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Investigation of Antibody Response to *Anaplasma phagocytophilum* Immunodominant Targets During Early Phase Seroconversion in Cats and Dogs from Europe

Andrea E. Arguello-Marin

A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

March 2024

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Abstract

Anaplasmosis is a tick-borne infection caused by the bacterium *Anaplasma phagocytophilum*, transmitted by *Ixodes* spp. ticks. It infects humans and a wide variety of domestic and wild animals worldwide. Anaplasmosis in humans is known as Human Granulocytic Anaplasmosis (HGA). It is characterized by severe systemic flu-like illness with hematologic abnormalities and mild hepatitis. HGA can have a severe effect on persons with underlying health conditions as well as immunocompromised and older adults. Early detection and treatment when antibacterial therapy is most effective has proven to have a positive impact on humans affected by tick-borne diseases. (Rikihisa, 2011)

Infected dogs and cats serve as sentinels for the presence of these pathogens in ticks in their geographical area and indicate if there is risk of people exposed to that same tick vector. Dogs and cats can also serve as a useful animal model for tick transmission. Emerging infections with *Anaplasma* species have become more frequently diagnosed in humans. Since animal reservoirs and tick vectors have increased in numbers, the threat to public health is increasing with newly emerging *Anaplasma* agents in areas where reservoir and tick population are high (CDC). However, there is little progress to improve early detection and early treatment of animals that are infected. Early signs and symptoms of these illnesses are nonspecific or mimic other illnesses, which can make diagnosis challenging.

Current options for diagnosing anaplasmosis in companion animals include blood smear analysis, molecular and serologic testing. Each have their own benefit, cost, and expertise requirement to obtain accurate results, but not all are available to veterinarians.

Inductive reasoning was used to determine that a second-generation of 4Dx® Plus SNAP® improves early detection of anaplasmosis relative to the prior generation of 4Dx® Plus SNAP® in cats and dogs with positive *Anaplasma* PCR results in European countries. The early detection with this enhanced diagnostic technology is due to the recognition of IgM and IgG by immunodominant targets as measured on ELISA and IFA of a geographically diverse population of serum samples. This improved detection gives a more representative estimate of the extent of anaplasmosis in the pet population and regional tick vectors.

Based on current companion animal health care practices in Europe, testing for vector borne diseases generally occurs when the animals are exhibiting clinical signs versus wellness visits in the United States. This study will be part of an educational campaign to promote tick-borne screening and regular application of tick preventative to drive awareness of locally relevant tick-borne diseases. (Schäfer et al., 2022; Sainz et al., 2015)

Awareness by veterinarians and pet owners helps to modify behaviors which limit contact with vectors or prevent transmission via vaccination.

Dedication

This effort is dedicated to my past and future, Abuelo-Buely and Athena.

Acknowledgments

I would like to express my deepest gratitude to my Thesis Research Director and mentor, Melissa Beall, for her invaluable patience, guidance, and encouragement. I am also grateful to Jill Saucier, Chandra Chandrashekar, Phyllis Tyrrell, Jackie Franco Marmolejo, Joshua Boucher and Christine Lemieux, who generously provided knowledge, expertise, and late-night feedback sessions.

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I am also grateful to my teammates, Brendon Thatcher, Jillian Laliberte, Tori Denis, Beverly Anderson, Tyler Bauersfeld and to the entire IDEXX Reference Laboratories Sample Management Team for their off-duty support.

Lastly, I could not have undertaken this journey without the support of my wife Stacy, my family, and friends. I cannot thank you enough for the encouragement and support you have provided me during this master's journey. And to Zeva, even though you barked during my Zoom classes, you were always providing emotional support. Gracias Totales.

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Chapter I.

Introduction

The following section details the foundation of this study. It describes *Anaplasma phagocytophilum* pathogenesis, and the importance of early detection.

Pathogenesis

Anaplasma phagocytophilum is an astute obligate intracellular small Gram-negative pathogen. It is transmitted by *Ixodes* spp. and can infect a variety of mammalian species including human beings, dogs, cats, horses, deer, sheep, and cattle. Strain variation has been reported to contribute to host pathogenicity (Massung, et al., 2009). The white footed mouse, short-tailed shrew, and the eastern chipmunk have been suggested to be competent reservoir hosts for tick transmission (Keesing, et al., 2012). *Anaplasma phagocytophilum* is a successful pathogen because it can adapt to changing environments by cycling between tick vectors and mammalian hosts. It accomplishes this adaptation by regulatory systems that sense osmolarity and urea concentration change, all carried by the outer membrane proteins.

After binding to scaffold proteins on the host neutrophil via their outer membrane proteins (Park, et al., 2004; Rikihisa, et al., 2011; Felek, et al., 2004) it establishes vacuoles within the host cell to survive and hide within its inclusions and escape humoral immune response. Once inside AnkA is secreted by the bacteria, it then translocates to the nucleus of infected cells regulating host cell transcription to facilitate bacterial survival and growth. It prevents production of reactive superoxide anion to survive in

neutrophils and delay the apoptosis of infected neutrophils to allow its replication. (Zhi, et al., 2002; Rikihisa, et al., 2011). It then induces a proinflammatory response leading to neutrophil recruitment, primarily due to the release of cytokines such as IL-8 and IFN-gamma, which contributes to continuous tissue injury. Despite the abundant production of cytokines, infected neutrophils are prevented from exerting an effective antimicrobial response. These cytokine-driven mechanisms explain the clinical manifestations such as a fever, pancytopenia, liver dysfunction or more severe manifestation such as shock or organ failure. *Anaplasma phagocytophilum* then divides until cell lysis or when the bacteria leave to infect other cells. (Zhi, et al., 2002).

Background of the problem

A. phagocytophilum has been detected across Europe in previous studies of dogs and cats. Blood samples have been screened by PCR and molecular seroprevalence rates in dogs and cats from European countries have been reported, demonstrating continuous growth, and a concerning indication that cats and dogs living in the close vicinity of their owners can act as direct sentinels for the infection of humans (Skotarczak, 2018; Andre, 2018; Schäfer, et al., 2022).

There has also been an increase in cases presented to veterinary clinics of cats and dogs in need of evaluation due to fever, lethargy and loss of appetite. (Lappin, et al., 2020; Balboni, et al., 2021; Duplan, et al., 2018). These early signs are nonspecific or mimic other illnesses, which can make diagnosis challenging (Lappin, et al., 2020). Guidelines for veterinary practitioners in Europe are available to provide support on the diagnosis, treatment, and prevention of anaplasmosis in cats and dogs in Europe. (Pennisi, et al., 2017; Lappin, et al., 2020; Sainz, et al., 2015).

However, the extent to which *A. phagocytophilum* can persist in the host and contribute to subclinical, acute, or chronic disease manifestations in dogs and cats has been a controversial issue. (Foley, et al., 2009). Clinical signs may vary or disappear spontaneously, even without treatment. Not only can this convolute the diagnosis of anaplasmosis but depending on the diagnostic methodology available to the veterinarian or the capacity of the pet's owner to afford the testing, it can further delay proper treatment.

Currently, there are three diagnostic methodologies recommended: direct blood smear, polymerase chain reaction (PCR), and serology. Each have their advantages and disadvantages and are best suited for testing during certain stages of the disease. Starting with a direct blood smear detection method for morulae. Morulae can be observed in neutrophils in up to 60% of clinical cases. However, the particular species of bacteria producing the morulae cannot be distinguished by microscopy. PCR should follow as a confirmatory test. It is more sensitive, and the detection of pathogenic DNA should be considered as evidence of an active infection. Cost, turn-around time, and equipment requirements for this testing can also delay a proper anaplasmosis diagnosis. False-negative results can also occur due to the absence of pathogens in the sample, potentially incorrect selection of primers could also affect the results. It is best to assess the PCR results in combination with an evaluation of antibody titers, clinical signs, and abnormal laboratory findings.

Antibodies to *Anaplasma phagocytophilum* can be detected by Immunofluorescence Assay (IFA) and enzyme-linked immunosorbent assay (ELISA) techniques, both have respective sensitivity and specificity limitations in detecting

evidence of *Anaplasma* infection in cats and dogs with early clinical signs. IgM is the largest immunoglobulin and typically the first antibody to be produced after pathogen exposure, with a short-term activity since their concentration usually decreases within several weeks of exposure. It is considered that the presence of IgM confirms an early infection in the host. Following, are IgG antibodies, they are generated in larger quantities and of higher infinity during slightly later stages of infection or as a secondary/anemnsetic immune response.

Detection of antibodies to *A. phagocytophilum* can be influenced by the targets utilized and the assay format. For instance, IFA has been shown to detect antibodies earlier than a rapid ELISA method in a canine experimental infection study (Scorpio, et al., 2011). IFA uses *A. phagocytophilum*-infected neutrophils to detect a polyspecific polyclonal response from the host as well as both IgM and IgG antibodies with and anti-canine immunoglobulin fluorescent conjugate. While this appears to provide enhanced sensitivity, IFA has demonstrated lower specificity when tested with patient samples from related infections (Qurollo, et al., 2014). The rapid ELISA method used in the Scorpio et all., study used a single synthetic peptide from the P44 immunodominant protein of *A. phagocytophilum* for both antibody capture and detection in the rapid assay format.

For this study, the immunodominant major outer membrane proteins, P44 (44-kDa), encoded by the p44 polymorphic multigene family, was considered as the foundation for this hypothesis. (Zhi, et al., 2002). This family of proteins has been shown to be immunodominant in HGA and recombinant proteins effective for serologic testing These proteins are genetically diverse, have conserved amino and carboxy domains and a

variable central region (Rikihisa, 2011). These proteins can induce production of proinflammatory cytokines by the hosts peripheral blood leukocytes after the pathogen synthesizes it at the early stage of the infection. This was confirmed on prior studies done with a synthetic peptide (design to mimic p44-18). After inoculation on a horse, IgM antibody specific to this peptide was detected 4 days post inoculation, with a peak of titer on day 12. (Zhi, et al., 2002)

Other major surface proteins have been studied. Recently, MSP5 was utilized to create a serologic indirect ELISA diagnostic method to analyze IgG and IgM antibodies of *Anaplasma* spp. in animals from the Qinghai–Tibetan Plateau region. The overall positivity for *Anaplasma* IgG (14.6%) and IgM (7.9%) antibodies against the recombinant MSP5 ELISA was reported. This is an indicative of a threat of possible tick bites and zoonotic pathogen infection in the vicinity of human and animal activities in the tested areas. (Zhang, et al., 2022).

From these findings, it was concluded that the use of a broad range of genus and species-specific immunodominant peptides in diagnostic tests would perform more like IFA and would allow veterinarians to determine *Anaplasma* species early exposures in dogs and cats in their region.

One of the commercially available rapids tests for detecting antibodies to vector-transmitted is the SNAP® test, manufactured by IDEXX Laboratories, Inc. (Westbrook, Maine, USA). This diagnostic kit utilizes an ELISA-based platform with bidirectional fluid flow. (O'Connor, 2015). Reagent for antibody detection, primarily synthetic peptides of immunodominant proteins, are deposited on the solid surface of the device. The patient's sample is mixed with a liquid conjugate containing the same synthetic

peptides covalently coupled to horseradish peroxidase. Once mixed, the liquid is poured into the samples well of the device and it flows across the matrix and detection spots to an activation circle. When the fluid wave reaches this spot, the device is depressed allowing the reverse flow of wash buffer and enzyme substrate to an absorbent pad that has been brought in contact with the solid surface (matrix) of the device. This process allows the sample a second opportunity to interact with the detection reagents, clears the solid surface of residual blood products, and enables the precipitant substrate to interact with any bound HRPO-conjugate. Results are read at 8 minutes and the presence of any blue color as clearly defined spot is considered positive. Absence of color is interpreted as a negative result. While IDEXX has had several versions of this device available to veterinarians since the early 2000's, the current version (SNAP® 4Dx® Plus Test) is licensed for the detection of antibodies to *Borrelia burgdorferi, Ehrlichia canis, Ehrlichia ewingii, Anaplasma phagocytophilum, Anaplasma platys,* and detection of antigen from *Dirofilaria immitis* in dogs.

It is important to note that the liquid conjugate does not use an anti-species antibody, which means antibody detection is possible for variety of species. By using the specific synthetic peptides as a conjugate, the antibodies in the patient's sample are responsible for bridging the solid phase detection peptides with the conjugate peptide. It is not known if both IgM and IgG antibodies perform equally well in this bridging reaction. (Figure 1)

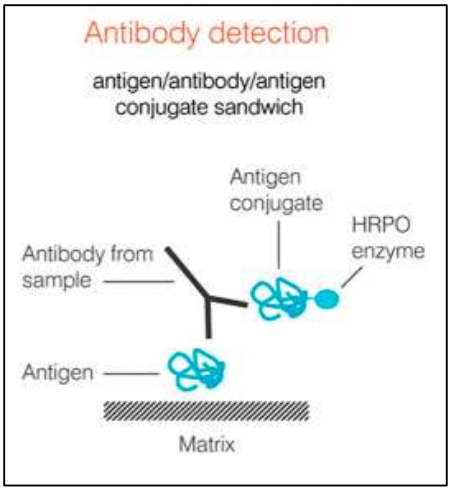


Figure 1. Immune complexes formed on the SNAP® flow matrix.

