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Silicon surface multiplex immunoassay (Accuplex) for antibodies against *Anaplasma* spp and *Ehrlichia* spp is a robust option for tick-borne pathogen screening in dogs

Michelle D. Evason, DVM, DACVIM[1*]; Graham Bilbrough, VetMB, MA,[1]; Jennifer L. Willcox, DVM, DACVIM[1]; Gregory Freeman, MBA[1]; Jere W. McBride, PhD[2]; Michael R. Lappin, DVM, DACVIM, PhD[3]; Christian M. Leutenegger, DrMedVet, PhD[1]

[1]Antech Diagnostics, Mars Petcare Science and Diagnostics, Loveland, CO
[2]Department of Pathology, Center for Biodefense and Emerging Infectious Diseases, Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX
[3]Department of Clinical Science, Center for Companion Animal Studies, Colorado State University, Fort Collins, CO

*Corresponding author: Dr. Evason (michelle.evason@antechdx.com)

Objective
To evaluate the diagnostic performance of a point-of-care (POC) test (SNAP 4Dx Plus; Idexx Laboratories Inc) versus a proprietary silicon surface multiplex immunoassay (SSMIA) test (Accuplex; Antech Diagnostics Inc) with indirect fluorescent antibody as the reference method (REFERENCE) for detecting antibodies against *Anaplasma* spp and *Ehrlichia* spp in dogs and to compare SSMIA test results with period prevalences reported by the Companion Animal Parasite Council (CAPC).

Methods
Anonymized retention-released serum samples from dogs in the US submitted to a commercial laboratory between July 1, 2023, and September 30, 2024, were classified as true positive or true negative for each antibody via REFERENCE. Performance metrics (sensitivity, specificity, and Cohen κ) were calculated (REFERENCE as the standard) for SSMIA versus POC. Additional separate serum samples from dogs in Connecticut, Massachusetts, New Jersey, New York, and Pennsylvania underwent SSMIA testing alone in July 2024, and results were compared with respective period prevalence data reported by the CAPC.

Results
Test methods did not differ significantly for detection of antibodies against *Anaplasma* spp (n = 310 samples). Silicon surface multiplex immunoassay was significantly more sensitive than POC for *Ehrlichia* spp antibody detection (n = 309 samples). Another 2,031 samples underwent SSMIA testing alone, and positivity rates were generally higher for SSMIA detection as compared to CAPC reporting.

Conclusions
These findings support SSMIA as a robust option for routine tick-borne pathogen exposure screening in dogs.

Clinical Relevance
Our work provides veterinarians with field and comparative performance data on 2 commercially available tests, informing evidence-based contextualized diagnostic options.

Keywords: *Anaplasma* spp, *Ehrlichia* spp, proprietary silicon surface multiplex immunoassay, Accuplex, point-of-care test

With the continued global expansion and growing abundance of tick vectors and their transmitted pathogens, effective and comprehensive surveillance for endemic, emerging, and evolving pathogens is essential. Tick-borne disease pathogens can have both veterinary and human health importance. Examples of these pathogens in North America include the intracellular, gram-negative bacteria *Anaplasma* spp (*Anaplasma phagocytophilum* and *Anaplasma platys*) and *Ehrlichia* spp (*Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*). In dogs, serology-based (antibody) screening remains a reliable and recommended method for identifying exposure to tick-borne pathogens of veterinary and One Health significance.[1–3]

Veterinary teams routinely perform annual surveillance for *Borrelia burgdorferi* (Lyme borreliosis), *Anaplasma* spp, and *Ehrlichia* spp antibodies, typically in

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conjunction with heartworm (*Dirofilaria immitis*) antigen testing, as advised by expert- and evidence-based veterinary groups, like the Companion Animal Parasite Council (CAPC).[1–4] Similar test methodologies (ie, immune migration and ELISA) for these tick-borne pathogens are commonly utilized by veterinary clinics, with various test options currently available at the point of care (POC) and reference laboratories.[5–9] The most used commercially available serologic tests for tick-borne pathogen exposure in North America include a proprietary silicon surface multiplex immunoassay (SSMIA) (Accuplex; Antech Diagnostics Inc) and the POC tests SNAP 4Dx Plus (Idexx Laboratories Inc) and VetScan FLEX4 Rapid Test (Zoetis Services LLC).[5–9]

