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Application of Diagnostic Tests for Mycoplasmal Infections of Desert and Gopher Tortoises, with Management Recommendations

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ABSTRACT. – **Mycoplasmosis is a transmissible upper respiratory tract disease that has affected plans for management and conservation of wild desert and gopher tortoises in the United States. Although impact of mycoplasmosis on populations of desert and gopher tortoises is unknown, increased prevalence of seropositive animals as well as field observations of clinically ill tortoises have occurred in association with declining populations. In order to help in the identification of potentially infected animals, three tests have been developed to diagnose mycoplasmal infections of tortoises: 1) direct mycoplasmal culture; 2) detection of mycoplasmal chromosomal DNA by polymerase chain reaction (PCR); and 3) detection of anti-*Mycoplasma* antibodies in tortoise plasma by enzyme-linked immunosorbent assay (ELISA). Each test provides different, complementary information that collectively can be used to define tortoise mycoplasmal infection status. The types of samples required, the predictive value, interpretation, and cost vary among tests. These assays have been used for epidemiological surveys and in decision making for relocation, repatriation, or captive management of tortoises to minimize the risk of outbreaks of mycoplasmal respiratory disease and spread of the causative agent of this disease. Certain features of mycoplasmal infections of tortoises and other animals create a diagnostic dilemma. Multiple *Mycoplasma* species can cause respiratory disease with identical clinical presentations. Further, individual strains of a given species may vary with respect to their virulence potential, and some species may be commensals rather than pathogens. Current diagnostic tests may not differentiate among mycoplasmal species or strains or permit determination of pathogenicity of individual isolates. Thus, the information provided by testing is not a simple "positive" vs. "negative" issue. While these tests provide much needed information on the exposure of tortoise populations to *Mycoplasma* species, they do not provide a complete picture of the overall health status of individual tortoises or populations. Unfortunately, test results are often used to make life and death decisions concerning disposition of tortoises being displaced by land development without a complete understanding of the limitations of the diagnostic tests or any consideration of other infectious agents that might be present.**

KEY WORDS. – **Reptilia; Testudines; Testudinidae; *Gopherus agassizii*; *Gopherus polyphemus*; tortoise; *Mycoplasma*; respiratory disease; diagnostics; management**

In 1988, a chronic upper respiratory tract disease (URTD) was recognized in desert tortoises (*Gopherus agassizii*) in the Desert Tortoise Natural Area, Kern County, California (Jacobson et al., 1991). Partially because of this disease and reported population declines in the western Mojave Desert, desert tortoises north and west of the Colorado River were listed as Threatened by the U.S. Department of Interior (1990). Desert tortoises with signs of URTD have been seen at other locations in California (Homer et al., 1998). The disease is characterized clinically by serous, mucous, or purulent nasal and ocular discharge, conjunctivitis, and palpebral edema. Clinical signs may be present intermittently, thus an infected tortoise may appear clinically ill or healthy at any given time. Characteristic microscopic lesions include infiltration of the nasal cavity mucosa and submucosa with inflammatory cells accompanied by hyperplasia and degeneration of upper respiratory tract epithelium (Jacobson et al., 1991, 1995; Brown et al., 1994). A similar appearing disease has been seen in free-ranging gopher tortoises (*Gopherus polyphemus*; Beyer, 1993; Epperson, 1997; McLaughlin, 1997; Smith et al., 1998; Diemer-Berish et al., 2000; McLaughlin et al., 2000) that have legal protection as a Species of Special Concern in Florida, and in a variety of species of captive, non-native tortoises in the pet trade (Jacobson et al., 1991).

Electron microscopic examination of the upper respiratory tract mucosa of free-ranging desert and gopher tortoises with URTD demonstrated the presence of cell-membrane associated bacteria with features compatible with *Mycoplasma* (Jacobson et al., 1991; McLaughlin et al., 2000). A previously undescribed species, *Mycoplasma agassizii,* was subsequently cultured from nasal lavages of affected desert and gopher tortoises (Brown et al., 1995, 2001b). Subsequent experimental infection studies of desert (Brown et al., 1994) and gopher (Brown et al., 1999b) tortoises demonstrated *Mycoplasma agassizii* to be an etiologic agent of URTD. These findings led to the development of optimized culture, polymerase chain reaction (PCR; Brown et al., 1995), and enzyme-linked immunosorbent assay (ELISA; Schumacher et al., 1993) tests for *M. agassizii* to determine the infection and antibody status of asymptomatic and URTD-symptomatic desert and gopher tortoises. Because these tests are currently being used to determine the disposition of displaced desert and gopher tortoises in certain parts of their range, the limitations of each test and the information they provide collectively need to be understood. While these assays can be used for epidemiological surveys and in decision-making for relocation, repatriation, or captive management of desert and gopher tortoises to minimize the risk of spread of mycoplasmal respiratory disease, they do not provide a complete picture of the health status of a seropositive or infected tortoise.

Overview of Mycoplasmal Disease. — Mycoplasmas are the etiologic agent of respiratory disease in a number of hosts, including humans, swine, cattle, laboratory rodents, and poultry. In most animals, respiratory mycoplasmosis is typified as a slowly progressing, chronic, and seemingly clinically silent infection which may be exacerbated by environmental factors, stress, or other microbial agents (Cassell et al., 1985; Schoeb et al., 1985; Weinach et al., 1985; Simecka et al., 1992). Most hosts have difficulty in eliminating the *Mycoplasma*, even in the presence of a strong immune response, and as a general rule, the animal is presumed to be infected for life. In fact, the host immune response is critical for development of lesions (Simecka et al., 1992). Respiratory mycoplasmosis in most species, including desert and gopher tortoises, is characterized by increased numbers of inflammatory cells, particularly in foci, and lymphoid hyperplasia (Simecka et al., 1992; Brown et al., 1994, 1999b; Homer et al., 1998; McLaughlin et al., 2000). Although overt clinical signs may be inapparent, lesions can range from microscopic to gross, with eventual loss of the normal respiratory epithelial architecture (Jacobson et al., 1991; Simecka et al., 1992; Brown et al., 1994; Homer et al., 1998; McLaughlin et al., 2000). For most mycoplasmas, morbidity is quite high, but mortality is not common, at least during acute infection in adults. Mortality may occur in the late stages of chronic disease, however.