Following the antibody detection assay by utilizing an immobilized antigen and an antigen-HRPO conjugate. From "SNAP Assay Technology" by Thomas P. O'Connor, 2015, Topics in Companion Animal Medicine, Volume 30, Issue 4, December 2015, Pages 132-138. Copyright 2015 by Elsevier Ltd.

In addition to the commercial product, IDEXX Laboratories, Inc. also developed a SNAP® device for research use only, SNAP® M-A, to broadly detect multiple species of *Anaplasma* and *Ehrlichia* antibodies on canines. This was used as a tool to characterize regional trends in seroprevalence to specific vector-borne pathogens in dogs. (Hegarty, et al., 2015; O'Connor, et al., 2005) This assay was developed as a canine assay. Like the commercial product, this SNAP® assay does not use a host species-specific conjugate and can be used on a research basis to screen other mammalian species. (Lappin, et al., 2015; Stillman, et al., 2014). Results from studies using the research SNAP® M-A suggested that the two different peptides from the same immunodominant P44 protein of *A. phagocytophilum* had different detection capabilities with one peptide demonstrating earlier antibody detection post-infection than the other in both cats and dogs.

(Chandrashekar, et al., 2017).

A second-generation of SNAP® 4Dx® Plus Test was released in 2022 which has been shown to improve early detection of anaplasmosis relative to the first generation of SNAP® 4Dx® Plus Test in cats and dogs with positive *Anaplasma* PCR results or to the point of clinically apparent infection. (Beall, 2023). While the early detection is enhanced with the addition of the second P44 peptide, it is not known if this enhances diagnostic detection is due to the preferential recognition of IgM versus IgG with the inclusion of specific peptide targets.

The second-generation test utilizes *Anaplasma* genus EENZ1/APH-1 peptides to broadly detect *Anaplasma* spp. antibodies, (it detects specific and cross-reactive antibodies against *A. phagocytophilum* and *A. platys*) and a *A. phagocytophilum* p44-4 (APH-4) peptide as the species-specific and early detection peptide. Theses peptides were previously utilized in the SNAP® M-A research done in 2015. (Hegarty, et all., 2015; O'Connor, et al., 2005). Extensive testing has been done on these species-specific peptides (Qurollo, et al., 2020; Beall, et al., 2023; Lappin, et al., 2015; Stillman, et al., 2014) and as a result, these ELISAs have enhanced specificity compared against whole organism-based IFA and rarely cross-react with antibodies against other *Anaplasma* spp. This study will focus on the role of IgM vs. IgG in facilitating earlier detection with APH-4 peptide. The IgM and IgG reactivity patterns may provide a better understanding of the immune response to acute anaplasmosis and the immunodominant targets.

This second-generation SNAP® of 4Dx® Plus Test is the improved point of care test that will benefit veterinarians in European countries in proactively testing for vector borne diseases instead of waiting to test when the animals are exhibiting clinical signs.

This improved point of care ELISA provides a fast, accurate and user-friendly method to screen cats and dogs for vector-borne disease exposure and infection. And from a One Health perspective, modifying their current testing practices will generate large data sets that help to establish the seroprevalence of canine tick-borne infections. This study will be part of an educational campaign to promote tick-borne screening and regular application of tick preventative to drive awareness of locally relevant tick-borne diseases. (CDC; Skotarczak, 2018).

Chapter II.

Research Materials and Methods

The following section details the materials and methods used throughout the study. This research does not involve human subjects. There is no interaction or intervention with living individuals. Neither the provider of the specimens and data, nor the recipient, can link these specimens and data with identifiable individuals.

Samples

Samples utilized were from cats and dogs and testing performed was developed on animal samples only. All procedures for the use of animals to generate specific antisera were approved by an internal animal welfare committee and in accordance with the IDEXX Animal Welfare Policy.

Two canine and two feline *A. phagocytophilum* sera-positive and negative by IFA and PCR were utilized as controls.

Serum samples from eight beagle dogs that were experimentally infected with wild-caught adult *Ixodes scapularis* from Rhode Island, an endemic region of the USA, were utilized to assess the sera-reactivity to *A. phagocytophilum* of dogs on peptide targets of suspected 'early' (APH-4) IgM response and 'late' (APH-1) IgG immune response. These dogs were previously infected with a protocol approved by the Institutional Animal Care and Use Committee by the research facility (# 187.004) on 8 January 2012. (Chandrashekar, 2017). Prior tick exposure, all eight dogs tested negative

for active or previous *A. phagocytophilum* infection by PCR and serology. Tick infestation occurred from day 0 to day 7 where a total of 25 ticks were used (13 females and 12 males). Canine vector borne disease clinical signs were monitored daily. Serum samples taken at day 0, prior tick placement, and days 3, 7, 10, 14, 17, 21, 24, 28, 35, 42, 49, 56, 63, 70, 77, and 84 post tick placement and removal, in graphing these samples will be referred as day-post infection (DPI). These aliquots were stored at -20°C until assessed in the serologic assays. From a previous study (Chandrashekar, et al., 2017) it was determined all eight dogs were PCR positive for *A. phagocythophilum* DNA by day seven.

Remnant field samples were obtained from the IDEXX Laboratories, Inc Reference Laboratory Network following completion of all testing and prior to discard. According to the diagnostic service terms, samples that have completed all testing and are being discarded may become the property of the service provider. Both canine and feline serum samples were obtained from laboratories in the following countries: United States, Germany, Spain, and the United Kingdom. Samples were tracked by laboratory accession number and had no patient or owner identification information associated with them other than species and originating country location.

Measurement of IgM vs. IgG reactivity to *A. phagocytophilum* P44

To assess the seroreactivity in experimentally infected dogs with PCR- positive results for *A. phagocytophilum* on p44 peptide targets of 'early' (APH-4) and 'late' (EENZ1/APH-1) immune response, an optimized indirect ELISA microtiter plate was utilized. *Anaplasma* specific IgM and IgG response were determined by P44 recombinant protein-based ELISA (rP44). These rP44 results serve as a guide for the epitope mapping

using a defined set of peptides. When appropriate, samples were blinded and randomized. The polypeptide/antibody complex formed helped determine the proper combination of conjugate and peptide concentration after determining the optical density at 650nm. A cutoff value was determined from the mean optical density plus 3 standard deviations.

Each of the outer-surface membrane (P44) associated peptides were first dissolved in Dimethyl sulfoxide (Invitrogen™) then individually diluted to 0.25 µg/mL for the APH-4 peptide and 0.5 µg/mL for the EENZ1 peptide with 0.05M sodium carbonate buffer (NaCO3), pH 9.6 and 100 µL were coated overnight on separate labeled 96-well microtiter Immulon 4HBX plates ELISA (Thermo Scientific™). Similarly, the recombinant *A. phagocytophilum* p44 (rP44) protein (Diarect AG, Germany) was diluted to 0.5 µg/mL with 0.05M NaCO3 (pH 9.6) and used as a confirmatory orthogonal confirmatory test method, 100 µL per well coated plate. All three sets of plates were washed with phosphate buffered saline solution containing Tween-20 and blocked utilizing 300 µl/well of 0.1M Tris buffered solution (pH 7.4) containing Tween-20 for 2 hours at room temperature. Plates were dried and stored desiccated in heat sealed Mylar bags at 2-7°C.

Canine experimentally infected samples and control samples were diluted in duplicate to 1:100 in sample dilution buffer with detergent and blocking protein. 100 µl/well were added to each coated plate type and incubated for 30 minutes at room temperature. Plates were then washed with phosphate buffered saline solution containing Tween-20. Peroxidase-conjugated AffiniPure Rabbit Anti-Dog IgG Heavy and Light chain (H&L) purchased from Jackson Immuno Research Laboratories Inc. and Goat Anti-

Dog IgM Heavy and Light (H&L) Peroxidase-conjugated (Abcam) were utilized as the conjugates. Each conjugate was diluted to a previously determined optimal concentration for each peptide and recombinant coated plates. On the APH-4 and EENZ1 peptide plates the IgG conjugate was diluted to 1:4000 and the IgM conjugate was diluted to 1:2000. And on the recombinant p44 plates, the IgG conjugate was diluted to 1:2000 and the IgM conjugate diluted to 1:1000 in conjugate dilution buffer with detergent and blocking protein. 100 µl per well of the corresponding conjugate was added to each plate and incubated for 30 min at room temperature, then washed with PBS based buffer with Tween-20. Followed with 50 µl/ per well of 3,3´,5,5´-tetramethylbenzidine (TMB) substrate solution and incubated at room temperature for 10 minutes, then stopped with 50 µl/ per well of stop solution buffer. Optical density (OD) was measured at 650 nm. For the rP44 plate, EENZ1 and APH-4, a cutoff value of 0.497, 0.272 and 0.188, respectively, was confirmed from the OD plus 3 standard deviations of previously tested canine negative population. (Beall, et al., 2022)

Capture of *A. phagocytophilum* specific canine IgM and IgG antibodies in infected dogs

This study was designed to see if it was possible to enhance detection of *A. phagocytophilum* specific IgM on the APH-4 peptide plate or recombinant p44 plates by depleting IgG from the serum sample prior to incubation. The concern was that IgG would quickly out-compete IgM capture lowering the resulting signal. A Protein A/G Magnetic Beads (Pierce ™) product was selected due to its convenience of IgG binding domains of both Protein A and Protein G on one bead and could also be used on feline and canine samples. Out of the eight experimentally infected dogs, two were selected;

one each that had the highest and lowest IgM response on the APH-4 peptide plate. Two negative canine samples served as controls.

The Pierce Protein A/G Magnetic Bead Protocol for Antibody purification from serum was followed as indicated by the manufacturer. Six sets of 50 µl of beads were washed with 150 µL of the provided Binding/Wash buffer and gently vortexed to mix (Four samples and two controls). A magnetic stand was used to collect the beads and the wash step was repeated. The samples were diluted 1:100 with Binding/Wash Buffer (Protocol deviation to match ELISA sample dilution). Then gently mixed for 1 hour with the washed beads at room temperature. A magnetic stand was then used to separate the beads from the supernatant. It was suspected the supernatant should contain the sample's unbound IgM. This supernatant was the used as the sample for the ELISAs described on this section. (Figure 2)

Secondly an IgM and IgG capture assay was designed to improve detection of IgM and IgG specific reactivity for *A. phagocytophilum* targets. But first, to avoid depleting serum of IgG, it was important to demonstrate an effective way of capturing IgM independent of specific *A. phagocytophilum* reactivity. To test this, Goat anti-Dog IgM H&L (Abcam) and for IgG, Affini Pure Rabbit Anti-Dog IgG H&L (Jackson Immuno Research Laboratories Inc) were diluted to 1 and 0.5 µg/mL with 0.05M Sodium Carbonate buffer (pH 9.6) and 100 µL were coated overnight with similar protocol as the EENZ1, APH-4 peptide and recombinant p44 plates.

Selected canine Protein A/G treated samples and the neat samples and controls were added or diluted in duplicate to 1:100 in sample dilution buffer with detergent and blocking protein. A total volume of 100 µl/well was added to each coated plate type and

incubated for 30 minutes at room temperature. The same ELISA protocol was followed as the EENZ1, APH-4 peptide and recombinant p44 plates.

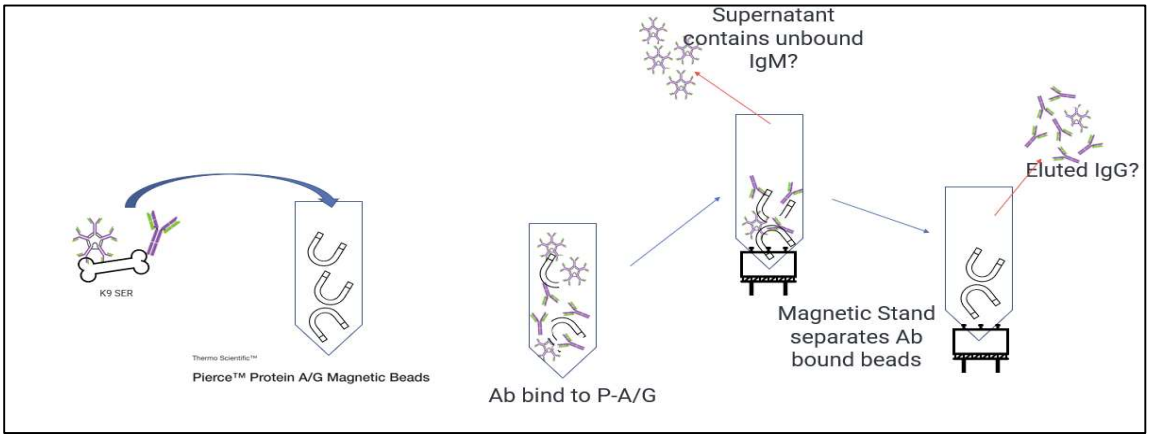


Figure 2. Modified Pierce Protein A/G Magnetic Bead Protocol

Utilized for the Antibody purification from serum.

