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RELATIONSHIP BETWEEN CLINICAL SIGNS OF UPPER RESPIRATORY TRACT DISEASE AND ANTIBODIES TO *MYCOPLASMA AGASSIZII* IN DESERT TORTOISES FROM NEVADA

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ABSTRACT: Plasma samples collected in 1990 from free-ranging desert tortoises (*Gopherus agassizii*) with and without clinical signs of upper respiratory tract disease (URTD) from Las Vegas Valley, Clark County, Nevada (USA), were tested by enzyme-linked immunosorbent assay (ELISA) for antibodies to *Mycoplasma agassizii*, a causative agent of URTD. The relationship between clinical signs and ELISA test results was evaluated. Of the 144 tortoises tested, 45 (31%) had clinical signs while 72 (50%) were seropositive. Presence of clinical signs of URTD was positively related to positive ELISA results ($P < 0.0001$) regardless of sex or age of the animal. Eighty-four percent of animals with clinical signs tested seropositive. Mucous nasal discharge, the most severe and obvious of the clinical signs, was highly predictive for exposure to *M. agassizii* based on the ELISA. Ninety-three percent of tortoises with mucous nasal discharge tested seropositive. Serologic testing for *M. agassizii* antibodies supported clinical signs as useful indicators of URTD, but it also detected potential subclinical infection in 34 (34%) of 99 animals without clinical signs.

Key words: Desert tortoise, *Gopherus agassizii*, *Mycoplasma agassizii*, ELISA, Upper Respiratory Tract Disease, URTD, Mycoplasmosis.

INTRODUCTION

The Mojave population of the desert tortoise (*Gopherus agassizii*), occurring north and west of the Colorado River in Utah, California, Arizona, and Nevada (USA) is listed as threatened by the U.S. Department of Interior (1990). *Mycoplasma agassizii* is an etiologic agent of a contagious upper respiratory tract disease (URTD) in the desert tortoise (Brown et al., 1994). Upper respiratory tract disease was first observed in desert tortoises two decades ago and may have contributed to the decline of this species (Jacobson et al., 1991). In its early stage URTD is characterized by palpebral edema and serous nasal and ocular discharge while intermittent episodes of mucous nasal and ocular discharge are seen in its chronic stage. The contagious nature of URTD complicates tortoise management decisions. Due to land development, relocation of tortoises is often employed as a conservation effort. Thus, a reliable diagnosis of mycoplasma infection is essential in curbing the spread of URTD. Chronically infected tortoises with URTD have intermittent clinical signs, thus making a diagnosis of this disease by clinical signs alone unreliable. Enzyme-linked immunosorbent assays (ELISA) measure antibodies that are produced in response to mycoplasma exposure and thus have the potential to detect subclinically infected tortoises. Diagnosing URTD by clinical signs alone would miss silent carriers, possibly resulting in the spread of URTD into a naive tortoise population. While serological testing cannot distinguish between an active infection and exposure to a pathogen, it is still the method of choice for screening large numbers of samples. In this study we examined the relationship between clinical signs of URTD and exposure to *M. agassizii* as measured

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by ELISA (Schumacher et al., 1993) in a free-ranging desert tortoise population.

MATERIALS AND METHODS

In 1990, blood samples were collected by jugular venipuncture (Jacobson et al., 1992) from wild, free-ranging desert tortoises in the Las Vegas Valley, Clark County, Nevada (35°57′N, 115°15′W). Blood was originally collected for complete blood counts and plasma biochemistry to establish baseline health profiles for 277 tortoises prior to relocation into a nearby conservation research facility (D. B. Hardenbrook, unpubl.). Surplus plasma samples from 144 tortoises were initially frozen in liquid nitrogen and then stored at −80 C until June 1993, when they were shipped to the University of Florida, Gainesville, Florida (USA) and tested for antibodies to *Mycoplasma agassizii* using an ELISA (Schumacher et al., 1993). The samples represented 61 adult males (43%), 55 adult females (38%), and 28 (19%) immature tortoises. Adults were defined as tortoises with a median carapace length (MCL) ≥204 mm whose sex could be determined using secondary sexual characteristics (Berry, 1984).

