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Evanescent-wave biosensor for field serodiagnosis of tortoise mycoplasmosis

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Abstract

Disease has become an increasingly important issue for wildlife management over the past two decades. Adequate surveillance is fundamental for disease prevention and control, thus there is an increasing need for diagnostic assays for wildlife management. The objective of this study was to evaluate the performance of a field-portable biosensor adapted for rapid detection of specific antibodies in tortoise plasma that reflect a history of exposure to *Mycoplasma agassizii*, which is an agent of tortoise upper respiratory tract disease. Banked plasma samples were tested in two blinded trials, and the parameters that define the reliability of a diagnostic test were estimated based on externally validated tortoise plasma controls. The mean sensitivity of the biosensor (ability to identify exposed tortoises in the group of all exposed individuals) was 78%; the mean specificity (unexposed individuals with negative test result, out of all unexposed individuals tested) was 73%; the mean positive predictive value (exposed individuals with positive test, out of all individuals with positive test) was 82%; the mean negative predictive value (unexposed individuals with negative test, out of all individuals with negative test) was 68%. In a 15-min field-portable format, the biosensor was able to discriminate between true seropositive ($n = 34$) and true seronegative ($n = 23$) tortoise plasma with overall accuracy of 84%. The goals established for the tortoise population can help managers decide whether potential diagnostic errors should impact management decision-making, and whether the benefits of the field-portable format of the biosensor assay outweigh any potential disadvantages.

Keywords: Biosensor; Management; Mycoplasmosis; Serology; Tortoise; Wildlife

1. Introduction

Disease has become an increasingly important issue for wildlife management considerations over the past two decades. Adequate surveillance is fundamental for disease prevention and control, thus there is an increasing need for the development of diagnostic assays for wildlife management. A potentially

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debilitating communicable upper respiratory tract disease (URTD; Wendland et al., 2006; Brown et al., 2005) of desert (*Gopherus agassizii*) and gopher (*Gopherus polyphemus*) tortoises is thought to have contributed to population declines over parts of those species' natural ranges during the past two decades (Jacobson et al., 1991, 1995; Berry et al., 2002). The bacteria *Mycoplasma agassizii* and *Mycoplasma testudineum* naturally infect tortoises, and were shown by experimental infection studies of *G. agassizii* and *G. polyphemus* tortoises to be primary agents of URTD (Brown et al., 1994, 1999b, 2001, 2004). Mycoplasmosis of tortoises elicits an IgM Ab response approximately 4 weeks after exposure, which shifts to a long lasting, predominantly IgY Ab response approximately 8 weeks after exposure (Schumacher et al., 1993; Brown, 2002). Re-exposure can further increase IgY Ab levels to a plateau (McLaughlin, 1997). Serological monitoring therefore may be valuable for epidemiologic studies of mycoplasmal diseases of tortoises (Lance, 1992, 1994; Brown et al., 1999a, 2002). Plasma from infected tortoises was used previously to develop a quantitative ELISA for monitoring exposure to mycoplasma among free-ranging tortoises, and to aid decision-making to control the spread of mycoplasmal URTD (Schumacher et al., 1993, 1997; Wendland et al., 2007).

Tortoise conservation and recovery plans now formally include testing for URTD (U.S. Fish and Wildlife Service, 1994; Savignano, 1996). Detection of specific Abs may be used to diagnose infection and immune status of tortoises for decision-making, especially with regard to management and conservation of legally protected species such as *Gopherus*. However, the ELISA and other laboratory-based assays require that plasma samples be kept cool in the field, and shipped cold (increasing the per-sample cost) to a laboratory for testing. In practice, the minimum turnaround time from sample collection to data reporting can be several days. In some cases, this time frame may complicate timely management decision-making (Brown et al., 2002), or create an added burden on management staff when attempting to minimize the risk of spread of mycoplasmosis before results are obtained. One of our overall objectives is to improve the diagnosis of infectious diseases in tortoises. The specific objective of this project was to evaluate the performance of a field-portable evanescent-wave affinity biosensor (Gooding, 2006; Bosch et al., 2007) adapted for rapid diagnosis of tortoise exposure to *M. agassizii*. A standard protocol for using the biosensor and interpreting the test results was developed. Banked plasma samples were tested in two blinded trials under laboratory conditions, and then from those data the parameters that define the reliability of a diagnostic test were estimated.

