Enzyme-Linked Immunosorbent Assay for Detecting Herpesvirus Exposure in Mediterranean Tortoises (Spur-Thighed Tortoise [*Testudo graeca*] and Hermann's Tortoise [*Testudo hermanni*])†

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An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies to a herpesvirus associated with an upper respiratory tract disease in Mediterranean tortoises [spur-thighed tortoise (*Testudo graeca*) and Hermann's tortoise (*Testudo hermanni*)]. This serodiagnostic test was validated through a hyperimmunization study. The mean of the A_{405} readings of the plasma samples collected at time zero of the hyperimmunization study plus three times the standard deviation was used as the cutoff for seropositivity in tortoises. ELISA results were compared to serum neutralization (SN) values for the same samples by using the McNemar test. The results obtained by SN and ELISA were not significantly different ($P > 0.05$). This new ELISA could be used as an important diagnostic tool for screening wild populations and private and zoo collections of Mediterranean tortoises.

Herpesviruses are well-known infectious agents with remarkably wide host ranges. Starting in 1975 (33), several reports have documented the presence of herpesvirus-like particles in land tortoises and freshwater and marine turtles (5, 7–9, 11, 15, 17, 19–23, 27–29, 30, 38). Recent investigations have revealed an association between the presence of herpesvirus and an upper respiratory tract disease in Mediterranean tortoises [spur-thighed tortoise (*Testudo graeca*) and Hermann's tortoise (*T. hermanni*)] (5, 8, 9, 17, 20, 23, 27–29, 30).

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 with herpesvirus infection (17).

Eosinophilic intranuclear inclusions, often seen in multiple tissues, are particularly prominent in tortoises with pharyngitis and glossitis. As seen with transmission electron microscopy, inclusions consist of numerous viral particles. The morphology and morphogenesis have been used to categorize the virus as herpesvirus.

A diagnosis of herpesvirus infection is often made based solely upon light or electron microscopy findings. Antemortem diagnosis can be made using biopsy specimens of oral lesions. A serum neutralization (SN) test has been developed but is

limited in its application since it is only available in a few research laboratories in Europe (10). In addition, time is a limiting factor with the SN test. Ten to 14 days are required to obtain the final reading and a laborious procedure is required. An easier and faster but equally reliable serodiagnostic test is needed. In this report, we describe the development of an enzyme-linked immunosorbent assay (ELISA) that can be used to monitor the exposure to herpesvirus of free-range, private, and zoo collections of tortoises.

MATERIALS AND METHODS

Viruses. Herpesvirus isolates HV1976 and HV4295/7R/95 were used as antigens in the ELISAs and immunoblotting. HV1976 was isolated from a captive Hermann's tortoise from the United States (Washington), while HV4295/7R/95 was isolated from a captive Hermann's tortoise in Germany during a herpesvirus outbreak in a private collection (27).

Antigen preparation for ELISA. The herpesvirus isolates were grown in terape heart cell monolayers (TH-1; ATCC-CCL 50 Sub-line B1; American Type Culture Collection, Rockville, Md.) in T-150 plastic flasks with ventilated caps (Corning, Rochester, N.Y.) for use as ELISA antigens. The TH-1 cells were grown in Dulbecco's modified Eagle's medium F12 (Gibco BRL, Grand Island, N.Y.) with 5% fetal bovine serum (Sigma, St. Louis, Mo.), gentamicin (60 mg/liter) (Sigma), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter), and amphotericin B (300 µg/liter) (ABAM; Sigma). Infected cell monolayers were scraped and collected with the culture medium after 7 days. The cell suspension was then frozen at -80°C and thawed three times, and the supernatant was clarified by centrifugation at $4,500 \times g$ for 30 min at 4°C . The clarified supernatant was centrifuged at $53,664 \times g$ at 4°C for 3.5 h to pellet the virus. The resuspended pellets were purified on 20-to-60% sucrose continuous gradients in TNE (100 mM Tris, 2 M NaCl, 10 mM EDTA, pH 7.4) and centrifuged at $156,194 \times g$ for 2 h at 4°C . A total of nine fractions of about 1 ml each were harvested from each gradient. The amount of the virus contained in each of the fractions was assessed by three methods: (i) a protein assay (Bio-Rad, Hercules, Calif.); (ii) an evaluation of the cytopathic effect (CPE) titer in TH-1 cells cultured in 96-well plates (according to the method of Spearman and Karber [18]); and (iii) negative-staining electron microscopy. The fractions richest in virus (assessed as described above) were used for the production of two rabbit polyclonal antibodies (raised against HV4295/7R/95 and HV1976) and for the hyperimmunization study. The antigen used in the ELISA was selected as well

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from the gradient fractions richest in virus but treated differently from above. These fractions were resuspended in 10 volumes of TNE and repelleted by centrifugation at $53,664 \times g$ for 3.5 h at 4°C. The pellet was then resuspended in phosphate-buffered saline (PBS; pH 7.2) and stored at -80°C.

