

Polymerase Chain Reaction (PCR) for the Detection of Herpesvirus in Tortoises

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ABSTRACT. A polymerase chain reaction (PCR)-based method using a herpesvirus consensus primer was assessed for the identification of herpesviral infections in tortoises. A single band of about 230 bp was detected in PCR products from two out of twenty swabs taken from the oral cavity, three out of three paraffin-embedded tissue sections from the liver (two cases) and oral mucosa (one case), and one out of two fresh tissue samples from the oral mucosa. Nucleotide sequencing of these PCR products indicated that the herpesvirus present in these tortoises might belong to the alphaherpesvirinae. PCR using swabs and biopsy tissues was a sensitive and highly specific method for the diagnosis of herpesviral infections in tortoises.

KEY WORDS: Alphaherpesvirus, PCR, tortoise.

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Herpesviral infections have been reported in a variety of tortoise species [2, 4, 6, 7, 9, 12, 13]. At least three large outbreaks have been documented [6, 7, 13] and in one case the morbidity level was reported to be more than 50% [7]. Several virological and serological surveys have also revealed a high prevalence of herpesvirus infections in tortoises [4, 8]. In a study of neutralising antibodies to tortoise herpesvirus in 617 samples, percentages of seropositivity in seven tortoise species ranged from 18.5 to 65.2% [4]. Herpesviral infection may thus be considered as one of the important epidemic diseases which can be life-threatening in captive tortoises. Detection of intranuclear inclusion bodies by light microscopy and of viral particles by electron microscopy [7, 12] have been the dominant methods used for the diagnosis of herpesviral infections in chelonians. Recently herpesviruses have been isolated from pharyngeal swabs in tortoises [8], and herpesviral DNAs extracted from tumor tissues in marine turtles have been amplified by PCR [10]. Virus isolation is another important method for the diagnosis of herpesviral infections in living chelonians, but usually it is difficult to obtain fresh materials for virus isolation, and to maintain the necessary cultured cell populations. Serological neutralization tests [4] can only be used in acute cases, or after an outbreak has begun. No adequate means for the detection of possible latent infections and virus carriers has yet been reported. In the present study, we tried to evaluate the usefulness of PCR-based methods for the diagnosis of herpesviral infection in tortoises.

In the present study, a consensus primer PCR method developed by VanDevanter *et al.* [14] was applied for the detection of diverse species of herpesvirus. The assay was based on a nested PCR format, and used two pairs of degenerate primers directed at the gene-encoding herpesviral DNA polymerase. PCR was first performed for DNA extracted from paraffin-embedded tissue from three species of tortoises (*Malacochersus tornieri*, *Testudo horsfieldii* and *Testudo graeca*). Each case examined had been histopathologically diagnosed as herpesviral infectious disease. PCR products extracted from the tissue of each of these three tortoises

yielded a discrete band about 230 bp in length (Fig. 1), which is the size to be expected for a segment of herpesviral DNA. To identify and characterize the segments obtained, segments from two of the three PCR products from the tortoise's tissues (in one case from oral mucosal tissue and in the other from liver tissue), and two PCR products from the swabs were cloned into pUC119 vectors and sequenced in both directions using an automated sequencer (373A, Applied Biosystems, Foster, CA, U.S.A.). Both segments were 181 bp in size excluding the primer-annealing regions, and were identical in nucleotide sequence. The sequence is available, using accession number AB027762, in the DDBJ/EMBL/GenBank DNA databases. The amino acid sequences determined showed close similarity to a part of the DNA polymerase from various herpesvirus species, especially human herpesvirus 1, with which the amino acid sequences shared 60% homogeneity, according to the protein database search. But the nucleotide sequences showed little homogeneity when compared with DNA databases using a BLAST2.0.7 program [1]. The nucleotide sequences obtained in the present study also showed only 57% homogeneity with those reported in the cases of green turtle fibropapillomatosis [10]. This fact suggests that the sequence determined in the present study may be ascribed to a new type of tortoise herpesviral DNA, which most likely belongs to the alpha subfamilies of herpesviridae [11], judging from its amino acid sequence.

The same method was then applied to determine the nature of DNA samples extracted from swabs of the oral or pharyngeal mucosa in 20 tortoises (3 *Malacochersus tornieri*, 6 *Testudo horsfieldii*, 1 *Geochelone pardalis* and 10 *Geochelone carbonaria*), which had been exposed to a colony affected with herpesviral infection. For extraction of DNA from the swabs, InstaGene-DNA purification matrix (Bio-Rad Laboratories, USA) was used. Amplified 230 bp-bands were seen in PCR products from 2 of 20 samples, both of which were from *Testudo horsfieldii* tortoises (Fig. 2). The same method was also applied to DNA samples extracted from fresh oral mucosal tissue in two other *Testudo horsfieldii* tortoises