2. Materials and methods

2.1. Evanescent-wave biosensor system

The biosensor (cat. no. 7000-115-200-01, Research International, Monroe WA, USA) is a portable, four-channel fluorometric assay system that has been used for high-sensitivity monitoring of various analytes (Anderson et al., 1996; Lim, 2000, 2003; Hoyle, 2001; Jung et al., 2003; Tims and Lim, 2003; Kramer and Lim, 2004; Nanduri et al., 2006). This and similar instruments have been used to detect specific Abs (Nath et al., 1997; Anderson et al., 1998; Marks et al., 2002; Leshem et al., 2004; Wei et al., 2007), but never before used for seroepidemiology of wildlife. It represents the integration of optics, microfluidics, electronics, and software into a compact and rugged instrument for use in laboratory settings and field assays (Ouellette, 1998). The unit can automatically perform a user-defined, multi-step assay protocol while simultaneously tracking evanescent fluorescence that is dependent upon immunochemical reactions occurring on the surface of each of the system's four fiber optical sensors (Hutchinson, 1995; Taitt et al., 2005). All liquids needed to perform an assay, with the exception of the sample, are contained in the unit. Reactants are held in a pre-cooled phase-change module, minimizing deterioration of thermally labile reagents. For these studies, the biosensor was operated on a laboratory benchtop using line power, and controlled by connection to a desktop computer.

2.2. Optical sensor coating and assembly

The disposable polystyrene fiber optical sensors were handled carefully by their mounting flange using serrated 5-in. dressing forceps having tips covered with soft Tygon tubing. New fibers were cleaned by washing in organic solvent according to manufacturer's instructions, followed by rinses with water, and air drying. The distal tip of each fiber was painted to create an optical sink. *M. agassizii* type strain PS6 (cat. no. 700616, American Type Culture Collection [ATCC], Manassas VA, USA) whole-cell lysate Ag was prepared in ATCC medium 988 supplemented with glucose and 20% (v/v) FBS (Schumacher et al., 1993), and stored in polypropylene cryovials at −80 °C at a stock concentration of 200 μg protein/ml. The PS6 Ag was diluted

1:5 in PBS, pH 7.2, to a final coating concentration of 40 μg/ml. To coat the cleaned and painted fiber optical sensors to be used for specific Ab capture, individual fibers were immersed up to the hub of the mounting flange in clean polypropylene 22 gauge 2.5 cm hypodermic needle caps filled with approximately 500 μl PS6 Ag for either 2 h at room temperature or overnight at 4 °C. As a positive control, optical fibers were coated directly with tortoise plasma diluted 1:10 in PBS. As a negative control, optical fibers were coated with SuperBlock (Pierce, Rockford IL, USA), which is a buffered proprietary irrelevant protein solution used for blocking excess binding sites in immunoassays. A separate disposable plastic assay cassette containing four mounted fiber optical sensors was assembled for each specimen by the procedure recommended by the manufacturer. Each four-channel cassette included two PS6 Ag-coated sensors, and positive and negative control sensors.

2.3. Secondary antibody

Tortoise anti-*M. agassizii* Abs bound to the PS6 Ag were detected with cyanine Cy5-labeled anti-tortoise Ig mouse IgG mAb HL673, which was prepared by the University of Florida Hybridoma Core Facility (Herbst and Klein, 1995). For conjugation to Cy5 (cat. no. PA25000, Amersham Biosciences, Piscataway NJ, USA) by the procedure recommended by the manufacturer, the dye was added to 1 ml of mAb HL673 (1 mg protein/ml) and incubated for 30 min, with agitation every 10 min. Labeled Ab was separated from excess unconjugated dye by low-pressure gel filtration chromatography through 16 mm × 60 mm Sephadex G-50 resin columns using PBS as the elution buffer. The fraction (approximately 1 ml collected) containing purified Cy5-labeled mAb HL673 was stored at 4 °C. That stock solution was diluted 1:125 in PBS to a final working concentration of approximately 8 μg/ml.