Although the pathogenic mechanisms of mycoplasmosis are not fully defined, a concept for pathogenesis has been proposed which is relevant to most mycoplasmal infections and fits the profile of disease observed in tortoises (Razin et al., 1998). Disease is dependent upon attachment and association of the *Mycoplasma* with the host surface, elicitation of an immune response, which may be both detrimental and beneficial to the host, and interaction with the host immune system which may enhance chronicity. Once attached to the host, mycoplasmas cause cell injury by a variety of methods, including but not limited to ciliostasis, hydrogen peroxide production, and toxin production (Razin et al., 1998). In some cases, as in URTD, the entire respiratory architecture can be changed. The interaction between mycoplasmas and other infectious agents may be synergistic (Cassell et al., 1985; Schoeb et al., 1985; Weinach et al., 1985). When this occurs, the *Mycoplasma* appears to be the initial colonizer of the respiratory tract and may predispose the host to subsequent secondary infections.

Mycoplasmal Disease in Other Wildlife Species. — Research on the impact of mycoplasmosis on wildlife has been limited, but reports of recent disease outbreaks in different wildlife species are provoking interest in mycoplasmosis as a newly emerging (or at least newly recognized) disease threat. The most publicized disease outbreak has been seen in *Mycoplasma gallisepticum* infection of house finches, goldfinches, and blue jays (Ley et al., 1996; Luttrell et al., 1996; Nettles, 1996; Hochachka and Dhondt, 2000). In 1993, an epizootic of polyarthritis caused by *M. crocodyli* occurred in juvenile farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe (Kirchoff et al., 1991; Mohan et al., 1995). In 1995, we characterized a systemic infection of captive adult American alligators associated with a previously unrecognized species of *Mycoplasma*. Unlike the outbreak in crocodiles, the disease in alligators was characterized by septic arthritis and pneumonia with atypically high acute mortality (> 70%) associated with *M. alligatoris* (Brown et al., 1997, 2001a; Clippinger et al., 2000).

Tortoise Mycoplasmas and URTD

URTD and Mycoplasmosis. — URTD refers to one or more clinical signs of illness seen in tortoises, including nasal discharge, palpebral edema, conjunctivitis, and ocular discharge. Isolation of *M. agassizii* and subsequent transmission studies confirmed *M. agassizii* to be a cause of URTD in desert and gopher tortoises (Brown et al., 1994, 1999b). While *M. agassizii* is the only confirmed etiologic agent of URTD to date in wild desert tortoises, other pathogens, including other species of *Mycoplasma*, may cause overlapping signs (McLaughlin, 1997). In preliminary experimental infections of gopher tortoises, *M. cheloniae* proposed sp. nov. caused URTD (McLaughlin, 1997). Further, this species has been isolated from clinically ill, wild desert tortoises. An iridovirus was reported in a gopher tortoise with URTD (Westhouse et al., 1996). A wild desert tortoise with signs of respiratory disease was found to have a fungal pneumonia (Homer et al., 1998). In Europe, Hermann's and Greek tortoises (*Testudo hermanni* and *T. graeca)* with clinical signs of URTD have been associated with a herpesvirus by electron microscopy and serology

(Muller et al., 1990), and cases of herpesvirus infection have been reported in captive desert tortoises (Harper et al., 1982; Pettan-Brewer et al., 1996). However, to date experimental transmission studies resulting in clinical signs compatible with URTD have been performed only for *M. agassizii* in desert and gopher tortoises (Brown et al., 1994, 1999b), *M. cheloniae* proposed sp. nov. in gopher tortoises (McLaughlin, 1997), and for a herpesvirus in Greek tortoises (Origgi, 2001).

The current evidence supports horizontal transmission of *Mycoplasma* in desert and gopher tortoises, most likely through direct contact of tortoises in the wild and captivity. Mycoplasmas lack a cell wall and are susceptible to desiccation; therefore, they do not normally persist in the natural environment for any appreciable length of time. A critical concern is contaminated fomites such as field equipment that might contribute to the spread of the organism, especially when mucous and organic contamination is present and proper disinfection techniques are not used. Fomite transmission does occur for other mycoplasmal infections, particularly in food and fiber animals (McMartin et al., 1987). A limited experimental study suggests that environment transmission does not occur in gopher tortoises (McLaughlin, 1997). Seropositive, clinically ill tortoises were housed in outdoor enclosures, these tortoises were removed, and seronegative gopher tortoises were immediately placed in the enclosures (McLaughlin, 1997). None of these tortoises seroconverted or developed URTD. Vertical transmission of mycoplasma is known to occur in poultry, but the rate of transmission is low (Lin and Kleven, 1982; Yoder, 1984). In a study of a very limited number of infected gopher tortoises, no egg transmission was documented (McLaughlin, 1997). Because the sample size was so small, egg transmission cannot be ruled out. Further, as will be discussed later, maternal antibody is present in the egg and persists in hatchlings, potentially confounding diagnostic tests (Schumacher et al., 1999).

Under experimental conditions, the onset of clinical signs in desert and gopher tortoises occurs as early as 2 weeks after infection. However, seroconversion lags behind clinical signs, with reliable detection by 8 weeks after infection. Affected desert and gopher tortoises are assumed to be capable of transmitting *Mycoplasma* through direct contact during the time between onset of signs and seroconversion, because we have found large numbers of *Mycoplasma* in the upper respiratory tract and nasal secretions of experimentally inoculated gopher tortoises during this time.

Clinical signs of URTD such as nasal discharge and conjunctivitis may reflect non-specific host responses to infection (inflammation, mobilization of phagocytes) as well as specific responses (immune-mediated complement activation, formation of immune complexes), although these have not been investigated. Signs may intensify and then abate in cycles, reflecting the progression of URTD. Based upon our transmission studies, our findings suggest that infection and expression of URTD occurs in the following steps: 1) initial colonization with *Mycoplasma*; 2) host response which reduces the population of *Mycoplasma* and simultaneously causes illness and signs of disease; 3) progression to chronic disease with intermittent expression of clinical signs and shedding of *Mycoplasma*. The presence of specific antibody to *M. agassizii* was associated with clinical signs of URTD in wild desert tortoises (Schumacher et al., 1993). In a desert tortoise population with natural infection, some initial fluctuation in antibody levels was observed but once an animal had a strong seroconversion, the antibody levels persisted for years (Brown et al., 1999a). This is consistent with mycoplasmal infections in other hosts.

