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Seroprevalence of *Mycoplasma agassizii* and tortoise herpesvirus in captive desert tortoises (*Gopherus agassizii*) from the Greater Barstow Area, Mojave Desert, California

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Abstract

Upper respiratory tract disease (URTD) has been implicated as a cause of decline of wild populations of desert tortoises, *Gopherus agassizii*, in the western Mojave Desert. One explanation for outbreaks of disease may be the release or escape of diseased captive tortoises into naïve wild populations. Because *Mycoplasma agassizii* and tortoise herpesvirus have surfaced as important pathogens, 179 captive tortoises were evaluated in the greater community of Barstow, San Bernardino County, California during 2000 and 2001 to determine pathogen exposure. An indirect enzyme-linked immunosorbent assay (ELISA) was performed to detect antibodies against *Mycoplasma agassizii* ($n = 179$) and tortoise herpesvirus ($n = 109$). Anti-mycoplasma antibodies were present in 82.7% of the tortoises while anti-herpesvirus antibodies were detected in 26.6%. A positive association was found between tortoises with anti-mycoplasma antibodies and severity of clinical signs of URTD ($p = 0.001$) and with age categories, with adults being more likely to be positive ($p < 0.001$). Neither association was found with herpesvirus exposure. No association was found between gender and pathogen exposure or between being positive for exposure to both

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pathogens. Findings suggest that captive tortoises can be a source of infection for free ranging desert tortoises.

Keywords: Herpesvirus; *Mycoplasma agassizii*; URTD; Desert tortoise; *Gopherus agassizii*

1. Introduction

The desert tortoise occupies a unique position as the state reptile of California. In addition to state protection as a threatened species in California, populations are listed by the United States (US) government as threatened; other populations north and west of the Colorado River in Nevada, Arizona, and Utah (US Fish and Wildlife Service (USFWS), 1994) are also protected by the US government. In addition to predation, habitat degradation and poaching, disease has been associated with major declines of desert tortoises (USFWS, 1994). A chronic upper respiratory tract disease (URTD) was first seen in captive desert tortoises in the 1970s (Fowler, 1980; Snipes and Biberstein, 1982) and subsequently identified in wild populations in the western Mojave Desert (Jacobson et al., 1991). While initial studies with affected captive tortoises failed to identify a specific pathogen, a *Mycoplasma* sp. was detected in close association with epithelial cells lining the nasal cavity of wild tortoises showing signs of URTD (Jacobson et al., 1991). This new mycoplasma was described as *Mycoplasma agassizii* (Brown et al., 2001) and through transmission studies, found to be a causative agent of URTD (Brown et al., 1994). Epidemiological studies of wild desert tortoises in the western Mojave Desert from 1992 to 1995 showed a 37% increase (from 5% to 42%) in seropositive testing for *M. agassizii* (Brown et al., 1999). Recently, a herpesvirus has been identified in tissues of tortoises manifesting overlapping clinical signs of URTD as seen with experimental infection with *M. agassizii* (Jacobson et al., 1985; Pettan-Brewer et al., 1996; Drury et al., 1998; Muro et al., 1998; Johnson et al., 2005). Based on these observations, URTD could in some instances be caused by agents other than mycoplasma or could be multi-factorial with concurrent infections creating synergistic effects. For instance, in rats, concurrent infection with *Mycoplasma pulmonis* and Sendai virus has been determined to significantly increase clinical signs and histopathologic lesions of respiratory disease (Schoeb et al., 1985).

Several different explanations have been proposed to explain outbreaks of URTD in free-ranging populations of desert tortoises. Along with predisposing factors such as drought and resulting degradation of forage (Jacobson et al., 1991), the release or escape of captive tortoises infected with a pathogenic strain of mycoplasma may be responsible (Jacobson et al., 1995) for outbreaks in certain locations. Releases of captive desert tortoises and discovery of formerly captive desert tortoises in the wild have been reported, with some showing signs of disease (USFWS, 1994). Complicating this situation is the mixing of different species of turtles and tortoises in captivity. Exotic tortoises may carry exotic pathogens such as intranuclear coccidia (Garner et al., 1998; Jacobson et al., 1999), iridovirus (Westhouse et al., 1996; Marschang et al., 1999) and herpesvirus (Muro et al., 1998). Herpesvirus has been shown to be a significant problem in exotic tortoises imported for the pet trade with mortalities reaching as high as 100% (Jacobson et al., 1985; Drury et al., 1998; Une et al., 1999). Since these pathogens infect multiple species of tortoises, desert tortoises are likely susceptible and herpesvirus-like particles have been identified in

captive desert tortoises (Harper et al., 1982; Pettan-Brewer et al., 1996; Martinez-Silvestre et al., 1999). A novel herpesvirus has recently been sequenced from the tissues of a captive desert tortoise from San Diego, California (Johnson et al., 2005). Because of this, previously captive animals could serve as a source of infection for wild populations.