In conjunction, a Crosslink Magnetic IP/Cp-IP kit ((Pierce ™) was utilized to further explore IgG or IgM sample depletion on both coated peptide plates. From the manual, A. Binding of Antibody to Protein A/G Magnetic Beads and B. Crosslinking the Bound Antibody protocol was followed. Goat anti-Dog IgM (Abcam), Rabbit IgG, Dog IgG and Rabbit anti-Dog IgG were purchased from Jackson Immuno Research Laboratories Inc. were crosslinked into properly labeled Protein A/G Magnetic beads. Similar ELISA protocol was followed as the EENZ1, APH-4 peptide plates. (Figure 3)

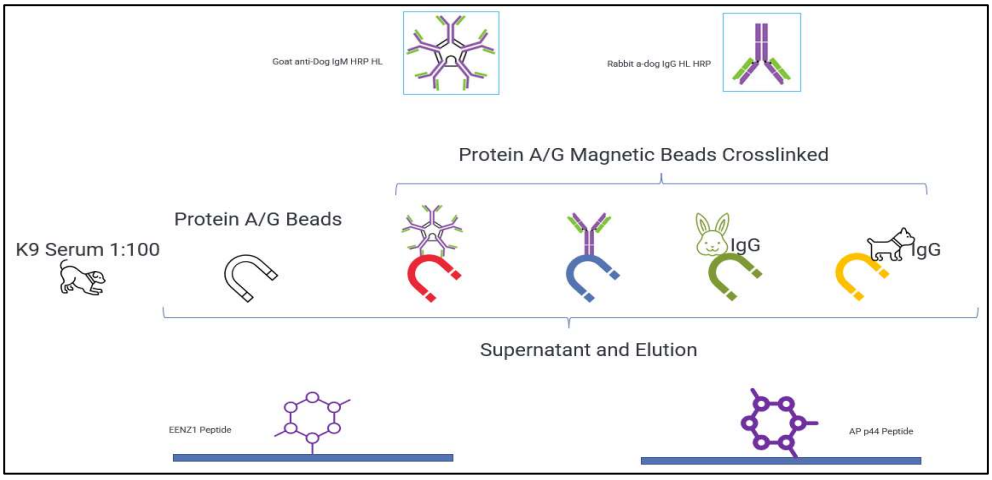


Figure 3. Modified Crosslink Magnetic IP/Cp-IP kit protocol layout.

Including the coated peptide and selected conjugate utilized for this experiment.

SNAP® detection of target-specific IgM vs. IgG

The second-generation of SNAP® 4Dx® Plus Test utilizes the same reagents and same peptides as the above-described ELISA plates. *A. phagocytophilum* EENZ1/APH-1 and APH-4 peptides. Both are derived from the immunodominant p44 protein of *A. phagocytophilum.* SNAP® 4Dx® Plus Test incorporates a bidirectional chromatographic flow of sample-conjugate mix and automatic sequential flow of wash solution and enzyme substrate amplification format that produces visible blue spots when antibody is present. These kits are currently commercially available and were utilized to determine if the SNAP is sufficient in detecting IgM and IgG based on antibody bridging. Samples that had an IgM only time point from the experimentally infected dogs were selected.

Individual EENZ1 and APH-4 conjugates were diluted to 0.75 µg/mL in conjugate dilution buffer with detergent and blocking protein. Included SNAP® 4Dx® Plus Test conjugate was also utilized as a control. IDEXX developed quality control

panels were utilized to confirm conjugates were performing according to previously established quality control standards.

Kit components were equilibrated to room temperature for 30 minutes before testing. Three drops of sample were dispensed utilizing the kit's transfer pipette. Four drops of conjugate were added to the sample tube and mixed as recommended in the kit's instructions. Sample-Conjugate mix was added to the Sample well and the device was activated when the mix appeared in the activation circle (Figure 4a). Results were read at 8 minutes, and to compensate the risk of lower seroreactivity, longer read times were also performed at 10- and 15-minutes post-activation. A positive result was described as the Positive control present, and the *A. phagocytophilum/A. platys* spot with a clear blue color development in the Result Window (Figure 4b).

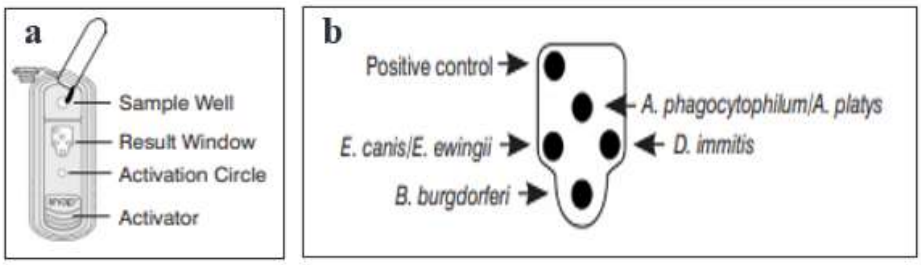


Figure 4. SNAP® Protocol schematic.

(a) SNAP® assay device diagram. (b) Results spot location, for this study the positive control and A. phagocytophilum/A. platys spot will be observed. From Heartworm Antigen-Anaplasma-Borrelia Burgdorferi-Ehrlichia Antibody Test Kit Insert. © 2022 IDEXX Laboratories, Inc. All rights reserved.

Follow up testing was performed on forty-four canine and twenty-nine feline PCR positive and negative serum samples for *Anaplasma phagocytophilum* submitted to IDEXX Reference laboratories in Germany and USA. These samples were utilized for the SNAP® detection of target-specific IgM vs. IgG by using individual EENZ1 and APH-4 conjugates diluted to 0.75 µg/mL. Each conjugate was blinded (A and B) and samples were aliquoted in duplicates. A blinded and randomized testing order was followed.

Serosurvey for European Canine and Feline samples

Two hundred and thirty feline and two hundred and nineteen canine serum samples submitted to IDEXX Reference laboratories in Germany, Spain and United Kingdom for a General Chemistry Panel testing were utilized for the Serosurvey Study. Samples arrived frozen and were properly transferred to sample storage tubes, and a new identification number was assigned for blinded and randomized testing.

These samples were used for the serology testing of anaplasmosis in dogs and cats using enhanced IDEXX ELISA diagnostic technology to provide an up-to-date geographical distribution of *A. phagocytophilum* in Europe.

Individual EENZ1 and APH-4 conjugates were diluted to 0.75 µg/mL in conjugate dilution buffer with detergent and blocking protein. IDEXX developed panels were utilized to confirm conjugates were made accordingly. Samples were aliquoted in duplicate. Conjugates were blinded and a randomized testing order was followed. SNAP® 4Dx® Plus protocol was followed as previously described, and results were read at 8 minutes.

Chapter III.

Results

The following section details the results of this study.

Investigation of IgM vs. IgG reactivity to *Anaplasma phagocytophilum*

The first objective of this study was to assess the seroreactivity of experimentally infected dogs to the APH-4 peptide targets, a presumed early detector, and EENZ1/APH-1 as the late detector of immune response. The second objective was to determine if IgM and IgG reactivity patterns provide a better understanding of the immune response to the immunodominant targets during acute anaplasmosis. A summary of the seroreactivity of the experimentally infected dogs can be found in Table 1.

On the APH-4 peptide ELISA plates four dogs had a stronger IgM response based on the ELISA's Average Optical Density at 650nm (OD values) vs. day drawn post infection (DPI). Three dogs had a higher IgG response, and one had a consistent response for both IgM and IgG. Results for the APH-4 peptide plates were grouped by similar immune response. Dogs A0110, A0510, A0710, and TIRO had an earlier stronger IgM reaction, for the first three dogs, a higher IgM signal was observed by day 10, and for dog TIRO on day 17 (Figures 8, 9, 11, and 14). IgG had a positive signal by day 14 for the first 3 and for dog TIRO by day 21. IgM signal peaks on day 14 for Dogs A0510, A0710 and A0110. And for dog TIRO, IgM peaks on day twenty-eight. Dog A0110 has the weakest IgM signal on the APH-4 peptide plate for this group (Figure 5). In this group of dogs, IgG signal peaks followed or coincided with IgM signal peak and were more variable with respect to DPI, A0510 IgG response peaks on day 17, Dog A0710

IgG response peaks on day 21, dog A0110 IgG signal surpasses IgM on day 17 and response peaks on day 24, and dog TIRO peaks on day 28.

Dogs A0610, THRO and SLRO were grouped as having higher IgG response in comparison to IgM at the time when IgM showed as a positive signal. Dog A0610 and THRO had a stronger IgM positive signal on day seventeen, for dog A0610 both IgG and IgM peaked on day twenty-one and dog THRO peaked for both IgG and IgM on day 24. (Figures 6 and 13). Dog SLRO had no increase in signal until day twenty-eight, IgG had a stronger signal than IgM with a lower average OD650 response than the other dogs and a dip on both signals on day 35 (Figure 7). Dog SQQ0 was the only dog that had a consistent response for both IgM and IgG response on the APH-4 peptide plates on day seventeen. IgG response peaked earlier on day twenty-one and on day 24 for IgM (Figure 12).

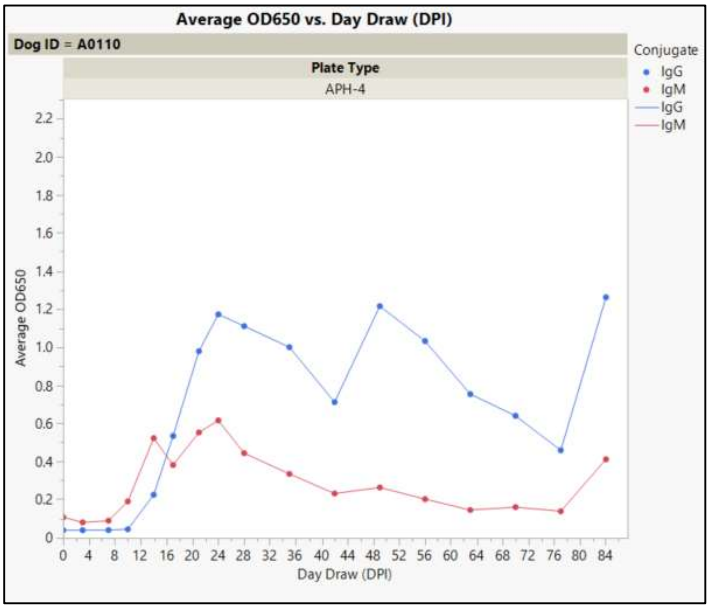


Figure 5. APH-4 ELISA plate results for experimentally infected dog A0110

Dog A0110 has the weakest IgM signal on the APH-4 peptide plate for the group of dogs with the earlier IgM signal.

On the EENZ1 peptide ELISA plates, IgG had the stronger overall response, based on the ELISA's Average Optical Density at 650nm (OD values) vs. day drawn post infection (DPI). The IgM response was earlier in five dogs but with a lower response signal than IgG. Three dogs had an earlier IgG positive signal response than IgM. Results for the EENZ1 peptide plates were grouped by similar immune response. Dogs A0510, A0710, THRO, TIRO and SQQ0 had an earlier IgM positive response on day 7, 10, 14, 17 respectively (Figures, 9, 11, 13, 14 and 12). IgG positive response was observed on day 14 for A0510 and A0710, day 17 for THRO, on day 21 for TIRO and SQQ0. IgM signal peaked on day fourteen for A0710, on day twenty-one for A0510, THRO, and for dog TIRO plateau, and on day 24 for dog SQQ0. IgG signal peaked on day twenty-eight for dogs A0510, A0710, THRO and TIRO, and SQQ0 reached a plateau on day twenty-four. Dogs A0110, A0610 and SLRO had earlier IgG positive responses compared to IgM. For dog A0110 IgG was positive on day ten, A0610 was positive on day twenty-one and SLRO on Day 28 (Figure 8, 10 and 7). Dog A0110 had a higher background signal for IgM, signal peaked on day fourteen and stayed consistent on the remaining DPI. IgM peaked on day twenty-one for dog A0610 and on day twenty-eight for dog SLRO. SLRO had the lowest IgM reactivity of all dogs on the EENZ1 peptide plates (Figure 7).

In seven of the eight experimentally infected dogs, an early strong IgM response was observed on the recombinant *A. phagocytophilum* p44 (rP44) protein ELISA based on the Average Optical Density at 650nm (OD values) vs. day draw post infection (DPI).