For ELISA, a plasma sample was considered to be positive if the absorbance at 405 nm of either one or both of its dilutions (two-fold dilution and 10-fold dilution) was greater than twice the absorbance of the negative control plasma at the same dilution. Samples with values equal to or lower than twice the negative control were considered negative.

At the time of blood collection, a physical examination was conducted. The following clinical signs were recorded: moisture around the nares, nasal discharge (ranging from serous leakage to a mucous discharge from the nares), occluded nares, labored breathing, wheezing, and palpebral edema. Based on clinical signs two groups were established. The first group consisted of tortoises with either only one or any combination of the above described clinical signs. The second group was a subgroup of the first group and consisted of tortoises with mucous nasal discharge only or in combination with any of the other clinical signs.

Data were analyzed using Chi-square analysis and Fisher's Exact *P*-value (StatView, Abacus Concepts, Inc., Berkeley, California). Positive and negative predictive values with 95% confidence intervals were calculated to identify the relationship between clinical signs and ELISA results (Smith, 1995). A kappa statistic (Rosner, 1995) was used to investigate the concordance of assessment of URTD status by clinical signs with assessment by ELISA. A kappa ≥0 and <0.4 indicated marginal concordance, kappa ≥0.4 and ≤0.75 indicated good concordance, and kappa >0.75 indicated excellent concordance. A $P > 0.05$ indicated statistical significance.

TABLE 1. The number of desert tortoises with and without clinical signs of URTD compared to the number of desert tortoises with and without antibodies to *M. agassizii* as measured by ELISA.

ELISA result	Clinical signs	No clinical signs
Seropositive	21/12/5[a]	17/15/2
Seronegative	1/5/1	22/23/20

[a] Number of adult male tortoises/number of adult female tortoises/number of immature tortoises.

RESULTS

Of the 144 tortoises tested for *M. agassizii* using an ELISA, 72 (50%) were seropositive and 72 (50%) were seronegative. Of 61 male tortoises, 38 (62%) were seropositive and 23 (38%) were seronegative. Of the 55 female tortoises, 27 (49%) were seropositive, and 28 (51%) were seronegative. Seven (25%) of the 28 immature tortoises were seropositive and 21 (75%) were seronegative.

Ninety-nine (69%) of the 144 tortoises did not have any clinical signs of URTD (Table 1). Sixty-five (66%) of these 99 animals without clinical signs were seronegative; yet 34 (34%) were seropositive, indicating exposure to *M. agassizii*. Thirty-eight (84%) of 45 animals with clinical signs were seropositive. Seven tortoises had clinical signs but were seronegative (9.7% of all seronegatives). Five of those seven tortoises (Table 1) had either moisture around the nares, had labored breathing, or were making wheezing sounds and two had mucous nasal discharge (Table 2). Clinical signs were significantly ($P < 0.0001$, Chi-square = 33.1) related to a seropositive ELISA result, regardless of sex and age (males $n = 22$, $P < 0.0001$, Chi-square = 16.1; females $n = 17$, $P = 0.044$, Chi-square = 4.55; immatures $n = 6$, $P = 0.0012$, Chi-square = 13.9). The probability for the examined population that a tortoise with clinical signs of URTD also tested seropositive (positive predictive value

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TABLE 2. The number of desert tortoises with and without mucous nasal discharge compared to the number of desert tortoises with and without antibodies to *M. agassizii* as measured by ELISA.

