A point-of-care dot blot ELISA assay for detection of protective antibody against canine adenovirus, canine parvovirus, and canine distemper virus is diagnostically accurate

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OBJECTIVE
To determine diagnostic accuracy of a point-of-care antibody-screening test by determining sensitivity, specificity, and overall accuracy when compared to reference standard tests for antibody against core vaccine viruses canine adenovirus (CAV), canine parvovirus (CPV), and canine distemper virus (CDV). A further aim was to provide the practitioner with information to guide selection of vaccinal antibody testing methods.

SAMPLES
Canine sera from across North America were submitted to a fee-for-service titer-testing laboratory. Samples came from healthy pet dogs with known core vaccination history (n = 431) as well as unvaccinated dogs held in isolation (132). This study examined a total of 563 samples for CDV/CPV and 183 for CAV.

PROCEDURES
Serum virus neutralization assays determined antibody titers for CDV and CAV. Hemagglutination inhibition assay determined antibody titers against CPV. All sera were also tested by point-of-care dot blot ELISA (index test).

RESULTS
For all 3 viral antigens, the index test provided sensitivity ranging from 96.03% to 96.75% and specificity ranging from 87.50% to 94.33%. Overall accuracy ranged from 93.43% to 95.91%.

CLINICAL RELEVANCE
The index test correlates well with reference standard tests and is a reliable, rapid screening test for detection of protective vaccinal antibody against CAV, CDV, and CPV in healthy dogs over 20 weeks of age. An accurate assessment of immunity allows clinicians to administer core vaccines appropriately as needed, avoiding unnecessary risk of adverse vaccine events.

Antibody testing as a method to determine the potential benefit of administration of core canine viral vaccines is rapidly becoming a standard practice in veterinary medicine. The 2017 American Animal Hospital Association canine vaccine guidelines includes a section regarding the use of antibody testing. The World Small Animal Veterinary Association also includes antibody testing as part of their vaccine guidelines.

Indications for core vaccinal antibody testing include adult dogs that are due for "booster" vaccination, any dog that has experienced an adverse reaction to vaccination in the past or other health issue, dogs that have an unknown vaccination history, puppies at least 2 weeks after the completion of the initial vaccination series, dogs in shelter outbreak situations, and nomograph analysis for the breeding bitch.

An array of antibody testing options is available to the veterinary clinician. These include laboratory-based tests, which provide quantitative end point titers, as well as rapid, point-of-care, antibody-screening tests. The objective of this study was to determine the diagnostic accuracy of the point-of-care dot blot ELISA (the index test) in comparison to reference standard laboratory-based assays to help guide the veterinary clinician in decisions regarding test choice.

Materials and Methods

Animals
Samples were submitted from across the US and Canada for quantitative testing by the Companion Animal Vaccines and ImmunoDiagnostic Service (CAVIDS) Laboratory, a fee-for-service titer-testing laboratory in...
the University of Wisconsin-Madison School of Veterinary Medicine. Sample submission and request for testing constituted informed owner consent. The IACUC of the University of Wisconsin School of Veterinary Medicine determined that IACUC oversight was not required for this retrospective study. The sample population included 431 pet dogs from a range of ages and breeds with known history of vaccination against canine adenovirus, canine parvovirus, and canine distemper virus as provided on the submission form. Mixed-breed dogs and 73 unique pure breeds were included. Mixed-breed dogs made up 22% of the samples. Golden Retriever and Labrador Retriever samples made up 12% and 10%, respectively. For the purposes of this study, Labradoodles and Goldendoodles were classified as mixed breed. A subset of 105 pet dogs were known to have received the Merial/Boehringer-Ingelheim combination CAV/CPV/CDV vaccine. The remaining 326 pet submissions did not specify vaccine manufacturer. Median age was 8.65 years (range, 0.2 to 17.1 years). Samples came from intact and spayed females (33% and 21%, respectively) and intact and neutered males (23% and 21%, respectively). No sex was indicated for 1% of the submissions. Sera from dogs indicated to have chronic systemic disease were not included in the data set. A set of 132 archived samples from purpose-bred specific pathogen–free (spf) Beagle dogs unvaccinated against CPV-2 or CDV and held in an isolation facility (Ridglan Farms Inc) were also included.