Dogs A0510, A0710, A0610 and A0110 had a similar IgM response with high positive IgM signal on Day 10 (Figures 9, 11, 10 and 8). Positive IgG signal was detected on day fourteen for A0510, A0710 and A0110, and on day seventeen for A0610. IgM signal peaked on day fourteen for A0510 and A0710, for A0610, and at day twenty-four for dog A0110. IgG signal reached a maximum plateau on day twenty-one for dogs A0510, A0610 and A0110, and on day twenty-eight for A0710. Dogs THRO and SQQ0 had a high positive IgM signal on Day 14, followed by a positive IgG signal on day 17 (Figures 13 and 12). IgM signal peaked at day fourteen for THRO and at day twenty-four for SQQ0. IgG signal reached a plateau on day 21for both dogs. Dog TIRO had a slower reaction to IgM and IgG, on day seventeen the positive signal was stronger for IgM and a lower signal for IgG. IgM signal peaked on day twenty-four and reached a plateau on day twenty-eight for IgG (Figure 6). Dog SLRO was the only dog that had a stronger IgG response before IgM, observed on day fourteen. IgG signal reached a plateau on day thirty-five and IgM signal peaked on the same day (Figure 7).

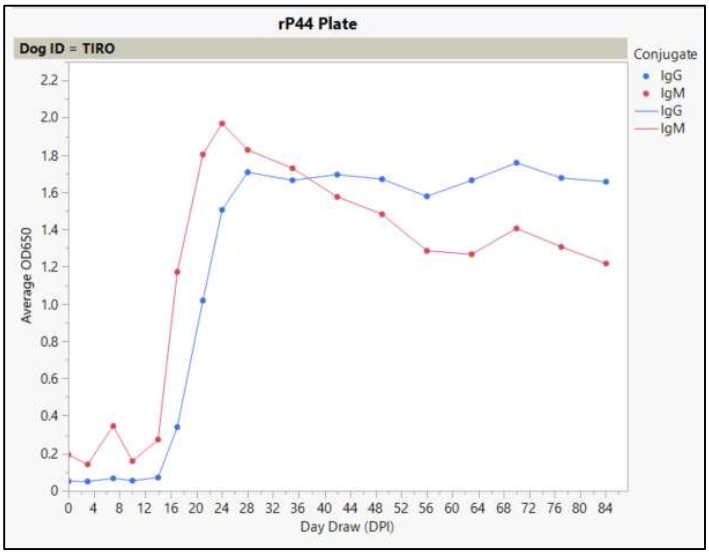


Figure 6. Recombinant A. phagocytophilum p44 (rP44) protein ELISA results for dog TIRO

This shows slower reaction to IgM and IgG, by day 17 the positive signal was stronger for IgM and a lower signal for IgG. IgM signal peaked on day 24 and reached a plateau on day 28 for IgG.

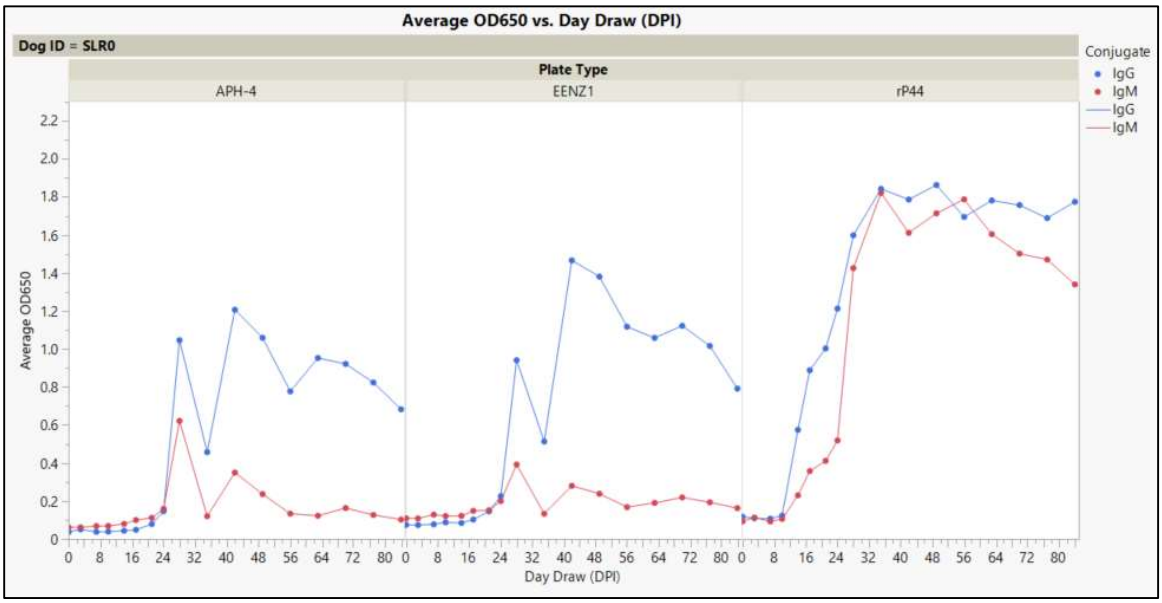


Figure 7. All three ELISA plate results for experimentally infected dog SLRO

IgM is typically the first antibody produced in response to a newly recognized foreign microorganism, and levels can be elevated even when no disease is present. However, the humoral immune response to the P44 *A. phagocytophilum* immunodominant protein, regardless of the specific target (APH-4, EENZ1, or recombinant), was not uniform among this group of experimentally infected, beagle dogs. (Table1). This made it difficult to confirm that APH-4 peptide was acting as a consistent and reliable early target of the IgM immune response. The rP44 protein, with its multiple

antigenic targets, demonstrated more consistent early detection of the IgM immune response in this population of dogs.

Table 1. Seroreactivity of Experimentally infected dogs

Dog	ELISA APH-4 Antibody	DPI	EENZ1 Antibody	DPI	rP44 Antibody	DPI
A0110	IgM *	10	IgG	10	IgM	10
A0510	IgM	10	IgM	7	IgM	10
A0610	IgG	17	IgG	21	IgM	10
A0710	IgM	10	IgM	10	IgM	10
SQQ0	IgM/IgG	17	IgM	17	IgM	14
TIRO	IgM	17	IgM	17	IgM*	17
SLRO	IgG*	28	IgG*	28	IgG*	14
THRO	IgG	17	IgM	14	IgM	14

*Note. * Indicate results that are described in Figures 5,6 and 7.*

Capture of *A. phagocytophilum* specific canine IgM and IgG antibodies in infected dogs

The focus of this study was to see if it was possible to enhance detection of *A. phagocytophilum* specific IgM on the APH-4 peptide plate or recombinant p44 plates by depleting IgG from the serum sample prior testing in the assay. From the initial set of experiments, dog A0610 and, THRO revealed stronger IgG responses to the immunodominant peptide targets despite having an early IgM response to the rP44 immunodominant protein. It is possible that the specific IgG response was outcompeting the IgM capture and resulting signal.

Out of the eight experimentally infected dogs, two were selected to explore method development; one each that had the highest (Dog A0510 on day 14) and lowest IgM response (Dog SLRO on Day 28) on the APH-4 peptide plate along with two negative controls. Pierce Protein A/G Magnetic Bead treated and untreated samples were

tested on both peptide plates with a Goat anti-Dog IgM H&L conjugate antisera to determine if IgG depletion improved IgM detection (Figure 2).

These results were compared to the untreated diluted samples tested on both peptide ELISA plates with the Affini Pure Rabbit Anti-Dog IgG Heavy and Light conjugated antisera (Figure 15). Figure 15 (a) depicts the results for the Goat anti-dog IgM H&L conjugate while Figure 15 (b) shows the results for the untreated samples with the Rabbit anti-dog IgG H&L conjugate for comparison. No difference in Average OD650 between the treated (Pierce Protein A/G Magnetic Bead) and untreated samples on the peptide ELISA plates was observed indicating that the attempt to deplete IgG from the sample was not effective at enhancing IgM signal drawing into question the usefulness of the method or specificity of the Pierce Protein A/G Magnetic Bead for immunoglobulin depletion. One of the negative samples, NA25505, had a spike in signal on the EENZ1-IgM conjugate combination which was unexpected and not evaluated further.

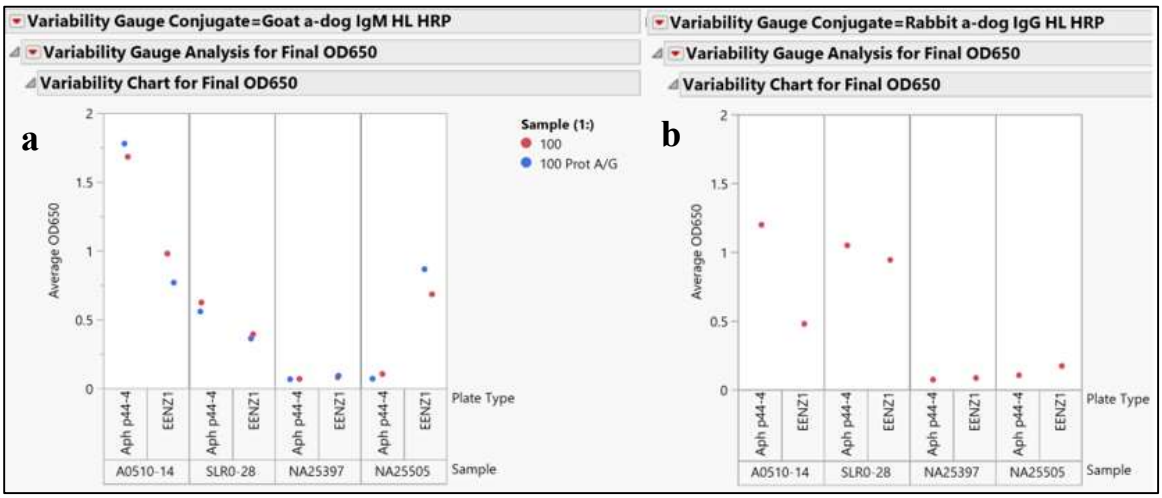


Figure 15. Capture of A. phagocytophilum specific canine IgM and IgG antibodies in infected dogs A0510 (DPI 14) and SLR0 (DPI 28).

(a) Results for the Goat anti-dog IgM H&L conjugate, (b) results for the untreated samples with the Rabbit anti-dog IgG H&L conjugate.

The next experiment was designed to demonstrate if it was possible to capture IgM independent of specific *A. phagocytophilum* reactivity, determine what is binding to the Protein A/G beads and demonstrate a differentiation between IgM vs. IgG circulating in the sample with specific conjugates. Unfortunately, there was very little difference between the diluted samples, which served as controls, and those treated with the Protein A/G Magnetic Beads, either as bound and eluted or as supernatant. (Figure 16). The average OD650 from the ELISA plates was higher on the Goat anti-Dog IgM coated plate (a) when performing the assay with the Goat a-dog IgM H&L conjugate, confirming the assay detects the presence of IgM in the samples. In comparison, lower signal was observed when the reaction was carried with the Rabbit a-Dog IgG H&L conjugate, indicating the assay still detects IgG present in the sample. Likewise, the IgM H&L conjugate detected antibody present in the anti-dog IgG capture ELISA (b). Together, the data suggested that this method was unlikely to be useful for further investigations as it did not demonstrate sufficient differentiation between the sample treatment groups. (Figure 16).

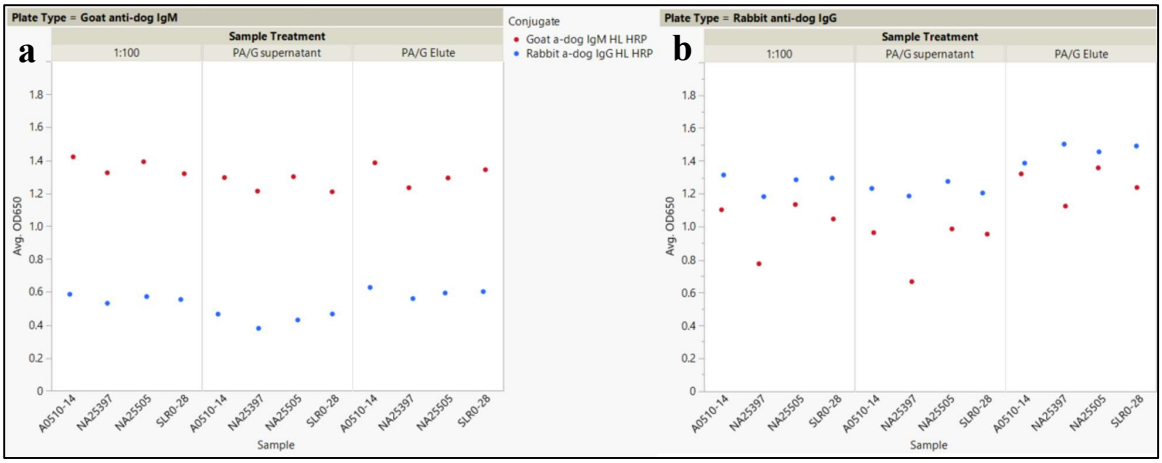


Figure 16. Capture of IgM present in the diluted, Protein A/G supernatant and eluting serum sample of experimentally infected dogs A0510 (DPI 14) and SLR0 (DPI 28).

a) Results with the Goat anti-dog IgM H&L conjugate, (b) results for the untreated samples with the Rabbit anti-dog IgG H&L conjugate.