Antigen preparation for immunoblotting. TH-1 cells infected with either HV4295/7R/95 or HV1976 and uninfected TH-1 cells were used for immunoblotting. Infected cells were harvested when they showed 80 to 90% CPE, while uninfected cells were harvested at confluency. The cell monolayer was washed with PBS and cells were scraped. Cells were then harvested, centrifuged at $250 \times g$, and resuspended in 1 ml of $1 \times$ lysis buffer solution (0.04% NP-40, 0.04% Tween 20, 0.01% sodium dodecyl sulfate). The final protein concentration was determined and the antigen preparations were stored at -80°C.

Tortoise plasma samples. Blood samples were collected from tortoises in a rehabilitation facility in France and from the tortoises in the hyperimmunization study described below. Blood was placed in lithium-heparinized plastic tubes (Becton Dickinson, Rutherford, N.J.), and plasma was obtained by low-speed centrifugation of the tubes at $350 \times g$ in a TRIAC centrifuge (Clay Adams Becton Dickinson and Company, Parsippany, N.J.) for 5 min at room temperature. The plasma samples were stored at -80°C.

Samples from Mediterranean tortoises in France. Plasma samples were collected from a group of 175 captive Mediterranean tortoises in France. All samples were previously tested by SN using three herpesvirus isolates recovered from Mediterranean tortoises in Europe (HV770/95, HV2245/92, and HV17/96 [K. Mathes, personal communication]). The tortoises were considered seropositive when their plasma successfully neutralized at least one of the herpesvirus isolates (27). The tortoises were considered seronegative when no neutralization activity was detected against any of the isolates used in the test.

Samples from hyperimmunized tortoises. Five adult male spur-thighed tortoises that were SN negative for exposure to tortoise herpesvirus and culture negative for tortoise herpesvirus were purchased from a reptile dealership and used in the hyperimmunization study. Seven days before hyperimmunization, the tortoises were separated into individual pens. The tortoises were randomly assigned to one of two treatment groups: (i) Group 1 (tortoises no. 1 and 3) were hyperimmunized with HV4295/7R/95 (passages 19 to 26) (European isolate) ($n = 2$); (ii) Group 2 (tortoises no. 2 and 4) were hyperimmunized with HV1976 (passages 14 to 15) (American isolate) ($n = 2$). The remaining tortoise served as a hyperimmunization control. For each hyperimmunization group, 15,000 50% tissue culture infection doses (TCID₅₀) in 0.4 ml of PBS was delivered either intramuscularly (i.m.) (tortoises no. 3 and 4) or intranasally (i.n.) (tortoises no. 1 and 2). Tortoise no. 1 was delivered an additional dose of virus (15,000 TCID₅₀) 3 months after the first hyperimmunization with HV4295/7R/95 because ELISA or SN detected no seroconversion after the first hyperimmunization. The control tortoise (no. 5) received 0.4 ml of PBS both i.n. and i.m. Blood samples were obtained immediately before virus administration (time zero) and subsequently every 2 weeks for a total of 17 and 15 weeks, respectively, for the tortoises infected i.n. (no. 1 and 2) and i.m. (no. 3 and 4). Starting at week 18 or 16 after i.n. or i.m. infection, respectively, the tortoises were hibernated for 6 weeks. Following hibernation, blood samples were obtained every 4 to 5 weeks. Plasma was tested for the presence of neutralizing antibodies and with the ELISA as described below.

Virus isolation was attempted from each tortoise in the hyperimmunization study. Briefly, starting 7 days after hyperimmunization and every 2 weeks thereafter until the hibernation period (17 or 15 weeks after i.n. or i.m. hyperimmunization, respectively), viral isolation was attempted from nasal flushes and pharyngeal swabs. After collection, the samples were stored in cell culture medium and kept on ice until delivered to the laboratory. Subsequently, the medium was filtered with 0.45- μ m-pore-size syringe filters (Fisher Scientific, Pittsburgh, Pa.), added to fresh TH-1 confluent monolayers in cell culture flasks, and incubated at 28°C. After 1 h, the medium was discarded and fresh medium containing 5% fetal bovine serum was added. Cells were monitored daily for CPE. One blind passage was performed after 7 days. No CPE detection after 2 weeks was considered a negative result.