To determine the relative disease risk of accidental or intentional release of tortoises into the wild, a study was undertaken to evaluate captive desert tortoises in the greater community of San Bernardino County, California. Here, we present serologic findings for exposure to *M. agassizii* and tortoise herpesvirus in 179 captive desert tortoises during the summers of 2000 and 2001.

2. Materials and methods

2.1. Animals

Samples were collected from privately owned desert tortoises from the National Training Center, Fort Irwin southwest to Hesperia, San Bernardino County, California, a range spanning over 100 km with Barstow at its centre. All captive tortoises resided within or were contiguous with the historic range of the desert tortoise in the Mojave Desert. Between June and August of 2000 and 2001, 179 tortoises were sampled. Owners volunteered their tortoises for testing based on newspaper ads, presentations at turtle and tortoise club meetings, through veterinary clinics, friends and from humane societies. Owners were made aware of the cause of URTD, clinical signs associated with URTD, and the purpose of the study. As a result, they would be informed of the herpesvirus and mycoplasma test results of their tortoise(s). A basic history was obtained and a physical examination was performed on each tortoise, which included gender (male, female or undetermined), age class (adult, sub-adult), other species of tortoises kept, and presence of clinical signs of upper respiratory disease. A subjective evaluation of clinical signs of URTD including serous or mucopurulent nasal discharge, ocular discharge, conjunctivitis, palpebral and periocular oedema was recorded in addition to any oral plaques, a clinical sign of herpesvirus. A rating system as described by Berry and Christopher (2001) was used to classify the tortoise's clinical signs as mild, moderate, or severe. In 2001, owners of tortoises sampled in 2000 were contacted to determine if any of the sampled tortoises had escaped or been released since that time and if so, had they been found, and if they had encountered any exotic species of tortoise during that time in the wild.

2.2. Blood collection

Blood was collected from either the jugular vein or the brachial vein and samples were immediately placed in 2 ml sodium heparin vacutainers (Fisher Scientific, Pittsburgh, PA) and gently mixed. Samples were stored on ice or refrigerated until centrifugation. Samples were centrifuged within 6 h of collection at 3000 rpm for 5 min. The plasma was removed and frozen at $-20\,^{\circ}C$ until shipment to the University of Florida. Samples were shipped within two months of collecting.

2.3. Serology

All samples were shipped on ice packs overnight to the Mycoplasma Research Laboratory, University of Florida, Gainesville, Florida. Upon completion of testing for

mycoplasma, samples were transferred on ice to the Reptile Serology Laboratory, University of Florida, College of Veterinary Medicine for herpesvirus testing. Methods previously reported for indirect ELISA to detect anti-mycoplasma and anti-tortoise herpesvirus-1 (THV-1) antibodies were used (Schumacher et al., 1993; Origgi et al., 2001). Although the ELISA to detect anti-THV-1 antibodies has not been validated for the desert tortoise, it has been adapted for use in detecting exposure. The herpesvirus ELISA utilizes two different viral antigens originally isolated from Hermann's tortoises (*Testudo hermanni*) and validated for Greek tortoises (*Testudo graeca*) (Origgi et al., 2001). The test has been modified from the validated test by using mouse anti-desert tortoise monoclonal antibodies instead of mouse anti-Greek tortoise monoclonal antibodies at the same concentration.

