Accuracy of a point-of-care ELISA kit for predicting the presence of protective canine parvovirus and canine distemper virus antibody concentrations in dogs

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Abstract

Canine parvovirus (CPV) and canine distemper virus (CDV) are highly infectious and often fatal diseases with worldwide distributions and are important population management considerations in animal shelters. A point-of-care ELISA kit is available to detect serum antibodies against CPV and CDV and presumptively to predict protective status. The aim of this study was to determine the diagnostic accuracy of the test against CPV hemagglutination inhibition titers and CDV serum neutralization titers determined by a reference laboratory, using sera collected from dogs housed at animal shelters. The ELISA was used under both field and laboratory conditions and duplicate specimens were processed using an extra wash step.

The test kit yielded accurate results (CPV: sensitivity 92.3%, specificity 93.5%; CDV: sensitivity 75.7%, specificity 91.8%) under field conditions. CDV sensitivity was improved by performing the test under laboratory conditions and using an optical density meter (laboratory performed 94.0%; optical density meter 88.1%). Point-of-care ELISA testing for serum CPV and CDV antibody titers was demonstrated to be a useful tool for determining antibody status when making decisions regarding population management in animal shelters and the need for CPV and/or CDV vaccination.

Keywords: Antibody; Canine distemper; Canine parvovirus; Diagnosis; ELISA
Introduction

Canine parvovirus (CPV) and canine distemper virus (CDV) are highly infectious and often fatal canine infections with worldwide distributions (Beineke et al., 2009; Goddard and Leisewitz, 2010; Pesavento, 2011; Greene and Decaro, 2012). Despite widespread vaccination, CPV and CDV remain major causes of morbidity and mortality, particularly in unvaccinated dogs housed in pet shops, puppy mills and animal shelters (Beineke et al., 2009; Goddard and Leisewitz, 2010; Steneroden, 2011). Although immunological resistance to CPV and CDV is multifactorial, moderately to markedly increased serum antibody responses distinguish resistant from susceptible animals (Karkowka et al., 1975; Noon et al., 1980; Carmichael et al., 1983; Winters et al., 1983; Rima et al., 1991). Therefore, measurement of serum antibody titers can be a useful tool for determining the need for vaccination (Tizard and Ni, 1998) and for making population management decisions in shelters (Newbury et al., 2009; Lecher et al., 2010).

Predicting protective status using antibody titers can be challenging. The virulence of the viral strain and the size of challenge dose, as well as the adequacy of T-helper cell-mediated immunity, immune-mediated cytotoxicity and the persistence of memory cells (which might prevent infection despite a decline in serum antibody concentrations) are unpredictable variables when determining whether a dog is protected from disease. Additionally, recommendations for test interpretation are usually based on information from laboratory challenge studies, where the infective doses used could far exceed what is usual under field conditions. However, in a shelter or disease outbreak (where errors could place animals at unnecessary risk of disease) conservative interpretation of test results is needed to minimize this risk (Crawford, 2010).

Serum CPV titers can be measured by ELISA, indirect fluorescent antibody assays (IFA) or by hemagglutination inhibition (HI) or virus neutralization (VN) tests. CPV challenge studies have demonstrated an adequate antibody response to vaccination and associated protection with an HI serum antibody titer ≥1:80 (Carmichael et al., 1983; Twark and Dodds, 2000). CDV titers are measured using ELISA, IFA and serum neutralization (SN) tests (Tizard and Ni, 1998). Early CDV challenge studies using SN determined that titers of 1:30-1:100 were protective (Gillespie, 1966; Appel, 1969), while a later study reported that titers of ≥1:32 (equivalent to an IFA result ≥1:5) indicated a sufficient antibody response to vaccination (Twark and Dodds, 2000).

The Synbiotics TiterCHEK CDV/CPV test is a point-of-care ELISA kit marketed for rapid determination of protective serum antibody concentrations in dogs against CPV and CDV (Carmichael, 2005). Results are interpreted as ‘positive’ or ‘negative’ for each virus, with the package
insert claiming that a positive result for CPV indicates an antibody titer equivalent to a CPV HI titer ≥1:80 and a positive for CDV indicates an antibody titer equivalent to a CDV SN titer ≥1:16. The aim of the present study was to determine the sensitivity and specificity of the test kit when compared to CPV HI titers and CDV SN titers measured by a reference laboratory, using sera collected from dogs housed at animal shelters.