ELISA result	Mucous nasal discharge	No mucous nasal discharge
Seropositive	17/7/2[a]	21/20/5
Seronegative	0/1/1	23/27/20

[a] Number of adult male tortoises/number of adult female tortoises/number of immature tortoises.

of clinical signs or sensitivity of the ELISA test) was 84% with a confidence interval of 70 to 93%. Positive predictive values of clinical signs and the corresponding confidence intervals for adult male, adult female, and immature tortoises were 96% (77 to 100%), 71% (44 to 90%), and 83% (36 to 100%), respectively. The negative predictive value for clinical signs or the specificity of the ELISA test for the total population was 66% with a confidence interval of 55 to 74%. Negative predictive values of clinical signs and the corresponding confidence intervals for adult male, adult female, and immature tortoises were 56% (40 to 72%), 61 (43 to 76%), and 91% (71 to 99%), respectively. Kappa for concordance of clinical signs with ELISA results for the entire population was 0.43 (P = 0.0001). For adult male, adult female, and immature tortoises, kappa was 0.48 (P = 0.0001), 0.28 (P = 0.017), and 0.7 (P = 0.0001), respectively.

Twenty-six (93%) of 28 tortoises whose clinical signs included mucous nasal discharge tested positive by ELISA for exposure to *M. agassizii* (P = < 0.0001, Chi-square = 25.5) (Table 2). The remaining two tortoises with mucous nasal discharge were negative, and represented 2.8% of the 72 seronegative tortoises. The relationship between mucous nasal discharge and a positive test result was significant for both male tortoises (P < 0.0001, Chi-square = 14.3) and female tortoises (P = 0.025, Chi-square = 5.52). In immature tortoises no relationship between mucous nasal discharge and a positive test result (P = 0.15, Chi-square = 3.11) was detected. The probability for a tortoise with mucous nasal discharge also being seropositive in the ELISA test was 93% with a confidence interval of 77 to 99% for the total population. In males it was 100% with a lower confidence limit of 84%, in females 88% (47 to 100%), and in immatures 67% (9.4 to 99%). Kappa for concordance of mucous nasal discharge with ELISA results for the entire population was 0.33 (P = 0.0001). For adult male, adult female, and immature tortoises, kappa was 0.38 (P = 0.0001), 0.23 (P = 0.0094), and 0.29 (P = 0.039), respectively.

DISCUSSION

For the tortoises examined from Las Vegas Valley, there was a positive relationship between clinical signs of URTD and exposure to *M. agassizii* as measured by ELISA. All clinical signs recorded at the time of blood collection were compatible with URTD (Jacobson et al., 1991). Clinical signs reported in this study were good indicators for *M. agassizii* infection in adult tortoises, as most adult tortoises with clinical signs of URTD were also *M. agassizii*-positive by ELISA. The small number of seropositive immature tortoises in this study caused a wide confidence interval which made it impossible to predict the serological status of immature tortoises by clinical signs. Mucous nasal discharge, alone or in combination with other clinical signs, was the most reliable indicator of a *M. agassizii* infection in adult tortoises. All male and most female tortoises with nasal discharge were accurately predicted to be positive for mycoplasma infection by visual inspection for mucous nasal discharge. The status of immature animals was impossible to predict, because of the small number of seropositive immature tortoises.

Results of the visual inspection of tortoises for clinical signs of URTD proved to be very specific in detecting mycoplasma infection in the adult tortoises of the examined population. However, absence of