Testing methodologies for tick-borne disease pathogens differ in myriad aspects, such as which pathogens (and pathogen species and subspecies) are offered on a specific platform and what type and number of antibodies are detected (ie, single or multiple). With the emergence of different species and subspecies of *Anaplasma* and *Ehrlichia* in the US and Canada in recent years and the number of positive tests increasing rapidly in novel regions, many commercial test platforms have been modified in the past decade (eg, addition of *A platys*, *E ewingii*, and *E chaffeensis* to existing tests).[5–9]

Silicon surface multiplex immunoassay is utilized by veterinarians to detect C6 antibodies to *B burgdorferi* and screen for antibodies to 2 *Anaplasma* spp (*A phagocytophilum* and, *A platys*) and 3 *Ehrlichia* spp (*E chaffeensis*, *E ewingii*, and *E canis*), as well as antigens for *D immitis* (heartworm). This test platform, which consists of a proprietary SSMIA, has undergone several significant modifications, expansions, and enhancements in recent years to meet veterinary and One Health needs. Consequently, there is a need for performance and field validations of the currently offered platform to provide veterinarians with peer-reviewed evidence for clinical decision-making and accessible options for testing. Additionally, there is increasing recognition that contextualized diagnostics that allow for incorporation of spectrum of care in day-to-day practice are required to assist veterinarians in meeting pet owners where they are. This rising need requires a foundation of evidence and data on existing screening test options, optimally in the form of a current comparative evaluation of the 2 most used *Anaplasma* spp and *Ehrlichia* spp testing methodologies (ie, the SSMIA [Accuplex] and POC [SNAP 4Dx Plus]).

The primary objective of our study was to perform a comparative evaluation using remnant canine clinical samples and estimate diagnostic-test performance metrics for *Anaplasma* spp and *Ehrlichia* spp. These performance metrics included sensitivity, specificity, and the Cohen κ (as compared to the indirect fluorescent antibody [IFA] reference method [REFERENCE]), along with direct test-versus-test (SSMIA vs POC) comparisons. Point-of-care testing was used, as no validation publications for Lab 4Dx Plus were available. A secondary objective was to perform a field validation of the updated SSMIA screening platform for *Anaplasma* spp and *Ehrlichia* spp peptides and compare these test results to public period prevalence data obtained from the CAPC website for the same time frame and states.[1]

Methods

For the primary objective, deidentified, retention-released remnant serum samples taken from dogs and originally submitted by US veterinary clinics to Antech Diagnostics for routine SSMIA analysis were eligible for inclusion in the comparison studies. Collection for the *Ehrlichia* spp comparison took place from the start of July to the end of August 2023, inclusive, with samples from the southeastern US targeted. Collection of samples for the *Anaplasma* spp comparison took place between the summer through fall 2024 (start of July to end of September, inclusive) from the northeastern US. All samples were stored at −20 °C after clinical testing until study use.

Silicon surface multiplex immunoassay is a proprietary multiplex platform using a silicon wafer disc as substrate for the detection of antigens and antibodies from serum samples including *Anaplasma* spp[10] and *Ehrlichia* spp.[11] On the silicon wafer, a 288-well pattern is printed onto the surface using a hydrophobic mask. Using a contact protein printer, the recombinant proteins are used to print 64 spots in a specific spot pattern in every well. The finished disc can hold up to 274 patient samples along with 8 positive controls and 6 negative controls spread around the disc. Initial SSMIA results were used to preliminarily screen and satisfy the minimum positivity rate for sample size requirements. Indirect fluorescent antibody testing (SLD-IFA-AP and -EC, screening titer 1:64; Veterinary Medical Research and Development [VMRD Inc]) was subsequently used to classify *Anaplasma* spp and *Ehrlichia* spp samples as true positive and true negative as described herein. Once the full sample set was assembled, operators were blinded to the original SSMIA results when comparison testing was performed. The VMRD Inc IFA slides, according to the technical insert, were created using *E canis* or *A phagocytophilum* cultures and have stated cross-reactivity to *E chaffeensis* and *E ewingii* and to *Anaplasma* spp other than *A phagocytophilum*. Cutoff values for *Ehrlichia* spp IFA were set at > 1:80 and for *Anaplasma* spp at > 1:40.