Upon re-exposure to *Mycoplasma*, previously infected gopher tortoises may develop clinical signs of URTD more rapidly than naïve animals. Under experimental conditions, the clinical signs were more severe, higher numbers of *Mycoplasma* were recovered from nasal flushes, and the antibody response was more rapid than after first exposure (McLaughlin, 1997). Thus upon re-exposure to the pathogen, previously infected gopher tortoises may actually experience exacerbated signs of illness rather than immune protection. This is consistent with the immunopathology associated with most mycoplasmal infections, where the host immune response is a key component in determining disease severity. While this evidence is limited to gopher tortoises, we expect a similar response in other tortoises infected with *Mycoplasma*. It is not clear if naturally infected tortoises develop a protective immune response. Our limited data in experimentally infected tortoises suggests this does not occur. However, there are populations with seropositive animals and no overt disease. Seronegative animals in these populations do often seroconvert, suggesting that *Mycoplasma* are still present in the population but the extent of carriers is unknown. At this point, there are insufficient data to determine if a protective immune response exists in tortoises.

Most hosts do not typically clear mycoplasmal infections, however numbers of mycoplasmas may be substantially lowered during chronic phases of infection in the absence of clinical signs. It is possible that this may occur in the tortoise as well. An additional consideration is the particular strain of *Mycoplasma* present in the individual or population. Strains of most mycoplasmal species vary in virulence. We have limited evidence in the gopher tortoise that suggests strains of *M. agassizii* differ in the minimum dose required to colonize and cause disease. Knowledge of the virulence potential of different strains will be important for risk assessment for future management decisions and also for evaluating the role of the microbe in disease transmission.

Mycoplasma agassizii can cause severe changes in the mucosal epithelium of the upper respiratory tract of desert and gopher tortoises (Jacobson et al., 1991; Homer et al., 1998). These lesions disrupt the normal epithelial arrangement of the tissues and more than likely compromise their function. We have seen a reduction in appetite and other changes in behavior in experimentally challenged tortoises (McLaughlin, 1997). Experimentally infected desert and gopher tortoises often remained in their burrows for extended periods of time once clinical signs of illness developed. Further, irregular basking and burrowing behaviors have been noted in a limited number

of animals in the wild (J. Berish, *pers. comm.*). The full impact of URTD on behavior and the implications of altered behavior on disease transmission and individual health and survival remains to be determined.

Differences among Mycoplasmal Isolates. — Mycoplasmas have been recovered by culture or detected by PCR in the following species of tortoises: desert tortoise (*Gopherus agassizii*), Texas tortoise (*Gopherus berlandieri*), gopher tortoise (*Gopherus polyphemus*), Chaco tortoise (*Geochelone chilensis*), leopard tortoise (*Geochelone pardalis*), Indian star tortoise (*Geochelone elegans*), radiated tortoise (*Geochelone radiata*), African spurred tortoise (*Geochelone sulcata*), Travancore tortoise (*Indotestudo travancorica*), spider tortoise (*Pyxis arachnoides*), flat-tailed tortoise (*Pyxis planicauda*) and spur-thighed tortoise (*Testudo graeca*). *Mycoplasma* has also been detected in Florida box turtles (*Terrapene carolina bauri*). Experimental infection studies have established that two isolates (PS6 and 723) of *M. agassizii* can cause URTD in desert tortoises (Brown et al., 1994) and gopher tortoises respectively (Brown et al., 1999b). The *M. agassizii* isolate 723 used in the gopher tortoise transmission studies (Brown et al., 1999b) was obtained from a very ill, naturally infected gopher tortoise. This particular strain appears to be highly virulent as evidenced by the fact that initial infective doses of only 10 colony forming units (CFU) were capable of causing both clinical disease and severe lesions. Preliminary results also suggested that a second genetically distinct species, *Mycoplasma cheloniae* proposed sp. nov., represented by tortoise isolate H3110 (American Type Culture Collection accession 700618), can cause URTD (Brown et al., 1995). We have preliminary evidence that other strains of *M. agassizii* do not cause overt clinical disease, even in relatively high doses. These observations of strain variability are similar to those observed for respiratory mycoplasmosis in rodents and poultry (Simecka et al., 1992). Additionally, the infectious dose required to establish colonization and to produce clinical disease may differ among strains. Therefore, it is likely that the strain of *Mycoplasma* present in a given population will influence both the clinical course and transmission of URTD.

Mycoplasma isolates are identified on the basis of 16S rRNA gene restriction fragment length polymorphism (RFLP) patterns. To date, at least five different patterns of *Mycoplasma* have been detected in tortoises. Three of these variants are now recognized as distinct species. The first species, *M. testudinis*, was originally isolated from the cloaca (Hill, 1985). *Mycoplasma agassizii* is recognized as a new species (Brown et al., 1995, 2001b), and *M. cheloniae* is proposed as a new species. Both of these species caused URTD in experimentally infected tortoises (McLaughlin, 1997). The biochemical properties, pathogenic capabilities, and species status of the other isolates remain to be determined, but at least some of the isolates appear to have serological cross reactivity with *M. agassizii*. The implications of serologic cross reactivity are discussed in the *Mycoplasma* diagnostic section below.

Mycoplasma Diagnostics

Electron microscopy provided the first evidence of *Mycoplasma* involvement in URTD (Jacobson et al., 1991). This was followed by the development of additional diagnostic tests that could be used to determine antemortem exposure/infection status in individuals and, ultimately, populations of tortoises. Culture, PCR, and ELISA have become the essential diagnostic tools for determination of the mycoplasmal status of tortoises.

Mycoplasma Culture. — *Mycoplasma agassizii* was first isolated from desert tortoises with signs of URTD by culture in SP4 medium (Brown et al., 1994). The *Mycoplasma* grows slowly (up to 6 weeks for primary isolation) in SP4 broth or on SP4 agar at an optimal temperature of 30°C, and ferments glucose under aerobic conditions resulting in an acidic pH shift in the medium. On agar, *M. agassizii* may assume either a classical "fried egg" or a mulberry or cauliflower-like colonial morphology. It is important to note that *M. agassizii* does not grow at higher temperatures. Additionally, ideal culture conditions may vary among species and strains of *Mycoplasma*. Different lots of culture medium may profoundly affect success of isolation, therefore quality control is essential.

