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UPPER RESPIRATORY TRACT DISEASE AND MYCOPLASMOSIS IN DESERT TORTOISES FROM NEVADA

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ABSTRACT: A population of desert tortoises (*Gopherus agassizii*) at Yucca Mountain (Nevada, USA) was monitored during four sampling periods using enzyme-linked immunosorbent assays (ELISA) to determine the percentage of individuals that had been exposed to *Mycoplasma agassizii*, a causative agent of upper respiratory tract disease. Respiratory tract disease has been considered a significant factor in the decline of desert tortoise populations in the Mojave Desert (USA). Few differences between sexes in ELISA values or percentages testing positive were noted. From 15 to 23% of samples per period tested positive for exposure to the mycoplasma. However, we noted few clinical signs of upper respiratory tract disease. This is in contrast to an earlier study which reported a similar proportion of seropositive tortoises as well as a high percentage of tortoises with clinical signs. However, our results are consistent with that study's conclusion that seropositivity for *M. agassizii* was a poor predictor of the likelihood to exhibit clinical signs of upper respiratory tract disease. Earlier reported epizootics of mycoplasma-associated respiratory disease occurred mainly during times of drought. Our samples were collected during a period of average to above-average rainfall, suggesting that manifestation of clinical signs of the disease may depend upon the physiological condition of tortoises which, in turn, is related to environmental conditions.

Key words: Clinical signs, desert tortoise, ELISA, *Gopherus agassizii*, *Mycoplasma agassizii*, mycoplasmosis, upper respiratory tract disease.

INTRODUCTION

The Mojave Desert population of the desert tortoise (*Gopherus agassizii*) is listed as threatened and was given Federal protection because of reported population declines (U.S. Fish and Wildlife Service, 1989, 1990; Corn 1994). Disease-related mortality has been considered a major factor contributing to these declines, and upper respiratory tract disease (URTD) has been cited as the most significant disease involved (U.S. Fish and Wildlife Service, 1994). Monitoring the disease status of tortoises throughout their range is considered important for understanding the dynamics of URTD in wild populations (U.S. Fish and Wildlife Service, 1994).

Recently, *Mycoplasma agassizii* has been shown to be a causative agent of URTD in desert tortoises (Brown et al., 1994). This disease is manifested as rhinitis accompanied by varying levels of serous to mucopurulent discharge from the nares, and conjunctivitis with varying levels of ocular discharge and palpebral edema. Additional symptoms include sunken eyes and dull skin and scutes (Jacobson et al., 1991; Brown et al., 1994). Little is known about the origin or spread of URTD in wild populations. Upper respiratory tract infections are common in captive tortoises (Jacobson et al., 1991), and there has been speculation that released captives have introduced particularly pathogenic strains of *M. agassizii* into wild populations (Jacobson et al., 1995). For example, comparatively high rates of antibody detection in Las Vegas Valley (Nevada, USA) were prevalent in areas where captives historically have been released (Jacobson et al., 1995).

As part of a study to assess the effects of increased human activities at Yucca Mountain (Nevada, USA), we monitored the disease status of desert tortoises. Our objectives were to determine if tortoises had been exposed to *M. agassizii* and to assess the relationship between the antibody status of individuals and observations of clinical signs of URTD. Further, some tortoises were tested for antibodies four times in 3 yr to determine how antibody status changed over time.

MATERIALS AND METHODS

Desert tortoises were studied at Yucca Mountain (Nye County, Nevada, 36°50′N, 116°25′W), approximately 150 km northwest of Las Vegas (Nevada, USA). This is an area of transition between the Mojave and Great Basin deserts, and is near the northern edge of the desert tortoise's range (Bury et al., 1994). Ecological studies were conducted at Yucca Mountain from 1989 to 1995 on a population of desert tortoises that were fitted with radiotransmitters (model HLPB 2114, Wildlife Materials, Inc., Carbondale, Illinois, USA; or custom built by AVM Instrument Company, Ltd., Livermore, California, USA).

All tortoises used in this study were >140 mm carapace length (CL). Sex was determined using morphological characteristics for individuals >200 mm CL (Woodbury and Hardy, 1948), or by measuring plasma testosterone levels using radio-immunoassay techniques for smaller individuals (Rostal et al., 1994).

Whenever tortoises were handled in the field or laboratory, they were examined for clinical signs of URTD. In particular, any type of mucous discharge from the nares or eyes, whether or not the nares were occluded, and any conjunctivitis or palpebral edema, was noted.