Hybridoma preparation. Mouse monoclonal antibodies (MAbs) against the Mediterranean tortoise immunoglobulin Y (IgY) heavy chain [IgY(H)] (13, 25, 39) were produced by following a standard hybridoma protocol (24, 26, 35) in the University of Florida Hybridoma Core Laboratory. IgY was purified from Mediterranean tortoise plasma (1). The MAb specific for Mediterranean tortoise IgY(H) was designated HL1546. MAb HL1546 was isotypized as IgG1 by using a mouse MAb isotyping kit (Iso Detect mouse isotyping kit; Stratagene, La Jolla, Calif.). The HL1546 MAb was purified on a protein G affinity column (Hi-Trap Protein G Sepharose High Performance; Pharmacia LKB, Uppsala, Sweden) and biotinylated with EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, Ill.) (12).

ELISA procedure. Each well of a microtiter plate (Maxisorp F96; Nunc, Kamstrup, Denmark) was coated with 50 μ l of antigen at a concentration of 5 μ g/ml in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% NaN₃ (PBS-A), and the plates were incubated at 4°C overnight (34). The wells were washed four times with PBS-A containing 0.05% Tween 20 (PBS-T) and then blocked with PBS-A containing 5% nonfat dry milk per well at room temperature for 60 min or at 4°C overnight. After four more washes, 50 μ l of plasma diluted 1:25 in blocking buffer was added in duplicate (for the end point titration, plasma was diluted up from 1/12.5 to 1/25,600), and the plates were incubated at room temperature for 60 min. The wells were washed again, and 50 μ l of the biotinylated MAb HL1546 diluted at a final concentration of 1 μ g/ml in PBS-A was added to the appropriate wells. The wells were incubated at room temperature for 60 min and washed. Fifty microliters of alkaline phosphatase (AP)-conjugated streptavidin (1:5,000 dilution in PBS-A; Zymed Laboratories, Inc., San Francisco, Calif.) was added to each well, and the plates were incubated at room temperature for 90 min. After washing the wells four times with PBS-T, 100 μ l of *p*-nitrophenyl phosphate disodium (1 mg/ml prepared in 0.01 M sodium bicarbonate buffer [pH 9.6] containing 2 mM MgCl₂; Sigma) was added to each well and the plates were incubated in the dark at room temperature for 90 min. The A_{405} of each well was measured in an ELISA plate reader (EAR 400 AT; SLT-Lab Instruments, Salzburg, Austria).

Initially, an SN-negative plasma sample from a Mediterranean tortoise from France served as a negative control. Pooled plasma from five Mediterranean tortoises that were SN positive for herpesvirus antibodies and had clinical signs consistent with herpesvirus infection served as the positive control. Subsequently, plasma collected at time zero from the herpesvirus SN-negative and culture-negative clinically healthy spur-thighed tortoises from the hyperimmunization study (described above) served as the negative control "gold standard" for determining the cutoff for positive samples. Positive and negative controls were included on each plate to determine interplate variation. Antibody level was expressed as the A_{405} of the sample. Plasma was considered positive for tortoise herpesvirus-specific antibody when the A_{405} of the samples was greater than the mean of the A_{405} plus three standard deviations of the negative control plasma (from the tortoises in the hyperimmunization study at time zero) (16).

SN assays. Plasma collected from the Mediterranean tortoises in the hyperimmunization study was tested for the presence of neutralizing antibodies against both the HV4295/7R/95 and HV1976 herpesvirus isolates, following an established protocol (4). The plates were read after 10 to 14 days and the neutralizing titer was defined as the highest dilution that did not show any CPE. Additionally, the 175 plasma samples from France were tested in another laboratory in Europe (Staatliches Veterinaruntersuchungssamt, Detmold, Germany) by using a similar technique with three different herpesvirus isolates (HV2245/92, HV770/95, and HV17/96) (K. Mathes, personal communication).

A random sample of five SN-positive and five SN-negative samples tested in Europe were also tested for SN using the HV4295/7R/95 and HV1976 herpesvirus isolates, utilizing the procedure described above.

Rabbit hyperimmune sera. Rabbit hyperimmune sera were raised against herpesvirus isolates HV4295/7R/95 and HV1976. Two specific-pathogen-free rabbits were immunized against HV4295/7R/95 and two other specific-pathogen-free rabbits were immunized against HV1976. An initial dose of 100 μ g of purified viral antigen was administered to each rabbit. An equal amount of purified viral antigen was used to booster the rabbits in the study for a total of eight times (every other week for a total of 16 weeks). The immunization procedure was performed by Pel-Freez Biologicals (Rogers, Ark.).

Immunoblotting. Hyperimmune tortoise plasma and rabbit sera raised against either the HV4295/7R/95 or HV1976 herpesvirus isolates were tested for their ability to detect viral proteins in an immunoblotting format. Infected and uninfected TH-1 cell lysates were used as antigens.