All ELISA results were expressed as ratios. A ratio signifies the optical density of the sample compared to a control negative sample. Control negative samples are wells coated with cell antigen that has not been infected with the pathogen, to provide the value for background binding. Ratios for anti-mycoplasma antibodies >3 were considered positive, >2 and $\leqslant 3$ were suspect and ratios $\leqslant 2$ were considered negative. Ratio reference values were determined when the ELISA was developed using known healthy tortoises and tortoises infected experimentally with *M. agassizii* (Schumacher et al., 1993). The results of the ELISA detecting anti-herpesvirus antibodies were reported as either positive ($\geqslant 0.5$) or negative (<0.5) as described by Origgi et al. (2001). No suspect range has been established for herpesvirus testing.

2.4. Statistical analysis

Statistical analysis was performed using SPSS (2001, vol. 11.0) to determine if associations exist between having a positive mycoplasma or herpesvirus ELISA result to severity of clinical signs of URTD, gender, age, or having a positive result for the other pathogen. Tortoises testing suspect for mycoplasma were excluded from the mycoplasma analyses and tortoises of undetermined sex were excluded from gender analysis. The Mann–Whitney *U*-test was used to look at severity of clinical signs to presence of pathogen antibodies and to determine whether there was a significant difference between age classes (sub-adult or young adult of undetermined sex versus adult) in having positive ELISA results for both pathogens. The χ^2 test was used to determine if there was an association between positive serology and gender. The Fisher's Exact test was used to determine if there was an association between having a positive herpesvirus ELISA in tortoises that were positive for mycoplasma.

3. Results

3.1. History

The tortoises sampled came from 45 households from the cities, towns and settlements of Barstow (52), Hesperia (44), Apple Valley (39), Victorville (25), Helendale (6), Fort Irwin (5), Hinkley (5), Phelan (2), and Oak Hills (1). Twenty-seven tortoises were young adults or sub-adults of undetermined sex, 95 were males, and 57 were females (Table 1). Clinical signs of URTD ranged from none to severe (Table 1) with the most tortoises

Table 1
Distribution of gender, clinical signs of URTD, mycoplasma and herpesvirus ELISA in sampled desert tortoises

	Frequency	Percent
Sex (n = 179)		
Undetermined	27	15.1
Male	95	53.1
Female	57	31.8
Clinical signs (n = 179)		
None	61	34.0
Mild	81	45.3
Moderate	29	16.2
Severe	8	4.5
Mycoplasma ELISA results (n = 179)		
Negative	23	12.8
Suspect	8	4.5
Positive	148	82.7
Herpesvirus ELISA results (n = 109)		
Negative	80	73.4
Positive	29	26.6

showing mild signs of disease (45.3%). No oral plaques were observed in any of the tortoises.

Seven of the 45 owners (15.5%) had multiple species of turtles and tortoises. Exotic species owned with desert tortoises included western ornate box turtles (*Terrapene ornata ornata*), eastern box turtles (*Terrapene carolina carolina*), three-toed box turtles (*Terrapene carolina triunguis*), Russian tortoises (*Testudo horsfieldi*), leopard tortoises (*Geochelone pardalis*), African spur-thigh tortoises (*Geochelone sulcata*), and red-footed tortoises (*Geochelone carbonaria*). Four of the seven owners had exotic species in direct contact with desert tortoises. Four (2.2%) tortoises that were owned in August of 2000 escaped when owners were contacted during June 2001. Two Russian tortoises were found wild during the same time.

3.2. Serology and statistical analysis

3.2.1. Mycoplasma results

One hundred forty-eight desert tortoises (82.7%) were positive for having anti-mycoplasma antibodies. Eight (4.5%) tortoises were suspect and 23 (12.8%) were negative (Table 1). There was a statistically significant positive association between severity of clinical signs and having a positive ELISA result (Mann–Whitney, $Z = -4.397$, $p = 0.001$, Tables 2 and 3). Adult desert tortoises were also more likely to have a positive mycoplasma ELISA result than sub-adults or young adults of undetermined sex (Mann–Whitney, $Z = -4.026$, $p < 0.001$, Tables 2 and 4). No association was found for ELISA results with gender (χ^2, $df = 1$, $\chi^2 = 0.026$, $p = 0.662$, Tables 2 and 4) or with having a positive herpesvirus ELISA result (Fisher's Exact, $p = 0.452$, Tables 2 and 5).