The previous Protein A/G treated samples were tested on the APH-4 and EENZ1 peptide plates to further explore the idea that enriching IgM in the sample could improve detection of IgM-specific reactivity for *A. phagocytophilum.* The format of these peptide ELISAs used a peptide conjugate which matched the peptides coated on the ELISA plates. Although this represents the same ELISA assay format of the SNAP® 4Dx® Plus Test, as far as reagents, they have different reaction mechanisms. The ELISA plate is a two-step assay, in comparison SNAP® 4Dx® Plus Test, is a one-step assay. As a result, no specific signal was detected by the ELISA plate assay used in this experiment. (Figure 17).

Figure 17. ELISA peptide plate assay results of Protein A/G supernatant and eluting serum sample of experimentally infected dogs A0510 (DPI 14) and SLR0 (DPI 28).

a) Results of ELISA EENZ1 peptide plate with EENZ1 peptide conjugate, (b) results of the ELISA APH-4 peptide plate with APH-4 peptide conjugate.

The same positive samples were then run on the SNAP® 4Dx® Plus Test utilizing the same separate peptide conjugates utilized on the peptide plate. The two SNAP® devices on the left represent experimentally infected dog A0510 day 14 results for the Protein A/G supernatant. The EENZ1 peptide conjugate was utilized on the first SNAP® (1+E) and the APH-4 peptide conjugate on the second SNAP® device (1+A). The third and fourth SNAP® devices represent the results for dog SLRO on day 28 using the Protein A/G supernatant with the EENZ1 conjugate (2+E) or the APH-4 Conjugate (2+A) (Figure 18.). While both SNAP® 4Dx® Plus Tests with the EENZ1 conjugate produced a negative result, positive results were obtained with the APH-4 Conjugate for both samples. For these time points, both IgM (higher) and IgG were detected on the APH-4 peptide ELISA plate. Even though both, the plate ELISA and SNAP® assays

were performed with the same reagents they performed differently suggesting that they have different reaction mechanisms. Despite the use of Protein A/G supernatants, these two canine samples are probably very similar to the diluted, native serum sample (based on the experiment above) and presumably reflect a higher level of reactivity, either IgM or IgG or both, to the Aph-4 peptide at the respective time points. (Figures 7 and 9).

Figure 18. Images of SNAP® 4Dx® Plus Tests. Results for dog A0510 (DPI 14) and SLR0 (DPI 28).

1+E and 1+A are result for A0510 DPI 14 and 2+E and 2+A are results for dog SLRO DPI 28.

Crosslink Magnetic IP/Cp-IP kit Goat anti-Dog IgM, Rabbit anti-Dog IgG, Rabbit IgG, and Dog IgG were crosslinked into properly labeled Protein A/G Magnetic beads. (Figure 3) Dog A0510 day 14 sample, and one of the negative samples were incubated with each of the Crosslinked Magnetic Beads.

Results on Figure 19a represents how the samples interacted on the APH-4 peptide plate with Goat anti-dog IGM H&L conjugate in red, and Rabbit anti-Dog IgG H&L conjugate on blue. Figure 19b are the results for the interaction on the EENZ1 coated peptide plates (Figure 19). First section on each graph are the Protein A/G magnetic treated beads followed by the crosslinked beads results. The supernatant sample represents the unbound material (IgG or IgM) and the Elution represent what was previously bound to the beads and then eluted per the protocol (either IgG or IgM). On the APH-4 peptide plate, there is no significant difference between the Goat anti-Dog IgM, Rabbit anti-Dog IgG, and Rabbit IgG crosslinked beads in comparison with Protein A/G treated samples. There is a difference in signal between elution and supernatant sample, indicating binding of IgG or IgM does occur and that there is a reaction to the APH-4 peptide in the ELISA. The last crosslinked set on the right of the graph, represent the results of Protein A/G Crosslinked beads with Dog IgG where the Elution sample in this case does not show a reactivity on the APH-4 peptide plate, a potential indicator of non-specific *A. phagocytophilum* IgG depletion from the sample. The high levels of signal detected in all supernatant conditions from the experimentally infected dog, A0510, at day 14 demonstrate an excess of immunoglobulin reactive to APH-4 that is not being captured by the beads regardless of format. This is contrast to what was observed for this dog when performing a similar experiment with the EENZ1 ELISA Plate.

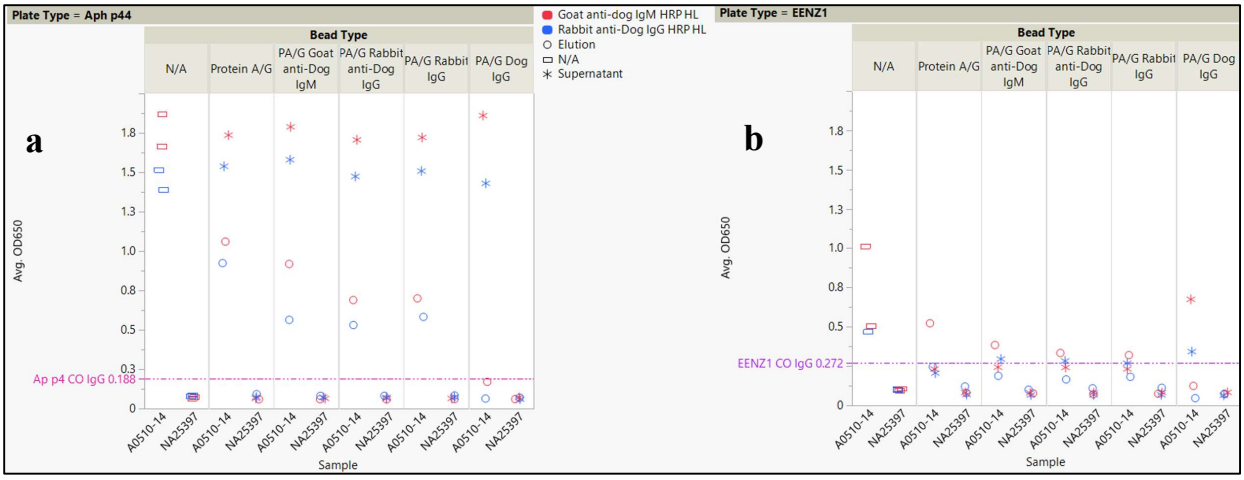


Figure 19. Result of the Crosslinking of Magnetic IP/Cp-IP kit of the treated supernatant and elution sample of the experimentally infected dog A0510 DPI 14.

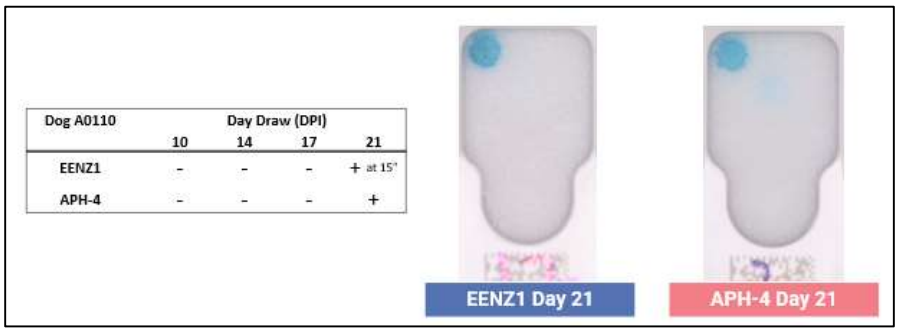
a) Results on the ELISA APH-4 peptide plate and b) EENZ1 peptide plate.

On the EENZ1 peptide plate the signal is much lower than on the APH-4 Peptide plate. In this case a depletion in signal is seen when compared to the non-treated sample. The best results appear to be with the Protein A/G beads, however that format provided challenges when evaluated previously. Concentration of specific antibody may be a critical factor for selecting the most appropriate method as antibody in excess of the beads absorptive capacity can results in poor performance. Likewise, lower signal in the ELISA with low concentration of antibody makes the interpretation of results more challenging as it is difficult to appreciate difference with the control included in this experiment. For example, the rabbit Ig looks similar to the rabbit anti-dog Ig. Between the supernatant and eluted sample, a potential depletion of non-specific *A. phagocytophilum* IgM and IgG is seen.

SNAP® detection of target-specific IgM vs. IgG

This experiment was designed to determine if the SNAP® is sufficient in detecting IgM and IgG based on antibody bridging. Day draw samples that had a positive IgM only time point from the experimentally infected dogs on the recombinant P44 ELISA plate were selected. Individual EENZ1 and APH-4 conjugates were utilized. Results were read at 8-, 10-, and 15-minutes post-activation minutes, to compensate the risk of lower seroreactivity. A positive result was described as the Positive control present, and the *A. phagocytophilum/A. platys* spot with a clear blue color development in the Result Window.

Out of the eight experimentally infected dogs, two had the same positive results on both peptide conjugates. For Dog A0110 day 21 was weak positive at 15-minute read time on the EENZ1 peptide conjugate and positive at 8-minute read time on APH-4 peptide conjugate (Figure 20). Dog A0510 day 17 was positive on the EENZ1 peptide conjugate and weak positive on the APH-4 peptide conjugate (Figure 21).



Dog A0110	Day Draw (DPI)			
	10	14	17	21
EENZ1	-	-	-	+ at 15'
APH-4	-	-	-	+

Figure 20. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog A0110.

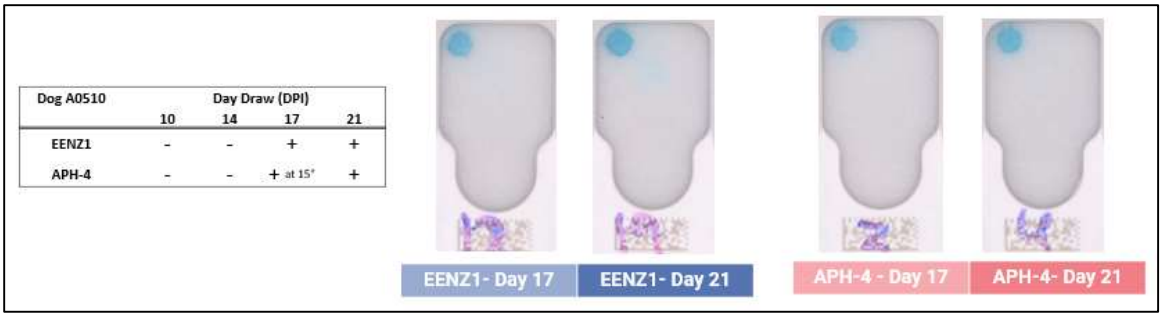


Figure 21. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog A0510.

The six remaining experimentally infected dogs had an earlier positive result on the APH-4 peptide conjugate. Dogs A0610 and TIRO were not positive on the selected time points on the EENZ1 peptide conjugate, but it was weak positive on day 17 at 8 minutes with the APH-4 peptide conjugate (Figures 22 and 23).

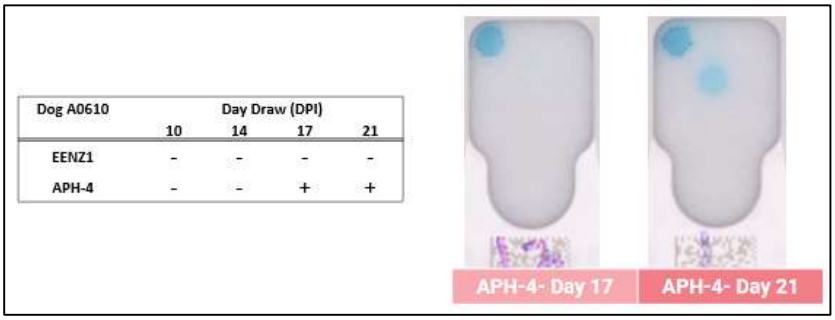


Figure 22. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog A0610.

Dog TIRO	Day Draw (DPI)			
	10	14	17	21
EENZ1	-	-	-	-
APH-4	-	-	+	+

Figure 23. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog TIRO.

Dogs A0710, THRO, SQQ0 were EENZ1 peptide conjugate positive on day 21. Weak positive for dog A0710, and positive on APH-4 peptide conjugate on day 14 for A0710 and THRO and day 17 for dog SQQ0 (Figures 24, 25, 26).