Samples (80 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and then transferred to a nitrocellulose membrane (0.2 μ m) (Bio-Rad) by standard methods (14, 37). The nitrocellulose was blocked with PBS containing 5% nonfat dry milk, washed three times with PBS, and placed into a Fisher nylon cutter (Fisher, Pittsburgh, Pa.) to obtain 25 separate strips (70 by 5 mm). The hyperimmune plasma diluted in blocking buffer (diluted 1/25 for tortoise plasma and 1/500 for rabbit plasma) was loaded in each channel of the incubation tray and incubated at 28°C (tortoise plasma) or 37°C (rabbit sera) overnight on a Hematek shaker (Miles Laboratories, Elkhart, Ind.). The biotinylated mouse anti-Mediterranean tortoise immunoglobulin MAb HL1546 (diluted to 1 μ g/ml in PBS-A) was then added into each channel and incubated for 120 min at room temperature on a shaker. Subsequently, the nitrocellulose strips were incubated either with AP-streptavidin (diluted 1/5,000 in PBS-A; Zymed Laboratories, Inc.) (tortoise) or with AP-conjugated labeled goat anti-rabbit antibody (1/10,000; Sigma) (rabbit) at room temperature for 60

TABLE 1. Summary of ELISA and SN results for the French tortoise plasma samples^a

ELISA result	SN result ^b		Total
	+++	---	
++	34	3	37
±	1	1	2
±	0	4	4
=	0	132	132
Total	35	140	175

^a The symbol +++ indicates a positive SN test against all three tested herpesvirus isolates. The symbol --- indicates a negative SN test against all three tested herpesvirus isolates. The symbol ++ indicates a positive ELISA result against both herpesviruses tested. The symbol ± corresponds to a sample positive only against isolate HV4295/7R/95 by ELISA. The symbol ± corresponds to a sample positive only against the HV1976 isolate. The symbol = indicates a negative ELISA result against both herpesviruses tested.

^b The SN titer against this isolate (HV17/96) was not available for sample K20.

min. The blot was finally developed with substrate buffer (0.1 M Tris HCl, 1 mM MgCl₂ [pH 8.8]) containing 44 µl of nitroblue tetrazolium chloride (NBT) and 33 µl of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) per 10 ml of substrate buffer. NBT and BCIP were obtained commercially (Promega, Madison, Wis.).

Statistical analysis. The McNemar test for nonindependent samples was used for comparing the ELISA results to those of the SN test (3). A *P* value of <0.05 was considered significant.

RESULTS

Determination of ELISA parameters. An end point titration curve was plotted for antiherpesvirus antibodies against the two herpesvirus strains (HV4295/7R/95 and HV1976) used in this study. A final plasma dilution of 1:25 was considered optimal for the ELISA based upon the A_{405} values obtained for the positive and negative controls.

The mean A_{405} (0.2014) and the standard deviation (0.097) of the plasma samples collected from the spur-thighed tortoises in the hyperimmunization study at time zero were used in determining the cutoff for the positive samples in the ELISA. The cutoff was the mean A_{405} plus three standard deviations (16). This resulted in a final cutoff A_{405} value of 0.48.

Evaluation of plasma samples by ELISA and SN. A total of 175 plasma samples from Mediterranean tortoises in France, previously tested for the presence of neutralizing antibodies, were tested with the ELISA. Twenty percent of the samples tested positive with the SN and 80% tested negative. When the same samples were tested by ELISA, 24.6% tested positive either for the European (HV4295/7R/95) or the American (HV1976) herpesvirus isolate, while 21.1% tested positive for both the European and American herpesvirus isolates. When tested against both the American and European isolates, 75.4% were negative. Table 1 summarizes the SN and ELISA results for the plasma samples obtained from the tortoise collection in France. Arbitrarily, a sample was considered positive with the ELISA when it was positive using both ELISA anti-

The samples positive by both ELISA and SN had mean A_{405} values of 1.57 and 1.53 when tested against the HV4295/7R/95 and HV1976 viral isolates, respectively ($n = 34$ samples). The mean A_{405} values for the ELISA-negative samples were 0.172

FIG. 1. Western blot comparison of the antigens of HV4295/7R/95 and HV1976 using hyperimmune tortoise plasma. Lanes 1 to 3 were developed using tortoise plasma raised against HV4295/7R/95; lanes 4 to 6 were developed using tortoise plasma raised against HV1976. Lanes 1 and 4, HV1976; lanes 2 and 5, HV4295/7R/95; lanes 3 and 6, TH-1 cell lysate. Few antibody reactions are detectable, and a very faint band of approximately 20 kDa is observable in the infected cells when stained using anti-HV1976 polyclonal tortoise sera. An antibody reaction with the same molecular mass was also detectable in the HV4295/7R/95-infected cells by using anti-HV4295/7R/95 polyclonal tortoise sera. The arrows show the approximate molecular masses of the different antibody reactions.

and 0.168 ($n = 132$ samples) when tested against the HV4295/7R/95 and HV1976 viral isolates, respectively. These values excluded the samples that were positive by ELISA and negative by SN ($n = 3$) and the samples that were positive by ELISA for only one of the viral antigens ($n = 6$) in the assay (Table 1).

