JOURNAL OF CLINICAL MICROBIOLOGY, June 1993, p. 1454–1460
0095-1137/93/061454-07$02.00/0

Vol. 31, No. 6

Detection of Antibodies to a Pathogenic Mycoplasma in Desert Tortoises (*Gopherus agassizii*) with Upper Respiratory Tract Disease†

ISABELLA M. SCHUMACHER,[1]* MARY B. BROWN,[2] ELLIOTT R. JACOBSON,[1] BOBBY R. COLLINS,[1] AND PAUL A. KLEIN[3]

Department of Small Animal Clinical Sciences[1] and Department of Infectious Diseases,[2] College of Veterinary Medicine, and Department of Pathology and Laboratory Medicine,[3] College of Medicine, University of Florida, Gainesville, Florida 32610

Received 21 December 1992/Accepted 16 March 1993

***Mycoplasma agassizii* (proposed species novum) is the etiologic agent of an upper respiratory tract disease in the desert tortoise (*Gopherus agassizii*), which is threatened in most of its range. An enzyme-linked immunosorbent assay (ELISA) for the detection of *M. agassizii*-specific antibodies in desert tortoises was developed with a monoclonal antibody with specificity for desert tortoise immunoglobulin light chain. Plasma samples from one group of tortoises were tested immediately before and 1 month after challenge either with nasal exudate containing *M. agassizii* or with a purified preparation of *M. agassizii*. Plasma samples from a second group of known healthy and sick tortoises were also tested. In the first group, the ELISA detected seroconversion in individual tortoises following challenge with *M. agassizii*. In the second group, ELISA results were positively correlated with the health status of the tortoises, as determined by clinical and pathologic findings. In addition, the ELISA revealed that tortoise antimycoplasma antibodies were specific for *M. agassizii* when samples were assayed against *M. agassizii*, *M. pulmonis*, *M. testudinis*, and *M. gallisepticum* antigens. The observed direct correlation between the presence of nasal mucosal lesions and *M. agassizii*-specific antibodies proved that the ELISA reliably diagnosed *M. agassizii* infection in desert tortoises and advocates its use for monitoring *M. agassizii*-induced upper respiratory tract disease in free-ranging desert tortoises.**

Over the past two decades an upper respiratory tract disease (URTD) in the desert tortoise (*Gopherus agassizii*) has contributed significantly to the decline of this species (15). Tortoises with URTD characteristically show clinical signs of rhinitis with clear to purulent nasal discharge, palpebral edema, dehydration and cachexia in the late stages, and a high mortality rate. In April 1990, desert tortoises in areas north and west of the Colorado River in the United States were listed as threatened by the U.S. federal government (7).

During the course of recent studies on the pathology of URTD, a new *Mycoplasma* strain was isolated from several clinically sick tortoises (4, 15). This strain, designated *Mycoplasma agassizii* (proposed species novum), was shown to be the principal etiologic agent of URTD by fulfillment of Koch's postulates in recent experimental transmission studies (4, 5). At present, there is no rapid and reliable diagnostic procedure that can be used to detect *M. agassizii* infection in desert tortoises, thus severely limiting the availability of the critical epidemiologic information needed for the management of URTD in free-ranging desert tortoise populations.

Serologic tests are most frequently used in the diagnosis of mycoplasmal infections, especially when isolation of the *Mycoplasma* species is time-consuming or difficult because of the fastidious growth requirements of the mycoplasma. In this report, we describe an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *M. agassizii* in desert tortoises (*G. agassizii*). This ELISA is the first immunological test which can be used to monitor a large free-ranging reptile population for an epidemic disease.

MATERIALS AND METHODS

Antigen preparation for ELISA and immunoblotting. *M. agassizii*, *M. gallisepticum*, *M. pulmonis*, and *M. testudinis* were used as antigens in ELISAs and Western blots (immunoblots). *M. agassizii* was isolated from a desert tortoise with clinical signs of URTD (15). *M. pulmonis* X1048, a clinical isolate, was obtained from Maureen Davidson, University of Alabama at Birmingham. *M. gallisepticum* 19610 and *M. testudinis* 43263 were obtained commercially (American Type Culture Collection, Rockville, Md.).

Antigen preparation for ELISA. For antigen preparation for ELISA (6), a 1-liter culture of each mycoplasma was grown in SP4 supplemented with 20% fetal bovine serum (25) and was harvested in the mid-logarithmic growth phase, as indicated by a color change of the medium, by centrifugation at 10,000 × *g* for 30 min at 4°C. The pellet was washed three times with sterile phosphate-buffered saline (PBS; pH 7.3). Bacterial contamination was monitored by inoculation of brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.). The washed pellet was suspended in sterile PBS to give a final protein concentration of 5 mg of protein per ml, as determined by the protein assay of Bradford (3). Cells were lysed by incubation in 20 volumes of 0.05 M carbonate-bicarbonate buffer (pH 10.0; lysing buffer) for 30 min at 37°C and neutralized with 2.2 g of boric acid per 100 ml of lysing buffer, as described by Horowitz and Cassell (14). The lysate protein content was determined by the protein assay of Bradford (3). All antigens were stored at −70°C.