Various sampling techniques have been employed to obtain specimens for culture. The most common of these include flushing the nares with 0.5–5.0 ml sterile saline or PBS, or with 0.5–1.0 ml sterile SP4 broth. Electron microscopic studies by the authors have identified preferentially colonized sites on the mucosal surface of ventrolateral depressions in the tortoise upper respiratory passages that are not accessible by swabbing. Therefore nasal swabs are likely to be uninformative unless a nasal discharge is present or when taken from a sectioned head with exposed nasal cavity at necropsy. Samples can be frozen at –20°C or lower for shipping to a testing laboratory, although each freeze-thaw cycle may reduce the number of viable mycoplasmas by as much as 10-fold. If sterile saline or PBS is used as the collection fluid, a cryopreservative such as sterile bovine serum albumin, horse or fetal calf serum, SP4 medium, or glycerol added at a final concentration of 10% prior to freezing may maximize recovery of *Mycoplasma*.

The limit of detection by culture in broth is 1 color change unit (CCU), the number of bacteria necessary to produce enough acidic end-products of fermentation to change the color of a pH indicator in a standard volume of broth in a fixed period of time. The limit of detection by culture on agar is 1 CFU, the number of bacteria necessary to form a colony on the plate large enough to be visible by light microscopy. However, even in pure culture, the number of bacteria in a CCU or a CFU is inconsistent because of the tendency of *Mycoplasma* cells to adhere to each other.

Clinical samples very frequently are contaminated heavily with bacterial and fungal members of the normal tortoise upper respiratory microbiota, as well as environmental contaminants. Many of these, especially fungi, rapidly overgrow the cultures, and these samples will be false

negative or uninformative by culture. To circumvent this problem, we routinely pass a portion of each sample through a 0.45 µm filter to minimize contaminants. Even with uncontaminated samples, the slow growth of primary isolates of *M. agassizii* often makes it difficult to detect low numbers of mycoplasmas, because broth cultures may evaporate and agar cultures can desiccate before mycoplasmal growth is detected. PCR can be performed on broth samples after incubation to increase the probability of detecting low numbers of mycoplasmas in culture.

Culture alone cannot differentiate among *Mycoplasma* species since their fastidious growth requirements preclude biochemical classification schemes and no differences are seen in colonial morphology. The classical method for *Mycoplasma* species identification is serology, most commonly growth inhibition, metabolic inhibition, or immunofluorescence using species-specific polyclonal antisera, usually of rabbit origin. Serological cross-reactivity is marked for some species of *Mycoplasma* because of shared antigens, but growth of *M. agassizii* was not inhibited by antisera against more than 70 other species of *Mycoplasma,* suggesting that cross reactivity is not a major concern for this species (J.G. Tully, *pers. comm*).

The advantages of diagnosing infection by culture are noninvasive sampling, simple technique, direct proof of infection at the time the sample was taken, theoretical low limit of detection, and generation of viable bacterial isolates for future study. Disadvantages of culture include the high proportion of false negative or uninformative samples because of microbial contamination, the high cost of SP4 medium, the very slow growth rate of some primary isolates of *M. agassizii*, and poor ability to identify *M. agassizii* specifically. The slow growth rate, variable colony morphology, and growth temperature restrictions may lead to misinterpretation of culture results in some instances.

Mycoplasma PCR.—Some of the limitations of detection by culture necessitated the development of an alternative diagnostic test for the presence of *Mycoplasma* in nasal lavage samples from tortoises. The principle of the PCR test is to synthesize an easily detectable number of DNA copies of a segment of the mycoplasmal chromosome by using *Mycoplasma*-specific primers. The segment of the *M. agassizii* chromosome selected for analysis, the 16S ribosomal RNA gene, contains conserved (genus-specific) DNA sequences and intervening variable (species-specific) DNA sequences. Thus a primer pair consisting of 1 genus-specific primer and 1 species-specific primer can very reliably distinguish among organisms. Alternatively, the presence of other *Mycoplasma* species can be determined by using generic genus-specific PCR primers. Species identification for such samples usually requires further nucleotide sequence analyses, however.

Nasal lavage samples or cultures can be analyzed for presence of *Mycoplasma* by PCR. *Mycoplasma* need not be viable to be detected by PCR. The sensitivity of the assay can be increased by culturing nasal flush samples before analysis. Excluding culture and lysis steps which may take up to 6 weeks, a batch of 60 samples can be processed in 2 working days. Contamination by other sources of DNA does not interfere with the PCR. However, blood, calcium alginate swabs, and other usually unidentified agents can inhibit the reaction.

PCR has excellent ability to distinguish among organisms for identification of *M. agassizii*. In part this is due to the specific affinity of the DNA primers. Also, the synthesized DNA can be further analyzed to confirm species-specificity. The most rigorous method for species identification is complete determination of the species-specific nucleotide sequences of the DNA (Brown et al., 1995). Alternatively, once the unique DNA sequences are known for a species, a characteristic DNA fingerprint for that species may be generated by treating the product of the PCR reaction with restriction endonuclease enzymes. The electrophoretic pattern of the treated DNA serves as independent species confirmation for a positive PCR signal, and also will reveal if more than 1 type of *Mycoplasma* are present in the sample. This additional analysis extends sample processing time by 1 working day.

The practical limit of detection by PCR when test results are visualized by fluorescence after agarose gel electrophoresis is about 1000 *Mycoplasma* (chromosomes) per 0.5 ml aliquot of broth. The PCR can approach the theoretical limit of detection by culture (1 viable bacterial cell), and in practice PCR may be able to detect a smaller number of *Mycoplasma* than culture can, because clumping of the *Mycoplasma* has no effect, and the cells do not have to be viable to be detected by PCR.