For determining the epidemiological parameters of specificity, sensitivity, positive and negative predictive values (PPV and NPV, respectively), and prevalence (3), the SN test was considered the gold standard and the 34 samples positive for both of the antigens by ELISA were considered true positives. The specificity of the test was 98% and the sensitivity was 97%. The PPV was 92% and the NPV was 99%. Seroprevalence and prevalence were 21 and 20%, respectively.

Immunoblot evaluation of viral antigens. Infected and uninfected TH-1 cells were evaluated by immunoblotting. As shown in Fig. 1, hyperimmune tortoise plasma raised against either HV1976 or HV4295 detected proteins spanning from about 20 kDa to more than 250 kDa. Two bands of 140 and 75 kDa in the uninfected TH-1 cells, which were faintly visible in the virus-infected cells, were considered nonspecific because they were recognized by both the secondary antibody HL1546 in the absence of primary antibody and the preexposure tortoise plasma (data not shown). The hyperimmune plasma against both viruses recognized a band at approximately 200 kDa. A band greater than 250 kDa was detectable by both hyperimmune plasma samples, but only in the HV4295/7R/95-infected cells. A very weak band at about 20 kDa was detectable in both the HV1976- and HV4295/7R/95-infected cells when the anti HV1976 tortoise polyclonal plasma was used. A similar but even weaker antibody reaction was also detectable in the HV4295/7R/95-infected cells when the anti-HV4295/7R/95 tortoise polyclonal plasma was used.

FIG. 2. Western blot comparison of the antigens of HV4295/7R/95 and HV1976 using rabbit polyclonal antibodies against HV1976 (STF) (lanes 1, 2, and 3) and HV4295/7R/95 (FOF) (lanes 4, 5, and 6). Lanes 1 and 4, HV1976; lanes 2 and 5, HV4295/7R/95; lanes 3 and 6, TH-1 cell lysate. Several low-molecular-mass antibody reactions can be seen in lanes 1, 2, 4, and 5 and are absent in lanes 3 and 4 (uninfected TH-1 cells). FOF serum was raised against HV4295/7R/95, while STF was raised against HV1976. The arrows show the approximate molecular masses of the different bands.

Rabbit polyclonal anti-HV1976 and anti-HV4295/7R/95 sera reacted against bands spanning from about 20 to 35 kDa for both viral isolates (Fig. 2). Anti-HV1976 showed a stronger reaction against these bands than did anti-HV4295/7R/95. A low-molecular-mass band (approximately 16 kDa) was detected with anti-4295/7R/95 but not with anti-HV1976. Both sera showed a strong reaction against many proteins from both isolates spanning from about 50 kDa to more than 250 kDa, with two major bands recognizable at approximately 75 and 140 kDa. The former was very weakly recognized in the uninfected TH-1 cells.

Both the tortoise hyperimmune plasma and rabbit antiherpes polyclonal antibodies reacted against a protein of approximately 20 kDa.

Hyperimmunization study. (i) Clinical findings. All tortoises ($n = 4$) hyperimmunized with HV4295/7R/95 or HV1976, either i.m. or i.n., showed clinical signs consistent with herpesvirus infection. The clinical signs were more severe when the tortoises were hyperimmunized i.m., while the tortoises hyperimmunized i.n. showed milder conjunctivitis with less ocular discharge. No other clinical signs were observed. Only one of the tortoises with clinical signs (tortoise no. 1) remained seronegative for antiherpesvirus antibodies by both ELISA and SN.

(ii) Serology. The plasma from tortoises hyperimmunized with HV1976 (no. 2 and 4) had an A_{405} reading of >0.48 at 4 weeks after hyperimmunization when tested using HV4295/7R/95. When these samples were tested against HV1976, only tortoise no. 4 was positive (for tortoise no. 2, the A_{405} against HV1976 was 0.46) (Fig. 3A and B). At that time point, neither of the two tortoises hyperimmunized with HV4295/7R/95 had an A_{405} of >0.48 . Seroconversion was detected in tortoise no. 3 at 7 weeks posthyperimmunization. At this time, tortoises no. 2, 3, and 4 showed an A_{405} of >0.48 when both viral antigens (HV4295/7R/95 or HV1976) were used. Tortoise no. 1 never seroconverted, either by ELISA or by SN. Beginning at 9 weeks postexposure, the A_{405} readings for plasma samples

from tortoises no. 2, 3, and 4 were never <1.3 when tested against either viral antigen. Table 2 summarizes the mean A_{405} readings obtained for the tortoises in the hyperimmunization study.