Antigen preparation for immunoblotting. One liter of each

* Corresponding author.

† This report is published as University of Florida Experiment Station journal series no. R-02817.

mycoplasma strain in mid-logarithmic growth was centrifuged at 12,000 × *g* for 30 min. The pellet was washed three times with sterile PBS (pH 7.3) and then suspended in 2 to 5 ml of sterile distilled water. The protein concentration was determined by the protein assay of Bradford (3) and adjusted to 2 to 5 mg/ml with sterile distilled water. An equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing bromphenol blue was added (10). The antigen preparations were stored at −20°C and were used at a concentration of 1 mg/ml.

Desert tortoise plasma samples. Blood samples were collected from the jugular vein of each tortoise and were placed in sodium-heparinized glass tubes (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) to prevent clotting. Plasma was obtained by low-speed centrifugation of the tubes at 350 × *g* in a Beckman GPR centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 10 min at room temperature. All plasma samples were stored at −20°C in a manual defrost freezer until the analyses were performed.

Plasma samples were collected from a group of 24 desert tortoises which were part of a comprehensive anatomical, histopathological, microbiological, and electron microscopic study of URTD at our institution. Plasma samples from URTD-free and URTD-infected tortoises, as determined by the presence or absence of clinical signs and pathologic lesions typical of URTD, were used for the present study. In addition, plasma samples were obtained from a separate group of 12 tortoises which were being used in a transmission study. Four of these tortoises were challenged intranasally with crude nasal exudates from desert tortoises with signs of URTD, and eight were challenged with pure *M. agassizii* (derived from a single colony and used for inoculation after the third passage in SP4 broth). The plasma samples used were obtained from these tortoises immediately before intranasal challenge and at 1 and 3 months after challenge.

Conjugates. Mouse monoclonal antibodies (MAbs) against desert tortoise immunoglobulin (IgY) (light and heavy chain) (9, 19) were produced by following a standard hybridoma protocol (16, 20, 23). Briefly, IgY was purified from desert tortoise plasma (2) and was used to immunize 6- to 8-week-old female BALB/c mice. The spleen cells of the hyperimmune mice were fused with myeloma cells (Sp2/O). Supernatants from the resulting hybridoma cells were screened by ELISA and Western blotting for antibodies specific for desert tortoise IgY light chain [IgY(L)] and IgY heavy chain [IgY(H)], respectively. After intraperitoneal injections of the selected cloned hybridomas into BALB/c mice, ascitic fluid samples containing the desired MAbs were harvested. The MAb specific for desert tortoise IgY(L) was designated HL673, and the MAb specific for desert tortoise IgY(H) was designated HL665. MAb HL673 was isotyped as an immunoglobulin G1 (IgG1) and MAb HL665 was isotyped as an IgG2b by using a mouse MAb isotyping kit (Amersham Mouse Monoclonal Antibody Isotyping Kit Code RPN.29; Amersham International plc, Amersham, United Kingdom). MAbs were purified from the ascitic fluid samples on protein G affinity columns (Protein G Sepharose Fast Flow; Pharmacia LKB, Uppsala, Sweden) and biotinylated with Immuno Pure NHS-LC-Biotin (120 μg of biotin per mg of antibody; Pierce, Rockford, Ill.) (8). Polyclonal rabbit anti-Russian tortoise (*Testudo* [*Agrionemys*] *horsfieldii*) (polyclonal anti-*T. horsfieldii*) IgY and IgM, specific for *T. horsfieldii* IgY(H) and IgM(H), respectively, obtained from H. Ambrosius, Leipzig, Germany, were tested for their cross-reactivities with desert tortoise plasma and were used in the initial stage of the study to verify the subclasses of the purified desert tortoise immunoglobulins. Aliquots of affinity-purified polyclonal rabbit anti-*T. horsfieldii* IgY(H) and polyclonal rabbit anti-*T. horsfieldii* IgM(H) were biotinylated.

