Mycoplasma agassizii Strain Variation and Distinct Host Antibody Responses Explain Differences between Enzyme-Linked Immunosorbent Assays and Western Blot Assays

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Received 20 May 2010/Returned for modification 21 June 2010/Accepted 25 August 2010

The precarious status of desert (Gopherus agassizii) and gopher (G. polyphemus) tortoises has resulted in conservation efforts that now include health assessment as an important component of management decision-making. Mycoplasmal upper respiratory tract disease (URTD) is one of very few diseases in chelonians for which comprehensive and rigorously validated diagnostic tests exist. In this study, serum samples obtained from eight Gopherus tortoises documented at necropsy to (i) be enzyme-linked immunosorbent assay (ELISA) seropositive using the PS6 antigen, (ii) be infected with Mycoplasma agassizii as indicated by direct isolation of the pathogen from the respiratory surfaces, and (iii) have histological lesions of mycoplasmal URTD were used to evaluate four distinct clinical isolates of M. agassizii as antigens for ELISA and Western blot analyses. Each animal sample reacted in the Western blot with its homologous M. agassizii strain, but recognition of heterologous M. agassizii strains was variable. Further, individual animals varied significantly with respect to the specific proteins recognized by the humoral immune response. An additional 114 Gopherus serum samples were evaluated using ELISA antigens prepared from the four distinct M. agassizii strains; A_s values were significantly correlated (r² goodness of fit range, 0.708 to 0.771; P < 0.0001) for all antigens tested. The results confirm that strain variation is responsible for the observed differences between Western blot binding patterns. Thus, reliance on a single M. agassizii strain as an antigen in Western blot assays may provide false-negative results. This could have adverse consequences for the well-being of these environmentally sensitive hosts if false-negative animals were relocated to sites consisting of true-negative populations.

Over the past two decades, disease has become an increasingly important issue for wildlife management. Disease surveillance is fundamental for disease prevention and control. Thus, the development of diagnostic assays will be critical to research and manage wildlife populations effectively. Diseases in free-ranging animal populations are often managed by isolation or culling, predominantly because treatment of individuals is impractical and vaccination programs can be instituted only in limited situations, if at all. As available habitat shrinks, relocation or relocation may be used as management tools, especially for “at-risk” species, and the use of diagnostic tools to minimize the risk of pathogen transmission will increase. It is critical that tests be appropriately validated and have quality control mechanisms established, as there may be adverse consequences for animals with positive diagnostic test results for infectious agents, as well as for naïve animals that might be exposed to pathogens by the relocation of diseased animals.

Mycoplasmal upper respiratory tract disease (URTD) (11, 13) is one of very few diseases in chelonians for which comprehensive and rigorously validated diagnostic tests exist. Current diagnostic methods include culture, PCR, and enzyme-linked immunosorbent assay (ELISA) serology (9, 42, 43).

Obtaining an adequate nasal flush or swab sample for culture and PCR is difficult, and both culture and PCR are significantly less sensitive when animals are not exhibiting overt clinical signs (9, 27, 42). The Mycoplasma agassizii ELISA (43) has been extensively validated, using controlled experimental infection studies of both desert and gopher tortoises (total n = 75) to establish sensitivity (0.983 to 0.985) and specificity (0.999 to 1) values. A sizeable serum bank from desert (n = 4,830) and gopher (n = 1,124) tortoises was used to establish standard curves and quality control measures. Importantly, the presence of specific antibody has been highly correlated with the presence of histopathological lesions of the upper respiratory tract as well as the presence of clinical signs, especially a nasal discharge (8, 27, 36, 42).

Recently it has been reported that the M. agassizii ELISA may misidentify true-negative tortoises as seropositive due to the presence of natural antibodies and that Western blot assays should be used as a confirmatory test for M. agassizii exposure (21). In that study, a single isolate of M. agassizii (PS6) was used as an antigen for immunoblotting. However, most mycoplasmas exhibit extensive intraspecies genotypic and phenotypic variability that can be manifested as antigenic variation in the context of immune recognition (2–5, 14, 17, 18, 22, 24, 30). This heterogeneity can confound analysis of mycoplasmal immunogen recognition when only a single mycoplasmal isolate is used as an antigen.