Dog A0710	Day Draw (DPI)			
	10	14	17	21
EENZ1	-	-	-	+
APH-4	-	+	+	+

Figure 24. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog A0710.

Figure 25. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog THRO.

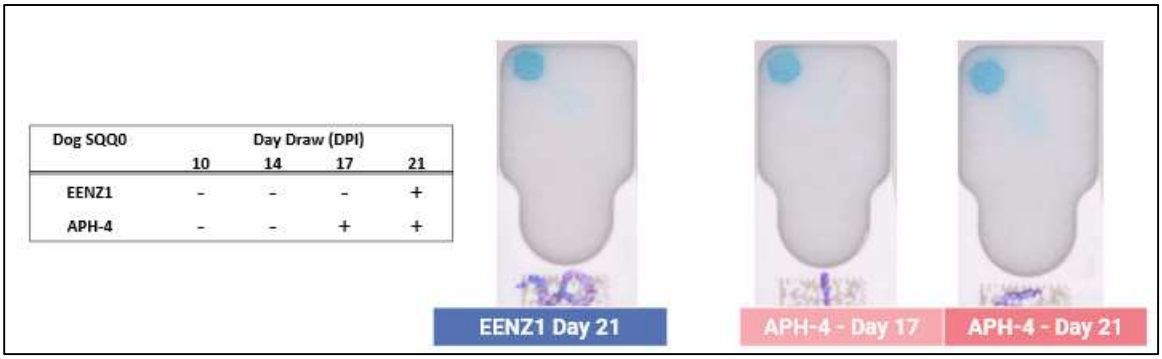


Figure 26. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog SQQ0.

Dog SLRO showed positive on the EENZ1 peptide conjugate on day 28 and weak positive on day 24 at 8 minutes on day 24. (Figure 27)

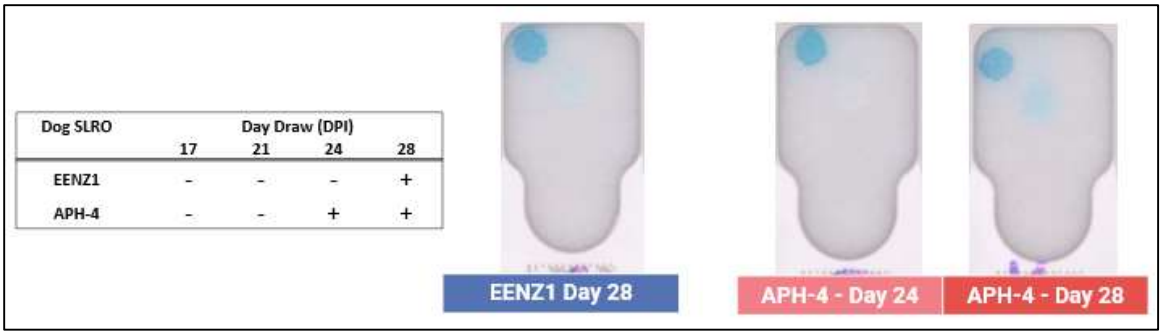


Figure 27. Images of SNAP® 4Dx® Plus Tests and visual results table for experimentally infected dog SLRO.

Results for dogs A0510 at day 14 and SLRO at DPI 28 differed slightly from the previous experiment using the Protein A/G supernatant. More specifically, A0510 did not test positive for antibodies to APH-4 at day 14, and SLOR did test positive for antibodies to EENZ1 at day 28. Possible reasons for these differences include samples type (Protein A/G supernatant vs. diluted serum), test-to-test variability (SLRO day 28 reactivity on EENZ1 is very weak positive), variation in conjugate materials (additional conjugate was made with a different batch of peptide). Overall, the results demonstrate the variation in the immune response to be immunodominant peptides of P44 in this group of experimentally infected dogs.

PCR Results

Follow up testing was performed on forty-four canine and twenty-nine feline samples with an *Anaplasma* spp. RealPCR Positive or Negative result that had been submitted to the IDEXX Reference laboratories in Germany and USA. A positive PCR result indicates that the DNA of *A. phagocytophilum* was detected in the sample. A Negative PCR results indicates that the DNA was not detected in the sample submitted,

the number of organisms may have been below the limit of detection, a decreased number of organisms may have occurred following treatment, or it was undetected due to strain variation. Out of the forty-four canine samples, twenty were *A. phagocytophilum* PCR Positive and twenty-four were PCR Negative. Out of the twenty PCR positives, eight samples were also positive on both peptide conjugates. One additional sample was positive on the EENZ1 only conjugate and a different sample was positive on the APH-4 peptide conjugate. Out of the negative PCR samples, one sample was positive on both peptide conjugates and two additional samples were positive on the APH-4 peptide conjugate only (Table 2 a).

Table 2a. SNAP® 4Dx® Plus Tests results of canine PCR positive and negative samples.

A. phagocytophilum PCR		Peptide Conjugate			
		EENZ1 +	APH-4 +	EENZ1/APH-4 +	Negative
Positive	20	1	1	8	10
Negative	24	0	2	1	21

Out of the twenty-nine feline samples, sixteen were *A. phagocytophilum* PCR Positive and thirteen were PCR Negative. From the sixteen PCR positives, one sample was also positive on both peptide conjugates, and three different samples were positive on the APH-4 peptide conjugate. Out of the negative PCR samples, one sample was positive on the APH-4 peptide conjugate only (Table 2b).

Table 2b. SNAP® 4Dx® Plus Tests results of feline PCR positive and negative samples.

A. phagocytophilum PCR		Peptide Conjugate			
		EENZ1 +	APH-4 +	EENZ1/APH-4 +	Negative
Positive	16	0	3	1	12
Negative	13	0	1	0	12

While these samples from cats and dogs had seroreactivity to the two immunodominant peptides of *A. phagocytophilum* P44, the APH-4 peptide immunoassay detected more patients that were being tested for a vector-borne infection by PCR. This test is typically used by veterinarians for acutely ill patients or as follow-up to assess the response of treatment,

Serosurvey for European Canine and Feline samples

Canine and feline samples from across Europe were evaluated with the two immunoassay formats to assess the frequency of positive results in the pet-owned population, From the two hundred and nineteen canine samples, seven samples were positive on the individual EENZ1 peptide conjugate and twenty-one on the APH-4 peptide conjugate. Forty-five were positive on both peptide conjugates. And from the two hundred and thirty feline samples only eight were positive with the individual APH-4 peptide conjugate. The remaining canine and feline samples were negative (Table 3). Results by country are available in Table 4 and 5.

Table 3. Serosurvey results for European canine and feline samples.

	POSITIVE			NEGATIVE	TOTAL
	EENZ1	APH-4	EENZ1/APH-4		
Canine	7	21	45	146	219
Feline	-	8	-	222	230

Results Summary

It is difficult to characterize IgM and IgG immune response to immunodominant peptides even in experimentally infected dogs, though several different enrichment strategies for class-specific immunoglobulin were attempted. Results suggest that there is a difference in how or when infected dogs recognize these two immunodominant peptides. Finally, field samples reveal importance of including both peptides for broadest detection of current or previous infection.

Chapter IV.

Discussion

This study showed that the humoral immune response to *A. phagocytophilum* regardless of the immunodominant target utilized in the assay, was not uniform among the group of experimentally infected beagle dogs. This indicated that it would be very unlikely to see a consistent pattern to the immune response to these immunodominant targets in the field population of dogs. Based on the literature, immune responses to the pathogen can be variable. During persistent infection there is reduction in immune responses, perhaps due to the immune evasion. For example, *A. phagocytophilum* infection causes immune suppression by reducing T-cell population in sheep and cattle and inhibits the phagosome-lysosome fusion to delay the apoptotic death of neutrophils (Woldehiwet, 2008). The infection stimulates quick short-lived production of plasma cells that stay within the spleen instead of long-lived plasma cells that migrate to the bone marrow (Zhuang, et al. 2007).

The lack of proper antigen or T-cell stimulation of response mature IgM may reduce isotype switching and not all vector-transmitted pathogens elicit a measurable IgM response prior to IgG response, for example Leishmania (Rodriguez-Cortes, et al. 2007). This demonstrates the potential for variability in IgM antibody levels and measurement. In field situations, ticks may contain other organisms that can be transmitted, like *Borrelia burgdorferi,* causing a mounted immune response to these other pathogens and this could influence *A. phagocytophilum* immune response (Thomas, et al. 2001; Holden, et al. 2005). Any previous exposure to similar antigenic epitopes could trigger a cross-reactive memory response, more likely in field cases where conserved

proteins, like heat shock proteins, contain shared epitopes across pathogens like *Borrelia* and *Anaplasma* (Bunnell, et al. 1999).

Regardless, determining if the APH-4 peptide was as an earlier detector of IgM turned out to be more complicated than anticipated. Some of the experimentally infected dogs had a clear IgM response before IgG response to the peptide markers while others had a more similar reaction or abbreviated response. On an individual dog basis, there is a rough correlation between when IgM and IgG appears, IgM signal is slightly higher at first, and on some dogs, it continues to stay elevated until there is a crossing or intersection point with the IgG signal curve that seems to indicate a class switch or decline of IgM present in the sample. If a uniform immune response was not observed in this well-controlled population, describing the response in a field population will be challenging. Perhaps this is due to the controlled number of male and female ticks utilized, or the duration of infestation, without the opportunity of re-infection or continuous exposure.

The non-uniformity of the immunogenic response to *A. phagocytophilum* makes enriching for particular subtypes of Ig challenging as demonstrated by the capture of *A. phagocytophilum* specific canine IgM and IgG antibodies in infected dogs utilizing the Protein A/G Magnetic Beads. This experiment showed that the excess antibody present in the samples causes havoc in the capture of either *A. phagocytophilum* specific IgM or IgG. Is almost as if they get tangled up, and not sure how to physically disrupt it, or prevent IgM to stick to IgG. Perhaps treating the samples to Protein A only, could be an alternative to clear some of the excess antibodies present in this sample. Although this

methodology could also provide a small percentage of affinity loss of IgG (18%) and IgM (33%). (Scott, et al. 1997)

Utilizing different targets and different testing platforms for detection and characterization of the immune response to *A. phagocytophilum* specific canine IgM and IgG antibodies in infected dogs was useful. Performing a time course of infection on plate assays with three different targets and multiple conjugates was new in literature, and it allowed me to narrow in on a range of important time points for further testing and determine which targets to use. While comparing the ELISA peptide plate assay with peptide conjugates against the SNAP® 4Dx® Plus Tests provided opposite results, I realized, even though both assays were performed with the same reagents they have different reaction mechanisms. The antibody, either IgM or IgG present in the sample has a high affinity for the antigen present, and in a two-step assay (plate assay) the solid phase binds all the targets present in the sample, leaving no open binding sites for the conjugate to detect it. In the SNAP® platform, a one-step assay, the binding between the solid phase and conjugate occurs at the same time with the sample, giving you a positive result if the target antibodies are present in the sample.

While both SNAP® 4Dx® Plus Tests with the EENZ1 conjugate produced a negative result, positive results were obtained with the APH-4 conjugate for both samples. For these time points, both IgM (higher) and IgG are detected on the APH-4 peptide ELISA plate. This perhaps is an indication that the EENZ1 peptide has no problem detecting IgG, and it can also detect high levels of IgM on the SNAP platform. However, APH-4 is better in recognizing lower levels of IgM, IgG, or a potential subclass or multifamily. But there is not a clear answer to the question if the

improvements to this second-generation SNAP® 4Dx® Plus Tests facilitated a diagnosis of anaplasmosis at an earlier post-infection time points due to the recognition of IgM. In part, APH-4 recognizes IgM and IgG, as it does EENZ1. What I think it could be the answer is that APH-4 has a higher affinity of detection to IgM, or class-switch to IgG when levels of response are lower, and EENZ1 has a higher affinity of detection to IgG, but neither are exclusive. However, by combining both peptides, this second-generation SNAP® 4Dx® Plus Tests, has enhanced the earlier detection by increasing the recognition of IgM not previously seen in the first generation due to the EENZ1 peptide's lower affinity for IgM.

This second-generation SNAP® 4Dx® Plus Test showed surprising sensitivity compared to the peptide ELISA, by adding that additional peptide and the lower assay time was a very useful platform for this investigation and it allowed me to test the two-hundred and nineteen canine samples and two-hundred thirty feline samples obtained from the European IDEXX Laboratories, Inc. This small serosurvey demonstrates the presence of *A. phagocytophilum* in companion animals and the need for continued monitoring for their health and well-being and offers a tool for the diagnostic of sick pets. This study agrees with prior evidence of feline exposure and infection with vector-borne pathogens that commonly infect dogs and humans with the positive results obtained.

Appendix 1.