ELISA procedure. Each well of a microtiter plate (Maxisorp F96; Nunc, Kamstrup, Denmark) was coated with 50 μl of antigen at a concentration of 10 μg/ml in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% NaN_3 (PBS/A), and the plates were incubated at 4°C overnight (11). The wells were washed four times with PBS/A containing 0.05% Tween 20 (PBS-T) with an automatic ELISA washer (EAW II; LT-Labinstruments, 5082 Groedig/Salzburg, Austria) and then blocked with 250 μl of PBS/A containing 1% bovine serum albumin per well at room temperature for 60 min or at 4°C overnight. After four more washes, 50 μl of plasma diluted 1:2 and 1:10 and then up to 1:3,200 with PBS/A was added to individual wells in duplicate, and the plates were incubated at room temperature for 60 min. The wells were washed again as described above, and 50 μl of the biotinylated MAb HL673 diluted 1:500 in PBS/A or biotinylated polyclonal anti-*T. horsfieldii* IgM(H) or biotinylated polyclonal anti-*T. horsfieldii* IgY(H), both diluted 1:50 in PBS/A, was added to the appropriate wells. The wells were incubated at room temperature for 60 min and washed as described above. The wells were filled with 50 μl of alkaline phosphatase-labeled streptavidin (AP-streptavidin; 1:1,000 dilution in PBS/A; Zymed Laboratories, Inc., San Francisco, Calif.), and the plates were incubated at room temperature for 60 min. After washing the wells four times with PBS-T, 50 μl of *p*-nitrophenyl phosphate disodium (1 mg/ml prepared in 0.01 M sodium bicarbonate buffer [pH 9.6] containing 2 mM $MgCl_2$; Sigma, St. Louis, Mo.) was added to each well, and the plates were incubated in the dark at room temperature for 60 min. The A_{405} of each well was measured in an ELISA plate reader (EAR 400 AT; SLT-Labinstruments, 5082 Groedig/Salzburg, Austria). In each assay, the blank was the mean value for two wells coated with antigen and incubated with the conjugate and the substrate only. Plasma from a desert tortoise that was culture negative for *M. agassizii* and free of lesions indicative of URTD served as a negative control. Plasma from a desert tortoise infected with *M. agassizii* and with lesions indicative of URTD was the positive control. Positive and negative controls were included on each plate to determine interplate variation. All antibodies used in the ELISAs were biotinylated. Antibody levels were expressed as the ratio of the A_{405} of the sample to the A_{405} of the negative control. Plasma was considered to be positive for *M. agassizii*-specific antibody when the enzyme immunoassay (EIA) ratio (the optical density at 405 nm [OD_{405}] of the sample/OD_{405} of the negative control) was above the value derived by the ratio of two times the mean of the negative control OD_{405} to the mean of the negative control OD_{405} (two times the mean of the negative control OD_{405}/mean of the negative control OD_{405}). This translates into cutoffs of EIA ratios of 2 for either dilution, which corresponds to cutoffs of 0.690 OD_{405} for the 1:2 plasma dilution and 0.618 OD_{405} for the 1:10 plasma dilution.

Immunoblotting. To demonstrate the specificities of our MAbs, pooled desert tortoise plasma (diluted 1:20 in PBS/A) was separated by SDS-PAGE under reducing conditions by using a precast 10% Tris-glycine gel (Novex, San Diego, Calif.) as described previously (18). The proteins were then electrophoretically transferred from the gel to a nitrocellulose sheet (BA-S 83; Schleicher & Schuell, Inc., Keene,

N.H.) (12, 24) by using the Novex Western transfer apparatus and the instructions provided by Novex. A Tris-glycine buffer (pH 8.3; Novex) in 20% methanol was used as the transfer buffer. The blotting time was 120 min at a constant voltage of 30 V. Once the transfer was complete, the nitrocellulose was blocked immediately with PBS/A containing 5% nonfat dry milk and the mixture was incubated at room temperature on an orbital shaker overnight. The nitrocellulose was then washed three times (5 min per wash) with PBS-T and placed into a trough manifold (PR 150 Mini Deca Probe; Hoeffer Scientific Instruments, San Francisco, Calif.). A total of 300 μl of biotinylated anti-desert tortoise immunoglobulin MAb HL673 and MAb HL665 (diluted 1:500 in PBS/A) and polyclonal anti-*T. horsfieldii* IgY(H) (diluted 1:50 in PBS/A) was loaded into each channel and were incubated on the nitrocellulose for 90 min at room temperature on an orbital shaker. After three more washes with PBS/A, the nitrocellulose was removed from the manifold and was incubated with AP-streptavidin (diluted 1:1,000 in PBS/A) at room temperature for 90 min. After three more washes with PBS/A, the blot was developed with substrate buffer (0.1 M Tris HCl, 1 mM $MgCl_2$ [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 (Immunoselect, GIBCO BRL, Gaithersburg, Md.). All antitortoise antibodies used for immunoblotting were biotinylated as described before.

Statistical analysis. Differences in the levels of specific antibody in challenge plasma were determined by Student's paired *t* test. The relationship between specific antibody levels and nasal mucosal lesions was analyzed by chi-square analysis with Yate's correction factor. A P value of <0.05 was considered significant.

RESULTS

Development of MAbs specific for desert tortoise immunoglobulins. MAbs specific for desert tortoise immunoglobulins were developed to ensure the long-term availability of highly specific secondary antibodies for testing of plasma from desert tortoise populations by ELISA. Figure 1 shows the Western blot reactivity on desert tortoise plasma of the IgY(L)-specific MAb HL673 and the IgY(H)-specific MAb HL665. Lanes 2, 4, and 6 were negative controls in which PBS/A was substituted for primary antibody. The blot (lane 7) shows that MAb HL673 reacted with a single band at approximately 27,000 Da corresponding to desert tortoise IgY(L). Mab HL665 reacted with a single band at approximately 65,000 Da (lane 5) corresponding to desert tortoise IgY(H) (1). Polyclonal anti-*T. horsfieldii* IgY(H) and polyclonal anti-*T. horsfieldii* IgM(H) were tested on the same immunoblot for their cross-reactivities with desert tortoise plasma in order to determine whether these antibodies, although available in very limited supply, could serve as polyclonal reference reagents for the newly developed MAbs. Polyclonal anti-*T. horsfieldii* IgM(H) (lane 1) reacted with one major dark band at approximately 74,000 Da which corresponds to desert tortoise IgM(H). Polyclonal anti-*T. horsfieldii* IgY(H) (lane 3) reacted with a single band of approximately 65,000 Da which corresponds to desert tortoise IgY(H) (1). MAb HL673 also reacted with desert tortoise IgM(L), as determined by ELISA on IgM-rich fractions of desert tortoise immunoglobulins (22).