Sample selection
Serum samples from 431 pet dogs and 132 spf dogs (n = 563 sera) were selected to provide a range of quantitative antibody titers against CPV and CDV (Figures 1 and 2). A subset of 183 pet dogs from this group provided a range of quantitative titers for CAV antibody (Figure 3). Antibody titers above previously determined protective thresholds were present in 83% (CAV), 86% (CPV), and 90% (CDV) of the pet samples. Samples were stored frozen at −20 °C after reference standard testing and were thawed immediately before index testing. Personal information regarding individual pet dogs and their owners was not included in the data set to protect client privacy.

Serology
The reference standard test for antibody against CPV-2 is the hemagglutination inhibition assay as described by Carmichael et al. Briefly, sera are doubly diluted in duplicate in round-bottom 96-well plates in a PBS solution containing bovine serum albumin and sodium azide. Dilutions begin at 1:10 and continue to 1:20,480. An extract containing 32 HA units of CPV type 2b (Schultz isolate) is added to all but the first dilution of all test wells. The first dilution serves as a serum control. After a 1-hour incubation, a cold suspension of washed 1% porcine erythrocytes is added to all wells. A control plate includes reference positive and negative sera diluted in duplicate, as well as viral back titration and cell controls. Positive and negative control sera were fully characterized previously by CAVIDS Laboratory. Plates are incubated at 4 °C overnight and then read for inhibition of viral–induced agglutination. Titer is reported as the reciprocal of the highest dilution that shows inhibition of viral agglutination of porcine erythrocytes.

The reference standard test for antibody against CDV and CAV is the serum virus neutralization (SVN) assay. In this assay, sera are doubly diluted in duplicate in flat-bottom 96-well tissue culture plates with minimal essential media (15-010-CM; Corning...
Inc) containing growth factors fetal bovine serum (35-010-CV; Corning Inc), L-glutamine, (25-005-CI; Corning Inc), and antibiotic/antimycotic solution (30-004-CI; Corning Inc). Dilution range covers 1:2 through 1:4,096. The reference viruses used are CDV Onderstepoort and CAV-1 Mirandola. An aliquot of reference virus is removed from –80 °C storage, thawed, and diluted as predetermined to reach 200 TCID50. This fixed amount of infectious virus is added to all test sera dilutions and incubated for 1 hour. A control plate includes reference positive and negative sera diluted in duplicate, as well as viral back titration and cell controls. Positive and negative control sera have been fully characterized by CAVIDS Laboratory previously. After initial incubation, susceptible tissue culture cell suspension is added to all wells. Cell lines used are Vero green monkey kidney for CDV and Madin-Darby canine kidney for CAV. The plates are further incubated for 4 days at 5% CO2 and 37 °C to allow for tissue culture cell growth and viral infection. After incubation, wells are read microscopically for cytopathic effect typical for the reference viruses. Titer is reported as the reciprocal of the highest dilution of serum that shows neutralization of virus, detected by lack of cytopathic effect.

Antibody against CAV is cross-reactive between CAV types 1 and 2. Canine core vaccine contains CAV-2 to provide protection against both strains without adverse effects such as uveitis. Infectious canine hepatitis caused by CAV-1 is a more severe disease with higher mortality than the respiratory disease caused by CAV-2. For this reason, only CAV-1 reference virus (Mirandola strain) is used in the SVN assay in our laboratory.