Additional Figures

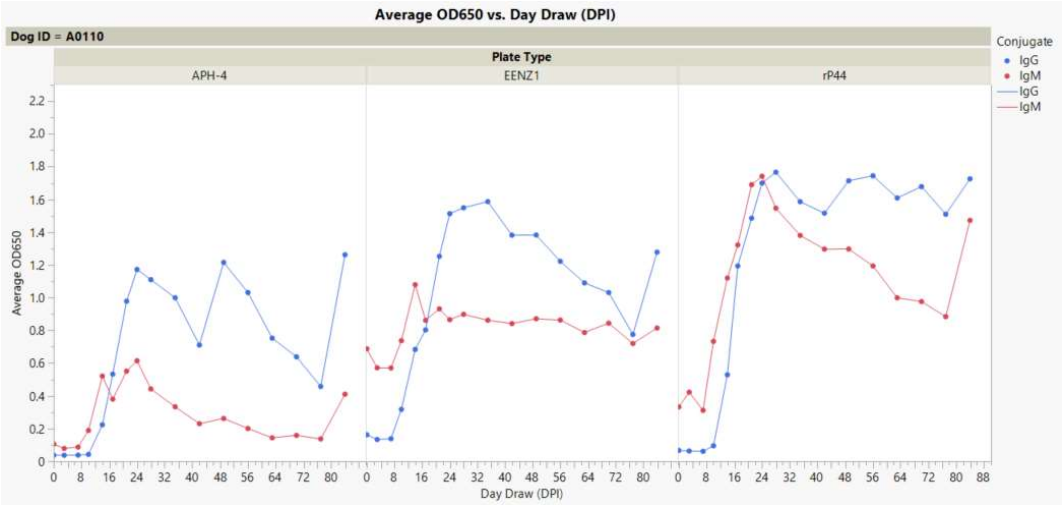


Figure 8. All three ELISA plate results for experimentally infected dog A0110.

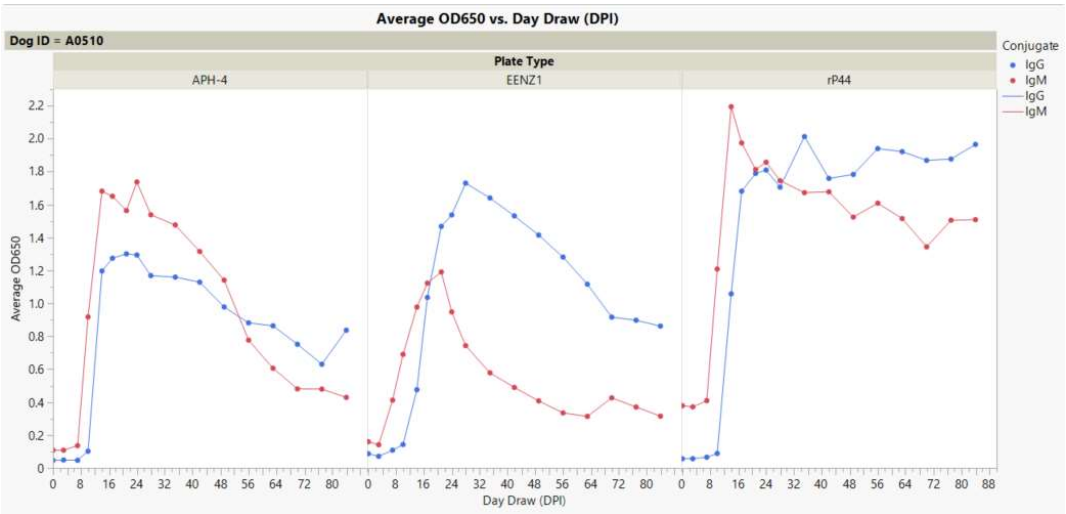


Figure 9. All three ELISA plate results for experimentally infected dog A0510.

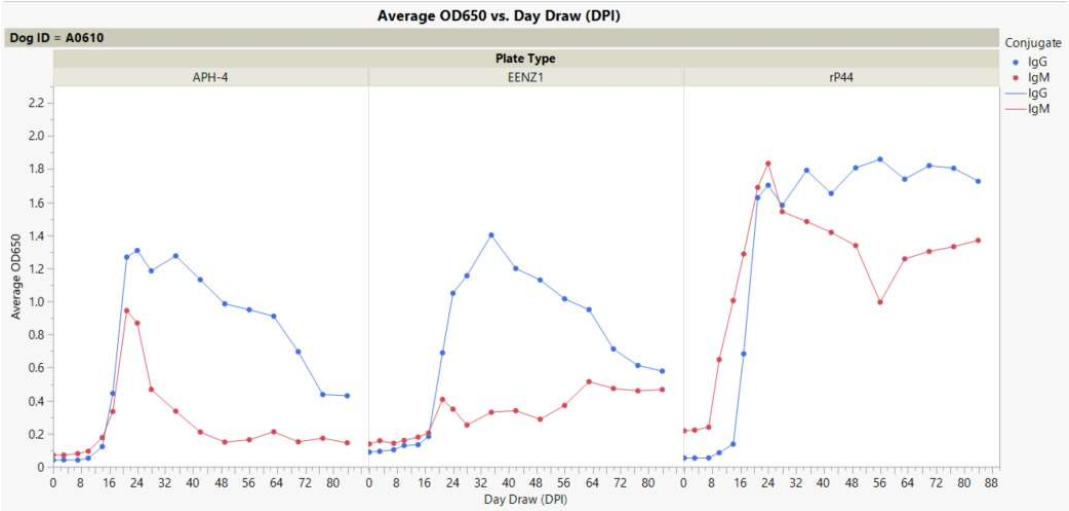


Figure 10. All three ELISA plate results for experimentally infected dog A0610.

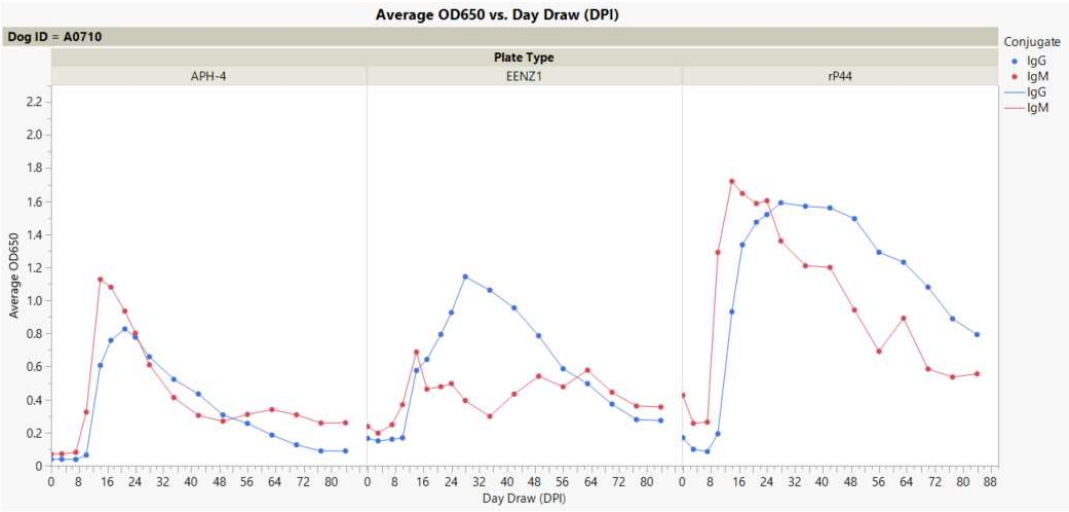


Figure 11. All three ELISA plate results for experimentally infected dog A0710.

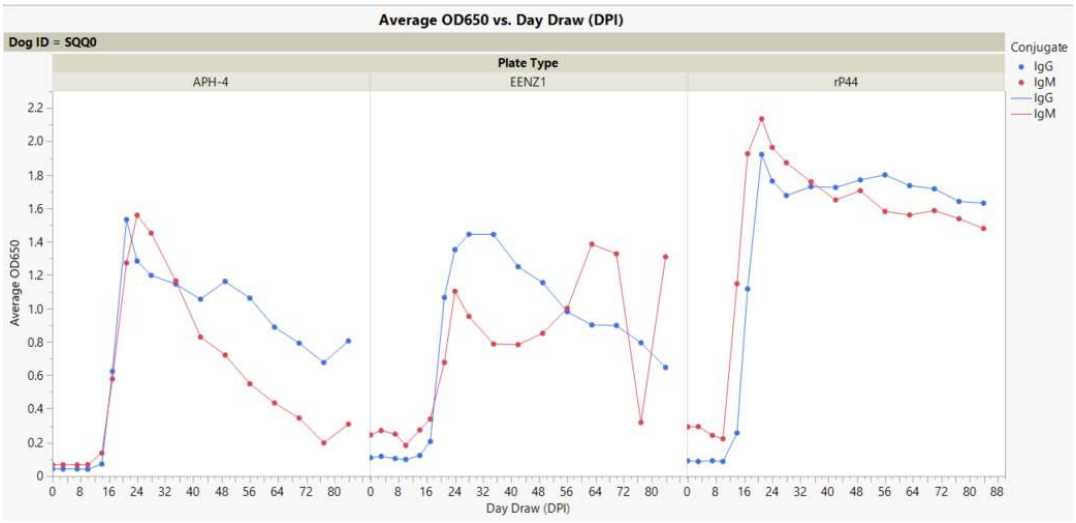


Figure 12. All three ELISA plate results for experimentally infected dog SQQ0.

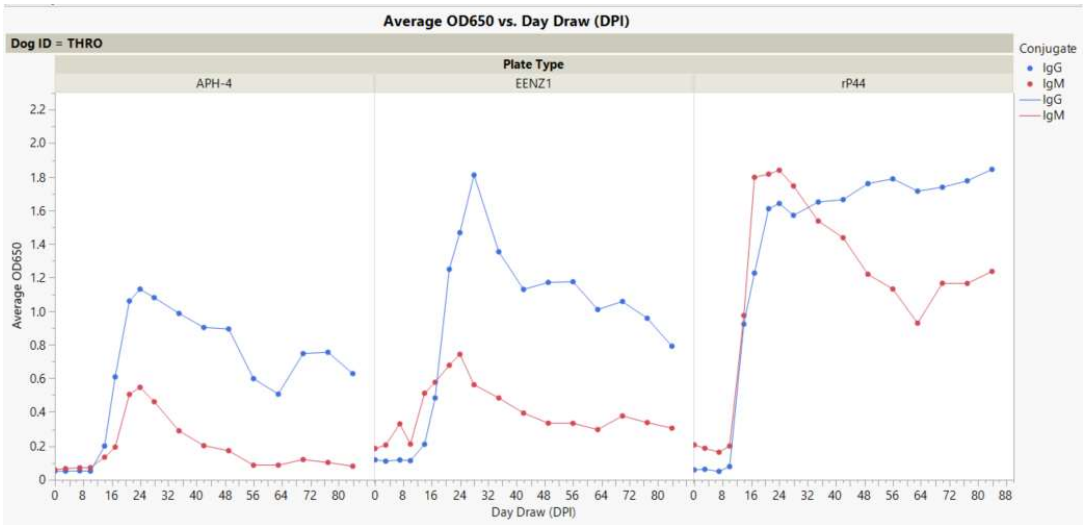


Figure 13. All three ELISA plate results for experimentally infected dog THRO.

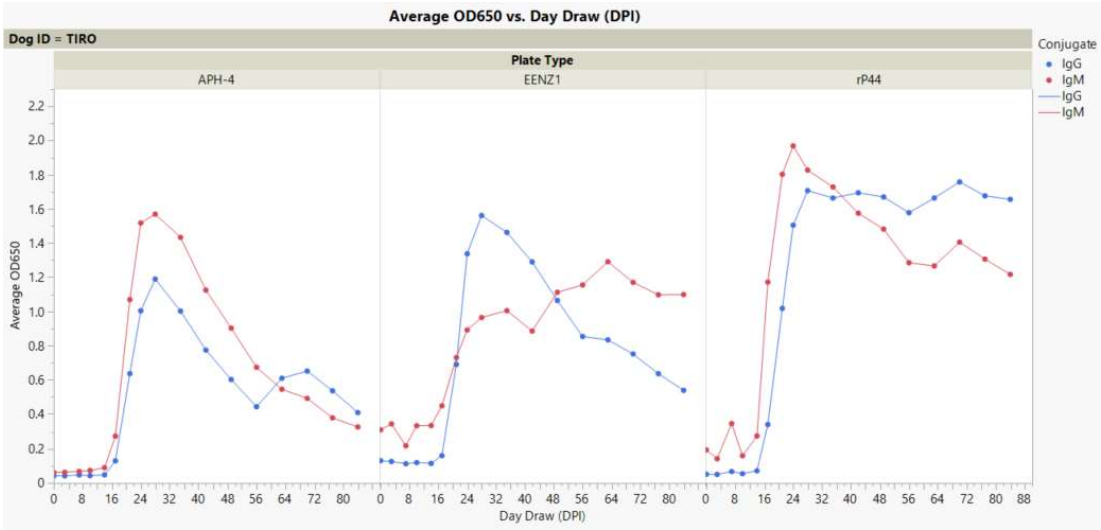


Figure 14. All three ELISA plate results for experimentally infected dog TIRO.