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clinical signs in a tortoise did not prove that it had not been exposed to *M. agassizii*; sensitivity of visual inspection for clinical signs was poor. One-third of the tortoises in this study appeared clinically healthy at the time of blood collection but were seropositive. We have seen captive tortoises with confirmed (by ELISA and culture) *M. agassizii* infections to be clinically normal for more than 1 yr before suddenly developing clinical signs of URTD. This can be attributed to the chronic nature of URTD, which causes intermittent appearance of clinical signs throughout the course of the disease. The serological status of these animals typically remained positive for antibodies to *M. agassizii* during the subclinical periods. Jacobson et al. (1995) reported on subclinical mycoplasmosis in a tortoise population in Las Vegas Valley. The environmental and behavioral stressors which may cause a latent *M. agassizii* infection to become clinically manifest have not been determined. Tortoises without clinical signs of URTD but seropositive for *M. agassizii* may be silent carriers and act as reservoirs for the pathogen. In some tortoises antibodies to *M. agassizii* may develop before any clinical signs of disease are seen, as demonstrated in a recent transmission study in which desert tortoises were inoculated with *M. agassizii* (Brown et al., 1994). Some tortoises may clear the mycoplasma infection while remaining seropositive; others may have been exposed to the pathogen without becoming infected. Unfortunately, logistical and funding constraints precluded a systematic follow-up in terms of the development of clinical signs of URTD in our tortoises. When a newly developed polymerase chain reaction (PCR) (Templeton, 1992) was used to screen presumed isolates of *M. agassizii,* 10 of 35 isolates did not have the same 16S rRNA sequence and represented a new species (Brown et al., 1995). However, when the new mycoplasma was used as the ELISA antigen in lieu of *M. agassizii,* the ELISA values obtained were virtually identical (I. M. Schumacher, unpubl.). Based on preliminary infection studies, the undescribed mycoplasma, like *M. agassizii,* can cause clinical signs of URTD in gopher tortoises (*Gopherus polyphemus*). To date the pathogenicity of the new mycoplasma has not been tested in the desert tortoise. There may be other agents that do not cause clinical disease, but share antigenic determinants with *M. agassizii,* thus causing tortoises exposed to those agents to react positive in the serologic test.

Of the tortoises with signs of URTD, seven were seronegative for exposure to *M. agassizii.* There are several possible explanations. Other pathogens, like *Pasteurella testudinis,* have been found in tortoises (Snipes and Bieberstein, 1982) and may cause clinical signs similar to those observed in the seven *M. agassizii*-negative animals. However, in a recent study, *Pasteurella testudinis* alone did not cause clinical signs (Brown et al., 1994). Also, some clinical signs reported may not have been indicative of illness but may have been caused by other stimuli. Wet nares in tortoises can be caused by eating or drinking, or in response to dust or other allergens. In some tortoises the appearance of clinical signs may precede the production of detectable levels of *M. agassizii* antibodies (I. M. Schumacher, unpubl.). Interpretation of ELISA results used in population management as a step to curb the spread of a contagious disease should err on the side of false positives rather than false negatives. However, the low cut-off in the ELISA used to define seropositive animals in order to avoid false negative results may still have been too high. Finally, the observed clinical signs in all or some of the seven tortoises may have been caused by one or several as of yet undiagnosed pathogens.

Although there was significant concordance between visual inspection for URTD and detection by ELISA of antibodies against *M. agassizii,* kappa was only between 0.2 and 0.4; thus only fair or good

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concordance occurred between the tests. This was a direct result of the way in which kappa is calculated. Kappa is derived by simultaneously taking into account the number of seropositive animals with clinical signs and the number of seronegative animals without clinical signs. Since the sensitivity of the ELISA (84%) was much better than the sensitivity of the visual inspection (53%), the overall concordance of the two tests was low. This reinforces the importance of serological testing of tortoises that have to be relocated in order to not miss animals that do not have clinical signs but that are infected with *M. agassizii* and able to spread the pathogen. Even the most careful observer may miss clinical signs; and there are cases where clinical signs are absent, either because infected chronically ill animals were visually assessed in between episodes of overt disease or because some tortoises were silent carriers that may, although infected, never show clinical signs.

The ELISA can be used to determine whether individual tortoises have been exposed to *M. agassizii* as well as the prevalence for *M. agassizii*-exposure within a population. But based upon a single blood sample, ELISA tests cannot be used to diagnose an active infection because the presence of *M. agassizii* organisms in an animal cannot be determined by serologic tests. Rising titers between paired samples taken approximately 2 mo apart would be evidence for a recent infection. Information on *M. agassizii* presence can be obtained by culture (Tully, 1977) or by PCR (Brown et al., 1995). These tests can be used to detect the microorganism or its genetic material in tissues or in nasal flushes. However, the sensitivity and specificity of the ELISA merits its use as the most reliable indicator of exposure to *M. agassizii* in free-ranging tortoise populations. Also, blood samples for ELISA testing are more conveniently obtained under field conditions and less costly than sterile nasal flush samples for culture.

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