Studies of swine, poultry, and ruminants (2–4, 18, 22, 24) document the necessity to use multiple strains of mycoplasmas...
as antigens in Western blots in order to avoid false negatives. Therefore, the objective of this study was to determine if strain variability impacted results obtained using Western blot analyses. Serum samples obtained from eight *Gopherus* tortoises (gopher tortoise [*G. polyphemus*] and desert tortoise [*G. agassizii*]) documented at necropsy to (i) be ELISA seropositive using the PS6 *M. agassizii* antigen, (ii) be infected with *M. agassizii* as indicated by direct isolation of the pathogen from the respiratory surfaces, and (iii) have histological lesions of URTD were used to evaluate four distinct clinical isolates of *M. agassizii* as antigens for Western blot analyses. We also compared the reactivities of tortoise sera (*n* = 114) in an ELISA using antigens prepared from the same four *M. agassizii* strains.

**MATERIALS AND METHODS**

Animals and clinical isolates. Eight adult tortoises (both gopher and desert) were submitted for necropsy at the University of Florida Veterinary Medical Teaching Hospital, Gainesville, FL. All eight tortoises (PS6, IR, 262, 723, 169, 185, 230, and 233) were confirmed to have active URTD by the following criteria: all had clinical signs of URTD, all were ELISA positive with *M. agassizii* Ps6 used as the ELISA antigen, all had *M. agassizii* isolated from the nasal cavity, and all had histological lesions of URTD at necropsy. We selected four clinical isolates of *M. agassizii* (strains PS6, 723, IR, and 262) for preparation of SDS-PAGE and ELISA antigen. Strain PS6 and 723 are the type strains used to fulfill Koch’s postulates in the desert and gopher tortoise, respectively (11, 13). Strains IR and 262 were isolated from gopher tortoises. Identity of all *M. agassizii* isolates was confirmed by PCR of the 16S rRNA followed by restriction fragment length polymorphism (RFLP) of the PCR product (8, 9); complete 16S rRNA sequencing was performed for *M. agassizii* strains PS6 and 723.

Monoclonal and polyclonal antibodies. Biotinylated mouse monoclonal antibody (MAB) HL673 and two biotinylated rabbit polyclonal antibodies (PAb) were selected for use. An IgG1 isotype, MAB HL673, was prepared against desert and gopher tortoise 27-kDa IgY light chain (26, 35, 37). This MAB has documented cross-reactivity with IgM light chain, and 2.5 ml of the affinity-purified tortoise immunoglobulin (0.18 mg/ml) was used to produce rabbit anti- *M. agassizii* *G. polyphemus* PAb (PAbGa/Gp), which binds gopher tortoise IgY heavy and light chains as well as desert tortoise IgY heavy chain, was prepared using affinity-purified gopher and desert tortoise immunoglobulins. Gopher tortoise serum and desert tortoise serum (2 ml each), representing 29 individual tortoises, were pooled and desert tortoise immunoglobulins. Gopher tortoise serum and desert tortoise serum (2 ml each), representing 29 individual tortoises, were pooled and diluted 1:1 in phosphate-buffered saline (PBS) with 0.02% sodium azide (PBS-AZ). Serum was recirculated over an MAB HL673 affinity column (no. 44990 Immunopure Protein G IgG Plus orientation kit; Pierce, Rockford, IL) for 1 h, and then eluted. Fractions 1 to 13 were determined to contain 26-kDa and 15-kDa light chain, and 2.5 ml of the affinity-purified tortoise immunoglobulin (0.18 mg/ml) was used to produce rabbit anti- *G. agassizii* *G. polyphemus* PAb (Strategic Biosolutions, Newark, DE). Anti-Agrionemys (*Testudo*) *horsfieldii* PAb (gift from Eva-Maria Andrea and Herwart Ambrosius, Karl-Marx Universität, Leipzig, Germany), prepared against Russian tortoise (*Agrionemys horsfieldii*) IgY heavy chain and with documented cross-reactivity with desert tortoise 65-kDa IgY heavy chain (27), served as a positive control.