Appendix 2.

Additional Tables containing results by country from the Serosurvey performed.

Table 4. Serosurvey results for European canine samples by country.

	POSITIVE				
COUNTRY	EENZ1	APH-4	EENZ1/APH-4	NEGATIVE	TOTAL
Austria	-	3	8	10	21
Czechia	-	-	1	-	1
Denmark	1	1	3	6	11
Finland	-	1	2	1	4
France	-	-	-	2	2
Great Britain	-	-	-	50	50
Germany	4	12	25	19	60
Greece	-	-	-	1	1
Italy	-	-	-	13	13
Luxembourg	-	1	-	3	4
Netherlands	2	1	1	4	8
Norway	-	-	-	1	1
Poland	-	-	-	2	2
Sweden	-	-	2	4	6
Slovenia	-	1	2	1	4
Slovakia	-	-	1	-	1
Spain	-	-	-	22	22
Switzerland	-	1	-	7	8

Table 5. Serosurvey results for European feline samples by country.

COUNTRY	POSITIVE			NEGATIVE	TOTAL
	EENZ1	APH-4	EENZ1/APH-4		
Austria	-	6	-	8	14
Finland	-	-	-	3	3
Great Britain	-	-	-	50	50
Germany	-	2	-	48	50
Hungary	-	-	-	1	1
Italy	-	-	-	4	4
Netherlands	-	-	-	8	8
Spain	-	-	-	100	100

References

Andre, M.R. (2018). Diversity of *Anaplasma* and *Ehrlichia/Neoehrlichia* Agents in Terrestrial Wild Carnivores Worldwide: Implications for Human and Domestic Animal Health and Wildlife Conservation. Front. Vet. Sci. 5:293. DOI: 10.3389/fvets.2018.00293

Balboni, A., Urbani, Lo, Morini, M., Dondi, F., Battilani, M., (2021). Molecular detection *Anaplasma phagocytophilum* in hair and spleen of cats revealed a possible underestimation of feline vector- borne pathogens. Research in Veterinary Science 137 92021) 144-149

Beall, M.J., Mainville, C.A., Arguello-Marin, A., Clark, G., Lemieux, C., Saucier, J., Thatcher, B., Breitschwerdt, E.B., Cohn, L.A., Qurollo, B.A., & Chandrashekar, R., (2022) An Improved Point-of-Care ELISA for the Diagnosis of Anaplasmosis and Ehrlichiosis During the Acute Phase of Tick-Borne Infections in Dogs. Topics in Companion and Med 51 (2022) 100735

Bunnell J.E., Magnarelli L.A., Dumler J.S. Infection of laboratory mice with the human granulocytic ehrlichiosis agent does not induce antibodies to diagnostically significant Borrelia burgdorferi antigens (1999) Journal of Clinical Microbiology, 37 (6), pp. 2077 - 2079, Cited 14 times. DOI: 10.1128/jcm.37.6.2077-2079.1999

CDC 'One Health" https://www.cdc.gov/onehealth/index.html

Chandrashekar, R., Beall, M.J., Thatcher, B., Saucier, J.M, Tyrrell, P., Lappin, M.R., (2017) Serologic responses to peptides of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in dogs infested with wild-caught *Ixodes scapularis*. The Veterinary Journal. Volume 226 (2017) Pages 6-11

Duplan, F., Davies, S., Filler, S., Abdullah, S., Keyte, S., Newbury, H., Helps, C. R., Wall, R., & Tasker, S., (2018) *Anaplasma phagocytophilum, Bartonella spp., haemoplasma* species and *Hepatozoan* spp. in ticks infesting cats: a large-scale survey. Parasites & Vectors (2018) 11:201

Felek, S., Telford, S., Falco, R. C., & Rikihisa, Y. (2004) Sequence analysis of p44 homologs expressed by *Anaplasma phagocytophilum* in infected ticks feeding on naive hosts and in mice infected by tick attachment. Infection and Immunity. PMCID: PMC321609 DOI: 10.1128/IAI.72.2.659-666.2004

Foley JE, Carrade DD, Borjesson DL, Sykes JE. Canine granulocytic anaplasmosis: a review. J Vet Intern Med. 2009 Nov-Dec;23(6):1129-41. doi: 10.1111/j.1939-1676.2009.0384. x. Epub 2009 Sep 16. PMID: 19761477.

Hegarty, B.C., Qurollo, B.A., Thomas, B., Karen Park K., Chandrashekar, R., Beall, M.J., Thatcher, B., & Breitschwerdt, E.B. (2015), Serological and molecular analysis of feline vector-borne anaplasmosis and ehrlichiosis using species-specific peptides and PCR. Hegarty et al. Parasites & Vectors (2015) 8:320 DOI 10.1186/s13071-015-0929-8

Holden, K., Hodzic, E., Feng, S., Freet, K., Lefebvre, R., Barthold, S., (2005), Coinfection with Anaplasma phagocytophilum Alters Borrelia burgdorferi Population Distribution in C3H/HeN Mice. ASM Journals. Infection and Immunity Vol. 73, No. 6

Keesing F, Hersh MH, Tibbetts M, McHenry DJ, Duerr S, Brunner J, Killilea M, LoGiudice K, Schmidt KA, Ostfeld RS. Reservoir competence of vertebrate hosts for Anaplasma phagocytophilum. Emerg Infect Dis. 2012 Dec;18(12):2013-6. doi: 10.3201/eid1812.120919. PMID: 23171835; PMCID: PMC3557888.

Lappin, M.R, Chandrashekar, R., Brett Stillman, B., Liu, J. & Mather, T.N. (2015) Evidence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* infection in cats after exposure to wild-caught adult *Ixodes scapularis*. Journal of Veterinary Diagnostic Investigation 2015, Vol. 27(4) 522– 525

Lappin, M.R., Tasker, S., Roura, X., (2020). ROLE OF VECTOR-BORNE PATHOGENS IN THE DEVELOPMENT OF FEVER IN CATS. Tick- and sandfly-associated diseases. Journal of Feline Medicine and Surgery (2020) 22, 41–48

Lin, Q. (2004) Mechanisms of the p44-multigene family expression and characterization of the p44 homologous gene msp2 expression in *Anaplasma phagocytophilum*. Dissertation for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University. Ohio State University (2004) UMI Number: 3119478

O'Connor, T.P. Jr, Chandrashekar, R., inventors; IDEXX Laboratories, assignee. Peptides for detection to *Anaplasma phagocytophilum*. US patent 6,964,855. Nov 15, 2005.

O'Connor, T.P. SNAP Assay Technology", 2015, Topics in Companion Animal Medicine, Volume 30, Issue 4, December 2015, Pages 132-138. Copyright 2015 by Elsevier Ltd

Park, J., Kim, K.J., Choi, K., Grab, D. J., Dumler, J. S. (2004). *Anaplasma phagocytophilum* AnkA binds to granulocyte DNA and nuclear proteins. *Cell Microbiol*. 2004 Aug;6(8):743-51. doi: 10.1111/j.1462-5822.2004. 00400.x

Pennisi, M.G., Hofmann-Lehmann, R., Radford, A.D., Tasker, S., Belák, S., Addie, D.D., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K., Horzinek, M.C., Hosie, M.J., Lloret, A., Lutz, H., Marsilio, F., Thiry, E., Truyen, U., & Möstl, K., (2017). *ANAPLASMA, EHRLICHIA* AND *RICKETTSIA* SPECIES INFECTIONS IN CATS European guidelines from the ABCD on prevention and management. Journal of Feline Medicine and Surgery (2017) 19, 542–548

Qurollo, B.A., Chandrashekar, R., Hegarty, B.C., Beall, M.J., Stillman, B.A., Liu, J., Thatcher, B., Pultorak, E., Cerrito, B., Walsh, M., & Breitschwerdt. E.B. (2014) A serological survey of tick-borne pathogens in dogs in North America and the Caribbean as assessed by *Anaplasma phagocytophilum*, *A. platys, Ehrlichia canis, E. chaffeensis, E. ewingii, and Borrelia burgdorfer*i species-specific peptides. Infection Ecology & Epidemiology, 4:1, 24699, DOI: 10.3402/iee. v4.24699

Qurollo, B.A., Stillman, B.A., Beall, M.J., Forster, P., Hegarty, B.C., Breitschwerdt, E.B., & Chandrashekar, R., (2020). Comparison of *Anaplasma* and *Ehrlichia* species–specific peptide ELISAs with whole organism–based immunofluorescent assays for serologic diagnosis of anaplasmosis and ehrlichiosis in dogs. AJVR • Vol 82 • No. 1 • January 2021

Rikihisa, Y. (2011). Mechanisms of Obligatory Intracellular Infection with Anaplasma phagocytophilum. CLINICAL MICROBIOLOGY REVIEWS, July 2011, p. 469–489

Rodríguez-Cortés, A., Ojeda, A., López-Fuertes, L., Timón, M., Altet, L., Solano-Gallego, L., Sánchez-Robert, E., Francino, O., Alberola, J., A long term experimental study of canine visceral leishmaniasis, International Journal for Parasitology, Volume 37, Issue 6, 2007, Pages 683-693, ISSN 0020-7519,

Sainz, A., Roura, X., Miró, G., Estrada-Peña, A., Kohn, B., Harrus, S., & Solano-Gallego, L., (2015) Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. Sainz et al. Parasites & Vectors (2015) 8:75 DOI 10.1186/s13071-015-0649-0

Schäfer, I., Kohn, B., & Müller, E. (2022) *Anaplasma phagocytophilum* in domestic cats from Germany, Austria and Switzerland and clinical/laboratory findings in 18 PCR-positive cats (2008–2020). Journal of Feline Medicine and Surgery 2022, Vol. 24(4) 290–297

Scott, M. A., Davis, J. M., Schwarts, K. A. (1997) *Staphylococcal protein A binding to canine IgG and IgM.* Vet Immunol Immunopathology 1997 Nov;59(3-4):205-12. doi: 10.1016/s0165-2427(97)00073-1

Skotarczak, B. (2018) The role of companion animals in the environmental circulation of tick- borne bacterial pathogens. Annals of Agricultural and Environmental Medicine 2018, Vol 25, No 3, 473–480

Stillman, B.A., Monn, M., Liu, J., Thatcher, B., Foster, P., Andrews, B., Little, S., Eberts, M., Breitschwerdt, E.B., Beall, M.J., & Chandrashekar, R., (2014). Performance of a commercially available in-clinic ELISA for detection of antibodies against *Anaplasma phagocytophilum, Anaplasma platys, Borrelia burgdorferi, Ehrlichia canis,* and *Ehrlichia ewingii* and *Dirofilaria immitis* antigen in dogs. J Am Vet Med Assoc. 2014 Jul 1;245(1):80-6. doi: 10.2460/javma.245.1.80.

Thomas, V., Anguita, J., Barthold, S., Fikrig, E. (2001). Coinfection with Borrelia burgdorferi and the Agent of Human Granulocytic Ehrlichiosis Alters Murine Immune Responses, Pathogen Burden, and Severity of Lyme Arthritis. ASM Journals. Infection and Immunity. Vol. 69, No. 5

Woldehiwet, Z., (2008). Immune evasion and immunosuppression by Anaplasma phagocytophilum, the causative agent of tick-borne fever of ruminants and human granulocytic anaplasmosis. Vet Journal. 2008 Jan;175(1):37-44. doi: 10.1016/j.tvjl.2006.11.019. Epub 2007 Feb 1

Zhang, J., Ma, H., Ai, J., Qi, T., Kang, M., Li, X., Sun, Y., (2022) Serological Analysis of IgG and IgM Antibodies against *Anaplasma* spp. in Various Animal Species of the Qinghai-Tibetan Plateau. Animals 2022, 12, 2723. https://doi.org/10.3390/ ani12192723

Zhi, N., Ohashi, N., Tajima, T., Mott, J., Stich, R.W., Grover, D., Telford III, S.R., Lin, Q., & Rikihisa, Y (2002) Transcript Heterogeneity of the p44 Multigene Family in a Human Granulocytic Ehrlichiosis Agent Transmitted by Ticks. INFECTION AND IMMUNITY, Mar. 2002, p. 1175–1184 Vol. 70, No. 30019-9567/02/$04.000 DOI: 10.1128/IAI.70.3.1175–1184.2002.

Zhuang, J., Furse, J. E., Brown, W.C., Brayton, K., A., Palmer, G. H., (2007) Maintenance of antibody to pathogen epitopes generated by segmental gene conversion is highly dynamic during long-term persistent infection. Infection and immunity. 2007;75: 5185–5190