2.4. Tortoise plasma samples

Banked (<1 year in polypropylene cryovials in a −20 °C non-defrosting freezer) lithium-heparinized ELISA-seropositive and -seronegative plasma samples, from free-ranging *Gopherus* tortoises previously analyzed by our laboratory, were the reagent controls (Feldkamp and Carey, 1997) used to explore various conditions of optical fiber preparation and executable step programming protocols. The ELISA status of each sample was re-confirmed to avoid potential freeze–thaw effects, then samples were blind-coded. In the first of two trials, six samples in each of four categories (seronegative [ELISA titer 1:<32], low-ELISA titer [1:64], mid-range ELISA titer [1:128], and high-ELISA titer [1:512]) were assayed in a blinded format. In the second trial, a different set of seropositive (ELISA titer 1:256; $n = 16$) and seronegative (ELISA titer 1:<32; $n = 17$) samples were assayed in a blinded format. After de-coding, the data were used to estimate the parameters that define the reliability of the biosensor for this application (National Committee for Clinical Laboratory Standards, 1994). Plasma was diluted 1:10 in PBS for biosensor analysis. The minimum amount of plasma needed for a biosensor assay was 100 μl.

2.5. Executable steps

The biosensor was programmed to run automatically with baseline and sample executable steps by following procedures recommended by the manufacturer. The instrument was calibrated with 1 ml of PBS serving as a sham negative sample. Specimens were introduced through the instrument's injection port using a disposable beveled 20 gauge, 3.8 cm hypodermic needle fitted to a 5 cm^3 disposable polypropylene syringe. To analyze a sample, the injected tortoise plasma was pumped into the cassette and incubated for 8 min, then the plasma was pumped out to waste and the optical fibers were rinsed three times with wash buffer (8.3 mM phosphate buffer, pH 7.2, plus 0.05% Triton X-100). The evanescent-wave fluorescence was measured in units of picoamperes (pA) and recorded at this mark (mark 1), when specific anti-*M. agassizii* Abs, that may have been present in the plasma, had accumulated on the PS6 Ag-coated Ab-capture fibers. Specific anti-*M. agassizii* Abs should not have accumulated on the positive or negative control fibers. The Cy5-labeled 2° mAb HL673 was then pumped into the cassette and incubated for 2 min, then the mAb HL673 was pumped back to its reservoir and the cassette was re-filled with wash buffer. The evanescent-wave fluorescence was also measured and recorded at this mark (mark 2). If specific anti-*M. agassizii* Abs were present in the plasma, then Cy5-labeled mAb HL673 bound to them and fluoresced where they had accumulated on the Ab-capture optical fibers. If no specific anti-*M. agassizii* Abs were present in the plasma, then no mAb HL673 should have bound to the PS6 Ag-coated Ab-capture fibers. Also at this mark, the mAb HL673 accumulated on the positive control optical fiber coated with irrelevant tortoise Ig, but it should not have accumulated on the SuperBlock-coated negative control fiber. The

difference (delta) between the laser-excited evanescent-wave Cy5 fluorescence at mark 1 and mark 2 was a measure of the amount of mAb HL673 binding and thus reflected the presence or absence of specific anti-*M. agassizii* Abs in the plasma. Excluding reagent setup and baseline calibration, a sample assay required about 15 min to complete, in contrast to 4–5 h for the standard ELISA. The assay results for the four channels were stored in flash memory for download to a desktop computer.

2.6. Statistical analyses

Two comparative trials of tortoise plasma samples were conducted. The objective of the first trial was to evaluate the congruence between the continuously distributed ELISA and biosensor values. The objective of the second trial was to compare the categorical classifications (seropositive vs. seronegative) observed using the biosensor with the outcomes expected based on the standard ELISA. Parameters that define the reliability of the biosensor (sensitivity, specificity, predictive values, and Youden statistic) were estimated for both trials.