During September 1993, June 1994, September 1994, and September 1995, radiomarked tortoises were captured and transported to a field laboratory in separate, clean, plastic boxes. Three to 4 ml of blood was collected by jugular venipuncture (Jacobson et al., 1992), transferred to a 5 ml tube containing lithium heparin, gently mixed, and centrifuged for 5 min to separate plasma. Tortoises were rehydrated with 6 to 12 ml (approximately two to three times the volume of blood drawn) of a 50 : 50 saline : dextrose solution injected subcutaneously into the spongy tissue of the axillary region. Tortoises were released at their point of capture, generally within 4 to 5 hr.

Plasma samples were stored at −78 C until they were shipped on dry ice to the University of Florida (Biotechnologies for the Ecological, Evolutionary, and Conservation Sciences, Immunological Analysis Laboratory, Gainesville, Florida, USA), where they were tested for the presence of antibodies to *M. agassizii* using enzyme-linked immunosorbent assays (ELISA) (Schumacher et al., 1993). Antibody levels were expressed as enzyme immunoassay ratios of the optical density of the sample to a negative control. Both five- and 10-fold dilutions of plasma were assayed. Tortoises were categorized as positive for presence of the antibodies if the ELISA value for either dilution was >3; negative if both dilutions were <2; and suspect if values for both dilutions were ≤3, and if at least one was >2. The cut-off points for these categories differ from those originally published for this test (positive >2, with no suspect category; Schumacher et al., 1993). Based on new information on the relationship between ELISA values and the manifestation of clinical signs, as well as recommendations from those authors, a value of >3 to delineate seropositive individuals was used. For comparison, we also considered the frequency of positive and negative tests using the original criteria.

Response categories (positive, suspect, or negative) and ELISA values were both used in statistical tests. ELISA values are ratios which are often problematic to analyze statistically because of uncertain distributions (Sokal and Rohlf, 1995). This was not a problem for these data because the negative control (the denominator of the ratio) had a very low variance and the same seronegative plasma was used as the control for all tests. Lilliefors' test was used to assess normality (Lilliefors, 1967) and F_{max} tests to test for variance homogeneity (Sokal and Rohlf, 1995). ELISA values were log transformed prior to analyses. Five- and 10-fold dilution ELISA values were highly correlated (Pearson product-moment correlation; Sokal and Rohlf, 1995; $r = 0.969$, $P < 0.001$) so only results of analyses on 10-fold dilutions are presented.

Because some tortoises were sampled during more than one period, the data lacked independence among sampling periods. Therefore, differences in ELISA values between sexes were tested for each sampling period using one-way analyses of variance. Chi-square tests were used to assess independence between ELISA category and sex. For 32 tortoises sampled during all four periods, repeated measures analysis of variance was used to evaluate changes in antibody levels over time. SYSTAT (1992) was used for all statistical procedures, and $\alpha = 0.05$ was the standard used to determine whether departures from null hypotheses were significant.

Tortoises were handled under permits PRT-683011 and PRT-781234 from the U.S. Fish and Wildlife Service (Portland, Oregon, USA) and S-0446, S-1595, S-3108, S-5041, S-6941, and S-9060 from the Nevada Division of Wildlife (Reno, Nevada, USA).

RESULTS

One hundred-five tortoises were sampled: 60 females, 44 males, and one of unknown sex. Thirty-two tortoises were sampled during all 4 periods, 31 three times,

TABLE 1. Proportion of female and male desert tortoises at Yucca Mountain (Nevada, USA) that tested positive, negative, or suspect for antibodies to *Mycoplasma agassizii* during four sampling periods (n = 282[a]).

Period	Sex	Total	Percent			χ^{2}[b]	P
			Positive	Negative	Suspect		
September 1993	F[c]	27	19	70	11	0.61	0.74
	M	22	14	68	18		
June 1994	F	32	19	44	38	0.82	0.67
	M	27	15	56	30		
September 1994	F	54	26	44	30	0.61	0.74
	M	37	19	49	32		
September 1995	F	46	22	61	17	2.32	0.31
	M	37	11	76	14		

[a] One tortoise tested in September 1995 was of unknown sex.
[b] Significances of differences between males and females were tested using chi-square tests.
[c] F = female, M = male.