Reactivity of tortoise sera with MAB and PAB. We confirmed the specificity and avidity of the MAB and PABs by both Western blot analysis and ELISA. For Western blot analysis, gopher and desert tortoise sera (diluted 1:50 in PBS-AZ) were separated by SDS-PAGE (23). Electrophoretic transfer of proteins onto nitrocellulose membranes and blotting of membranes were performed using previously described conditions (10, 37); each blot was reacted with a different biotinylated antibody (HL673, anti-*Agrionemys horsfieldii* PAb, or PAbGa/Gp, diluted to 1 µg/ml in PBS-AZ; 900 µl/m). To determine binding reactions in ELISA, serum pools from gopher and desert tortoises were diluted in serial 2-fold dilutions in PBS-AZ. Each dilution (50 µl/well) was coated in duplicate in a 96-well microtiter plate (Maxisorp; Nunc, Roskilde, Denmark), and two wells of every plate served as blanks (incubated with conjugate and substrate only). Plates were incubated overnight at 4°C and then washed four times with PBS containing 0.05% Tween 20 (PBST) in an automatic plate washer (ELX405; Bio-Tek Instruments, Inc., Winooski, VT) and blocked with 300 µl of PBST containing 5% nonfat dry milk (PBSTM) for storage at 4°C. Plates were washed as described above, and based on previously reported optimal ELISA dilutions (35, 43), biotinylated HL673 MAB (1.45 µg/ml), biotinylated anti-*Agrionemys horsfieldii* PAb (5 µg/ml), or biotinylated PAbGa/Gp (1.5 µg/ml) was added (50 µl/well; 1 h incubation). Addition of streptavidin-alkaline phosphatase and substrate buffer and measurement of subsequent color development were carried out as previously described (35, 43).

**Detection of specific antibody reactions to clinical isolates of *M. agassizii***. ELISA antigens were prepared as previously described (35, 43). For SDS-PAGE antigen, 1 liter of each mycoplasma strain was grown to mid-logarithmic phase in SP4 medium, and cells were centrifuged at 12,000 × g for 30 min. The pellet was washed three times in 200 ml sterile PBS (pH 7.3), protein concentration was adjusted to 2 to 5 mg/ml in sterile distilled water (7), and an equal volume of 2x SDS-PAGE electrophoresis sample buffer containing bromophenol blue was added. Antigen preparations were stored at −20°C.

For Western blot analysis, mycoplasmal antigens (1 mg/ml) were separated on a 10% Tris glycine gel, transferred to nitrocellulose, and washed and blocked using conditions previously described (39). Serum samples from the eight URTD-positive tortoises were diluted 1:50 in PBS-AZ containing 1% sterile SP4 broth to remove nonspecific reactions with medium components (19, 34) and were used to probe the blots. Binding of tortoise immunoglobulins IgY and IgM was detected with biotinylated MAb HL673 (1 mg/ml) as described above.

We also compared the reactivities of tortoise sera in an ELISA in which different strains of *M. agassizii* were used as antigens. *M. agassizii* strains PS6, 723, IR, and 262 were used as antigens to coat ELISA plates (50 µl/well at 10 µg/ml). Individual tortoise serum samples (*n* = 114) submitted to the University of Florida Mycoplasma Diagnostic Laboratory were run in an ELISA with *M. agassizii* strains PS6 (*n* = 52), 723 (*n* = 52), IR (*n* = 14), and 262 (*n* = 14). The assay was performed as previously described (35, 43, 42), with sera diluted 1:10 in blocking buffer. Linear regression analysis was performed using pairwise comparisons of *A*<sub>psp</sub> values between PS6 and the three additional ELISA antigens (Prism, GraphPad Software, Inc., La Jolla, CA), with a *P* value of <0.05 accepted as significant.

**RESULTS**

MAB HL673 and PAbGa/Gp react with both gopher and desert tortoise immunoglobulins in Western blot and ELISAs (Fig. 1). We confirmed that MAB HL673 binds with the 27-kDa gopher tortoise IgY light chain, as well as with the desert tortoise IgY light chain (26, 37). PAbGa/Gp recognized the 65-kDa IgY heavy chain of both the gopher and desert tortoise and the 27-kDa IgY light chain of the gopher tortoise. Use of an affinity-purified immunoglobulin as the antigen for preparation of PAbGa/Gp resulted in a reagent that had good specificity with no overt cross-reactions to the nonimmunoglobulin components of serum. In the ELISA (Fig. 1), both PAbGa/Gp and HL673 had curves similar to that of the reference PAb anti-*Agrionemys horsfieldii*. Both PAbGa/Gp and HL673 showed strong reactivity when either gopher or desert tortoise serum was used as the ELISA antigen (Fig. 1); however, the endpoint dilution for PAbGa/Gp was 5-fold greater.