In the first trial, the non-parametric Spearman rank correlation between the continuous ELISA optical density and biosensor fluorescence delta values was calculated. Also, before the samples were unblinded, inspection of the mean observed delta values of the samples and externally validated control tortoise plasma, traceable to tortoises experimentally inoculated with *M. agassizii* (Brown et al., 1994, 1999b), supported a subjective cutoff value of approximately 500 pA. Samples were scored as seronegative or seropositive using that cutoff, then unblinded for comparison to their ELISA classification by χ^2 analysis using StatView 5.0.1 (SAS Institute, Cary NC, USA). The null hypothesis was independence, i.e., a random relationship between ELISA results and biosensor results. In the second trial, the delta was normalized as a percent of the fluorescence pA at mark 1 (delta%). After unblinding the samples, the effect of ELISA status, i.e., either seronegative or seropositive, on delta% was analyzed by one-way ANOVA. Although the effect was significant ($P < 0.05$), since the F-test for equality of variance showed that the variance of delta% was not equal for ELISA-negative and ELISA-positive samples (0.114 and 0.956, respectively; $F_{16/15\ \text{d.f.}} = 0.119$, $P < 0.0001$; see Section 3), the non-parametric Mann–Whitney U-test was used for post hoc comparison. A subjective cutoff between positive and negative delta% was determined retrospectively by inspection of relative frequency histograms of delta% for ELISA-negative and ELISA-positive samples.

To estimate the reliability of the biosensor quantitatively (Schisterman et al., 2005), the sensitivity, specificity, optimal cut-point (c), and corresponding Youden indices (J) were calculated for each trial using SAS programming language (SAS 9.1.3 [2002–2004], SAS Institute). Before analysis, the data were transformed by taking the square root of the pA and delta% values; qq-plots and Shapiro Wilk goodness-of-fit tests were used to confirm normality (JMP IN 5.1 [2005], SAS Institute). Results from the laboratory-based ELISA were used as the gold standard for comparisons. The positive and negative predictive values (PPV, NPV) for each trial were determined using Bayes' rule (Smith, 1995). To demonstrate the impact of seroprevalence on predictive values, the PPV and NPV for seroprevalence levels from 0 to 100% were plotted for each trial.

3. Results

In the first trial, the Spearman rank correlation of ELISA optical density and biosensor fluorescence delta values was 0.804 (Z-value = 3.858, $P < 0.0001$). Using <500 pA = seronegative and ≥ 500 pA = seropositive as the observed outcome, and the ELISA classification as the expected outcome, 22 of 24 samples (92%) were classified accurately by the biosensor. The null hypothesis of only a random relationship between ELISA results and biosensor results was rejected ($\chi^2_{1\ \text{d.f.}} = 14.5$, $P < 0.0001$). The sensitivity, specificity, PPV, and NPV of the biosensor in this trial were 86, 68, 89, and 62%, respectively, and results for all but two samples were categorically identical to ELISA (Fig. 1).

In the second trial, the mean delta% of ELISA-negative samples (0.797 ± 0.092) was lower (Mann–Whitney $U = 249$; $P = 0.0002$) than that of ELISA-positive samples (1.563 ± 0.190). There was considerable overlap of the distribution of delta% of seropositive and seronegative plasma (Fig. 2), but a subjective cutoff at delta% = 1 was apparent. Using delta% ≤ 1 = seronegative and delta% > 1 = seropositive, and the ELISA classification as the expected outcome, 26 of 33 samples (79%) were classified accurately by the biosensor. The sensitivity of the biosensor was 70%, the specificity was 78%, the PPV was 75%, and the NPV was 74%. Thus, in general, false negatives were a worse problem than false positives.

The receiver operating characteristic (ROC) curves for both trials are shown (Fig. 3a and b). The c to maximize assay sensitivity and specificity for trial 1 was 850 pA, with a resultant sensitivity, specificity, and J of