Table 2
Statistical tests and results of statistical analysis demonstrating probability of associations between positive ELISA results and severity of clinical signs, gender, age and presence of antibodies to the other pathogen

Variable	Test	Mycoplasma			Herpesvirus		
		N	Test statistic	*p*	*N*	Test statistic	*p*
Clinical Signs of URTD	Mann–Whitney	179	$Z = -4.397$	0.001	109	$Z = -1.080$	0.280
Gender	χ^2	171	$\chi^2 = 0.026$	0.871	109	$\chi^2 = 0.825$	0.662
Age (adults vs young and sub-adults)	Mann–Whitney	171	$Z = -4.026$	<0.001	109	$Z = -0.766$	0.444
Presence of exposure to the other pathogen	Fisher's Exact	105	NA	0.452	105	NA	0.452

NA = not applicable.

Table 3
Correlation of severity of clinical signs to mycoplasma and herpesvirus ELISA results

Clinical signs	Mycoplasma			Herpesvirus		
	Negative	Positive	Total	Negative	Positive	Total
None	20	38	58	33	8	41
Mild	0	78	78	30	14	44
Moderate	1	27	28	13	5	18
Severe	2	5	7	4	2	6
Total	23	148	171	80	29	109

Tortoises with a suspect mycoplasma ELISA result are excluded. Values represent total numbers of tortoises.

3.2.2. *Herpesvirus results*

Of 109 samples analysed for anti-THV-1 antibodies, 29 (26.6%) were seropositive and 80 (73.4%) were negative (Table 1). No associations were found between positive ELISA result and severity of clinical signs (Mann–Whitney, $Z = -1.080$, $p = 0.280$, Tables 2 and 3), age class (Mann–Whitney, $Z = -0.766$, $p = 0.444$, Tables 2 and 4), gender (Chi-square, $\chi^2 = 0.026$, $p = 0.871$, Tables 2 and 4) or with a positive mycoplasma ELISA result (Fisher's Exact, $p = 0.452$, Tables 2 and 5).

4. Discussion

Over 200,000 desert tortoises are kept in captivity in the state of California alone (Jacobson et al., 1995). Results of this study showed that approximately 83% of the captive desert tortoises tested in the high desert of the western Mojave Desert in California were exposed to *M. agassizii*. This high level of prevalence may be somewhat positively biased as the majority of tortoises came from households in which more than one desert tortoise was owned and tortoises may be at higher rates of exposure as a result. However, another study with a smaller sample size, revealed a similar seroprevalence rate. Sixteen plasma samples from desert tortoises kept in a small park in the Fort Irwin Cantonment were evaluated and 13 (81%) were positive for exposure to mycoplasma (Jacobson et al., unpublished

Table 4
Distribution of mycoplasma and herpesvirus ELISA results by gender

Gender	Mycoplasma			Herpesvirus		
	Negative	Positive	Total	Negative	Positive	Total
Undetermined	9	15	24	13	3	16
Male	8	83	91	40	16	56
Female	6	50	56	27	10	37
Total	23	148	171	80	29	109

Tortoises with a suspect result on mycoplasma ELISA are excluded.

Table 5
Results for exposure to *M. agassizii*, in tortoises tested for exposure to tortoise herpesvirus

Mycoplasma ELISA result	Herpesvirus ELISA results		Total
	Negative	Positive	
Negative	14	6	20
Positive	63	22	85
Total	77	28	105

Tortoises not tested for herpesvirus, and those testing suspect for mycoplasma are excluded.

data). The seroprevalence of mycoplasma exposure in both these groups of captives is much higher than that seen in wild populations within and around the National Training Center, Fort Irwin, California, which is approximately 50 km northeast of Barstow and includes one of the healthiest remaining populations of desert tortoises in the western Mojave desert. In a 1993–1996 study on wild populations of desert tortoises at Fort Irwin, of 120 wild tortoises sampled, 10 (8%) were found to be seropositive for exposure to *M. agassizii* (Jacobson et al., unpublished data). Mycoplasmas are thought to persistently infect the host (Brown et al., 2002), which would make all mycoplasma seropositive tortoises a possible reservoir of infection for wild populations if they were to escape or be released.