The point-of-care dot blot ELISA (VacciCheck Biogal Laboratories LLC) contains specific antigens for all 3 viruses (CAV, CPV-2, and CDV) fixed onto a plastic surface shaped like a comb, with each individual serum sample given a separate tooth on the comb. After test sera are added to wells containing dilution buffer, the comb containing the antigens is incubated with the diluted sera in the first row. The comb is subsequently moved at specified intervals to successive wells containing wash buffer, conjugate, and chromagen. Antibody present in the test sera binds to the antigen(s), which is then bound by a conjugated anti-canine IgG antibody. After reaction with chromagen, the resulting color changes are visually compared against a single internal control on each tooth. A sliding scale is provided by the manufacturer to allow technicians to interpret the depth of color reaction as an “S” value in comparison with the internal control. Results are available in approximately 25 minutes. Samples can be run individually or batched up to 12 sera/kit.

In the current study, the index test was read by technicians who had no knowledge of the results of the reference standard tests or any clinical details regarding individual dogs. Reference standard tests were also read independently of clinical details.

Protective thresholds for the reference standard tests have been determined previously through challenge of immunity trials conducted by our laboratory. For this study, a titer of 1:8 or above (CAV and CDV) and a titer of 1:40 or above (CPV) were considered protective. For the index test, a threshold of S2 had been set by the manufacturer as the semiquantitative threshold for protection for all 3 antigens.

Five hundred sixty-three samples were tested for CDV and CPV-2 antibody and 183 samples for CAV (pet samples only).

**Analysis**

An online medical calculator (MedCalc Software Ltd) was used to determine sensitivity, specificity, and overall accuracy. Analyses of CDV and CPV antibody detection were determined on the basis of results of all 563 samples (pet dogs plus unvaccinated spf dogs). Analysis of CAV antibody detection was based on results for 183 samples (pet dogs only).

**Results**

The sensitivity, specificity, and accuracy analysis of the index test for all 3 viral antigens is shown (Table 1).

When used to detect protective antibody levels against CAV, the index test provided a sensitivity of 96.03% (95% CI, 91.55% to 98.53%), specificity of 90.62% (95% CI, 74.98% to 98.02%), and overall accuracy of 95.08% (95% CI, 90.87% to 97.73%). The index test produced 3 false-positive and 6 false-negative results over 183 samples tested. When used to detect protective antibody levels against CPV in 563 samples, the index test produced 11 false-positive and 12 false-negative results, giving it an overall accuracy of 95.91% (95% CI, 93.93% to 97.39%), with a

<table>
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<th>Virus</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Accuracy (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>CAV</td>
<td>96.03% (91.55%–98.53%)</td>
<td>90.62% (74.98%–98.02%)</td>
<td>95.08% (90.87%–97.73%)</td>
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<td>Pet dogs only</td>
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<td>n = 183</td>
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<tr>
<td>CPV</td>
<td>96.75% (94.39%–98.31%)</td>
<td>94.33% (90.08%–97.14%)</td>
<td>95.91% (93.93%–97.39%)</td>
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<td>Pet and spf dogs</td>
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<td>n = 563</td>
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<tr>
<td>CDV</td>
<td>96.12% (93.69%–97.81%)</td>
<td>87.50% (81.69%–92.00%)</td>
<td>93.43% (91.05%–95.33%)</td>
</tr>
<tr>
<td>Pet and spf dogs</td>
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<td>n = 563</td>
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spf = Specific pathogen–free.
sensitivity of 96.75% (95% CI, 94.39% to 98.31%) and specificity of 94.33% (95% CI, 90.08% to 97.14%). The index test produced 22 false-positive and 15 false-negative results over 563 samples when used to detect protective antibody levels against CDV. This provided a sensitivity of 96.12% (95% CI, 93.69% to 97.81%), specificity of 97.50% (95% CI, 91.69% to 92.00%), and overall accuracy of 93.43% (95% CI, 91.05% to 95.33%).

No differences were seen in detection of CDV and CPV-2 antibody for dogs vaccinated with Merial/Boehringer-Ingelheim core vaccine products as compared with dogs given other commercially available vaccines (data not shown). There were not enough negative samples in this subgroup to test specificity; however, no false positives were detected. False negatives were seen in 2 samples for CPV (sensitivity, 98.0%; 95% CI, 93.0% to 99.9%) and in 4 samples for CDV (sensitivity, 96.2%; 95% CI, 90.5% to 99.0%). None of these samples were tested for CAV antibody.