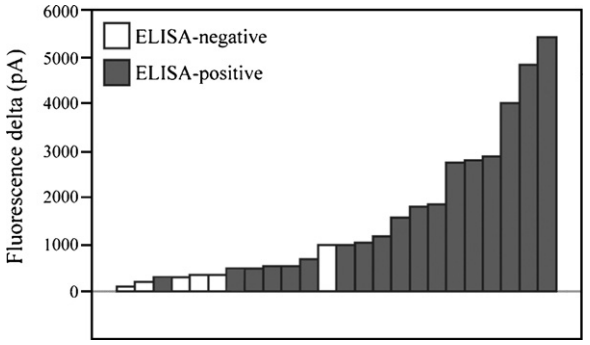

Fig. 1. Trial 1 comparison of laboratory-based ELISA to field-portable biosensor assays for anti-*Mycoplasma agassizii* Abs in plasma of *Gopherus* tortoises. Six samples in each of four categories (ELISA seronegative [titer 1:<32], low-ELISA titer [1:64], mid-range ELISA titer [1:128], and high-ELISA titer [1:512]) were assayed. Samples were scored as seronegative or seropositive using a subjective biosensor fluorescence cutoff of 500 pA, then unblinded for comparison with the ELISA category as the expected outcome and the biosensor result as the observed outcome (Spearman's $\rho = 0.804$, $P < 0.0001$; $\chi^2_{1\,\text{d.f.}} = 14.5$, $P < 0.0001$).

74, 93, and 67%, respectively. However, setting *c* at that value would have resulted in misclassification of four additional samples (i.e., false negative results) in the low-positive range. The discrepancy between the subjective cutoff point and the calculated *c* was due to one outlier in the ELISA-negative group that shifted *c* to a higher value. Adjusting the cutoff point to 500 resulted in increased sensitivity for the assay (86% vs. 74% for *c*), but at the expense of a reduced specificity (68% vs. 93% for *c*). The calculated *c* for trial 2 was delta% = 1.14, and was very similar to the subjective cutoff. The *c* resulted in sensitivity, specificity, and *J* of 63, 87, and 50%, respectively. For the combined trials of

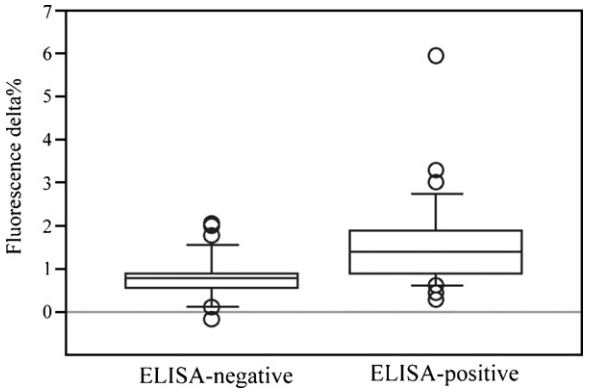

Fig. 2. Trial 2 distribution of normalized biosensor fluorescence of *M. agassizii* ELISA-negative (titer 1:<32; $n = 17$) and ELISA-positive (titer 1:256; $n = 16$) tortoise plasma samples. Box plots show medians, quartiles, and 90th percentiles.

57 tortoise plasma samples, with the ELISA classification as the expected outcome, overall accuracy of the biosensor was 84%. The mean sensitivity was 78%, the mean specificity was 73%, the mean PPV was 82%, and the mean NPV was 68%.

In the first and second trials the true seroprevalence was 75 and 48%, respectively. The effect of seroprevalence extremes on PPV and NPV is shown in Fig. 4. Graphs for subjective and *c* cutoff points illustrate the potential impact of outliers, and how shifting the cutoff point influences all measures of test reliability.

4. Discussion

Our long-term goals are to understand the impacts of diseases on free-ranging tortoises in order to improve the sustainability of managed tortoise populations. *M. agassizii* is a bacterial agent of upper respiratory tract disease suspected to have adverse effects on tortoise health at the population level (Jacobson et al., 1991, 1995). Detection of specific anti-mycoplasma Abs can be used to diagnose infection and immune status of tortoises for epidemiological studies of natural populations, and for decisions to minimize the risk of spread of mycoplasmosis (Brown et al., 2002; Wendland et al., 2007). Specific anti-*M. agassizii* Abs in the plasma of tortoises can be detected reliably by quantitative ELISA 8 weeks after experimental inoculation with the microorganism (Schumacher et al., 1993). There is a high-positive correlation between presence of specific Ab against *M. agassizii* in tortoise plasma and URTD (Schumacher et al., 1997), and seropositive status is a significant risk factor for transmission of URTD (Schumacher et al., 1997; Brown et al., 1999b). However, laboratory-based assays for specific anti-mycoplasma Ab are limited by sample-handling requirements, and by turnaround time which requires tortoise quarantine before decision-making.