Anaplasma spp (n = 310 samples) were processed using SSMIA, POC, and IFA (SLD-IFA-AP; VMRD Inc), following manufacturers' protocols for POC and REFERENCE and reference laboratory standard operating procedures for SSMIA, performed in parallel. Codetected *Ehrlichia* spp antibody samples on SSMIA or POC were excluded due to potential REFERENCE cross-reactivity.

Similarly, *Ehrlichia* spp samples (n = 309) were evaluated by SSMIA, POC, and REFERENCE (SLD-IFA-EC; VMRD Inc) performed in parallel. Similarly, codetected *Anaplasma* spp antibody samples on SSMIA or POC were excluded due to potential REFERENCE cross-reactivity.

For the secondary objective (field validation), deidentified remnant serum samples (n = 2,031) from dogs submitted for routine analysis were eligible for

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inclusion. These dog samples were from 5 northeastern American states (Connecticut, Massachusetts, New Jersey, New York, and Pennsylvania) and were submitted to a reference laboratory during July 2024 and evaluated using the SSMIA platform as previously described. Companion Animal Parasite Council datasets for the same 5 American states and time frame (July 2024) were obtained from the CAPC website and results compared to those evaluated by SSMIA.[1]

Statistical analysis

Sample sizes for both the *Anaplasma* spp and *Ehrlichia* spp comparisons were based on convenience as well as purposive sampling and calculated with a 95% CI, α = 0.05, and a disease prevalence set to 25%. Descriptive statistics (sensitivity, specificity, and Cohen κ) were calculated from per-animal test calls with the IFA assay (REFERENCE) as the standard. Ninety-five percent CIs for sensitivity and specificity were calculated by the Wilson score method, and 95% CIs for the Cohen κ were derived from the asymptotic SE. Test-versus-test comparisons (SSMIA vs POC) were performed on the same animals. Sensitivity and specificity were compared with the McNemar test for paired proportions (exact binomial form), applied separately within the REFERENCE-positive stratum (sensitivity) and the REFERENCE-negative stratum (specificity). Agreement (Cohen κ) was compared between tests with a paired bootstrap (20,000 resamples; fixed random seed), reporting the difference in the Cohen κ with its 95% CI. Two-sided $P < .05$ was considered statistically significant. All analyses were performed with standard software (Python, version 3.10.12; Python Software Foundation) using the SciPy (version 1.15.3), NumPy (version 2.2.6), and statsmodels (version 0.14.6) packages.

Results

For *Anaplasma* spp **(Table 1)** the REFERENCE identified 129 positives (42%) and 181 negatives (58%). Agreement (Cohen κ) for *Anaplasma* spp was 0.93 (95% CI, 0.89 to 0.97) for SSMIA and 0.94 (95% CI, 0.90 to 0.98) for POC as compared to REFERENCE, respectively. For *Anaplasma* spp, the SSMIA and POC performed equivalently, with no statistically significant difference in sensitivity or specificity.

For *Ehrlichia* spp **(Table 2)**, the REFERENCE identified 78 positives (25%) and 231 negatives (75%). Agreement (Cohen κ) for *Ehrlichia* spp was 0.92 (95% CI, 0.86 to 0.97) for SSMIA and 0.67 (95% CI, 0.57 to 0.77) for POC as compared to REFERENCE, respectively. For *Ehrlichia* spp, the SSMIA and POC tests differed significantly. The SSMIA test was substantially more sensitive ($P < .001$) and had significantly higher agreement (Cohen κ difference, +0.24; 95% CI, +0.14 to +0.35; $P < .001$), while POC was marginally more specific ($P = .031$).