Since herpesviruses can also persistently infect their hosts (Ahmed et al., 1996), and are significant pathogens in captive tortoises (Jacobson et al., 1985; Drury et al., 1998; Une et al., 1999), with several reports of desert tortoises with herpesvirus infection (Harper et al., 1982; Pettan-Brewer et al., 1996; Martinez-Silvestre et al., 1999; Johnson et al., 2005), we decided to survey the desert tortoises for exposure to herpesvirus. The test used was an indirect ELISA, with two isolates of THV-1 from Hermann's tortoises serving as the antigen. The value of this test in determining exposure of desert tortoises to herpesvirus was confirmed when the modified ELISA was able to show seroconversion between 4 and 6 weeks after plaques were first noted in the oral cavity of a captive desert tortoise in San Diego with confirmed herpesvirus infection (Johnson et al., 2005). The timing of seroconversion was consistent with an experimental infection of Greek tortoises, which demonstrated seroconversion between 4 and 7 weeks post-inoculation with purified

herpesvirus (Origgi et al., 2001). This indicated that the test can be used to detect THV-1 exposure in desert tortoises. In our study, 27% of the tortoises demonstrated previous exposure to a herpesvirus.

Overall, 155 tortoises (86.6%) were exposed to one or both antigens, indicating that the majority of captive tortoises in this area could be a source of mycoplasma and/or herpesvirus infection for naïve, wild desert tortoises. In addition to the threat of mycoplasma or herpesvirus introduction, these tortoises may pose a serious threat to wild populations of tortoises as a result of other pathogens exotic to native desert tortoises. Exotic tortoises may carry other pathogens such as intranuclear coccidia (Garner et al., 1998; Jacobson et al., 1999), and iridovirus (Westhouse et al., 1996, Marschang et al., 1999). Forty-five of the tortoises (25%) in this study were exposed to non-native species of tortoises.

This study also provided an estimate of the percent of tortoises that escape in a given year. Between 2000 and 2001, four (2.2%) captive desert tortoises that were tested in 2000 and were seropositive for exposure to mycoplasma escaped and were not located by the original owners as of the summer of 2001. In addition, two Russian tortoises were found wild and brought to an adoption agency. This percentage suggests, as worst case scenario, that if 200,000 tortoises are kept in captivity in California as has been previously estimated (Jacobson et al, 1995), approximately 4400 captive desert tortoises could escape in any given year.

Statistical analysis of the results demonstrated that there was no association between having a positive mycoplasma ELISA result and having a positive herpesvirus ELISA result. Concurrent infections of mycoplasma with other microbial agents may be synergistic (Schoeb et al., 1985). Lesions associated with mycoplasmosis in rats are more severe with concurrent Sendai virus infection than lesions associated with either agent acting alone (Schoeb et al., 1985). Kennel cough in dogs is an example of a disease in which two pathogens are needed to cause clinical signs in dogs and mycoplasma, parainfluenza virus or adenovirus is often isolated with *Bordatella bronchiseptica* in these animals (Ford and Vaden, 1998). Our results demonstrated that if *M. agassizii* is dependant on another organism, it is not dependant on THV-1 exposure for demonstrating clinical signs of disease. Mycoplasma was positively correlated with clinical signs of disease as has been seen previously (Jacobson et al., 1995), but herpesvirus was not. Of the 22 tortoises with positive results for both herpesvirus and mycoplasma, three had no signs of disease, 14 had mild signs and five had moderate signs of disease. All but one tortoise with severe signs of disease were positive solely for mycoplasma. Of the six tortoises positive for herpesvirus that were negative for mycoplasma, only one showed any clinical signs of disease and they were severe. This tortoise had severe metabolic bone disease, which may have contributed to overall appearance of ill-health, or signs could have been caused by the herpesvirus. Further diagnostic tests would have been needed to identify whether or not this tortoise had an active herpesvirus infection, as opposed to a latent infection.

Statistical analysis also demonstrated a positive correlation with age. Sub-adults or young adults of undetermined sex were less likely to have positive ELISA results than were adults. This indicates that escaped or released adults are at a higher risk of pathogen transmission than younger tortoises.

URTD continues to be seen in wild and captive tortoises and more then one pathogen may be involved (Brown et al., 2002). Although the exact number of tortoises released into the wild in the past remains unknown, this study indicated that release of desert tortoises

may and likely has resulted in the concomitant release of pathogens into the wild. Thus, there is an urgent need for owner education that addresses the topics of appropriate animal husbandry, proper enclosures that will minimize interactions between wild and captive tortoises and prevent escape, and the potential problems that might result from multi-species housing of tortoises.

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