The objective of the project described was to accumulate data on performance of a field-portable evanescent-wave biosensor for rapid detection of specific Abs in tortoise plasma that reflect a history of exposure to *M. agassizii*. The evanescent-wave biosensor tested is a laser-based polystyrene fiber optic sensor that can detect specific Abs bound to Ag (Nath et al., 1997; Anderson et al., 1998; Marks et al., 2002; Leshem et al., 2004; Wei et al., 2007). Under various experimental protocols, the signals from positive control tortoise plasma samples were up to seven times higher than the signals from negative control plasma samples, when using *M. agassizii* strain PS6 whole-cell lysate Ag-coated fiber optics and cyanine Cy5-labeled

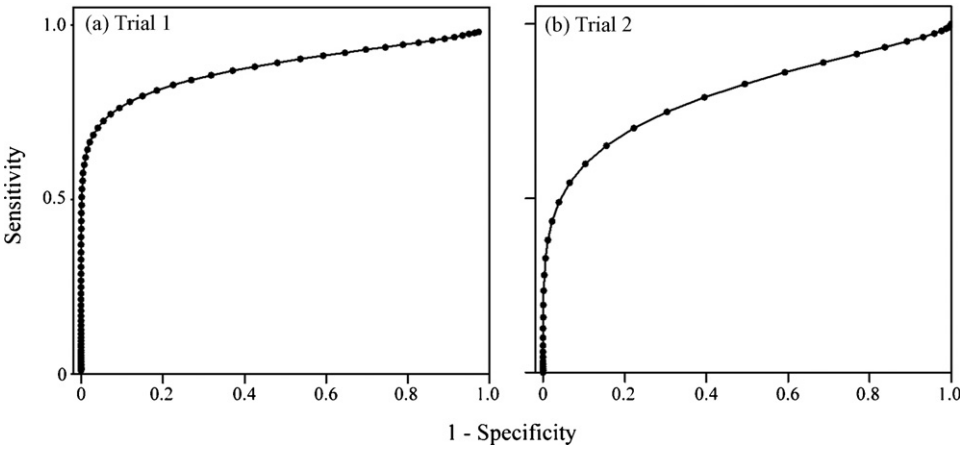

Fig. 3. Receiver operating characteristic (ROC) curves for biosensor trial 1 (a) and trial 2 (b). Higher sensitivity and specificity values obtained in trial 1 resulted in better discrimination between positive and negative samples (Youden statistic $J = 67\%$) than in trial 2 ($J = 50\%$).

anti-tortoise immunoglobulin 2° mAb HL673. The biosensor offers several benefits over standard laboratory-based ELISA as it could eliminate the need for plasma sample refrigeration and costly shipping, and most importantly, provide nearly instant information for management decision-making. Further, the test could be performed in the field using a pre-programmed biosensor according to a standard protocol by personnel