Determination of ELISA parameters. Plasma samples were obtained from desert tortoises before and after challenge with *M. agassizii* and nasal exudate from desert tortoises with URTD and from desert tortoises with and without clinical signs and pathologic lesions indicative of URTD. It was possible to detect *M. agassizii*-specific antibodies in these samples by using MAbs HL673 and HL665 or polyclonal anti-*T. horsfieldii* antibodies as secondary antibodies. The strongest signal, however, was obtained with biotinylated MAb HL673, which was used in all subsequent experiments. Figure 2 shows the anti-*M. agassizii* antibody titers measured in plasma samples from three different tortoises which were either (i) sick with signs and lesions indicative of URTD, (ii) healthy without signs or lesions indicative of URTD, or (iii) healthy (desert tortoise E2) immediately before and 1 month after challenge with nasal exudate from desert tortoises with URTD. Antibody levels against *M. agassizii* were high in the known URTD-positive tortoise and in tortoise E2 1 month after challenge. Antibody levels

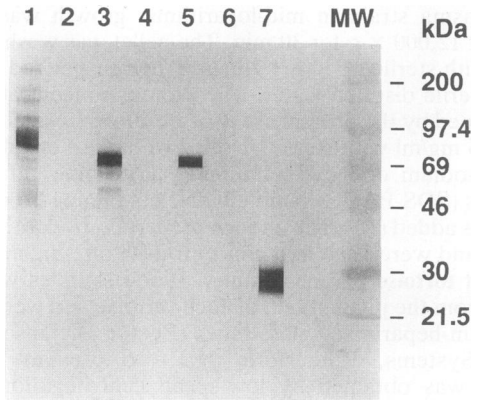

FIG. 1. Western blot analysis of polyclonal antibody and MAb reactivities on desert tortoise plasma. Lane 1, polyclonal anti-*T. horsfieldii* IgM(H); lane 3, polyclonal anti-*T. horsfieldii* IgY(H); lane 5, Mab HL665; lane 7, Mab HL673; lanes 2, 4, and 6, PBS/A controls. MW, molecular weight markers. Pooled desert tortoise plasma (1:20 dilution) was separated by SDS-PAGE. Polyclonal antibodies were used at a 1:50 dilution. MAbs were used at a 1:500 dilution.

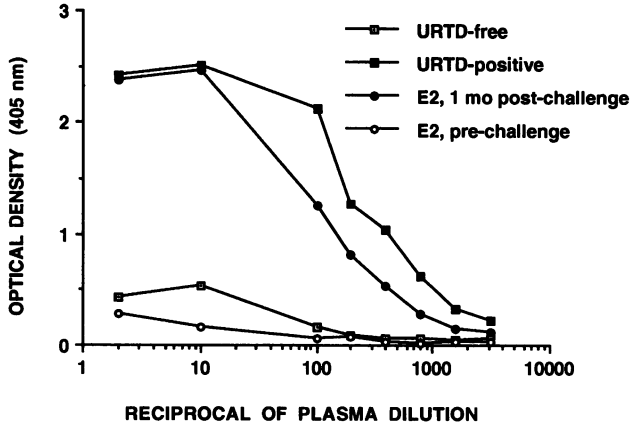

FIG. 2. Titration of desert tortoise plasma samples in the ELISA for antibodies to *M. agassizii*. Plasma samples were from a URTD-free animal and a URTD-positive animal and from a pre- and postchallenge tortoise (tortoise E2), as indicated. Biotinylated MAb HL673 was used as secondary antibody at a 1:500 dilution.

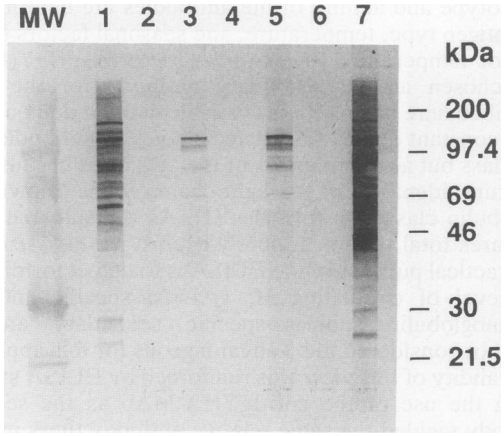

FIG. 3. Western blot analysis of desert tortoise plasma samples tested by the ELISA for which the results are shown in Fig. 2. Lane 3, plasma from tortoise E2 before challenge with *M. agassizii* antigens; lane 1, plasma from tortoise E2 1 month after challenge; lane 5, plasma from a URTD-free tortoise; lane 7, plasma from a URTD-positive tortoise; lanes 2, 4, and 6, PBS/A controls. MW, molecular weight markers. *M. agassizii* was used as antigen at a concentration of 1 mg/ml. Biotinylated MAb HL673 was used as secondary antibody at a 1:500 dilution.

