Simple Tests for Rapid Detection of Canine Parvovirus Antigen and Canine Parvovirus-Specific Antibodies

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Canine parvovirus (CPV) is the number one viral cause of enteritis, morbidity, and mortality in 8-week-old young puppies. We have developed twin assays (slide agglutination test [SAT] for CPV antigen and slide inhibition test [SIT] for CPV antibody) that are sensitive, specific, cost-effective, generic for all genotypes of CPV, and provide instant results for CPV antigen detection in feces and antibody quantification in serum. We found these assays to be useful for routine applications in kennels with large numbers of puppies at risk. The results of these assays are available in 1 min and do not require any special instrumentation. SAT-SIT technology will find applications in rapid screening of samples for other hemagglutinating emerging viruses of animals and humans (influenza virus and severe acute respiratory syndrome coronavirus).

Canine parvovirus (CPV) is the number one viral cause of puppy enteritis and mortality (8). Unique properties of CPV make it an emerging and reemerging pathogen of dogs worldwide (2, 5, 16, 17). Parvoviruses have a single-stranded DNA genome of 5,000 bases with a hairpin structure (4). Parvoviruses have exceptional evolutionary ability (10). Parvoviruses are extremely stable in the environment and relatively resistant to disinfectants because they are nonenveloped viruses (19). CPV multiplies in the rapidly dividing cells in the crypts of the intestine, leading to diarrhea and dehydration (4).

In the kennel environment, the availability of a large number of susceptible puppies, environmental stress, and unique properties of CPV combine to form an ideal scenario for the rapid spread of CPV. Effective commercial modified live virus vaccines that vary in the genotype (CPV type 2 [CPV-2] and CPV-2b) of CPV in the vaccine are available. There is currently no commercial vaccine with CPV-2e in the vaccine. However, induction of active immunity in puppies is blocked by maternal immunity in the puppies (18). The stability of CPV in the kennel environment and excretion of large amounts of CPV by sick puppies can expose susceptible puppies to massive infectious doses of CPV. This CPV susceptibility window coincides with weaning in puppies in the age group of about 6 to 8 weeks old. Eight weeks is the age when the largest number of puppies succumb to CPV. Moreover, there are variations in the decay of antibodies and induction of active immunity after vaccination directed by the genetic makeup (canine major histocompatibility antigens) of the puppies.

It would be clinically useful if there were diagnostic tests that could detect the amount of CPV in a sample, genotype the virus, and quantify the antibodies against different CPV subtypes quickly in the kennel environment. With this goal, we have developed and validated instant CPV antigen and antibody tests (slide agglutination test [SAT] for CPV antigen and slide inhibition test [SIT] for CPV antibody in serum). These tests are instant (real time), sensitive, quantitative, and generic for all the CPV types. We are confident that these safe, economical, and rapid tests will encourage timely use of the vaccines based on antibody decay in puppies and help manage outbreaks of CPV in kennels after an outbreak with minimum training of the personnel. There are a few tests that have been used for rapid detection of CPV in fecal samples and CPV antibodies. These tests include an immunochromatography assay (15), latex agglutination test (1, 20), and coagglutination test (21).

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MATERIALS AND METHODS

Clinical samples. All the samples that were submitted to the Oklahoma Animal Disease Diagnostic Laboratory (OADDL) were from puppies that had a history of vomiting and diarrhea. These animals were suspected to have canine parvovirus. Most animals had a history of hemorrhagic diarrhea, and a few had yellow diarrhea with mucus. Fecal samples and intestinal tissue samples from CPV suspect dogs were prepared as 10% (wt/vol) suspensions in phosphate-buffered saline (PBS) (pH 7.2) for this study. A total of 23 clinical field submissions (intestinal contents/feces/intestinal tissue homogenates) were evaluated. In addition, cell culture supernatants (n = 60) from dogs for which the CPV status was known based on conventional tests were also tested. The CPV status of all the samples used in development of this assays was confirmed by conventional assays, such as hemagglutination (HA) test and virus isolation followed by HA test for CPV quantification. The PCR (6) genotyping was done as described before (9).