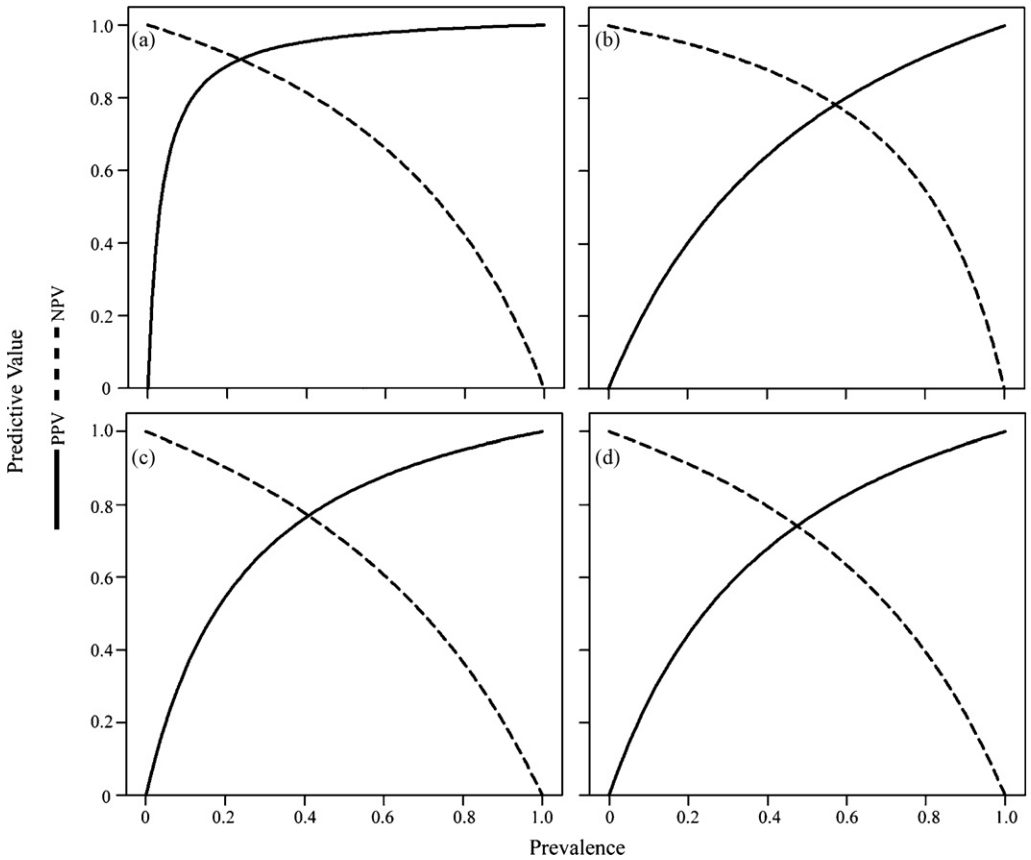

Fig. 4. Effect of seroprevalence on biosensor positive and negative predictive values for trial 1 (a and b) and trial 2 (c and d). Panels (a) and (c) were based on optimal cut-points calculated as described by Schisterman et al. (2005). Panels (b) and (d) were based on cutoffs estimated subjectively by inspection of the data shown in Figs. 1 and 2.

with no or limited training in immunoassay technology, and with a supply of consumables provided in the form of a kit.

The sensitivity of an immunoassay is the ability to identify exposed individuals in the group of exposed individuals, i.e., "if they were exposed, do they test positive?" It can only be estimated retrospectively by comparison to external standards, in this case the ELISA serostatus validated by comparison to plasma from experimentally inoculated tortoises. Nath et al. (1997) and Wei et al. (2007) achieved biosensor sensitivity approaching 100% for externally validated seropositive humans and rabbits exposed to *Leishmania donovani* and *Yersinia pestis*, respectively. The 78% combined sensitivity obtained in the current study was lower than the >98% sensitivity possible with the laboratory-based ELISA for tortoise mycoplasmosis (Brown et al., 2002; Wendland et al., 2007), but may be adequate depending on the goals of the user and conditions under which the biosensor is used. For example, the portable format of the assay makes it particularly useful in extremely remote areas or in other circumstances where transport of biological samples from the field to a testing laboratory may be problematic. If management decisions that will directly determine the survival (e.g., relocation) of an individual are to be made on the basis of test results, the traditional ELISA should be used for confirmation of the serological status as it will likely provide more reliable results. Potential future approaches to improving biosensor sensitivity by minimizing the mark 1 (background fluorescence) values include titering the Ag coating concentrations, incorporating a blocking step in the Ab-capture optical fiber preparation protocol, and incorporating additional wash steps before mark 1 fluorescence is recorded. Potential approaches to maximizing the mark 2 values include titering the Cy5 labeling protocol to increase the molar ratio of dye bound to 2° Ab molecules, using a different fluorophore (Anderson and Nerukar, 2002), titering the working concentration of fluor-labeled 2° Ab, and increasing the length of incubation with fluor-labeled 2° Ab before mark 2 fluorescence is recorded. Based on our experience, lot-to-lot variation in the fluor labeling of 2° Ab seems likely to remain a significant source of variation in reliability of the biosensor assay.