20 twice, and 22 once. Likelihood to test positive for mycoplasmal antibodies did not differ between sexes during any sampling period (all $P \geq 0.31$, Table 1). Means of the log-transformed ELISA values did not differ between sexes during September 1993, June 1994, or September 1994 (all $P \geq 0.34$), but did during September 1995 ($P = 0.02$), when females had higher antibody levels (Table 2). For all tests of ELISA values, about 6% of the variability in antibody level was explained by sex. For the 32 tortoises sampled four times (13 males, 19 females), no effects of sex were found ($F_{1,30} = 0.64$, $P = 0.43$). Because few differences were detected between males and females, and because the percentage of variation that was explained by sex in the significant case was low, males and females were pooled for all other analyses.

Pooled over sampling periods, 53 of 283 (19%) samples were seropositive (we failed to draw blood on four occasions). The percentage of seropositive samples in each period (9/93, 6/94, 9/94 and 9/95) was 15, 17, 23, and 17, respectively. Median untransformed ELISA values were 1.4, 1.6, 1.8, and 1.5, respectively, for those sampling periods. ELISA values for the 32 repeatedly-measured tortoises differed among periods ($F_{3,93} = 15.56$, $P < 0.01$); untransformed median ELISA values were 1.3, 1.8, 2.0, and 2.0, respectively.

The likelihood for an individual to test positive at least once appeared to increase with the number of times an individual was tested. For the 22 tortoises sampled once, 14% were seropositive. For individuals tested two, three, or four times, the percentage seropositive was 25, 26, and 38, respectively. However, these proportions did not differ ($\chi^2 = 4.35$, df = 3, $P = 0.23$).

TABLE 2. Sample sizes, mean untransformed ELISA values, and standard errors for male and female desert tortoises at Yucca Mountain (Nevada, USA) during four sampling periods (n = 282[a]).

Period	Males			Females			F[b]	P
	n	$\bar{x}$	SE	n	$\bar{x}$	SE		
September 1993	22	1.6	0.19	27	2.1	0.44	0.18	0.67
June 1994	27	1.9	0.27	32	2.2	0.34	0.91	0.34
September 1994	37	2.3	0.30	54	2.7	0.38	0.35	0.55
September 1995	37	1.8	0.30	46	3.0	0.51	5.40	0.02

[a] One tortoise tested in September 1995 was of unknown sex.
[b] Log-transformed means were compared using one-way analysis of variance.

Of 83 tortoises tested two or more times, antibody status changed in 40 individuals (48%). Most changed to or from the suspect category; only two changed from seropositive to seronegative, and four changed from seronegative to seropositive. Excluding the June 1994 samples, and comparing changes in ELISA values over consecutive years, values increased by <1 unit in 44 of 118 cases, and decreased by <1 unit in 43 cases. Values increased by >2 units nine times and five of these instances were increases of >5. Only two consecutive measurements decreased by >2.

The 105 tortoises were examined for clinical signs 287 times during blood sampling sessions and clinical signs of URTD were observed once in seven individuals (2%). In all cases, signs were minimal (slight nasal discharge, but no ocular discharge, palpebral edema, or conjunctivitis). Blood from three of these seven tortoises was tested during all sampling periods: one tortoise was always seronegative; the second was seronegative three times and suspect once; and the third was seronegative once, suspect twice, and seropositive once. This was the only case in which a tortoise that tested positive at any time during the study also displayed clinical signs during a laboratory examination. However, this tortoise was not seropositive at the time clinical signs were observed, but rather was seropositive one year later when clinical signs were not observed. Two of the seven tortoises showing clinical signs were tested three times, and all tests were negative. One tortoise was tested twice and was seronegative both times; the last individual was tested once and was suspect.

During other studies at Yucca Mountain, all tortoises handled in the field (n = 425) were examined for clinical signs. Of 1,294 observations (most individuals were examined multiple times) made between April 1990 and September 1995, signs of the disease were seen six times (0.5%) in six individuals. In all cases the symptoms were minimal. One of these individuals had clinical signs in August 1992, and was seronegative in September 1993 and seropositive in September 1995. Four individuals were tested once and all were seronegative. The sixth tortoise was not tested.

DISCUSSION

Of the 283 blood samples collected in this study, 19% were seropositive. This value was low compared to a similar study from Las Vegas Valley (Nevada, USA) where 144 tortoises were tested once and 50% were seropositive (Schumacher et al., 1997). However, much of this difference was due to different cut-off values used to delineate seropositive tortoises. If we had used a cut-off value of two, as Schumacher et al. (1997) did, then 43% of the Yucca Mountain samples would have been positive, a value that differs little from the 50% from Las Vegas Valley.