The advantages of diagnosing infection by PCR are noninvasive sampling, direct proof of infection at the time the sample was taken, the reaction is not inhibited by sample contamination with other microbes, the potential short sample turnaround time if the sample is not cultured before analysis, accurate identification of *M. agassizii* and other *Mycoplasma*, and a theoretical low limit of detection. Disadvantages of PCR include the need for specialized laboratory equipment and sophisticated and meticulous technique, the high cost of special reagents, the potential for false positive results caused by cross-contamination, uninformative samples caused by inhibitory substances in the reaction, and a portion of the sample is consumed in the reaction (it cannot be re-tested).

Mycoplasma ELISA. — The principle of the ELISA is to detect the presence of anti-*M. agassizii* antibodies in tortoise plasma (Schumacher et al., 1993). It is an indicator of prior infection with *M. agassizii* and of current anti-*M. agassizii* antibody status, but that by itself does not reveal if a tortoise was infected at the time the blood sample was taken.

Results of the ELISA are analyzed quantitatively but interpreted categorically (negative, suspect, or positive). Previously, results were reported as a ratio of the absorbance of a sample to that of the negative control. Using this system, ratios ≥ 3 were considered positive for antibody to *M. agassizii.* Ratios ≥ 2 and < 3 were considered suspect and ratios < 2 were considered negative. The cutoff points were chosen subjectively and conservatively, with the specific objective of minimizing the chance of false negative results.

The ELISA has since been refined based on the evaluation of over 6000 samples to include more stringent quality control measures, and to report results as a titer in order to make the assay more consistent with other serologic tests. Under the current system, a negative titer is < 32, a suspect titer is 32, and a positive titer is ≥ 64.

The monoclonal antibody used in the ELISA, HL673, is a mouse antibody against the light chain of both IgM and IgY tortoise immunoglobulins. IgY, the equivalent of mammalian IgG, is the class of tortoise antibodies produced during a chronic infection or during an immune memory response. IgY may persist, even after clearance of *Mycoplasma*. The specificity of the monoclonal is a critical component of the assay as it determines which tortoise species can be tested in the current ELISA. The monoclonal has been validated for both the gopher and desert tortoise, but we have evidence that this monoclonal does recognize IgY from other selected tortoise species.

Limited studies in desert tortoises showed that passive transfer of maternal antibodies occurred through the egg yolk and persisted in the hatchlings for up to 1 yr (Schumacher et al., 1999). To differentiate between maternal antibodies and those derived from active infection in a hatchling, samples should be taken at least 3 mo apart to detect a change in antibody titer over time.

Specificity (see below) of the ELISA depends on the affinity and avidity of antibodies produced by the tortoise in response to *M. agassizii* antigens. However, some *Mycoplasma* species may share immunogenic epitopes or antigens. For example, experimental infection with a genetically distinct *Mycoplasma* (*M. cheloniae* proposed sp. nov.) from a desert tortoise also caused URTD, induced antibodies that cross-reacted with *M. agassizii* whole-cell lysate antigens in Western immunoblot analyses, and produced positive ELISA results (Brown et al., 1995). This may have been due to antigens that are the same or very similar in the two species; however mycoplasmal growth was not inhibited by specific antisera in reciprocal tests (J.G. Tully, *pers. comm.*). The prevalence of tortoise infection with *M. cheloniae* is unknown, but this species has also been found in one population of gopher tortoises (Epperson, 1997). Documented cross reactivity by ELISA in this population has been limited (J. Berish, *pers. comm.*). The current assay uses antigen from *M. agassizii* only, and may miss infections by other species that also may be involved in URTD. Therefore, in an animal with clinical signs compatible with mycoplasmosis and a negative ELISA result, one must also consider the possibility of infection with other mycoplasmal species that do not cross react in the ELISA. Culture and PCR can help to clarify the clinical picture in these animals.

The advantages of diagnosing *Mycoplasma* exposure and potential infection by ELISA include high assay sensitivity (fewer false negatives; see below), relative ease of assay automation, more rapid results, antibody quantification by titer, and assay reproducibility. The major disadvantages of ELISA testing are invasive sampling and technical skill required to collect blood. A limitation of the assay is that a positive test indicates past exposure but not necessarily current infection. As with any serologic assay, there may be false positives caused by cross-reaction of tortoise antibodies to other bacteria with similar antigens. Conversely, there may be false negatives due to the lack of cross-reactivity with other *Mycoplasma* species that may cause URTD. Further, false negative results will occur in animals in the first 6 to 8 wks of initial infection, prior to initiation of the host humoral immune response. Although immune periodicity has been documented in other reptiles (Zapata et al., 1992), no clear pattern of seasonal fluctuation in *M. agassizii*-specific antibody concentrations has been reported even though individual fluctuations may occur (Brown et al., 1999a). Classification of a sample as "suspect" is problematic for decision making if the tortoise will not be re-tested. Hatchling tortoises with positive initial ELISA results should be retested in 3 mo to distinguish between maternal and hatchling antibodies; this is time consuming, expensive, and requires quarantine of the hatchling. It should be noted that these limitations are consistent with other serological tests, however.

Sensitivity, Specificity, and Predictive Values

The ability of serologic assays to distinguish between animals that are either exposed or non-exposed to a specific pathogen is expressed as the sensitivity, specificity, positive predictive value, and negative predictive value of the assay (Courtney and Cornell, 1990). These terms are generally used when describing the effectiveness of an assay to distinguish between exposed and non-exposed individuals in a population. The sensitivity of an assay answers the question: "if an animal is diseased, will it test positive?" Not all truly infected animals test positive; therefore the sensitivity is the ratio of the positive animals detected by the test compared to the total number of truly infected animals that were tested, including those animals with negative test results (Kenny, 1992). Conversely, the specificity of an assay is the ability of the test to identify individuals without disease in a healthy population and answers the question: "if they are healthy, do they test negative?" It should be noted that prevalence of disease influences these values. When disease is common, false negatives are more likely to occur. When disease is present in low levels, false positives are more likely to occur.

