were counterfixed with osmium tetroxide, embedded in epoxy resin, and stained with lead citrate and uranyl acetate.

Formalin-fixed, paraffin-embedded tissue from the liver of tortoise No. 2 and from the oral mucosal lesions of tortoise No. 5 was used for PCR analysis. Renal tissue from a dog and liver tissue from a cat infected with canine and feline herpesvirus, respectively, were used as positive controls. For negative controls, reagent only and liver tissue from a bird infected with a nonherpesvirus were used. DNA was extracted, a nest of primers was established, and PCR was carried out according to the method previously described by VanDevanter et al.¹²

At necropsy, both species of tortoise showed severe necrotizing lesions of the oral mucosa, with a yellowish-white pseudomembrane extending from the caudal half of the tongue to the caudal pharynx and epiglottal area (Fig. 1). In tortoise No. 1, enlargement and ecchymosis of the liver were also observed, and in tortoise No. 2, pseudomembrane formation was seen in the stomach. Despite small differences in the distribution and severity of lesions, all six tortoises shared the following histopathologic features. The mucosal epithelium from the tongue to the pharyngolaryngeal region showed diffuse areas of degeneration and necrosis, with an accumulation of necrotic cellular debris and fibrin on the surface, which formed a pseudomembrane. The mucosa was infiltrated by mixed inflammatory cells. Polymorphic eosinophilic or amphophilic inclusion bodies were visible in the nuclei of mucosal epithelial cells, in some cases occupying the entire nucleus (Figs. 2, 3). Similar lesions were also present in the liver, spleen, esophagus, stomach, cerebrum, and lungs of tortoise No. 1; in the liver, spleen, adrenal glands, kidneys, duodenum, jejunum, colon, and pancreas of tortoise No. 2; in the spleen and esophagus of tortoise No. 3; in the liver and esophagus of tortoise No. 4; in the trachea of tortoise No. 5; and in the spleen and pancreas of tortoise No. 6.

Ultrastructurally, in the nuclei of epithelial cells, close to the nuclear membrane, unenveloped electron-lucent and immature virus particles that measured about 99 nm in diameter (sometimes with a small, electron-dense core) were observed in large numbers. Accumulations of an electron-dense, granulelike substance were also visible within the nucleus. In the cytoplasm, virus particles of about 117 nm in diameter, with a thick capsid and electron-dense core, were observed (Fig. 4).

Using PCR analysis, products from extracts from the liver of tortoise No. 2, oral mucosal tissue from tortoise No. 5, and the feline liver and canine kidney tissue from herpesvirus-infected animals used as positive controls were detected as a single band located at about 210 base pairs (Fig. 5). No products were detected in either of the negative controls used.

In all six tortoises, necrotizing lesions of the mucosal epithelium were accompanied by intranuclear inclusion body formation. Under the electron microscope, in the same lo-

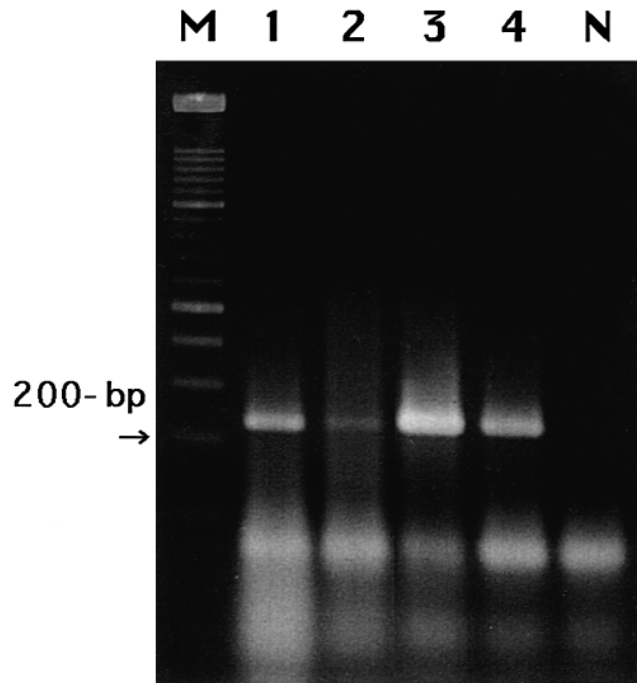


Fig. 5. Detection of herpesvirus DNA by the PCR method using the herpesvirus consensus primers. Lanes 1–4 show amplification of a 210-base pair (bp) herpesvirus DNA fragment. M = 100-bp scale molecular weight marker. Lane 1 = feline herpesvirus-positive control; Lane 2 = canine herpesvirus-positive control; Lane 3 = tortoise No. 2; Lane 4 = tortoise No. 4; N = reagent-only negative control.

cation, particles analogous to herpesviral particles were observed. These findings are in accord with those of previous reports of herpesviral infection in tortoises.^{1,4,6-9} In addition, PCR analysis using a herpesvirus consensus primer method¹² showed a characteristic herpesviral band. Diagnosis of herpesviral infection in the pancake and Horsfield tortoises was made on the basis of these observations.

In herpesviral infection in tortoises, oral lesions are reportedly most prevalent, extending to the upper digestive and respiratory tracts and especially to the glottis.^{1,4,6-9} Observations in the present study were in accordance with these reports and revealed that pancake tortoises are also susceptible to herpesviral infection.

The route of chelonian herpesviral disease transmission is unclear.¹¹ In the present study, lesions were centered in the oral cavity in all cases. Viral replication was also confirmed in the digestive tract, kidneys, and liver, especially in the pancake tortoises. Although vertical transmission cannot be ruled out, these findings strongly suggest horizontal transmission of the disease, with exudate-containing saliva, nasal secretions, urine, and feces as likely sources of viral contamination. Many of the Horsfield tortoises from the first consignment in the present study were already dead or were showing signs of disease at the time of import, which suggests that infection arose either before export or during transit. It is not known whether those pancake tortoises imported subsequently contracted the herpesviral infection before export, in transit, or in the facility in which they were main-

tained upon arrival in Japan. But herpesvirus has been isolated from cells of an apparently healthy iguana (*Iguana iguana*),² and long-term-resident turtles in an aquarium reportedly developed signs of disease after the introduction of other apparently healthy turtles.⁵ This suggests that tortoises with subclinical virus infection may have been present in the imported group. These tortoises may then have begun to show signs of disease as a result of transport stress or environmental change or may have passed on the infection to other, previously healthy tortoises that had become more susceptible to infection as a result of such stress or of environmental factors. Cases in which crowded conditions¹⁰ and environmental changes³ have been associated with the manifestation of herpesvirus infections have been reported in some reptiles. Whatever the case, more care must be taken in the administration and handling of chelonian imports.

VanDevanter et al. developed a consensus primer PCR method that amplifies a region of herpesviral DNA-directed DNA polymerase and that uses degenerate primers in a nested format.¹² By applying the assay to 22 species of herpesvirus (8 human and 14 animal viruses), they obtained PCR products for 21 of the 22 viruses. The developers concluded that consensus primer PCR targeted to herpesviral DNA polymerase may prove useful in the detection and identification of known herpesviruses in clinical samples.¹² Using this method, we also obtained PCR products from formalin-fixed, paraffin-embedded specimens of tissue lesions in tortoises. We therefore believe that PCR analysis, using the consensus primer method for herpesviral DNA, is a useful tool for diagnosing herpesviral infections in tortoises.

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