Materials and methods

**Serum samples**

The study protocol was approved by the Purdue University Animal Care and Use Committee (PACUC number 10-037). Sera were successively collected from dogs at the time of surrender or delivery by municipal authorities to two metropolitan animal shelters, namely, PAWS Chicago, a large adoption-guarantee shelter, and the Humane Society of Indianapolis, a limited admission shelter. Enrollment criteria were (1) an estimated age based on dentition ≥4 months in order to minimize the confounding influence of maternally-derived antibodies (2011 American Animal Hospital Association Canine Vaccination Guidelines), and (2) clinically healthy animals based on physical examination by the attending shelter veterinarian. Dogs were excluded if estimated to be <4 months of age or if they had clinical signs of systemic illness at the time of shelter intake. A target enrollment of 50 dogs per shelter was used so that sample size would permit statistical analysis of results, with several extra convenience samples collected on the final study day.

Blood was collected at the time of shelter intake and the test was performed on site. After initial blood collection, each dog was vaccinated using a modified live C5 vaccine (PAWS Chicago: Pfizer Duramune Max 5; Humane Society of Indianapolis: Pfizer Vanguard Plus 5). All dogs that were suspected not to have protective titers against both CPV and CDV by the TiterCHEK CDV/CPV test kit performed on day 1 were retested on days 6-8. Dogs that might not have had protective titers against both CPV and CDV at the day 6-8 recheck were retested from days 13-15. Duplicate serum samples were collected and stored at -80 °C at each time point for (1) determination of CPV HI titers and CDV SN titers by a reference laboratory (Cornell University Animal Health Diagnostic Center) and (2) submission to the Synbiotics Corporation for TiterCheck testing under laboratory conditions by one laboratory technician and by microplate reader (ELx800 Universal Microplate Reader, Bio-Tek Instruments).

**Point-of-care ELISA**

The ELISA (Synbiotics TiterCHEK CDV/CPV test) was used according to the manufacturer’s instructions, reporting results as either positive or negative. Each assay includes separate CPV and CDV rows, consisting of (from left to right) a positive control well, a negative control well, a single specimen well and, lastly, a duplicate positive control well (Fig. 1). To
simulate the variability inherent in point-of-care testing, specimens were processed at the shelters where they were obtained or at Purdue Veterinary Medicine by the co-authors or either of two laboratory technicians (‘field method’) rather than by a single individual.

In addition to the manufacturer's recommended interpretation of test results as ‘positive’ or ‘negative,’ results were reported using a semi-quantitative evaluation scheme. Negative results were categorised as follows: (1) NegNCV, no color visible; (2) NegVSC, very slight color but obviously less than positive control; (3) NegCCV, considerable color but clearly less than positive control; and (4) NegSPC, color appears to be similar but not equivalent to positive control. The positive results were differentiated as: (1) PosEPC, appears to be equivalent to positive control; (2) PosMMPC, marginally more color than positive control; and (3) PosSMPC, significantly more color than positive control.

Aliquots of sera from a subset of dogs from the Humane Society of Indianapolis were also processed using a modified method, whereby extra wash steps were added. In the ‘extra wash’ method, six individual washes (vs. three washes recommended by the manufacturer) were used for each of the two wash steps.

At the time that the tests were performed, personnel were masked to the results of the reference standard CPV HI and CDV SN tests.

*Reference standard measurement of CPV HI titers and CDV SN titers*

The SN test for CDV was done as previously described by Appel and Robson (1973) using Vero cells and the Onderstepoort strain of CDV. Sera were tested in duplicate in 96-well microtitre plates with microscopic detection of viral cytopathology after a 5 day incubation period. Antibody titer (reciprocal of the dilution at the end-point) calculations were based on serum dilutions (initial serum dilution of 1:4) and 50% end-point determinations (see Appendix A: Supplementary material).

Antibody titers against CPV-2 were determined by HI assays as described by Carmichael et al. (1980). All sera were adsorbed with a 50% suspension of porcine red blood cells to remove non-specific inhibitors. The initial serum dilution for the HI test was 1:10.

*Statistical methods*

Sensitivity and specificity for dichotomous data (positive/negative test results) were calculated using Win Episcope 2.0. Agreement of categorical data (semi-quantitative evaluation scheme) was calculated using simple linear regression after checking residual plots for normality (StatsDirect statistical software Version 2.7.8). Data were transformed for CPV HI titers.
by calculating the \( \log_2 \) of 0.1x the original titer and, for CDV SN titers, by calculating the \( \log_2 \) of 0.25x the original titer prior to linear regression.

Results
A total of 200 serum samples were collected from 108 dogs (PAWS Chicago: \( n = 51 \); Humane Society of Indianapolis: \( n = 57 \)). Among the study population, there were 69 mixed-breed dogs, 10 Beagles, 12 Maltese terriers, nine Pit bull terriers, five Labrador retrievers and three German shepherds. Ninety-three specimens were collected from dogs at PAWS Chicago (day 1, \( n = 51 \); days 6-8, \( n = 30 \); days 13-15, \( n = 12 \)) and 107 specimens from dogs at the Humane Society of Indianapolis (day 1, \( n = 57 \); days 6-8, \( n = 32 \); days 13-15, \( n = 18 \); Fig. 2). Results reported as either ‘positive’ or ‘negative’ were compared against the CPV HI titers and CDV SN titers to generate sensitivity and specificity data (Tables 1 and 2). Table 3 reports the results of linear regression performed to test agreement between semi-quantitative results and logarithmically transformed reference standard results. All correlation coefficients (\( r \)) values were significantly different from zero (\( P < 0.0001 \)).