Schumacher et al. (1997) observed clinical signs more frequently than we did, and they reported a high correlation between clinical signs and testing positive. Of all tortoises sampled in Las Vegas Valley, 31% (45/144) showed clinical signs, and 84% of those were seropositive. Comparatively, the number of tortoises exhibiting clinical signs at Yucca Mountain during field and laboratory examinations was low (<1%). Four tortoises at Yucca Mountain were seropositive during all sampling periods, but never exhibited clinical signs. One of these tortoises had particularly high antibody levels (i.e., four ELISA values of 9.8–16.6), and was examined >20 times during 1991 to 1995.

Regardless of the values used to categorize seropositive individuals, there was little association between testing positive and manifestation of clinical signs at Yucca Mountain. Whereas clinical signs, and in particular nasal discharge, were very good predictors of positive antibody status for tortoises sampled in Las Vegas Valley (Schumacher et al., 1997), this clearly was not the case at Yucca Mountain, primarily

because few clinical signs were observed at Yucca Mountain.

Changes in antibody category status occurred in nearly 50% of tortoises we tested two or more times. In most cases, switching occurred between adjacent categories due to only small changes in ELISA values. However, these results are particularly important to consider if management decisions, such as whether to translocate or euthanize individuals, are being based on ELISA outcome. It is important to recognize that a positive ELISA test only indicates past exposure to the pathogen. The presence of the mycoplasma can only be confirmed by culturing the organism or by detecting the organism's genetic material using polymerase chain reaction (Brown et al., 1995).

Antibody values in the 32 tortoises each tested four times suggested that ELISA values were increasing for repeatedly-sampled tortoises. The proportion of these 32 tortoises testing positive also increased. These findings could imply that additional handling resulted in additional exposure to causative agents. However, all of the radiomarked tortoises at Yucca Mountain were involved in a number of other ecological studies and the number of times blood was collected from an individual is not an accurate representation of the total number of times that a tortoise was handled. It would be advantageous to repeatedly sample individuals from another locale to further evaluate the effect of sampling blood.

Little is known about the progression of URTD in free-ranging tortoises once they have been exposed to *M. agassizii,* or what biotic or abiotic factors contribute to manifestation of URTD. Whereas some individuals exhibit severe signs of URTD following exposure, some may never show clinical signs (Brown et al., 1994). It has been suggested that URTD is clinically manifested during times of stress caused by factors such as drought, habitat degradation, or overcrowding (Jacobson et al., 1991). For example, Peterson (1994) reported high mortality rates during 1990, a period when tortoises were in very poor physiological condition due to prolonged drought, and some deaths in that study may have been due to URTD. Schumacher et al. (1997) also collected their data during a drought year. In contrast, we collected blood during a period of average or above-average rainfall, tortoises appeared to be in good physiological condition, and few clinical signs of URTD were observed. These results suggest that manifestation of clinical signs of the disease may depend upon the physiological condition of tortoises which, in turn, is related to environmental conditions.

It has been suggested that captive tortoises may be the source of an extremely pathogenic strain of *M. agassizii* (Jacobson, 1994; Jacobson et al., 1991, 1995). When released to the wild, captive tortoises may transmit *M. agassizii* to naive populations and may be responsible for URTD outbreaks. For example, higher percentages of tortoises with clinical signs of URTD were observed in areas of the Las Vegas Valley where captive tortoises were known to have been released (Jacobson et al., 1995), and the original reports of the disease in southern California came from areas where captive tortoises traditionally were released (U.S. Fish and Wildlife Service, 1994). If released tortoises are the source of a pathogenic strain, the likelihood of that strain occurring at Yucca Mountain is small because the area is remote and has been closed to the public for more than 30 yr.

Further research is needed to clarify the transmission, diagnosis, and etiology of URTD in wild populations. This is underscored by the fact that a new species of mycoplasma recently has been identified (Brown et al., 1995). This species cross reacts to ELISA testing with *M. agassizii,* and also causes clinical signs of URTD in the congeneric gopher tortoise (*Gopherus polyphemus*) (I. M. Schumacher, pers. comm.). Other causes of respiratory tract disease which result in clinical signs and

have not been elucidated may be present in desert tortoise populations. Continuation of long-term monitoring for signs of URTD in desert tortoises throughout their range is necessary to clarify environmental factors which contribute to the manifestation of the disease. Remote populations should be tested and compared to populations that have been subjected to urban expansion and development to assess how disturbances are influencing the disease status of individuals.

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