Evaluation of enzyme-linked immunosorbent assays based on monoclonal antibodies for the serology and antigen detection in canine parvovirus infections[1]

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SUMMARY *An enzyme-linked immunosorbent assay (ELISA) system was developed for the detection of canine parvovirus (CPV) or CPV antigen in dog faeces and two other ELISA systems were developed for the detection of CPV-specific antibodies in dog sera. The ELISA's were based on the use of CPV-specific mouse monoclonal antibodies, which recognise different epitopes of the haemagglutinin of CPV and which also neutralise the virus. A double antibody sandwich (DAS) ELISA for the detection of CPV in dog faeces was compared with the haemagglutination (HA) test. The DAS-ELISA proved to be more specific, sensitive and easier to perform than the HA assay. An indirect ELISA and a competitive ELISA for the detection of CPV-specific antibodies in dog sera were compared with the haemagglutination inhibition (HI) test.*

Both ELISA systems proved te be specific and easy-to-use methods for the detection of CPV-specific antibodies. The indirect ELISA, specially, proved to be more sensitive than the HI test.

The higher sensitivity and specificity of the ELISA's as compared to HA and HI tests, and their ease of use, make them suitable for routine use in the serology and diagnosis of CPV infections.

INTRODUCTION

Since the identification in 1978 of canine parvovirus (CPV) as a major causative agent of acute gastrointestinal and cardiac disease in the dog (1, 10), several methods have been developed for the diagnosis of CPV infections in dogs. For the demonstration of CPV or CPV antigen in faecal samples, haemagglutination (HA) assays, electronmicroscopy (EM) and virus isolation in cell cultures proved te be useful procedures (3, 4, 6). For the quantitation of CPV-specific antibodies, virus-neutralisation (VN) and haemagglutination-inhibition (HI) assays have been commonly used (4, 14). Recently, enzyme-linked immunosorbent assays (ELISA) have been described for the detection of CPV-specific antibodies in serum and for the detection of CPV antigen in faeces (5, 7, 8, 13).

[1] This work was supported by the 'Bond tot Bescherming van Honden', The Hague, The Netherlands, and by a research fund from the National Veterinary Institute, Uppsala, Sweden.

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The present paper describes the evaluation of three different ELISA systems for the detection of CPV antigen or CPV-specific antibodies, based on CPV-specific monoclonal antibodies (MoAbs), using a collection of faecal and serum specimens from kennels with problems of acute diarrhoea and from a specific-pathogen-free (SPF) dog colony. The results were compared with those obtained in HA and HI tests, respectively.
The ELISA procedures proved more specific, more sensitive and more suitable for incorporation in large-scale screening procedures than the HA and HI assays.

MATERIALS AND METHODS

CPV antigen

The continuous canine cell line A-72 (2) was cultivated in Eagles medium supplemented with Hanks salts, lactalbumin hydrolysate (5.23 gr/l), natrium carbonate 350 mg/l, glutamine 0.002 M, 100 IU penicillin/ml, 100 μg streptomycin/ml and 10% foetal calf serum in 150-cm^2 monolayer cultures at 37°C. Complete monolayers were trypsinised, washed and resuspended in pH 7.4 phosphate-buffered saline (PBS). The resuspended cells were infected with CPV (strain C780916, kindly supplied by Dr. L. E. Carmichael, Cornell University, Ithaca, New York, USA) at a multiplicity of infection of about 10^{-3} $TCID_{50}$/cell. The cell-virus suspension was incubated for 90 min at 37°C and after low speed centrifugation the cells were suspended in culture medium and cultivated at 37°C. Six to seven days after infection the cultures exhibiting complete cytopathic changes were frozen and thawed twice. The CPV suspension (HA titre $\geqslant$ 512) was clarified by centrifugation at 10,000 x g for 20 min. This preparation was used as non-concentrated CPV antigen. It was subsequently concentrated 50 to 100 times by centrifugation for two hours at 135,000 x g. The virus pellet was resuspended in 0.05 M sodium carbonate buffer, pH 9.6, sonicated and clarified by low-speed centrifugation. The supernatant was stored at – 70°C until used as concentrated CPV antigen preparation.

Mouse monoclonal antibodies and antibody conjugates

BALB/c mouse hybridomas producing MoAbs reactive with the haemagglutinin of CPV, which also neutralised the virus, were generated as previously described (12). Mouse ascitic fluids were produced in syngeneic BALB/c mice. For the tests described below, two MoAbs were selected which recognised two different epitopes as demonstrated in a competitive ELISA system (10): CPV H-1 (IgG2a) and CPV H-2 (IgG2b). The immunoglobulin fraction of the ascitic fluids were purified by affinity chromatography on a protein-A sepharose column (Pharmacia, Uppsala, Sweden). For the preparation of antibody conjugates, antibodies were coupled to horse-radish peroxidase (HRP) (type VI, Sigma Chemical Company, St. Louis USA) by the periodate method (9).