There were a number of discordances when results obtained using the regular method and reported as either ‘positive’ or ‘negative’ were compared against the reference standard CPV HI titers and CDV SN titers. For CPV, both specimens that yielded false positive results (\( n = 2/199; 1.0\% \)) had CPV HI titers within one dilution of the cut-off titer for protection (antibody titer = 80). For CDV, 6/7 false positive specimens (\( n = 7/200; 3.5\% \)) had CDV SN titers within one dilution of the cut-off titer used for protection by either the ELISA kit manufacturer (antibody titer = 16) or the reference laboratory (antibody titer = 32). For false negative results, 7/13 discordant CPV results (\( n = 13/199; 6.5\% \)) were within one dilution of the cut-off titer and seven of 28 discordant CDV results (\( n = 28/200; 14.0\% \)) were within one dilution of one of the two cut-off points. Using the regular method, the true prevalence of CPV was 84.4\% (95\% confidence intervals 79.4-89.5) and it was 57.5\% for CDV (95\% confidence intervals 50.6-64.4). Modification of the manufacturer’s recommended protocol via three extra washes minimally improved accuracy, sensitivity and specificity of the point-of-care test for detection of either serum CPV or CDV antibodies (Tables 1-3).

Discussion
The sensitivity and specificity of the ELISA when performed as a point-of-care test according to the manufacturer’s instructions under field conditions exceeded 90\% except for CDV protective antibody titer sensitivity, which was 75.7\% (95\% confidence interval 67.8-83.5\%). In general, the diagnostic accuracy for CPV was better than for CDV, although a comparison of the 95\% confidence intervals reveals that there is overlap when test results from the same methodology are compared between viruses, except for the sensitivity results for the regular method and the
extra wash method, both performed under field conditions (Tables 1 and 2). While this reduction in the ability to detect an animal with a positive CDV titer could lead to a decision to administer vaccination unnecessarily, this is far preferable to using a test with relatively poor specificity, which could result in exposing a susceptible animal to infection. This is particularly important in a shelter, where infection is more likely because of the combination of an increased environmental viral load and a population of animals with varying health status and vaccination histories (Lechner et al., 2010).

There was some discordance between serum ELISA results obtained using the regular method and reference standard CPV HI and CDV SN results. False positive ELISA results could occasionally occur when non-neutralizing antibodies result in a positive point-of-care test result, but are ineffective at HI or SN. False negatives could occur because of low ELISA sensitivity, particularly when low HI or SN antibody concentrations are induced by vaccination and yet are sufficient to induce protection from challenge. We presume that this is the reason why some dogs might have had undetectable or otherwise ‘negative’ results when tested by the ELISA at the 6-8 or 13-15 day time points, as this length of time should have been sufficient for seroconversion, antibody maturation and isotype switching to have occurred.

Field use of point-of-care tests requires that diagnostic accuracy remains high under highly variable conditions. To simulate these conditions and evaluate their effect, the ELISA results from three locations (both animal shelters and Purdue Veterinary Medicine), were compared with those obtained in a laboratory with a single technician. For CPV, point-of-care testing resulted in similar sensitivity, but superior specificity, to laboratory-performed testing, whereas for CDV point-of-care testing produced inferior sensitivity but superior specificity to laboratory testing (Tables 1 and 2). It is possible that these were chance occurrences associated with poor statistical power and inadequate sample size rather than real findings, since a review of the 95% confidence intervals reveals overlap for all except the CDV sensitivity results and the confidence intervals were relatively wide.

When the laboratory-performed test results were compared with the optical density meter results from the same laboratory, the results were very similar, leading to the conclusion that human eyes can discriminate color differences as accurately as calibrated optical devices and that subjective color assessment is not a major source of error when performing this ELISA. Similarly, the semi-quantitative method produced very good levels of agreement with the reference standard results (Table 3), but it appears unlikely that the semi-quantitative methodology appreciably improved diagnostic accuracy when compared with results reported as either
‘positive’ or ‘negative’, as the manufacturers recommend. The results for the regular method and the extra wash method were remarkably similar. This is most likely because the number of washes in the regular method was enough to rid the wells of unbound reagents so that they did not subsequently interfere with the binding of antibody or conjugate during the assay.