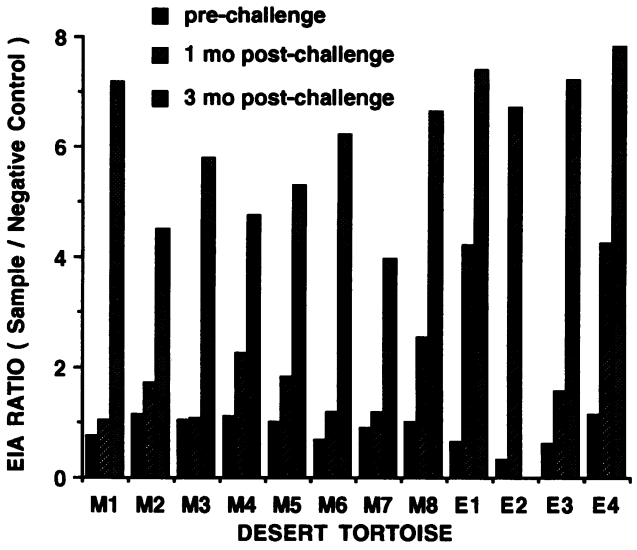

FIG. 4. Antibody levels to *M. agassizii* as detected by the ELISA in the course of a transmission study. Desert tortoise plasma samples were tested at a 1:10 dilution. M1 to M8, eight desert tortoises challenged with pure *M. agassizii* preparation; E1 to E4, four desert tortoises challenged with *M. agassizii*-positive nasal exudates from sick desert tortoises.

were very low in the URTD-free tortoise and undetectable in tortoise E2 before challenge.

Confirmation of antigen-antibody reaction in desert tortoise plasma samples by immunoblot analysis. In order to test the antigen-antibody reaction in an independent assay, Western blots were performed on antigen preparations of *M. agassizii* (1 mg/ml) separated by SDS-PAGE. The immunoblot was carried out by using the same pre- and postchallenge plasma from tortoise E2 and plasma from the same known URTD-positive and URTD-free tortoises tested in the ELISA. Figure 3 shows the reactions of plasma from the URTD-free tortoise (lane 5) and prechallenge plasma from tortoise E2 (lane 3) with *M. agassizii* antigens. Plasma from the URTD-positive animal (lane 7) and postchallenge plasma from tortoise C5 (lane 1) show strong antibody responses to multiple antigenic species in the *M. agassizii* preparation, which supports the results obtained previously in the ELISA (Fig. 2).

Detection of seroconversion in individual tortoises by the ELISA. The ability of the ELISA to detect specific antibody in desert tortoises exposed to *M. agassizii* was further evaluated in two groups of tortoises which were challenged either with pure *M. agassizii* or with nasal exudate containing *M. agassizii*. Antibody levels were measured in prechallenge plasma samples and plasma samples obtained 1 and 3 months after challenge. Plasma dilutions were 1:10. Figure 4 shows that small increases in the levels of *M. agassizii*-specific antibodies could be detected 1 month after challenge in most animals receiving either pure *M. agassizii* (animals M1 to M8) or nasal exudate (animals E1 to E4) ($P < 0.02$). Three months after the challenge, however, both groups of tortoises showed a further significant increase in levels of specific antibody in comparison with the prechallenge values ($P < 0.0001$). The tortoise in the exudate group with the highest antibody level (tortoise E2) died before it could be tested at 3 months postchallenge.

Specificity of desert tortoise antibody response for different *Mycoplasma* strains. The specificities of tortoise antimycoplasma antibodies for mycoplasma strains *M. agassizii*, *M. pulmonis* (mammalian), *M. testudinis* (reptilian), and *M. gallisepticum* (avian) were determined by the ELISA. Tests were carried out on the pre- and postchallenge plasma from tortoise E2 and plasma from the known URTD-positive and URTD-free tortoises tested previously (Fig. 2 and 3). With the exception of *M. pulmonis*, very low levels of antibody to antigens of the tested *Mycoplasma* strains were detectable in the prechallenge plasma and in the plasma of the URTD-free tortoise (Fig. 5). Postchallenge plasma and plasma from the URTD-positive tortoise showed high antibody levels to *M. agassizii* and low antibody levels to *M. gallisepticum* and *M. testudinis*. In both of these plasma samples, the antibody response to *M. pulmonis* was greater than the responses to *M. gallisepticum* and *M. testudinis*.