A high degree of cross-reactivity was detected in all the hyperimmune plasma for both isolates (HV1976 and HV4295/7R/95) used as antigens in the ELISA and SN test.

SN results paralleled the ELISA findings (Table 3 [SN results] and Fig. 3A and B [ELISA results]). For all the tortoises in the hyperimmunization study except for tortoise no. 1, neutralizing antibodies directed against both isolates were detected. SN titers were detected after 9 weeks posthyperimmunization for tortoise no. 2. However, no neutralizing activity was ever detected in the plasma of tortoise no. 1.

Both of the tortoises hyperimmunized i.m. were positive for the presence of SN antibodies. Plasma from tortoise no. 4 was SN positive against both isolates, while plasma from tortoise no. 3 was SN positive only for the homologous isolate (HV4295/7R/95) up to 21 weeks posthyperimmunization. After 25 weeks postimmunization, the first SN titer for the heterologous isolate (HV1976) was detected (Table 3).

The SN results obtained for the tortoises in France reflected the same immunological cross-reactivity pattern that was seen in the hyperimmunization study. All SN-positive samples collected in Europe reacted against all three isolates used in the test (HV770/95, HV2245/92, and HV17/96). The SN titers detected for the tortoise plasma samples from France were consistently higher than those detected in plasma of the hyperimmunized tortoises. One sample from France had a titer of 1:724 and several had a titer of 1:362. The highest titer for tortoises in the hyperimmunization study was 1:64.

The recovery attempts from the nasal washes and from the pharyngeal swabs for the viruses used as inocula were unsuccessful.

ELISA and SN cross-reactivity. By ELISA and SN, immunological cross-reactivity was recorded between the two herpesviral isolates used in the hyperimmunization study. In order to further evaluate cross-reactivity among chelonian herpesvirus antigen, random plasma samples that were SN negative ($n = 5$) and SN positive ($n = 5$) for HV770/95, HV2245/92, and HV17/96 from the sample group from France were tested for SN and compared to the isolates used in the ELISA (HV4295/7R/95 and HV1976). The results correlated 100% with the results obtained using the European herpesvirus isolates (HV770/95, HV2245/92, and HV17/96). These results demonstrated a high degree of cross-reactivity among the five herpesvirus isolates (HV770/95, HV2245/92, HV17/96, HV1976, and HV4295/7R/95) and were consistent with the cross-reactivity detected between the herpesviral isolates HV4295 and HV1976 by both ELISA and SN.

Reproducibility of the ELISA results. Interassay reproducibility was determined by calculating the mean A_{405} , the standard deviation, and the range for the negative and positive control of five ELISAs over a time period of 1 month, using the same batches of HV4295/7R/95 and HV1976 antigens, plasma, and MAb HL1546.

The mean A_{405} values of the negative control were 0.173 and 0.157 when tested against HV4295/7R/95 and HV1976, respectively. The standard deviations were 0.0063 and 0.01, respectively, for the two isolates. The mean A_{405} readings for the

TABLE 2. Mean A_{405} values of the immunized tortoises against HV4295/7R/95 and HV1976 viral antigens^a

Tortoise no. (treatment)	Infection route(s)	Antibody response (A_{405}) to:	
		HV4295/7R/95	HV1976
No. 1 (HV4295/7R/95 infected)	i.n.	0.168	0.154
No. 2 (HV1976 infected)	i.n.	1.72	1.58
No. 3 (HV4295/7R/95 infected)	i.m.	1.59	1.51
No. 4 (HV1976 infected)	i.m.	1.63	1.51
No. 5 (control, PBS)	i.n. and i.m.	0.16	0.25

^a Antibody responses (A_{405}) were determined 6 (i.n.) or 7 (i.m.) weeks after hyperimmunization.

positive controls were 1.596 and 1.581 when tested against HV4295/7R/95 and HV1976, respectively. The standard deviations were 0.0019 and 0.12, respectively, for the two isolates; the ranges varied between 0.024 and 0.341.

DISCUSSION

Herpesvirus is recognized as an important emerging pathogen in chelonians. Recent findings have placed this novel herpesvirus among the alpha subfamily (32, 36). Both the conservation importance of the tortoises in the genus *Testudo* and their extreme popularity as pets in Europe and in the United States were important factors to justify the development of the first ELISA for detecting herpesvirus exposure in tortoises.