The positive predictive value (PPV) of an assay is the ability of the test to distinguish true infections in a population of individuals with positive test results (diseased individuals with positive test, out of all individuals with positive test), i.e., "if they tested positive, is this is a true positive or a false positive result?" Similarly, the negative predictive value (NPV) is the ability to distinguish true negative individuals from diseased individuals that have a negative test result (healthy individuals with negative test, out of all individuals with negative test), i.e., "if they tested negative, are they really healthy?" For such calculations, a reference standard for diagnosis of disease that is independent of the assay being evaluated is required (Kenny, 1992). For pur-

poses of comparing the mycoplasmal diagnostic tests during their development, the presence of clinical signs and histopathological lesions were used as the reference standard (Brown et al., 1994, 1999b; Schumacher et al., 1997). Three controlled experimental infection cohort studies have been conducted that allowed calculation and comparison of the sensitivity, specificity, and predictive values of culture, PCR, and ELISA as diagnostics of mycoplasmal infections of tortoises (Table 1).

In the first study (Brown et al., 1994), 17 clinically healthy desert tortoises were experimentally inoculated with 2.5×10^8 or 5×10^8 CCU of *M. agassizii* strain PS6 alone or in combination with *Pasteurella testudinis*. The strain PS6 was obtained in pure culture from nasal lavage of a naturally infected desert tortoise with signs of URTD. Twelve control tortoises were inoculated with placebo (sterile SP4 medium) or untreated. Plasma samples were collected before inoculation and at 1, 3, and 6 mo postinoculation (PI) for ELISA, and ELISA results of samples taken 3 mo PI were used for comparisons (Table 1). Fifteen of 17 inoculated tortoises developed clinical signs of disease and all necropsied tortoises had histopathological lesions. All 17 inoculated tortoises seroconverted (either changed to an ELISA ratio > 2, or increased > 0.1 unit). Three control tortoises developed clinical signs of disease, but only 1 of these seroconverted. One control was seropositive by ELISA but showed no clinical signs of disease. Therefore, in this experiment the sensitivity of ELISA was 16/18 = 89%, and the specificity was 8/11 = 73% (Table 1). The PPV of ELISA was 16/19 = 84%, and the NPV was 8/10 = 80% (Table 1).

In the second study (Brown et al., 1999b), 9 clinically healthy, ELISA-negative gopher tortoises were experimentally inoculated with 1×10^8 CFU of *M. agassizii* strain 723 that was obtained from a naturally infected gopher tortoise with signs of URTD. Six control tortoises were inoculated with placebo. Plasma and nasal lavage samples were collected before inoculation and monthly for 4 mo PI for culture, PCR, and ELISA. Culture, PCR, and ELISA results for samples taken 3 mo PI were compared (Table 1). Eight of 9 inoculated tortoises developed both clinical signs of disease and histopathological lesions. No control tortoises developed clinical signs of disease or had positive culture, PCR, or ELISA test results at any sampling point. All 9 inoculated tortoises seroconverted (changed to ELISA ratio > 3). Therefore, in this experiment the sensitivity of ELISA was 100%, and the specificity was 86% (Table 1). The PPV of ELISA was 89%, and the NPV was 100% (Table 1). ELISA results were identical at 2, 3, and 4 mo PI. The 8 diseased tortoises were all culture-positive 3 mo PI; therefore the sensitivity, specificity, PPV, and NPV of culture were all 100% at that sampling point. Culture results were the same at 1 and 4 mo PI, but at 2 mo PI only 6 tortoises were culture-positive, lowering the calculated sensitivity (75%) and NPV (78%). Only 3 tortoises were PCR-positive 3 mo PI. The sensitivity of PCR was only 37%, but the specificity was 100%. The PPV of PCR was 100%, but the NPV was only 58%. However, 1 mo earlier, 5 tortoises were PCR-positive, increasing the calculated sensitivity (62%) and NPV (70%) at that sampling point.

In the third study (Brown et al., 1999b), 17 clinically healthy, ELISA-negative, culture-negative, and PCR-negative gopher tortoises were experimentally inoculated with 1×10^1 to 1×10^5 CFU of *M. agassizii* strain 723. Control tortoises were inoculated with placebo ($n = 4$); one tortoise was untreated and served as a sentinel. Plasma and nasal lavage samples were collected before inoculation and biweekly up to 8 wks PI and at 12 wks PI for culture, PCR, and ELISA. Culture, PCR, and ELISA results of 14 inoculated tortoises and 5 controls sampled 3 mo PI were compared; three individuals were not available for sampling at the 3 mo time point. All inoculated tortoises developed clinical signs of disease (confirmed by histopathological evaluation at necropsy for representative individuals). No control tortoises developed clinical signs of disease or had positive culture, PCR, or ELISA test results at any sampling point. Thirteen inoculated tortoises seroconverted (changed to ELISA ratio > 3). The sensitivity of ELISA was 93%, and the specificity was 100% (Table 1). The PPV of ELISA was 100%, and the NPV was 83% (Table 1). Culture results were identical to those for ELISA at that sampling point. At 2 wks PI, culture had a higher sensitivity (94%) than ELISA. Nine tortoises were PCR-positive 3 mo PI. The sensitivity of PCR was 64%, and the specificity was 100%. The PPV of PCR was 100%, but the NPV was only 50%. However, 6 wks earlier, the calculated sensitivity, specificity, PPV, and NPV of PCR were all 100% ($n = 22$ tortoises).

For controlled experimental studies where animals can be observed regularly, observation of clinical signs was fairly reliable for diagnosis of URTD. However, even under these controlled conditions, infected animals had intermit-

Table 1. Detection limitations and predictive values of ELISA, culture, and PCR for mycoplasmal infection status in experimentally infected desert and gopher tortoises. Results are based on experimental infections in three studies with a limited number of desert (study 1) or gopher (studies 2 and 3) tortoises of known infection status (study 1: infected, $n = 17$; controls, $n = 12$; study 2: infected, $n = 9$; controls, $n = 6$; study 3: infected, $n = 17$; controls, $n = 5$). PPV = positive predictive value; NPV= negative predictive value.