HA test. The hemagglutination test was performed as described by Carmichael et al. (3). The samples were serially diluted twofold in PBS (0.2 M) in V-bottom plates. First, 50 microliters of PBS was added to each well of the plate. In the first column, 50 μl of sample (fetal suspension or cell culture supernatant) was added. The sample was mixed five times, and 50 μl of PBS was added to the next well. Each sample was diluted from 1:2 through 1:4,096. Then, 50 μl of PBS was added to each well. The HA test was performed using porcine erythrocytes (0.5%). The corners of the plate were tapped four or five times to mix the erythrocytes. The plates were covered with lids and incubated at 4 to 7°C for 2 to 4 h. Positive agglutination was indicated by mat formation, and the button indicated lack of

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agglutination. The titer was calculated as the reciprocal of the last well with agglutination. After overnight incubation, some positive samples can elute.

SAT. For the slide agglutination test, the conditions of the test were standardized to obtain agglutination results in 30 to 60 s of mixing the reaction mixture components. The buffer was the same as for the HA test, PBS (0.2 M PBS [pH 7.2]). Cooled glass plates (catalogue number M 6225; American Scientific Products) were kept in the freezer compartment of the refrigerator, cleaned, and ready to use. Each plate had 30 circles. For the assay, each plate was wiped with a paper towel to remove the moisture and kept on Styrofoam to keep it cool during the procedure. Twenty microliters of unknown sample was added as a drop on the plate. To each well, 20 μl of porcine erythrocytes (2.5% [vol/vol]) suspended in 0.2 M PBS with 2% fetal bovine serum (FBS) were added as a separate drop in the circle. The total volume was made up to 50 μl with 10 μl of 0.2 M PBS as the third drop. Three drops were mixed with a wooden toothpick in a circular motion for 30 s. CPV-positive samples produced agglutination within 1 minute. Negative samples were homogeneous and showed no agglutination. However, all samples were further incubated in the refrigerator for an additional 5 min before the results were recorded and confirmed microscopically. CPV-positive samples showed large clumps of agglutination (Fig. 1), and CPV-negative samples showed single erythrocytes homogeneously spread in the well (Fig. 2). Partial agglutination was microscopically confirmed with smaller clumps of porcine erythrocytes. Weak CPV-positive samples can take up to 3 min to agglutinate in the refrigerator. For determination of the amount of the virus, the CPV-positive samples were diluted twofold in a U-bottom well plate (Linbro/ Titertek) (96-well U-bottom plate) (ICN Biologicals, Inc., Aurora, Ohio). Using the SAT procedure, the results were recorded as agglutination, no agglutination, and partial agglutination. The dilution of the sample that showed partial agglutination was recorded as 1 hemagglutinating unit (HAU). This convention and calculation were adopted from the hemagglutination inhibition assays for CPV.

The dilution that contained 1 HAU was divided by 8 to calculate the dilution within 1 minute. Negative samples were homogeneous and showed no agglutination. After overnight incubation, some positive samples can elute.

Virus isolation. CPV was isolated from clinical samples submitted to the OADDL. Intestinal tissue samples and fecal samples were used. Before inoculation, the samples were processed in two ways. One set was diluted to 10% (vol/vol) in PBS, centrifuged to remove the particulate material, filtered through a 0.2-μm filter to remove the bacteria, and about 1 ml was inoculated within 1 hour of freezing the Crandall Reese feline kidney (CRFK) cell line. The second set was extracted with an equal volume of chloroform and vortexed for 1 min. The samples were centrifuged for 10 min, and the clear supernatant was inoculated on cells after filtration through a 0.2-μm filter. The cells were plated at a density of 40%. After 1 h of inoculation, minimum essential medium containing 5% fetal calf serum was added. The cells were observed daily for 6 days postinoculation for cytopathic effects, such as rounding and detachment. The cells were freeze-thawed and centrifuged and checked for CPV by hemagglutination test using porcine erythrocytes (0.5%).