Discussion

The aim of the current study was to provide the veterinary clinician with an independent evaluation of a point-of-care dot blot ELISA antibody screening kit to aid selection of core canine vaccine antibody testing method. In addition to diagnostic accuracy, it is helpful to fully understand the strengths and drawbacks of antibody testing options to determine best applications for each indication.

Overall sensitivity values of approximately 96% indicate that the index test will falsely characterize 1 out of every 25 protected dogs as lacking antibody. These dogs would be unnecessarily revaccinated. However, this represents a refinement as 24 out of 25 protected dogs would be spared revaccination and associated risks.

Index test specificity in the range of 90% implies that 1 in 10 dogs that truly lack antibody protection will be falsely characterized as protected. It should be noted that sample selection inadvertently produced a sample set with a relatively high number of samples close to the test threshold for CDV antibody (Figure 2). This may have impacted the test specificity for CDV (87.50%).

It is important to note that, regardless of the testing method, a negative result indicates that further vaccination may provide a benefit to the patient by inducing a rise in antibody level, not necessarily that the adult dog is susceptible to disease.

The goal of core vaccination is to induce antibody levels that provide sterilizing immunity, which completely protects the vaccinee from infection. However, other aspects of immunity, such as cellular immune components and immune memory, could be activated if sterilizing immunity is not achieved and infection occurs. Unfortunately, there are no convenient tests for cellular immunity currently available to the veterinary clinician. Because humoral immunity stems from the complete activation of multiple components of the immune system, the presence of antibody can be correlated with immune memory. Known quantitative titer thresholds for sterilizing immunity have been determined through in vivo challenge of immunity studies. When antibody titer is above threshold, infectious virus is neutralized and cannot enter the animal’s cells to replicate. In other words, the virus is “sterilized.” Administering modified-live viral vaccine “booster” to an already actively immune dog provides no benefit in the face of sterilizing immunity. More doses of vaccine do not induce higher titers once an active immune response produces antibody above this threshold. However, due to vaccine excipients, risk of adverse reaction is present at every administration. Although the risk of adverse event is small, this risk is unwarranted in an already immune dog.

The index test in the current study was developed on the basis of known sterilizing immunity thresholds and was designed to correlate closely with the reference standard tests. The index test and other qualitative vaccinal antibody tests provide a result that is reported as adequate (meets or exceeds the threshold) or not adequate (does not meet threshold) on the basis of presence of color reaction. The dot blot ELISA test that was evaluated in the current study can also be read semi-quantitatively by measuring the relative depth of color reaction to get an “S” number. It is important to remember that this is not a titer and should not be used where a true numerical result is needed.

Quantitative assays, such as the reference standard tests hemagglutination inhibition and serum virus neutralization, provide a numerical end-point titer based on testing patient sera through multiple dilutions. These lab-based end point assays are also directly functional because the patient sera interact with and neutralize live infectious virus.

The index test is appropriate for routine antibody screening of healthy adult dogs to determine potential benefit of “booster” core vaccination, triage of dogs in shelters experiencing outbreaks, and puppies after the end of their initial vaccine series (at 20 weeks of age or older). At ages < 20 weeks, a negative index test result is meaningful, but a positive result can be confounded by maternal antibody still declining.

When it is necessary to know antibody levels in puppies younger than 20 weeks, laboratory-based gold-standard assays are indicated. Such a situation might arise in an outbreak or exposure, after an adverse vaccine reaction, or other health issue. Reverse half-life analysis is applied to resulting numerical titers to differentiate puppy active immune responses from maternal antibody still declining. To do this, puppy reciprocal antibody titer results are doubled for every half-life that has passed since birth to find the corresponding maternal titer transferred to the neonate. In the case of an actively immune puppy, resulting titer is many logs greater than normal maternal titer range.