SN has been considered the gold standard for the detection and quantification of antiviral antibodies (31) and has been used for detecting herpesvirus exposure in tortoises (10). The limitations of SN as a seroepidemiological tool include its laborious procedure, the long time required to determine the final result (10 to 14 days), and the absence of a universally recognized reference isolate. In contrast, the ELISA offers the advantage of a 1-day procedure with an overall simplicity of execution.

Our results indicated a good correlation between the ELISA and the SN test (Table 1). All the samples that tested positive for SN were positive by ELISA. The only exception was one SN-positive sample that was positive in the ELISA for only one of the isolates (HV4295/7R/95) (Table 1). The majority of the samples showed cross-reactivity, suggesting the presence of conserved antigenic motifs among the five herpesviral isolates used in the ELISA and SN tests. In determining the sensitivity and specificity of the ELISA, we used a conservative approach by considering samples as detected positive only if they were positive with both antigens.

In our study we considered the SN test as the gold standard, and the results obtained from this test were considered "true." Traditionally when a new diagnostic test is compared to a gold

TABLE 3. SN results for tortoises immunized with HV4295/7R/95 and HV1976 i.n. and i.m.^b

Time ^a (wk) after i.n./i.m. treatment	SN titer to HV4295/7R/95 in no.:				SN titer to HV1976 in no.:			
	1 ^c	3 ^c	2 ^d	4 ^d	1 ^c	3 ^c	2 ^d	4 ^d
0/NA ^e	—	—	—	—	—	—	—	—
2/0	—	—	—	—	—	—	—	—
4/2	—	—	—	—	—	—	—	—
6/4	—	—	—	—	—	—	—	—
9/7	—	—	1/8	1/4	—	—	1/8	1/8
11/9	—	1/4	1/32	1/8	—	—	1/4	1/4
13/11	—	1/4	1/32	1/16	—	—	1/4	1/8
15/13	—	1/2	1/32	1/16	—	—	1/8	1/16
17/15	—	1/4	1/32	1/32	—	—	1/16	1/4
23/21	—	1/4	1/64	1/64	—	—	1/8	1/32
27/25	—	1/2	1/64	1/16	—	1/4	1/16	1/32
31/29	—	—	1/8	1/8	—	1/4	1/16	1/32
36/34	—	1/2	1/16	1/16	—	1/8	1/16	1/64

^a Plasma samples were collected from the tortoises in the immunization study from time zero to 36 and 34 weeks after i.n. and i.m. hyperimmunization, respectively. —, negative result.

^b A total viral amount of 100 TCID₅₀ was used in the SN test. The control tortoise (no. 5) remained seronegative for the duration of the study and has not been included in the table. Results are shown as i.n. response/i.m. response.

^c HV4297/7R/95 was used as the hyperimmunization antigen.

^d HV1976 was used as the hyperimmunization antigen.

^e NA, not applicable.

standard, the two tests need to be biologically independent; that is to say, they need to measure different targets (3). In this study, while the SN test measured the presence of herpesvirus SN antibodies, the ELISA theoretically measured all the possible subclasses of IgY directed against herpesvirus. Statistical analysis showed no significant difference between the results of the ELISA and SN tests ($P > 0.05$).

The rationale for the low plasma dilution that was chosen for the ELISA (1:25) was to detect most of the positive samples. We considered this a priority for a test where the detection of some false positives has far fewer consequences than a false negative in relation to conservation issues. The statistical agreement of the results obtained by SN and ELISA supports the selection of this dilution.

The mean A_{405} values obtained in the hyperimmunization study and from the plasma samples of the tortoises from France were comparable. These results might be explained by antigenic similarities between the different isolates or because of a specific immunologic response to herpesvirus in tortoises.

None of the SN-positive samples from the hyperimmunization study had a titer of $>1:64$, while much higher values were detected in the SN-positive samples from Europe. There may be several explanations for this difference. In the hyperimmunization study, tortoises were exposed to the virus once (except for tortoise no. 1, which was boosted 3 months after the first

FIG. 3. ELISA results for tortoises hyperimmunized with HV1976 and HV4295/7R/95 i.n. (A) and i.m. (B). Data shown are A_{405} readings obtained for the plasma samples collected from the tortoises in the hyperimmunization study from time zero to 36 and 34 weeks posthyperimmunization i.n. and i.m., respectively. Each bar corresponds to a single sampling. On the left side of the plotted area of each figure are the results from the ELISA when the tortoise plasma samples were tested against HV4295/7R/95 used as antigen, while on the right side the results are shown when the plasma was tested against HV1976. On the far right of each figure, outside the plotted area, the time sequence of the sampling is reported. The values reported on the y axis are A_{405} readings. On the x axis, the tortoise animal numbers are reported along with the antigens used for the hyperimmunizations (HV4295/7R/95, tortoises no. 1 and 3; HV1976, tortoises no. 2 and 4; PBS, tortoise no. 5). On the top of the graphs, the antigens used in each ELISA are indicated.