Study	Test	Sensitivity	Specificity	PPV	NPV
Study 1	ELISA	89	73	84	80
	Culture[a]	-	-	-	-
	PCR[a]	-	-	-	-
Study 2	ELISA	100	86	89	100
	Culture	100	100	100	100
	PCR	37	100	100	58
Study 3	ELISA	93	100	100	83
	Culture	93	100	100	83
	PCR	64	100	100	50
Mean	ELISA	94	86	91	88
	Culture	97	100	100	92
	PCR	51	100	100	54

[a]Culture and PCR were not performed in study 1.

tent clinical expression of disease. Therefore because clinical signs may be mild, expressed intermittently, or have causes other than *Mycoplasma* infection, a reference standard other than clinical assessment will be more reliable for diagnosis of mycoplasmosis in wild tortoises. Changes in the calculated sensitivities, specificities, and predictive values of the different diagnostic tests at different sampling points PI reflect in part the progression of the disease. The sensitivity and PPV of culture and PCR can be better than ELISA for diagnosis in early stages of infection (2 to 6 wks post-exposure), because time is required for the tortoise to respond to infection immunologically. In contrast, the sensitivity and NPV of ELISA can be better than culture and PCR in later stages of infection. This may be due to the limitations of culture and PCR as discussed earlier.

In the studies described, the rate of false positives was 0% for culture and PCR, and from 0 to 27% for ELISA. The comparatively higher rate of false positives for ELISA reflected the conservative cutoffs established heuristically for interpretation of quantitative ELISA data. The rate of false negatives was from 0 to 17% for culture, 0 to 20% for ELISA, and 36 to 63% for PCR. Thus in general false negatives were more of a problem than false positives, and this was especially true for diagnosis by PCR. These studies were performed with known experimentally infected animals, and false negative results are more likely when the prevalence of infection is high. Thus our findings are not surprising.

Applications for Management and Conservation

Diagnostic Applications for Wild Populations. — Following the identification of mycoplasmosis as a health problem of wild desert tortoises, culture, PCR, and ELISA were developed to determine the infection/exposure status of wild desert and gopher tortoises. Although these tests do not give a complete picture of the type of infection (i.e., acute, chronic, subclinical, carrier) or relative virulence of the *Mycoplasma* present, they represent the best tests available for determining mycoplasmal status of tortoises. The tests can be used to determine the infection/exposure status of individuals, to monitor the progression of infection of individuals, and finally to determine prevalence within populations. It is imperative that decisions regarding wildlife population management be made on the basis of the best scientific information available. Given the importance of mycoplasmosis in wild desert and gopher tortoise populations, these diagnostic tests can provide important information to assist in the decision making process as it relates to issues such as relocation, repatriation, identification of healthy populations and those populations at risk for epidemics requiring specific protection or intervention.

The impact of infectious disease on wildlife populations is a relatively new consideration for wildlife management and ecology (Spalding and Forrester, 1993). However, it is now recognized that common management practices such as relocation and restocking can be compromised by infectious agents. Further, simply protecting habitat without regard to the health of populations residing in that habitat may be inadequate to ensure survival of those species. This has become evident in some protected tortoise populations that are now experiencing substantially increased mortality (Gates et al., 2002; B. Blihovde, *pers. comm.*). There must be constant awareness of new, previously unreported, or emerging diseases as well as noninfectious causes of mortality. Therefore, it is important to know the overall health status of individuals and populations. Ideally, general health evaluations should be incorporated into tortoise ecological studies, and mycoplasmal testing is only one component of such an assessment.

The use of specific *Mycoplasma* diagnostic tests will depend on the specific goals of the user, i.e., health assessment of an individual tortoise, population survey, long term population monitoring, and investigation of a mortality event. Ideally ELISA, culture, and PCR should be performed for all studies. If this is not possible, the next recommendation would be ELISA serology on all animals, coupled with culture and PCR on animals exhibiting clinical signs of URTD. If only a single test can be performed, then ELISA is the test of choice because it has a high sensitivity (> 90%) and both the PPV and NPV are similar (> 85%), meaning that the chance of either a false positive or negative result is similar and relatively low. Additionally, unlike culture and PCR, ELISA is not influenced by the low numbers of mycoplasmas in the nasal cavity when clinical signs are absent. A clear caveat of ELISA is that it will not detect early exposure (within 2–3 wks) prior to seroconversion. Culture and PCR are most likely to be informative if an animal is exhibiting clinical signs, especially a nasal discharge or if samples can be obtained directly from the nasal cavity at necropsy. Mortality events represent a unique situation. In such cases, if at all possible, moribund animals should undergo extensive diagnostic testing including blood profiles, toxicology screening, and full necropsies, including histological evaluation of all major organ systems and microbiologic evaluation of lesions.

For population monitoring, determination of the appropriate sample size is an important consideration. Valid population sampling will depend on the population size, the true prevalence of infection, and the sensitivity and specificity of each test. Sampling inadequate numbers of animals can seriously confound the data and lead to inaccurate conclusions. The minimum number of animals that need to be tested to give at least a 95% chance of detecting any infected animal at a given prevalence of infection has been determined for other mycoplasmal infections (Davidson et al., 1994). The sample size needed to accurately detect infection is a function of the true prevalence of the disease in the population. If the true prevalence is high, then a smaller number of animals will need to be tested. If the prevalence is low, then a higher number of animals will need to be tested. Formulas for calculating sample sizes are available (Martin et al., 1987; Davidson et al., 1994) One should assume that the disease is present at a low true prevalence for initial calculation of sample sizes. Once an estimate of the true prevalence is available, sample size can be adjusted if necessary.

Tortoise Relocations. — Relocation is a complex issue, and infectious diseases in general and URTD in particular are only one consideration. For example, in Florida over 25,000 gopher tortoises were permitted for relocation in the 1990s (Florida Fish and Wildlife Conservation Commission, J. Berish, *pers. comm.*). Although relocation has become a popular solution for political and moral reasons, it may have a number of detrimental biological effects. Principal concerns regarding relocation include alteration of local genetics, disruption of the normal population dynamics on the recipient site, and the potential for spread of infectious diseases (Burnhem and Anderson, 1984; Diemer, 1987; Burgman et al., 1993; Brown et al., 1999a).

Consistent with the objectives of tortoise management and conservation plans (U.S. Fish and Wildlife Service, 1994), efforts should be made to minimize the risk from *Mycoplasma* infections for tortoises that are candidates for relocation. It should be noted that other infectious agents cannot be excluded on the basis of *Mycoplasma* screening. Tortoises from both donor and recipient populations should be tested for *Mycoplasma* as described in the section above. Clearly, clinically ill animals or animals from populations experiencing unusually high mortality should not be relocated. Similarly, healthy animals should not be relocated to populations with extensive clinical disease or those undergoing increased mortality events.

