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Reintroduction of Clinically Healthy Tortoises: The Herpesvirus Trojan Horse

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ABSTRACT: Reintroduction programs of tortoises are often implemented for the recovering of natural populations. Introduced animals should be free of known diseases and pathogens, such as herpesviruses; these are well known to cause latent infections that may be reactivated under certain conditions. Thus, clinically healthy chelonians may carry and shed herpesviruses, posing a threat to naïve populations. From August 2006 to August 2007, blood and oral swabs were collected from 92 clinically healthy tortoises (Testudinidae), and a serum-neutralization test was performed to detect antibodies against tortoise herpesviruses. Oral samples were tested by polymerase chain reaction (PCR) for the presence of the tortoise herpesvirus. Anti-herpesvirus antibodies were detected in 9% of the tested animals, whereas 16% of the oral samples were positive for tortoise herpesvirus using PCR. The relatively high percentage of clinically healthy tortoises shedding herpesviruses suggests that, before reintroduction of tortoises, herpesvirus testing should be mandatory and that both serology and PCR should be applied.

Key words: Herpesvirus, PCR, reintroduction, serology, tortoises.

Reintroduction programs for tortoises are implemented for the recovery of chelonian populations or for releasing illegally caught tortoises (Pedrono and Sarovy, 2000; Milinkovitch et al., 2003; Bertolero et al., 2007). These reintroduction programs carry with them the risk of introducing respiratory infections, such as mycoplasmosis. Respiratory diseases have been associated with population declines of tortoises in the United States (USFWS, 1994), and in one study, signs of respiratory tract disease were reported in 35% of the relocated gopher tortoises (*Gopherus polyphemus*; Ashton and Burke, 2007).

In addition to *Mycoplasma* spp., herpesviruses are of major importance in respiratory diseases in marine turtles and terrestrial tortoises (Pasmans et al., 2007). In tortoises with herpesvirus infection, clinical signs range from a mild conjunctivitis to a severe stomatitis-glossitis and pharyngitis. Diphtheritic plaques can be observed on the dorsal surface of the tongue and on the hard palate of infected tortoises. Frequently, a clear serous to a mucopurulent nasal discharge is present. Signs of central nervous system disease have also been reported in Mediterranean tortoises (*Testudo* spp.) with herpesvirus infection (Heldstab and Bestetti, 1989). Some tortoises will succumb to infection within days, whereas more resistant species will develop antibodies and recover after some weeks. Antibodies prevent the spread and multiplication of the virus but do not eliminate it. Thus, every tortoise that has survived a herpesvirus infection has to be considered a latent carrier of herpesvirus and a threat to a naïve population, even if the animals appear perfectly healthy and active. Because of this, previously captive animals could serve as a source of infection for wild populations.

Herpesviruses have been found in all European and several species of exotic tortoises (Blahak, 2000) and should, therefore, be considered a risk for reintroduction projects all over the world. To determine the relative disease risk of release of captive tortoises in the wild, a study was undertaken to evaluate the presence of tortoise herpesvirus and antibodies against tortoise herpesvirus in captive tortoises.

During the months of August 2006 to August 2007, blood and oral swabs were collected from 92 tortoises (Table 1). Sixty-four animals were clinically healthy

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TABLE 1. Presence of tortoise herpesvirus, as tested by polymerase chain reaction (PCR), and antibodies against tortoise herpesvirus in captive tortoises.

Species	No. of animals	Serology positive	PCR positive
Geochelone sulcata	3	0	0
Stigmochelys pardalis	3	0	0
Chelonoidis carbonaria	7	0	2
Astrochelys radiate	1	0	0
Acdabrachelys gigantea	1	0	0
Indotestudo elongate	2	0	0
Testudo graeca	14	4 (strain 770)	4
Testudo marginata	11	2 (strain 770)	4
Testudo hermanni boettgeri	34	1 (strain 1432)	4
Testudo hermanni hermanni	9	0	1
Testudo kleinmanni	1	0	0
Testudo horsfieldii	6	1 (strain 1432)	0

and belonged to 11 private collections in Belgium. Twenty-eight animals were individual cases with no clinical signs presented in the Clinic for Avian and Exotic Animal diseases (Faculty of Veterinary Medicine, Ghent University) for a prehibernation control. A basic history was obtained, and a physical examination was performed on each tortoise.