Strain variation is responsible for differences in Western blot profiles of individual tortoises (Fig. 2). Strain-dependent results were observed in Western blot analyses of serum obtained from eight tortoises with documented URTD. These eight animals also were seropositive by ELISA that used *M. agassizii* strain PS6 as the ELISA antigen. Each animal reacted in the Western blot with its homologous *M. agassizii* strain, but recognition of heterologous *M. agassizii* strains was variable. As an example, serum from the gopher tortoise infected with *M. agassizii* strain IR did not recognize antigen prepared from *M. agassizii* strain PS6 or *M. agassizii* strain 262 but did react with antigen prepared from *M. agassizii* strain 723 and the homologous IR strain isolate. Tortoises infected with either *M. agassizii* strain PS6 or *M. agassizii* strain 262 had strong reactivity with *M. agassizii* IR as detected by Western blot. Thus, *M.
strain IR did not appear to have unique or missing antigens compared with PS6 and 262. Individual animal samples varied significantly with respect to specific proteins recognized by the humoral immune response. Although serum samples from all tortoises reacted with SDS-PAGE antigens prepared with the homologous strain of *M. agassizii*, reactions with SDS-PAGE antigens prepared from heterologous clinical isolates varied markedly. Individual tortoise samples responded to different proteins, as best demonstrated by the responses of tortoise samples to antigens prepared from *M. agassizii* strains 262 and IR. Serum samples from tortoises 262 and 723 recognized a wide range of proteins found in antigens prepared from *M. agassizii* strains 262 and IR, confirming that these strains did in fact contain these immunoreactive epitopes. However, serum samples from tortoises PS6, IR, 169, and 185 failed to recognize many of these same epitopes in *M. agassizii* 262 antigens. With *M. agassizii* strain IR antigen, tortoise PS6 had a greater and more intense banding pattern than that for tortoises 169, 185, 230, and 233.

Low-molecular-mass proteins (14- to 21-kDa range) were recognized by all tortoises except PS6; however, once again, the specific recognition pattern was dependent on the strain of *M. agassizii*. For *M. agassizii* strains PS6 and 262, serum samples from all tortoises except PS6 and IR had demonstrable bands in the 14- to 21-kDa range. Reactions with *M. agassizii* strain 723 antigen were observed only in serum samples from tortoises 262 and 723. For *M. agassizii* strain IR, significant banding reactions were observed only with serum samples from tortoises IR, 262, and 723.

Two of the eight tortoise samples tested using the PS6 antigen had limited to no reactions by Western blot analysis. A
limited repertoire of recognized proteins was also observed in two of eight tortoise samples when *M. agassizii* strain 262 was used and in four of eight tortoise samples with strain IR. *M. agassizii* strain 723 was widely recognized by this limited number of tortoise samples.

ELISA was less strain dependent. Unlike the Western blot analysis, the reactions of tortoise sera in the ELISA were similar, even when the antigens were prepared from different strains of *M. agassizii* (Fig. 3). Results obtained using the same sera reacted with ELISA antigens prepared from *M. agassizii* strains PS6, 723, IR, and 262 showed that the $A_{405}$ results obtained were significantly correlated ($r^2$ goodness of fit range = 0.708 to 0.771; $P < 0.0001$) for all antigens tested. Only 14 of 114 (12%) serum samples tested had discrepant results, where PS6 antigen would have classified the results differently from results obtained with a different antigen. In nine of these (8%), ELISA results with PS6 antigen were positive but tested negative using antigens prepared from *M. agassizii* strains 723 ($n = 2$), IR ($n = 5$), and 262 ($n = 2$). In 5 of 114 (4%) serum samples, ELISA results with PS6 antigen were negative but tested positive using antigens prepared from *M. agassizii* strains 723 ($n = 4$) and IR ($n = 1$).

DISCUSSION

Antigenic variation is perhaps the best-studied aspect of mycoplasmas. Their ability to rapidly alter surface antigens contributes to the evasion of host immune defenses and establishment of chronic disease. In fact, the high rate of antigenic variation among mycoplasma species and also among clinical isolates of individual species has been well documented (for examples, see references 15, 17, 18, 29–33, and 44). Importantly, this constantly changing surface pattern does not necessarily generate a protective antibody response.