Conclusions
The TiterCHEK CDV/CPV test is a useful in-clinic ELISA to determine CDV and CPV antibody status when used according to manufacturer’s instructions under field conditions and could be used in shelters for population management in a disease outbreak. However, a decline in serum protective antibodies to below those levels considered to be protective is not synonymous with vulnerability to infection; long-term protection (i.e. years beyond vaccination) from CDV or CPV infection is likely to persist due to long-lived undifferentiated T-memory cells, CD4+ T-helper cells (i.e. cell-mediated immunity) and CD8+ T-cells (i.e. cytotoxic T-cells). As such, the test is most suitable for point-of-care identification of dogs that do not require vaccination; negative results are most likely to be sensitive, but not specific, for identifying those dogs vulnerable to CDV or CPV infection.

Conflict of interest statement
Synbiotics Corporation supplied the ELISA kits used in this study. Maddie’s Fund and Synbiotics Corporation played no role in the study design nor in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A: Supplementary material
Supplementary data associated with this article can be found, in the online version, at doi: ...

References


Table 1
Diagnostic accuracy of Synbiotics TiterCHEK CDV/CPV for detection of serum canine parvovirus (CPV) antibody titers using results reported as either ‘positive’ or negative’. The results were compared against hemagglutination inhibition (HI) titers performed at a reference laboratory.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point-of-care testing using manufacturer-recommended protocol (n = 199)</td>
<td>92.3 (88.2-96.3)</td>
<td>93.5 (84.9-100.0)</td>
</tr>
<tr>
<td>Point-of-care testing using extra wash (n = 103)</td>
<td>94.2 (89.2-99.1)</td>
<td>94.1 (82.9-100.0)</td>
</tr>
<tr>
<td>Synbiotics laboratory-performed testing using manufacturer-recommended protocol (n = 136)</td>
<td>94.9 (91.0-98.9)</td>
<td>88.9 (74.4-100.0)</td>
</tr>
<tr>
<td>Optical density (OD) measurement (n = 136)</td>
<td>93.2 (88.7-97.8)</td>
<td>88.9 (74.4-100.0)</td>
</tr>
</tbody>
</table>

\(^a\) 95% CI, 95% Confidence interval.
Table 2
Diagnostic accuracy of the ELISA for the detection of serum canine distemper virus (CDV) antibody titers using results reported as either ‘positive’ or negative’. The results were compared against serum neutralization titers performed at a reference laboratory.

<table>
<thead>
<tr>
<th>Test Description</th>
<th>Sensitivity % (95% CI) (^a)</th>
<th>Specificity % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point-of-care testing using manufacturer-recommended protocol ((n = 200))</td>
<td>75.7 (67.8-83.5)</td>
<td>91.8 (85.9-97.6)</td>
</tr>
<tr>
<td>Point-of-care testing using extra wash ((n = 104))</td>
<td>76.3 (65.4-87.1)</td>
<td>93.3 (86.0-100.0)</td>
</tr>
<tr>
<td>Synbiotics laboratory-performed testing using manufacturer-recommended protocol ((n = 122))</td>
<td>94.0 (88.4-99.7)</td>
<td>85.5 (76.1-94.8)</td>
</tr>
<tr>
<td>Optical density (OD) measurement ((n = 122))</td>
<td>88.1 (80.3, 95.8)</td>
<td>89.1 (80.9, 97.3)</td>
</tr>
</tbody>
</table>

\(^a\) 95% CI, 95% Confidence interval.
Table 3
Agreement between the ELISA antibody test results reported using a semi-quantitative evaluation scheme and canine parvovirus (CPV) hemagglutination inhibition titers/canine distemper (CDV) serum neutralization titers performed at a reference laboratory.

<table>
<thead>
<tr>
<th>Test Description</th>
<th>CPV $r^2$ (n)</th>
<th>CDV $r^2$ (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point-of-care testing using manufacturer-recommended protocol</td>
<td>0.67 (199)</td>
<td>0.71 (200)</td>
</tr>
<tr>
<td>Point-of-care testing using extra wash</td>
<td>0.64 (103)</td>
<td>0.73 (104)</td>
</tr>
<tr>
<td>Synbiotics laboratory-performed testing using manufacturer-recommended protocol</td>
<td>0.56 (153)</td>
<td>0.72 (153)</td>
</tr>
<tr>
<td>Optical density (OD) measurement</td>
<td>0.51 (153)</td>
<td>0.63 (153)</td>
</tr>
</tbody>
</table>
Figure legends

Fig. 1. Commercially available ELISA used to detect serum antibodies against canine parvovirus (CPV) and canine distemper virus (CDV).

Fig. 2. Bar chart showing collection days and source of dogs for 200 serum samples analyzed. Black bars, PAWS Chicago; White bars, Human Society of Indianapolis.