Blood was collected from the dorsal tail vein, and samples were immediately placed in heparin containers. Samples were stored refrigerated until centrifugation (1,300×G for 5 min). The plasma was removed and frozen at −20 C until further processing. Oral swabs were stored at −20 C in medium containing Dulbecco's Modified Eagle Medium (Gibco, Merelbeke, Belgium) with 1% penicillin/streptomycin.

To determine the presence of herpesvirus antibodies, a serum neutralization assay was performed on the plasma. Briefly, the plasma samples were tested using two serologically different strains of tortoise herpesvirus (strains 1432/94 and 770/95). The plasma samples were serial-diluted twofold and incubated with the viruses in microtiter plates. The concentration of the viruses was 100 median tissue-culture infective doses. The test was read after 6 days. The titer was determined as the highest dilution that was able to inhibit virus growth in cell culture. Three collections, with serologically positive tortoises at the first sampling, were retested after 6 mo. To detect the presence of herpesvirus, the polymerase chain reaction (PCR) described by Van Devanter et al. (1996) was performed on the DNA samples.

Results of the serologic and PCR examination are shown in Tables 1 and 2. Overall, in 10 (11%) of the 92 animals, antibodies against herpesvirus were shown. Seventeen (17%) of the tested animals were positive in the PCR.

Nine animals, clinically healthy at the time of sampling, had a previous history of respiratory signs. None of these nine animals tested positive for herpesvirus antibodies, but two (22%) of them were positive by PCR.

In a collection containing two marginated tortoises (*Testudo marginata*), one Greek tortoise (*Testudo graeca*), one eastern Hermann's tortoise (*Testudo hermanni boettgeri*), one red-footed tortoise (*Chelonoidis carbonaria*), and one African spurred tortoise (*Geochelone sulcata*), the *T. graeca* tested antibody positive for the herpes tortoise virus strain 770. The same tortoise and one *T. marginata* were positive in the PCR test. Six months later, after hibernation, the *T. graeca* showed yellow plaques in its mouth and was still antibody and PCR positive; the *T. marginata* and all other tortoises in this collection

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TABLE 2. Summary of the serology and polymerase chain reaction (PCR) results of 92 clinically healthy tortoises tested for tortoise herpes virus.

Serology	PCR	No. of animals
Positive	Positive	5
Negative	Positive	10
Positive	Negative	3

tested antibody and PCR negative for this virus.

In a collection of three *Testudo horsfieldii*, one of the animals tested seropositive for strain 1432. The animal tested negative by PCR. In the retest, 6 mo later, none of the other animals had seroconverted.

In one collection, two *T. graeca* and two *T. marginata* were suspect for herpesvirus antibodies and were positive by PCR. After 6 mo, antibody titers increased in these animals and they remained positive by PCR. At both sampling times, these animals were clinically healthy.

This study shows that 16% of the clinically healthy tortoises randomly sampled for the presence of herpesvirus were shedding herpesvirus through oropharyngeal secreta as shown by PCR. Combined with the serologic results, 20% of the animals were herpesvirus positive. This remarkably high percentage underlines that, before a tortoise is released in the wild, testing for herpesvirus should be mandatory. Because only six animals were positive by both PCR and antibody tests, compared with 12 tortoises that tested positive in a single test, both serologic and PCR screening are highly recommended before releasing tortoises in the wild.

Several (3%) of the tested tortoises were negative by PCR reaction but antibody positive. These seropositive tortoises are considered to be latent carriers and, if released, could pose a threat to a naïve population even if not actively shedding the virus. Because of stress, or after hibernation, the virus can be reactivated, shed, and spread to naïve animals (Blahak 2000; Une et al., 2000; Blahak 2006).

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