The animals used in our study for Western blot analysis were rigorously identified as true URTD-positive tortoises. Individual variation in immune response among these animals, even to the same strain of *M. agassizii*, was common in Western blot analysis (Fig. 2). This was not surprising given the known propensity for high rates of antigenic variation in most myco-
The ability of clinical isolates of most mycoplasma species to express different surface proteins, the variability in host immune recognition of antigenic determinants, and the need for multiple mycoplasma strains as antigens in Western blot analysis of samples from naturally infected animals are well documented in the literature (1–6, 17, 18, 24, 25, 28, 29–31, 38–40). We observed similar heterogeneity in the response of individual tortoise samples to *M. agassizii*, with antigens prepared both from the homologous strain recovered from the animal and from heterologous strains. Western blot testing using a single antigen (PS6) failed to detect animals proven to have URTD in approximately 25% of cases, whereas ELISA using this same strain as an antigen reliably detected all infected animals. This result is comparable to the reported failure to detect banding patterns to *M. agassizii* strain PS6 that was attributed to natural antibodies (21). Based on our findings, Western blot as confirmation of infection status and ELISA results is appropriate only if the homologous strain antigen or multiple heterologous strain antigens are used. The usefulness of the Western blot is further limited by the difficulty in obtaining clinical isolates due to the fastidious nature of the organism and intermittent shedding by infected animals.

The method of antigen preparation used in this study was the same as that reported by Hunter et al. (21), and caveats associated with proteins prepared by SDS and boiling are common to both studies. Because SDS is an anionic denaturing detergent, it is an effective agent to release membrane-associated proteins but also does denature proteins by breaking protein-protein interaction via dissolution of disulfide bonds, clearly altering secondary and tertiary structure. Boiling could also result in damage to heat-labile proteins. Both treatments may affect nonprotein moieties that are associated with native proteins. However, many of the immunodominant antigens of mycoplasmas are membrane associated, and SDS/boiling is one of the most common methods of preparing whole-cell mycoplasmal antigens for Western blots.

For many mycoplasma species, detection of specific antibodies by ELISA is considered to be relatively strain independent, whereas other assays, such as Western blot, metabolic inhibition, and complement fixation, are documented to be strain dependent or best used for confirmation (1, 12, 16, 17, 22, 40, 41). For example, Kittelberger et al. (22) found that the Western blots gave highly variable results for different sera with respect to banding patterns and that ELISA had higher specificity and sensitivity than either Western blot or complement fixation assays. These differences are likely explained by the location of the antigens (surface exposed, membrane, or cytosolic), binding affinity to microtiter plates, degree of surface variation, biofunctional assays, and *in vivo* expression of antigens. Our findings that multiple strains are required for Western blot analysis but not for ELISA were consistent with what has been described for other mycoplasmal species.

The current ELISA is the only diagnostic test for mycoplasmal URTD that has undergone rigorous validation (43) and has been correlated with clinical disease, culture, and PCR as well as the presence of histological lesions in both naturally and experimentally infected animals (11, 13, 20,
26, 27, 36, 42, 43). The assay has a sensitivity of 0.98 and specificity of 0.99 (U statistic = 0.98), thus providing a clinically reliable diagnostic test for exposure to M. agassizii. Western blot analysis, particularly when only a single isolate is used as an antigen, is likely to lead to misidentification of approximately 15 to 25% of truly infected animals as negative (Fig. 3). Relocation of false-negative tortoises into naive populations is likely to contribute to spread of the pathogen. It is therefore critical that diagnostic tests be appropriately validated, have quality control mechanisms established, and be used in the manner most likely to yield reliable results for management decisions. Finally, it is important to recognize that the ultimate goal is protection of threatened tortoise populations. Therefore, collaborative, interdisciplinary efforts that build on the strengths of ecologists, field biologists, infectious disease experts, veterinarians, and wildlife managers are likely to have the greatest positive impact on conservation efforts to recover these species.

ACKNOWLEDGMENTS
We thank Eva-Maria Andreas and Herwart Ambrosius, Karl-Marx Universität, Leipzig, Germany, for their gift of polyclonal anti-\( \alpha \)-hors-fieldii sera. We also thank Linda G. Green and Diane G. Duke from the ICBR Hybridoma Core Laboratory, University of Florida, for their help in developing the monoclonal antibodies and Western blots. We thank Daniel Brown for critical review of the manuscript and helpful suggestions.

This work was supported by grants from the Walt Disney Development Company and the National Science Foundation Ecology of Infectious Diseases program (DEB-0224953). L. D. Wendland was supported by a National Institutes of Health K08 award (5K08AI57722).

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