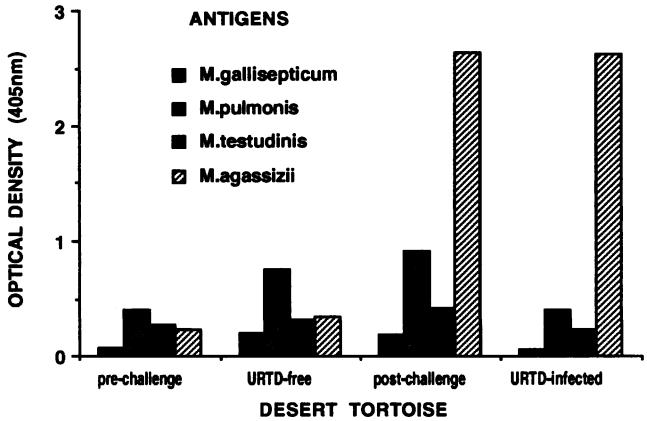

FIG. 5. Antibody levels against various *Mycoplasma* strains in 1:10 dilutions of desert tortoise plasma determined by the ELISA. The plasma samples were the same as those tested previously (see legends to Fig. 2 and 3). Biotinylated MAb HL673 was used as secondary antibody at a 1:500 dilution.

TABLE 1. Reproducibility of the ELISA[a]

Plasma sample	Dilution	Mean A_{405}[b]	SD	CV[c]
Negative control	1:2	0.345	0.077	22.30
	1:10	0.309	0.052	16.80
Positive control	1:2	2.621	0.114	4.35
	1:10	2.765	0.109	3.94

[a] Reproducibility is on the basis of 16 tests, each including duplicates of the negative and the positive control plasma at 1:2 and 1:10 dilutions.
[b] Mean A_{405} values of 16 observations.
[c] CV, coefficient of variation.

Reproducibility of the ELISA. An estimate of the reproducibility of the ELISA is given in Table 1. Interassay reproducibility was determined by calculating the mean A_{405}, the standard deviation, and the coefficient of variation for the negative and positive controls of 16 ELISAs over a time period of 2 months by using the same batch of *M. agassizii* antigen, plasma, and MAb HL673.

Correlation of the presence of *M. agassizii*-specific antibodies and the presence of URTD in desert tortoises. Lesions in the nasal mucosae were significantly correlated ($P = 0.0297$; chi-square analysis) with the detection of *M. agassizii*-specific antibody by ELISA (Table 2). *M. agassizii*-specific antibody was detected in 1:10-diluted plasma of all of the clinically sick animals with lesions. One clinically sick animal with *M. agassizii*-specific antibody did not have lesions. One clinically healthy animal exhibited lesions, but no *M. agassizii*-specific antibody could be detected in its plasma. *M. agassizii*-specific antibody was detected in two clinically healthy animals without lesions.

DISCUSSION

The study described here represents the first attempt to apply the tools of biotechnology to the development of a diagnostic serologic test for measuring pathogen-specific antibody in reptile plasma. The results demonstrate that the ELISA can reliably detect antibodies to *M. agassizii* in the desert tortoise (*G. agassizii*). The ELISA was found to have a high degree of specificity for the detection of *M. agassizii* antibodies in desert tortoise plasma and a high degree of sensitivity for distinguishing desert tortoises with antibodies to *M. agassizii* from desert tortoises without specific antibodies. The ELISA also can detect seroconversion and monitor the development of antibody responses to *M. agassizii* in newly infected tortoises.

In the tortoise, the dynamics of the immune response and the isotype and affinity of the antibodies are determined by the antigen type, temperature, and seasonal factors independent of temperature (1). Anti-desert tortoise IgY(L) MAb was chosen as the secondary antibody for the ELISA described here because it is available in unlimited quantities, has a constant quality, and detects not only antibodies of the IgY class but also antibodies of the IgM class because of the structural identities of the light chains of the known immunoglobulin classes in tortoises (1). As a result, the ELISA measures total plasma antibody against *M. agassizii*. Since the practical purpose of the ELISA is to detect tortoises with any level of circulating *M. agassizii*-specific antibodies, immunoglobulin subclass-specific secondary antibodies were not considered to be advantageous for this application. The validity of this view was reinforced by ELISA studies in which the use of the anti-IgY(L) MAb as the secondary antibody yielded the same relative antibody titers in plasma samples and detected all antibody-positive tortoises which could be detected by using either biotinylated polyclonal anti-IgM(H) antibody or polyclonal anti-IgY(H) antibody as secondary reagents (22). The future availability of MAbs specific for desert tortoise IgM will allow more detailed study of the antibody subclass response to *M. agassizii* in the desert tortoise.

TABLE 2. Correlation of the presence of URTD-related lesions with the presence of *M. agassizii*-specific antibodies in 23 individual desert tortoises diagnosed as clinically healthy or clinically sick by the absence or presence of nasal discharge

Diagnostic results	No. of desert tortoises ($n = 23$)[a]	
	Healthy	Sick
Lesion +, antibody +	4	11
Lesion +, antibody −	1	0
Lesion −, antibody −	4	0
Lesion −, antibody +	2	1

[a] Healthy, no nasal discharge; sick, nasal discharge present.