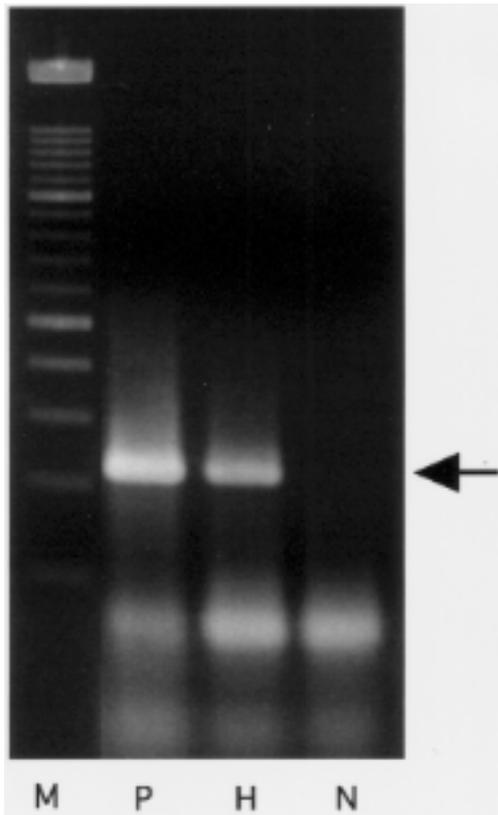


Fig. 1. Herpesviral DNA from paraffin-embedded sections of tortoise tissue (*Malacochersus tornieri*: P and *Testudo horsfieldii*: H) Consensus PCR method. PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide. The arrow designates an amplified band approximately 230 bp long. The size-marker lane is a 100-bp ladder. M = 100 bp scale molecular weight marker. N = negative control (reagent-only).

which had been exposed to the same colony of tortoises affected with herpesviral infection. An amplified 230-bp band was seen in PCR products from the tissue of one of these two tortoises. Neither of these two cases showed histopathological abnormalities characteristic of herpesviral disease, indicating that the tortoise in which the herpesvirus band was identified might be a carrier of the virus.

VanDevanter *et al.* [14] developed a consensus primer PCR method using degenerate primers in a nested format. Of 22 species in human and animal herpesviruses, PCR products from 21 herpesviruses were determined. VanDevanter *et al.* concluded that the consensus primer method targeted at herpesviral DNA polymerase may be useful for the detection of known herpesviruses using clinical samples. Quackenbush *et al.* [10], using the same kind of primers as those of VanDevanter *et al.* [14], amplified herpesviral DNA in tissue from fibropapillomatosis lesions in green turtles. Analysis of the nucleotide sequence showed that the 237-bp segment obtained was most similar to the DNA polymerases of bovine herpesvirus type 1 (BHV-1) and pseudorabies virus (PRV).

In the present study, analysis of the nucleotide sequence of the tortoise herpesviral DNA showed that the 230-bp sequence obtained in our study differed from that in a herpesvirus found in green turtle fibropapillomatosis [10], suggesting the possibility of a new type of tortoise herpesviral DNA, most likely belonging to the alpha subfamilies of herpesviridae [11], as mentioned above. The PCR method using a consensus primer may thus be another useful method for identifying herpesviral infections in tortoises, and especially effective in the diagnosis of early or latent infections in living tortoises. Only a tiny tissue sample obtainable by a non-invasive sampling technique is necessary for PCR study.

Necrotizing inflammation of the oral mucosa, or so-called "mouth rot", is one of a number of lesions well recognized in captive reptiles [3]. Possible causes of this lesion in European tortoises are thought to include viruses and/or Gram-negative bacteria including *Aeromonas* species [5]. In herpesviral

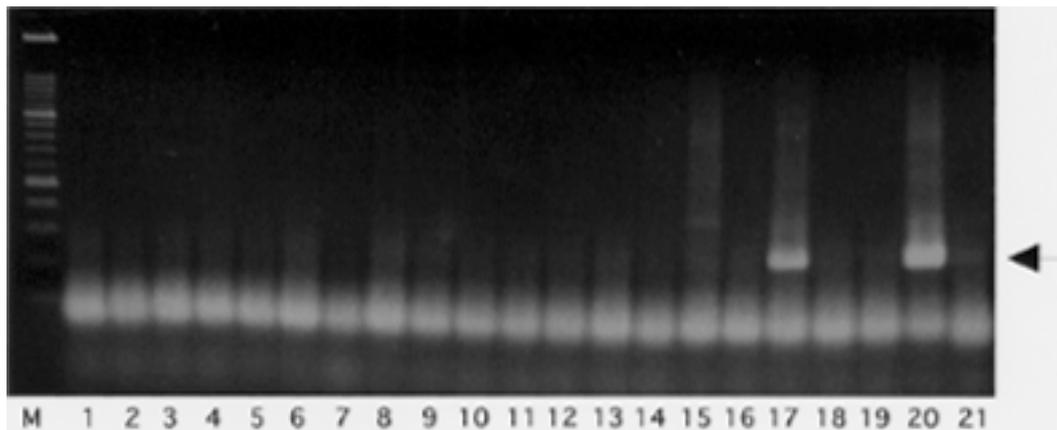


Fig. 2. See legend to Fig. 1 for the conditions of the PCR. The target amplified band is marked by an arrow. M= 100 bp scale molecular weight marker. Lane 1: negative control (reagent-only). Tortoise species: Lanes 2–11: *Geochelone carbonaria*, Lanes 12–14: *Malacochersus tornieri*, Lane 15: *Geochelone pardalis*, Lanes 16–21: *Testudo horsfieldii*. PCR analysis of swab extracts.

infection in tortoises, oral lesions are reported to be most common, usually involving to the upper digestive tract, especially the glottitis [6]. Quick diagnosis of the causes of "mouth rot" can facilitate proper treatment. Acyclovir has been reported to be effective for the treatment of herpesvirus infections [12]. Moreover, if the pathogen suspected can be identified, secondary opportunistic infection can be reduced.

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