For the field study validation, the updated SSMIA platform detected higher test positivity for all American states, aside from Connecticut, for *Anaplasma* spp as compared to CAPC data **(Table 3)**. For *Ehrlichia* spp, SSMIA demonstrated increased test positivity as compared to CAPC data for all states **(Table 4)**.

Table 1—Results for diagnostic performance metrics of a point-of-care (POC) test (SNAP 4Dx Plus; Idexx Laboratories Inc) versus a silicon surface multiplex immunoassay (SSMIA) test (Accuplex; Antech Diagnostics Inc) for detecting antibodies against *Anaplasma* spp in 310 anonymized retention-released serum samples from dogs in the US submitted to a commercial laboratory July 1 to September 30, 2023, with indirect fluorescent antibody test as the reference method (REFERENCE), which identified 129 positives (42%) and 181 negatives (58%).

Performance metric	SSMIA	POC	*P* value
Positives (n)	133	122	—
Negatives (n)	177	188	—
Sensitivity (95% CI)	98% (93%–99%)	94% (88%–97%)	.227 (SSMIA vs POC, REFERENCE-positive dogs)[a]
Specificity (95% CI)	96% (92%–98%)	99% (97%–100%)	.070 (SSMIA vs POC, REFERENCE-negative dogs)[a]
Agreement with κ (95% CI)	0.93 (0.89–0.97)	0.94 (0.90–0.98)	.900 (SSMIA vs POC)[b]

[a]McNemar test. [b]Difference in Cohen κ; paired bootstrap with 20,000 resamples.
— = Not calculated.

Table 2—Results for diagnostic performance metrics for the assays described in Table 1 for detecting antibodies against *Ehrlichia* spp in 309 anonymized retention-released serum samples from dogs in the US submitted to a commercial laboratory July 1 to August 31, 2023, with indirect fluorescent antibody test as REFERENCE, which identified 78 positives (25%) and (75%) 231 negatives.

Performance metric	SSMIA	POC	*P* value
Positives (n)	80	45	—
Negatives (n)	229	264	—
Sensitivity (95% CI)	94% (86%–97%)	58% (47%–68%)	< .001 (SSMIA vs POC, REFERENCE-positive dogs)[a]
Specificity (95% CI)	97% (95%–99%)	100% (98%–100%)	.031 (SSMIA vs POC, REFERENCE-negative dogs)[a]
Agreement with κ (95% CI)	0.92 (0.86–0.97)	0.67 (0.57–0.77)	< .001 (SSMIA vs POC)[b]

[a]McNemar test. [b]Difference in Cohen κ; paired bootstrap with 20,000 resamples.

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Table 3—Field validation of an updated SSMIA for *Anaplasma spp* (n = 2,031) and comparison to test positivity data obtained from the Companion Animal Parasite Council (CAPC) for the same month and state, expressed as percentages, July 2024.[1]

State	SSMIA	CAPC	Difference
Pennsylvania	16.1	13.2	3.0
New York	20.7	15.8	4.9
New Jersey	13.8	10.9	2.9
Massachusetts	26.1	23.7	2.4
Connecticut	24.8	27.1	–2.3
Mean	20.1	18.1	2.16

Table 4—Field validation of an updated SSMIA for *Ehrlichia spp* (n = 2,031) and comparison to test positivity data obtained from CAPC for the same month and state, expressed as percentages, July 2024.[1]

State	SSMIA	CAPC	Difference
Pennsylvania	2.6	1.1	1.6
New York	4.3	1.5	2.8
New Jersey	5.3	3.7	1.6
Massachusetts	4.3	1.9	2.4
Connecticut	4.3	1.7	2.6
Mean	4.2	2.0	2.19

Discussion

For *Anaplasma* spp and *Ehrlichia* spp antibody detection, both SSMIA and POC demonstrated high test specificity (> 90%). Performance metrics were mostly comparable between both tests. However, SSMIA was significantly superior to POC for *Ehrlichia* spp sensitivity and the Cohen κ.