There are inadequate scientific data to provide definitive guidelines for the disposition of seropositive tortoises . Potential options that have been considered include maintenance of tortoises on site, relocation to recipient populations with high prevalence of infection and with resident tortoises that already express clinical signs of URTD, admission into captive breeding programs, adoption as pets, or euthanasia. Each of these options has inherent problems. No single solution is applicable for every situation, therefore the unique circumstances of each population should be considered. Decision making pertaining to such animals should be dynamic, flexible and change as new scientific information becomes available.

Diagnostic Applications for Tortoise Captive Breeding. — ELISA, PCR, and culture can be used for monitoring tortoise breeding programs . If at all possible, only negative, healthy animals should be used for captive breeding programs. There may be unique situations where this is not feasible, however. We have examined approximately 200 eggs and hatchling gopher tortoises obtained from only a few females with mycoplasmosis, and we did not detect vertical transmission in this very limited sample (McLaughlin, 1997). This finding should be interpreted with extreme caution. Egg transmission is well established for avian mycoplasmas. However, transmission occurs at a low rate. For tortoises, a much larger sample of independent clutches, most likely in the hundreds, would be required to rule out ovarian transmission. Further, our studies are limited to gopher tortoises and additional studies would be needed in other tortoise species in order to make generalizations. If absence of vertical transmission is documented in tortoise species, then an alternative way to take advantage of the reproductive potential of affected tortoises would be through captive breeding. Adult female or male tortoises without clinical signs of URTD but with positive ELISA, culture, or PCR results may be useful as candidates for captive breeding programs. Although studies have not been conducted, logic would suggest that tortoises with severe clinical signs during the time of egg development may be more likely to shed higher numbers of *Mycoplasma*, thus potentially increasing risk of transmission to the egg and hatchling. It is essential that strict isolation be maintained between affected adults and offspring, since we do know that offspring housed with adults can develop severe and lethal disease (McLaughlin, 1997). The objectives of such a breeding program would be to conserve genetic diversity and produce mycoplasmal infection-free offspring for restocking depleted populations.

Ideally, any captive-bred candidates for release should be evaluated by ELISA, PCR, and culture and held in isolation until tests are completed. With large numbers of tortoises this may not be practical. In such cases, a statistically significant subset should be sampled. If other species

Table 2. Summary of major conclusions and areas where additional scientific data are needed.

It is certain that:
- *Mycoplasma agassizii* (strains PS6 and 723) is a cause of URTD.
- The pathology of mycoplasmosis involves hyperplastic and dysplastic lesions in the upper respiratory tract.
- Clinical signs of URTD vary in onset, duration, and severity.
- Mycoplasmosis is chronic and may be clinically silent (subclinical) in adult tortoises.
- Infection with *M. agassizii* elicits specific antibody responses that can be detected by ELISA.
- The current ELISA cannot detect exposure of all tortoises to mycoplasmas other than *M. agassizii*, although some cross-reactions do occur.
- The antibody responses to *M. agassizii* are reliably detectable by ELISA beginning 8 wks after experimental infection.
- Under experimental conditions, gopher tortoises become ill quicker after repeated exposure to *M. agassizii*.
- Colonization of the upper respiratory tract with *M. agassizii* may be detected by culture and PCR, but assay sensitivity is not as high as the ELISA.
- Mycoplasmosis is a horizontally transmissible disease.

It is probable, but not clearly established, that:
- Pathogenic and nonpathogenic tortoise mycoplasmas exist.
- There is variation among strains of *M. agassizii* in their ability to cause URTD.
- Other species of *Mycoplasma* (such as *M. cheloniae*) also can cause URTD.
- Specific antibodies against *M. agassizii* do not confer protective immunity.
- *Mycoplasma* can be transmitted by some forms of indirect contact.

We suspect the following:
- In gopher tortoises, if vertical egg transmission of *Mycoplasma* occurs, it does so at a relatively low rate.
- Mycoplasmosis can affect the survival and reproduction of individual tortoises.
- Mycoplasmosis is a multifactorial disease, interacting in some circumstances with other stressors to affect tortoise population dynamics and viability.
- Mycoplasmosis directly affects desert and gopher tortoise population dynamics and viability.

It is unlikely that:
- *Mycoplasma* can persist in burrows of infected tortoises.

are similar to desert and gopher tortoises, most hatchlings from *Mycoplasma*-positive females will have detectable levels of anti-mycoplasmal antibodies due to the transfer of maternal antibodies into the egg, potentially causing false-positive ELISA tests. Maternal antibodies in desert tortoise hatchlings may persist up to 1 yr (Schumacher et al., 1999), requiring the use of paired ELISA titers taken at a minimum interval of 3 mo. In uninfected hatchlings, antibody should decrease or remain stable during this interval; potentially infected hatchlings should have increased titers.

Euthanasia. — ELISA testing has been used as a convenient method for determining the ultimate disposition of gopher and desert tortoises in certain parts of their range. While convenient, this approach may in fact result in the elimination of tortoises that can potentially make reproductive and genetic contributions to wild populations. By the same token, relocation of seropositive tortoises may result in spread of disease to susceptible animals and could have detrimental impacts on recipient populations. Again, there are insufficient data to make conclusive recommendations regarding these management options. Euthanasia is politically, morally, and logistically problematic to implement in wild populations. Although alternatives to euthanasia should be considered, there are instances when euthanasia is the appropriate choice.

The statements in Table 2 are based on knowledge of tortoise mycoplasmosis accumulated over the last decade and represent our current understanding of the disease in gopher and desert tortoises. This table should be considered dynamic and will need to be updated and revised as additional data from stringent scientific studies are reported in peer reviewed literature.

Mycoplasmosis is a complex, likely multifactorial disease, associated with declines of desert tortoises in the southwest United States. Over the last 10 years several diagnostic tools have been developed to determine the serologic and infectious status of individual tortoises. On a broader level, these tests can be used in population monitoring. Each test alone provides certain information that when used collectively provides a more complete picture of the infection status of a tortoise. While these tests can be used to guide the development of recommendations on the disposition of tortoises, additional studies are necessary for making sound recommendations in the future. The immediate need is for the development of scientifically based recommendations for management of seropositive healthy and clinically ill wild tortoises to minimize the risks to both individuals and populations of uninfected tortoises.

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