RESULTS

For standardization of SAT, three porcine erythrocyte concentrations were tried, 0.625%, 1.25%, and 2.5%. For all the assays, equal amount of blood from two different pigs was collected in Alsever’s solution and used within 1 week. The clarity and visibility of erythrocyte clumps based on their size were used as the criteria for selecting the erythrocyte concentration. Based on an experiment using a CPV-positive sample and a CPV-negative sample, 2.5% porcine erythrocytes gave the clearest positive and negative results, and this concentration was used throughout the tests. Three different final volumes of reaction mixture were tried, 30 μl, 40 μl, and 50 μl per circle of the glass plate. We found that the final reaction mixture volume of 50 μl was the most suitable for the well size (circle) in our experiment. Our plates had circles with an internal diameter of 1.9 mm. This decision was made based on the amount of liquid that completely filled the plate circle without spilling outside during mixing with toothpicks using circular motion. We studied the effect of the FBS concentration in each PBS buffer on the size of the clumps and visibility of the agglutination reaction using three different concentrations of FBS, 1%, 2%, and 3%. We found that 2% FBS was suitable for the SAT. The final reaction mixture contained 20 μl of the
sample suspected of CPV, 10 μl of buffer (0.2 M PBS [pH 7.2], 2% FBS) 1, and 20 μl of 2.5% porcine erythrocytes.

We performed all the tests using reagents that were stored in a refrigerator and chilled on wet ice in a Styrofoam bucket. The solutions were kept chilled during use. The ambient temperature of the virology laboratory was set at 72°C and thermostatically controlled. However, the test also performed well on a brucellosis card test with teardrop-shaped wells with chilled reagents. A total of 23 chloroform-extracted fecal suspensions and 60 cell culture supernatants were tested by the SAT. The correct CPV status (positive/negative), CPV genotype if positive, and titer of the virus as determined by conventional plate HA was known for all samples. Further, virus isolation was performed on all the fecal samples on the CRFK cell line. After virus isolation, the amount of the CPV in cell supernatant was determined by the plate HA test for CPV. We found a very high correlation (100% positive/negative status for CPV) between the SAT results with conventional hemagglutination test (Fig. 3). Only samples with hemagglutination titers on the plate HA test equal to or below 40 were found negative in the SAT. A plate HA titer of 1:40 is considered negative. Thus, the SAT was found to be slightly less sensitive but more accurate in classifying the fecal samples for CPV status. The CPV samples that are less than 1:40 do not react in the SAT. When the endpoint titers for the same sample by SAT and conventional plate HA tests were compared, the sensitivity of SAT was lower than that for the standard hemagglutination test. However, the SAT classifies the fecal samples correctly on every clinical sample (n = 23) tested. A total of 60 cell culture samples were tested. Of these, 26 were positive by the conventional plate HA test and 34 were negative. The SAT missed six positive cell culture samples. Thus, for cell culture-propagated CPV isolates, we found a lower sensitivity by the SAT assay. This variation is probably due to differences in suitable pH requirement (pH 6.5 to 7.2) for carnivore parvoviruses propagated in cell culture (13).

In the SIT, the presence of antibody was indicated by inhibition of erythrocyte agglutination with hyperimmune serum against CPV-2b. We tested CPV-2b (n = 1), CPV-2c (n = 4), and a raccoon parvovirus isolate in the SIT assay. The SIT titers of hyperimmune serum were much higher for homologous CPV-2b isolate than for heterologous CPV-2c isolates. When the hyperimmune anti-CPV-2b serum was used against CPV-2c (sample 07080441), the SIT titer was 1:512. However, when CPV-2b (sample 07080274) was used as virus antigen, the same hyperimmune anti-CPV-2b serum gave an SIT titer of 1:4,096. Moreover, one raccoon parvovirus isolate (sample 08080274) was not inhibited by the standard CPV-2b hyperimmune serum, indicating that it was antigenically different from CPV isolates.

**DISCUSSION**

Canine parvovirus is the number one cause of viral enteritis and is responsible for significant morbidity and mortality (8). Due to the rapid evolution of CPV, monoclonal antibody-based diagnostic tests for field use need to be updated and evaluated for sensitivity against the current variants of CPV in the United States (CPV-2, CPV-2a, CPV-2b, and CPV-2c) (7, 9, 14). From February 2006 to October 2008, a total of 163 CPV samples have been genotyped at the OADDL. Of these samples, 13 were CPV-2, 82 were CPV-2b, 67 were CPV-2c, and 1 was mixed CPV-2b and CPV-2c (9; S. Kapil, unpublished data from OADDL records). There are other reports of mixed CPV-2 infections. Moreover, the cross species transmission of CPV or related viruses further threatens the sensitivity of the monoclonal antibody-based animal slide tests that are commercially available for field use. In this study, we have developed generic CPV detection tests for CPV antigen detection in the feces and CPV antibody quantification in serum.