A few studies,[5–9] published between 2014 and 2023, have provided information on POC test performance metrics, reporting over 90% sensitivity and specificity for both *Anaplasma* spp and *Ehrlichia* spp. In one of these studies,[5] the POC was established as diagnostically superior to the earlier-referenced commercially available POC test for both sensitivity and specificity of *Anaplasma* spp and *Ehrlichia* spp detection. Our work indicates that SSMIA has high sensitivity and specificity for *Anaplasma* spp (98% and 96%) and *Ehrlichia* spp (94% and 97%) antibody detection, respectively. Silicon surface multiplex immunoassay also showed excellent agreement (Cohen κ > 0.93) as compared to REFERENCE and was overall comparable to POC for *Anaplasma* spp screening.

In our work, the sensitivity of POC for *Ehrlichia* spp was found to be considerably lower than in past studies.[5–9] The reason for this low sensitivity (58%) for POC versus SSMIA (94%) is unclear; however, this may reflect differences in study or changes to test design. The marginal increase in POC specificity (100%) versus SSMIA (97%) for *Ehrlichia* spp may be due to the same reasons.

For the field study validation, the updated SSMIA platform detected higher test positivity for all American states for *Anaplasma* spp and *Ehrlichia* spp as compared to CAPC data, aside from *Anaplasma* spp in Connecticut. These higher test positivity findings may indicate increased "real-world" test sensitivity—particularly for *Ehrlichia* spp—or, potentially, a bias in sample population reflecting differences in reference lab–contributed samples versus a mix of in-hospital testing and reference laboratory samples.

Limitations of our work include those commonly associated with retrospective sampling studies, such as incomplete metadata; lack of control over sample selection; variation in sample handling, operator, or storage; and inability to standardize testing conditions across all samples. Additional limitations include potential sampling bias, as the samples included may have disproportionately represented certain geographic regions or pathogen strains, and the concern that IFA (REFERENCE) was used as the standard for ethical reasons for both tests versus the gold standard of full necropsy with additional confirmatory diagnostics. Lastly, there may have been limitations associated with convenience and purposive sampling, which may have implications on generalizability, and the lack of clinical data on dog samples or specific and individual performance evaluations on *Anaplasma* spp and *Ehrlichia* spp, such as *E ewingii*, *E chaffeensis*, and *A platys*. Importantly, comparative sensitivity estimates may be influenced by regional variation in the species, and subspecies, compositions of *Anaplasma* spp and *Ehrlichia* spp circulating in a given population.

The POC and SSMIA platforms employ different antibody targets; as such, a test with relatively higher sensitivity for *A platys* versus *A phagocytophilum* (or vice versa) could appear to perform differently depending on the local species prevalence, independent of any true difference in assay performance. This likely represents a limitation of both cross-platform comparisons and field-prevalence comparisons that cannot be resolved without species-level confirmation of positive samples. Future studies should include sampling from different geographic regions.

This research provides timely field and comparative performance information on 2 commonly used test platforms for *Anaplasma* spp and *Ehrlichia* spp serologic screening in dogs. Our findings serve to assure veterinary teams that the SSMIA is an excellent, accurate option for *Anaplasma* spp and *Ehrlichia* spp exposure detection in dogs and inform on the use of these tests for routine veterinary screening as advised by evidence- and expert-based guidelines and as part of annual *D immitis* (heartworm) antigen and *B burgdorferi* (Lyme borreliosis) C6-antibody screening.[1,2] In conclusion, SSMIA demonstrated high sensitivity and specificity for both *Anaplasma* spp and *Ehrlichia* spp serologic screening in dogs, with substantially superior *Ehrlichia* spp sensitivity compared to POC in this sample set. These findings support SSMIA as a robust option for routine tick-borne pathogen screening and provide peer-reviewed, evidence-based data to inform contextualized veterinary clinical decision-making and selection of diagnostics across the spectrum of care.

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Disclosures

Michelle D. Evason, Christian M. Leutenegger, Graham Bilbrough, Jennifer L. Willcox, and Gregory Freeman are employees of Antech Diagnostics (Mars Petcare Science and Diagnostics). The commercial silicon surface multiplex immunoassay test used in this study was Accuplex, an Antech Diagnostics (Mars Petcare Science and Diagnostics) product.

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