Although some immunological studies have been carried out by Ambrosius (1) on the tortoise *T. horsfieldii* and Hermann's tortoise (*Testudo hermanni*), no studies have evaluated the immune system of the desert tortoise (*G. agassizii*). In our study, the heavy and light chains of both the high-molecular-weight immunoglobulin (IgM-like) as well as the low-molecular-weight immunoglobulin (IgY) of the desert tortoise were found to be heavier than those reported for the respective immunoglobulins of the tortoise *T. hermanni* (1). Ambrosius (1) reported molecular weights of 70,000 and 23,000, respectively, for the heavy and light chains of the high-molecular-weight immunoglobulin and 63,000 and 23,000, respectively, for the heavy and light chains of the low-molecular-weight immunoglobulin. However, because of the intrinsic error in determining molecular weights by SDS-PAGE, these values should only be considered estimates (13).

An ELISA has never been used before to study an infectious disease in a free-ranging reptile. There was no alternative test which we could use as a reference. Mycoplasma culture alone was not reliable as a diagnostic tool because it is difficult to grow *M. agassizii* and growth of the organism requires 3 to 6 weeks for primary isolation. Since there were no preexisting control groups of confirmed negative and positive sera available, we had to confirm the perceived health status of individual tortoises by pathologic and histologic evaluations of necropsy specimens. Only 24 animals were made available for the necropsy studies described here since desert tortoises are listed as threatened by the U.S. federal government.

We had only one large plasma sample from a tortoise proven to be URTD-free and one large sample from a severely affected URTD-positive tortoise to serve as the negative and positive controls, respectively, during development of the ELISA. For routine diagnostic use of this ELISA in the future, large collections of both positive and negative plasma samples from necropsied animals need to be established for use as controls.

In three of the four plasma samples tested (Fig. 2), the OD for the 1:10 sample dilution was higher than that for the 1:2 sample dilution, demonstrating the presence of a prozone effect. This was similar to the results obtained recently by

Kao et al. (17) for an ELISA designed to measure anti-human leukocyte antigen antibodies in human blood transfusion recipients. In that assay, some false-negative results were observed with samples at a 1:2 dilution. In order to avoid this potential problem, each plasma sample should be assayed for the presence of *M. agassizii*-specific antibodies at 1:2 and 1:10 dilutions. This also ensures that animals with low levels of specific antibody will not be missed, which is important for monitoring animal populations. The criteria for positive and negative ELISA results will be refined once a larger number of tortoises with verified health status are available.

The ELISA was found to be both sensitive and specific for *M. agassizii*. The presence of low levels of antibody to *M. pulmonis*, a mammalian *Mycoplasma* strain commonly found in small rodents, may be due to the fact that small rodents are frequently found to live as commensal organisms in desert tortoise burrows (26). Therefore, it is possible that the tortoises were exposed to *M. pulmonis* and mounted a limited immune response. Alternatively, some common antigens may be shared between *M. pulmonis* and *M. agassizii*. Rodents infected with *M. arthritidis* react with the *M. pulmonis* ELISA antigen; however, animals infected with *M. pulmonis* do not recognize the *M. arthritidis* ELISA antigen (21).

The direct correlation between the presence of histopathologic nasal mucosal lesions and the detection of *M. agassizii*-specific antibodies by the developed ELISA justifies the use of this ELISA for the diagnosis of *M. agassizii* infections in the desert tortoise (*G. agassizii*). *M. agassizii*-specific antibody was detected in the plasma of all of the clinically sick animals with lesions. One clinically sick animal with *M. agassizii*-specific antibody did not have lesions. One clinically healthy animal exhibited lesions but had no *M. agassizii*-specific antibody in its plasma. *M. agassizii*-specific antibody was detected in two clinically healthy animals without lesions.

There are several explanations why a clinically healthy tortoise may have lesions of the nasal mucosa but no *M. agassizii*-specific antibodies. It is unlikely that the ELISA result was a laboratory error, since the same negative result was obtained when the test was repeated. The lesions could have been very old and the animal no longer had circulating antibodies. Alternatively, the lesions observed may have been caused by another pathogen. The two healthy tortoises and one sick tortoise without lesions but with detectable *M. agassizii*-specific antibody levels could have been in a very early stage of URTD, before lesions had developed. Alternatively, they could have recently recovered from URTD and had a regenerated or healed nasal mucosae on histologic examination. Although the mortality rate from URTD is high among desert tortoises, recovery of some individuals may occur.

The specificity and reproducibility of the ELISA for *M. agassizii*-specific antibodies make it a valuable tool for seroepidemiologic studies of URTD in free-ranging desert tortoise populations. The method is fast and reliable and renders itself to automation to accommodate large numbers of samples. After further development and evaluation, the ELISA can easily be modified to be used as a large-scale diagnostic test for the detection of *M. agassizii*-specific antibodies in desert tortoises. The test will eliminate the need to sacrifice tortoises in order to obtain a reliable diagnosis of URTD. In combination with careful observation for clinical signs of URTD, the test will become an important, noninvasive, and reliable way to assess the health status of desert tortoise populations threatened with URTD.