Lateral flow immunoassay (LFA) is a convenient format for front line diagnostics of viruses and antibodies. These tests are...
specific, sensitive, and easy to use and require minimum training. LFA can be developed for both antigen and antibody detection. However, they require at least one monoclonal or polyclonal antibody to manufacture the test kit. The only limitation of the LFA is that these assays can be cost prohibitive for routine use when very large numbers of samples have to be tested in kennel situation. Moreover, LFAs are qualitative or semiquantitative but still very useful screening diagnostic tools.

Enzyme-linked immunosorbent assays for both CPV antigen and antibody have been developed (12, 15). However, enzyme-linked immunosorbent assays with even short incubation times (5 to 10 min) require multiple washing steps and can be cost prohibitive for high volume use by kennel breeders for CPV antigen and antibody detection.

Agglutination assays have been tested for CPV and have traditionally used latex beads coated with monoclonal antibodies (20, 22). These assays have been found to be useful for CPV antigen detection. There is one report of latex beads for antibody detection (1). However, these assays are not commercially available in the United States, and agglutination tests have been evaluated in research laboratories (21). Moreover, continuous evolution of CPV will affect the usefulness of latex bead assays. There is a need for pan-CPV tests that can detect all genotypes and antigenic variants of CPV for field use.

In this study, we have used the intrinsic property of CPV-2 to agglutinate porcine erythrocytes and modified it to develop very rapid, generic, economical assays for CPV antigen and antibody measurements. The only potential limitation of our assay is the need to bleed a pig to obtain erythrocytes. However, it can be solved by properly fixing the swine erythrocytes that will provide a longer shelf life at room temperature (11). We have done a preliminary trial with formalin-fixed swine erythrocytes. We found that 4% formalin fixation can adversely affect the performance of the SAT for CPV detection (S. Y. Marulappa and S. Kapil, unpublished data). Thus, we are trying other fixatives to stabilize the porcine erythrocytes for the SAT. Fresh swine erythrocytes can be used for only 1 week and require refrigeration. However, for hemagglutinating viruses, such as CPV, SAT-SIT can be a very cost-effective alternative to field technologies, such as LFA.

We found these twin assays, SAT and SIT, to be very useful for field applications for the management of CPV outbreaks in kennels. The SIT assay can be used to refine the time of vaccination for CPV puppy shots. For example, if the puppy has high levels of maternal antibodies, the puppy can be vaccinated later. However, if there is no antibody titer, the puppy can be vaccinated after the negative or low CPV antibody check. CPV antigen and antibody monitoring using easy and economic assays will lead to better and timely CPV vaccination compliance and more effective take of the CPV vaccine antigens. The SAT assay can also be used to check the environmental swipes/swabs for CPV contamination quickly, and if CPV is present, the area can be cleaned and disinfected. The SAT assay can be used to verify the efficiency of decontamination of an area. We found both assays to be practical for kennel use. Because the reagents (PBS and porcine erythrocytes) are commonly available, the assays can be used in developing countries for CPV monitoring. The cost per test is very low, and all animals can be repeatedly checked for antibodies in serum against CPV. The conventional CPV HA test takes 2 to 4 h, and we have transformed the hemagglutination assays for CPV antigen and antibody into rapid and easy formats of SAT-SIT assays. Similarly, tests for the other significant hemagglutinating viruses, such as influenza A virus and severe acute respiratory syndrome coronavirus, can also benefit from modification to SAT-SIT format assays using the buffer conditions and erythrocyte conditions compatible with those viruses. Thus, SAT-SIT can replace the conventional slide hemagglutination tests as quick screening tests. SAT-SIT technology will be useful for any situation where rapid, low cost, low tech screening is needed for any hemagglutinating virus in outbreak situations in developing countries.

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