Antibody testing of the puppy after the initial core vaccine series is arguably the most important
indication for antibody testing, regardless of the method utilized. Failure of vaccine to immunize and subsequent development of disease is most likely to occur in the first year of life. Antibody testing of this population is an invaluable tool to avoid the preventable situation of a “fully vaccinated” patient that is in fact not immunized. Additionally, a positive antibody test result early in life proves immune competence—an important health baseline to document before the onset of old age, immune senescence, and geriatric health issues.

Quantitative, end point titer testing methods are required for nomograph calculations for a breeding bitch or prevaccination baseline maternally derived titers in puppies at high risk for parvovirus. In both situations, passive antibody half-life degradation analysis is applied to numerical titers to optimize a tailored vaccination schedule and timing of puppy follow-up testing. When maternal titers are low, follow-up testing for pups can be completed as early as 12 weeks of age.

Due to the higher sensitivity of laboratory-based testing, the authors prefer quantitative methods for canine patients that have a history of adverse vaccine reaction, autoimmunity, or other health issues. Alternatively, positive results obtained from the point-of-care test can be confidently interpreted as protective and “booster” vaccination avoided, while negative results for this high-risk group can be confirmed via gold-standard testing.

Screening tests such as the point-of-care dot blot ELISA have several advantages over lab-based end point titer testing. The greatest advantage is the speed of turnaround for results—approximately 25 minutes for the index test. This is the main reason this test is currently used extensively in animal shelters undergoing outbreaks of distemper or parvovirus. Rapid determination of protection allows shelter personnel to triage accurately and move dogs appropriately, ultimately saving lives and resources. With the recent increase in transport of dogs from high-population shelters to regions with higher demand for adoptable dogs, the authors urge the use of point-of-care testing pretransport to assure that dogs are protected before travel. While financial constraints may preclude such use, antibody screening of shelter dogs before transport would greatly decrease the spread of core vaccine-preventable viruses.

In the clinic setting, the index test can be completed while the patient and owner wait. This is very convenient for a client who will be traveling with their dog or boarding them soon. However, technician time can be used more efficiently by testing sera in batches, since the dot blot ELISA has multiple steps requiring distinct timings.

Some drawbacks of the current study included the small size of the Merial/Boehringer-Ingelheim vaccine group and lack of data for the manufacturers of vaccines given to the rest of the study population. The study did not include CAV results for the complete data set for all 563 dogs because this test is requested much less frequently. The relatively larger percentage of CDV titers close to the threshold cut-off could be considered a drawback of the study; however, the index test correlated well with the CDV reference standard despite this added challenge.

Strengths of the current study included the large sample size, known vaccination status of all dogs, exclusion of dogs that were indicated to have systemic disease at the time of sample collection, and wide variety of ages, breeds, and geographical locations included. This sample set is highly representative of healthy vaccinated pet dogs across North America. However, the percentages of dogs in this sample set with protective antibody levels are not typical. To ensure a robust evaluation of the index test, samples were selected to include a slightly higher percentage of unvaccinated dogs than has been reported in the literature. Future studies include analysis of longitudinal titer data for individual dogs which have been tested repeatedly over time.

Sensitivity, specificity, and diagnostic accuracy analysis for this point-of-care, dot blot ELISA test correlate very favorably with the reference standard assays. The overall accuracy of 93.43% to 95.91% for this test provides confidence in its reliable use in the routine screening of previously vaccinated dogs over 20 weeks of age to determine the benefit of administering a “booster” core vaccine. Use of this point-of-care test (or laboratory-based quantitative testing) to determine vaccinal antibody status allows the clinician to avoid the unnecessary medical procedure of core vaccine administration to an already immune pet. This evidence-based approach to appropriate canine core vaccination can improve client trust and decrease practitioner liability, while supporting the overarching goal of canine health and immunity against the core viruses.

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