exposure). Reexposure could have happened over time in the tortoise collection in France. Second, the tortoises were hyperimmunized with a dose of 15,000 TCID₅₀, whereas in natural transmission the tortoises may be exposed to a higher viral load. Possibly, the immunological response is dose dependent. Furthermore, the isolates used were passed several times in culture, whereas the tortoises from Europe were by definition infected with wild types. It is possible that the isolates used for the hyperimmunization were attenuated and less immunogenic compared to wild-type virus. This could account for the observed minimal stimulation of SN antibody production. Interestingly, when random samples from France were tested against the two herpesviral isolates used as antigens in the ELISA, the SN titers were higher than the titers from the hyperimmunized animals with the homologous isolates. These data support this hypothesis.

To validate the ELISA, we hyperimmunized a group of five spur-thighed tortoises using the same herpesvirus isolates that were used as the antigen in the other experiment (HV4295/7R/95 and HV1976). The limited number of animals in the study was due to several issues: their status as endangered animals, along with the necessity of using adult animals (kept in captivity mainly for breeding purposes), made it difficult to find experimental animals for this study. Importing tortoises from overseas was not considered an option.

The amount of live virus used for hyperimmunizing the tortoises in the study was deliberately low (15,000 TCID₅₀) because we wanted the tortoises to survive the hyperimmunization and develop an immune response. Both a natural route (i.n.) and an experimental route (i.m.) were used. The unsuccessful attempt in recovering the virus might in part depend on the small size of the inoculum and on the delayed beginning of the recovery attempts (7 days postinoculation). No data are available about the limit of sensitivity of the TH-1 cell line for tortoise herpesvirus isolation, and we cannot exclude that this factor could have influenced the results of our virus isolation attempts. A possible absence of virus shedding due to a latent infection also could be an explanation.

The results of this study indicated that seroconversion was not detectable earlier than 4 weeks postexposure when using either the ELISA or SN test. This response was earlier than that observed in desert tortoises (*Gopherus agassizii*) when immunized with *Mycoplasma agassizii* (seroconversion at 8 weeks) (34). Additionally, the ELISA detected the presence of antiherpesvirus antibodies 2 to 5 weeks earlier than the SN test. These results are not surprising, since an ELISA can detect antibodies binding to multiple antigenic determinants of a virus, while the SN test detects antiherpesvirus antibodies which are biologically active in neutralization and are directed against surface glycoproteins (40).

Immunological cross-reactivity was observed when comparing the two isolates used in our study with three additional isolates used in Germany. This has been reported for other herpesviruses, such as varicella-zoster herpesvirus, which is well known for showing a large degree of cross-reactivity in serological tests (2). Frost and Schmidt (10) concluded that, given the serological relatedness of the tortoise herpesviruses, it would be sufficient to employ a single isolate for the purpose of SN testing. However, serogroups of tortoise herpesvirus may exist (6).

Some of the clinical signs reported in the literature for tortoises with herpesvirus infection, such as monolateral or bilateral conjunctivitis (32), were seen in our hyperimmunized tortoises. A more severe conjunctivitis was observed in the two tortoises hyperimmunized i.m. compared to those hyperimmunized i.n. An i.n. route may allow the host to better react to the pathogen. No information is currently available about the possible existence of mucosal immunity in reptiles, but we cannot exclude its possible role in infection prevention.

We considered it important to evaluate the reactivity of the tortoise plasma against the herpesviral antigens in an immunoblotting format in comparison with rabbit polyclonal sera. It is common to observe in tortoises the presence of a strong plasma activity directed against only a very limited number of proteins following immunization or infection when compared to rabbit polyclonal sera raised against the same antigen (F. Origi, unpublished observation). The results we obtained in this investigation are consistent with these observations.

In summary, this study reports the development of an ELISA for detection of herpesvirus exposure in Mediterranean tortoises. Without affecting the consistency of the results, the ELISA was far more practical compared to the more laborious and time-consuming SN test. The ELISA was found to have high specificity, high sensitivity, and high positive and negative predictive values. The transmission study demonstrated that the ELISA detects seroconversion earlier than the SN test. Even with some of the problems associated with antigen preparation, the ELISA has broad application in seroepidemiological investigations of the prevalence of herpesvirus exposure in wild, private, and zoological collections of tortoises.

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