ACKNOWLEDGMENTS

We thank Eva-Maria Andreas and Herwart Ambrosius, Karl-Marx Universität, Leipzig, Germany, for their gift of polyclonal anti-*T. horsfieldii* sera. We thank Keith Harris from the Armed Forces Institute of Pathology, Washington, D.C., for providing the results on the histologic examination of the tortoise heads. We also thank Linda G. Green and Diane G. Duke from the ICBR Hybridoma Core Laboratory and the BEECS Immunological Analysis Laboratory, University of Florida, for their help in developing the MAbs to desert tortoise immunoglobulins. Special appreciation is given to The Living Desert and staff, and in particular to Terrie Correll, for maintaining the tortoises used in the present study.

This study was cooperatively supported by the Nevada Department of Wildlife, Las Vegas District of the U.S. Bureau of Land Management, and The Nature Conservancy under amended Federal Permit PRT-747182. Funding was provided through a grant (B812680) from The Nature Conservancy made possible by contributions from the development community in southern Nevada.

REFERENCES

1. **Ambrosius, H.** 1976. Immunoglobulins and antibody production in reptiles, p. 298–334. *In* J. J. Marchalonis (ed.), Comparative immunology. Blackwell Scientific Publications, Oxford.
2. **Andreas, E.-M., and H. Ambrosius.** 1989. Surface immunoglobulin on lymphocytes of the tortoise *Agrionemys horsfieldii*. Dev. Comp. Immunol. **13:**167–175.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Brown, M. B., I. M. Fuerst, L. Pathangey, P. Klein, H. Adams, and E. Jacobson.** 1992. Upper respiratory tract disease in the desert tortoise, abstr. PS1/56, p. 189. Abstr. 9th Int. Congr. Int. Org. Mycoplasmology 1992.
5. **Brown, M. B., I. M. Schumacher, K. Snipes, P. A. Klein, and E. R. Jacobson.** Unpublished data.
6. **Cassell, G. H., and M. B. Brown.** 1983. Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-mycoplasma antibody, p. 457–469. *In* J. G. Tully and S. Razin (ed.), Methods in mycoplasmology, vol. 1. Academic Press, Inc., New York.
7. **Department of Interior.** 1990. Endangered and threatened wildlife and plants; determination of threatened status for the Mojave population of the desert tortoise. Federal Register **55:**12178–12191.
8. **Goding, J. W.** 1986. Monoclonal antibodies: principles and practice, 2nd ed., p. 263–265. Academic Press, Inc., Orlando.
9. **Hädge, D., H. Fiebig, and H. Ambrosius.** 1980. Evolution of low molecular weight immunoglobulins. I. Relationship of 7S immunoglobulins of various vertebrates to chicken IgY. Dev. Comp. Immunol. **4:**501–514.
10. **Hames, B. D., and D. Rickwood (ed.).** 1981. Gel electrophoresis of proteins. Oxford University Press, New York.
11. **Harbeck, R. J., and P. C. Giclas.** 1991. General ELISA procedure for detection of miscellaneous antigens and antibodies, p. 173–183. *In* R. J. Harbeck and P. C. Giclas, Diagnostic immunology laboratory manual. Raven Press, New York.
12. **Harlow, E., and D. Lane.** 1988. Immunoblotting, p. 473–510. *In* Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Hjelmeland, L. M., and A. Chrambach.** 1981. Electrophoresis and electrofocusing in detergent containing media: a discussion of basic concepts. Electrophoresis **2:**1–11.
14. **Horowitz, S. A., and G. H. Cassell.** 1978. Detection of antibodies to *Mycoplasma pulmonis* by an enzyme-linked immunosorbent assay. Infect. Immun. **22:**161–170.
15. **Jacobson, E. R., et al.** 1991. Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). J. Wildl. Dis. **27:**296–316.
16. **Kao, K. J., and P. A. Klein.** 1986. A monoclonal antibody-based enzyme-linked immunosorbent assay for quantitation of plasma

thrombospondin. J. Clin. Pathol. **86:**317–323.
17. **Kao, K. J., J. C. Scornik, and S. J. Small.** 1993. Enzyme-linked immunoassay for anti-HLA antibodies: an alternative to panel studies by lymphocytotoxicity. Transplantation **55:**192–196.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
19. **Leslie, G. A., and L. W. Clem.** 1969. Phylogeny of immunoglobulin structure and function. III. Immunoglobulins of the chicken. J. Exp. Med. **130:**1337–1352.
20. **Liddell, J. E., and A. Cryer.** 1991. A practical guide to monoclonal antibodies. John Wiley & Sons, Ltd., Chichester, England.
21. **Minion, F. C., M. B. Brown, and G. H. Cassell.** 1984. Identification of cross-reactive antigens between *Mycoplasma pulmonis* and *Mycoplasma arthritidis*. Infect. Immun. **43:**115–121.
22. **Schumacher, I. M., and P. A. Klein.** Unpublished data.
23. **Simrell, C. R., and P. A. Klein.** 1979. Antibody responses of tumor-bearing mice to their own tumors captured and perpetuated as hybridomas. J. Immunol. **123:**2386–2394.
24. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
25. **Tully, J. G., R. F. Whitcomb, H. F. Clark, and D. L. Williamson.** 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. Science **195:**892–894.
26. **Woodbury, A. M., and R. Hardy.** 1948. Studies of the desert tortoise, *Gopherus agassizii*. Ecol. Monogr. **18:**145–200.