The specificity of an immunoassay is the ability to identify unexposed individuals, i.e., "if they were not exposed, do they test negative?" This too can only be estimated retrospectively by comparison to external standards. Wei et al. (2007) reported 94.7% biosensor specificity for externally validated seronegative rabbits, and Nath et al. (1997) achieved 100% specificity for a small number of negative human sera. The 73% combined specificity obtained in the current study also was lower than the >99% specificity possible with the laboratory-based ELISA (Brown et al., 2002; Wendland et al., 2007). However, actual false positive results among the samples were rare, and inspection of the raw data revealed that for each of those, one of the duplicate Ag-capture optical fibers gave anomalous results. This result points out another of the most significant sources of variation in reliability of the biosensor, which is its dependence on manual assembly of the delicate optical fibers into the cassettes. Any flexion of, or physical contact with, the fiber during coating or assembly may lead to unreliable results. Certain samples were false-negative for similar reasons. This effect seems likely to remain an operator-dependent source of variation in reliability of the biosensor.

With any diagnostic test, some results are expected to be false-positive, false-negative, or abnormal (Feldkamp and Carey, 1997). Predictive values provide a measure of confidence in diagnostic test results, and should always be considered when making management decisions based on surveillance evidence. Despite the slightly higher retrospective false-positive rate (27% vs. 22% false-negative) obtained in this study, the 68% combined NPV indicated that false-negatives can be expected to be a greater limitation of the biosensor than false-positives. In addition, predictive values of such immunoassays depend on the prevalence of seropositive individuals within the population. Plots such as those shown in Fig. 4 allow users to make decisions with a known level of confidence in the assay results based on seroprevalence. When conducting surveillance for exposure to *M. agassizii*, occasional false-negative results from a population known to have moderate to high seroprevalence seem unlikely to impact management decisions significantly. In populations with low seroprevalence, the likelihood of false-positive results increases. The most effective use of the biosensor therefore might be for screening to indicate the presence of seropositive tortoises in previously uncharacterized or suspected low-seroprevalence populations, or monitoring of seronegative populations for changes in serostatus. Positive results from a number of animals could be trusted collectively to indicate the presence of seropositive tortoises within the population, but any individual positive result, especially one from an adequately sampled population believed to have low seroprevalence, should be interpreted with caution because it has a greater risk of being a false-positive result. In such instances, confirmatory immunoassays

such as traditional ELISA, or Western blot (Wei et al., 2007), would be necessary before imposing management sanctions on an individual tortoise or the subpopulation from which it originated. Depending on the potentially adverse outcomes of management decisions following a diagnostic error (e.g., relocation of false-seronegative but actually infectious individuals to an unexposed population), any individual negative result should also be confirmed by independent means.

When making animal management decisions on the basis of infectious disease diagnostics, regardless of their format, it is critical to establish a priori goals for the population of interest and to determine sample sizes necessary to meet the goals for surveillance before implementing any policy (Wendland et al., 2007). Alternative recommendations for using mycoplasmosis serology to facilitate free-ranging tortoise management have differed in emphasis on either minimizing risk of transmission at population levels (Brown et al., 2002) or on the potentially grave consequences of management intervention to seropositive individuals or groups (Wendland et al., 2007). The goals established for the tortoise population can help managers decide how potential diagnostic errors should affect decision-making, and whether the benefits of the rapid field-portable format and potentially lower per-sample cost of the biosensor assay outweigh its disadvantages in capital cost and throughput capacity.

5. Conclusion

The evanescent-wave biosensor is capable of detecting specific Abs in tortoise plasma that reflect a history of exposure to *M. agassizii*, and for the majority of samples, under the conditions described, the biosensor system was able to discriminate between true seropositive and true seronegative tortoise plasma. In a laboratory setting the biosensor produced information similar to the traditional ELISA, with a protocol that could be performed in a few minutes by a minimally trained technician, but with less sensitivity and specificity. In addition to the refinements mentioned above, and further trials with larger sample sizes, two fundamental objectives remain: (1) assess the ability of the biosensor to discriminate tortoise Abs to *M. agassizii* from tortoise Abs to *M. testudineum* or other potentially cross-reactive Ags; (2) assess the effects of environmental and sample-handling conditions likely to be encountered in the field on the performance characteristics of the instrument. Those objectives represent further essential stages of validation necessary before eventual field application to tortoise serodiagnostics would be justified.

Brown et al. (2005).

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