Faecal and serum samples from dogs

Faecal and serum samples were collected through veterinary practitioners and clinics from dogs with a history of acute gastrointestinal disease in the open populations in the Netherlands and in Sweden and from dogs in a SPF colony in the Netherlands. Ten percent weight per volume faecal samples from dogs in kennels with problems of acute diarrhoea were prepared in PBS, pH 7.4. Debris was allowed to settle for one hour at 20°C and the supernatant fluids were used as samples in the respective tests. All samples were stored at – 20°C until use.

Haemagglutination (HA) and haemagglutination-inhibition (HI) tests

HA and HI tests were performed in 96 wells of round-bottomed microtitre plates as previously described (4, 6, 10) using African green monkey *(Cercopithecus aethiops)* or porcine erythrocyte suspensions. Specificities of HA reactions for the detection of CPV antigen in faecal samples were confirmed by inhibiting the reaction with a specific serum in alle positive cases.
Serum samples with HI titres $\leqslant$ 20 were considered to be negative.

Enzyme-linked immunosorbent assays (ELISA)

Double antibody sandwich (DAS) ELISA for the detection of CPV antigen

Microtitre plates (Nunc, Denmark) were coated with 250 ng of each of the two Protein-A purified MoAbs H-1 and H-2 in 0.1-ml volumes in 0.1 M carbonate buffer, pH 9.6, for 16 hours at 4°C. After washing with demineralised water containing 0.05% Tween 80 (Merck, Schuchardt, FRG) (DWT), plates were blocked with PBS containing 0.05% Tween 80, 1% bovine serum albumin (BSA) (Organon Teknika, Oss, The Netherlands) and 0.1% NaN_3.

The plates were sealed and stored at 4°C until use. Plates were washed with DWT and 50-μl volumes of 10% weight per volume suspensions of faecal samples were added simultaneously with 50 μl of a mixture of HRP-conjugated MoAbs H-1 and H-2, each at a dilution that has been proven to be optimal for the detection of CPV antigen. After incubation for 30 min at 20°C, the plates were washed twice and 100-μl volumes of substrate solution (0.1 mg/ml tetramethyl benzidin (TMB) and 0.003% H_2O_2 in 0.1 M NaAC buffer, pH 5.5) were added to each well. After incubation for 10 min at 20°C, 100-μl volumes of 2 M H_2SO_4 were added to stop the reaction. The absorbance was read in a Titertek Multiscan (Titertek, Flow Laboratories). Samples giving absorbance at 450 nm (A450 nm), exceeding three times the background values obtained with a panel ($n = 34$) of proven negative dog faecal samples, were considered to be positive (A450 nm > 0.2).

Indirect ELISA for the detection of CPV-specific antibodies

Microtitre plates (Type III, Titertek, Flow Laboratories) were coated with 250 ng of each of the two protein-A purified MoAbs H-1 and H-2 in 0.1-ml volumes in 0.1 M carbonate buffer, pH 9.6, for 16 hours at 4°C. Plates were washed twice with DWT and blocked with PBS containing 0.05% Tween 80 (Merck, Schuchardt, FRG), 1% (weight per volume) BSA (Organon Teknika, Oss, The Netherlands) and 0.1% NaN_3 (PBS-TB). The plates were then stored at 4° C until use. Prior to use, the plates were again washed twice as described above and incubated with 100-μl volumes of non-concentrated CPV antigen (HA $\geqslant$ 512) or with control antigen (culture supernatant of non-infected A-72 cells) for one hour at 37°C. After washing, 100-μl volumes of threefold dilutions of serum samples in PBS-TB were added per well, with a starting dilution of 1: 50, and the plates were incubated for one hour at 37°C. The plates were washed with DWT, 100-μl volumes of a goat anti-dog IgG horseradish peroxidase conjugate (Cappel, Cooper Biomedical, Malvern, USA) dilution were added and the plates were incubated for one hour at 37°C. Then 100-μl volumes of TMB-substrate solution were added to each well. After 10 min at 20°C the colour reaction was stopped by adding 2 M H_2SO_4. The A450 nm was read in a Titertek Multiscan (Titertek, Flow Laboratories). Serum titres were given as the reciprocals of the dilutions still giving more than three times the background absorbance values, obtained on control antigen-coated plates.

Competitive ELISA for the detection of CPV-specific antibodies

Microtitre plates (Nunc, Denmark) were coated with concentrated CPV antigen at a dilution of 1: 300 in 0.1 M carbonate buffer, pH 9.6, for 16 hours at 4°C. The plates were washed with DWT and blocked with PBS-TB, and 50-μl volumes of fivefold serial dilutions of serum samples were added, with a starting dilution of 1: 10, together with 50 μl CPV-specific MoAbs H-1 and H-2 conjugated to HRP at a dilution giving 75% of maximum binding.

After incubation for one hour at 37°C the plates were washed with DWT and developed using the TMB-substrate, and the A450 nm was read as described above. Percentage inhibition was calculated according to the formula

$$\% \text{ inhibition} = \frac{\text{A450 nm not inhibited - A450 nm inhibited}}{\text{A450 nm not inhibited}} * 100$$

Dilutions of serum samples giving more than 50% inhibition were considered to be positive.

RESULTS

Comparison of HA and double antibody sandwich (DAS) ELISA

To compare the HA and the DAS-ELISA for the detection of CPV antigen in faeces from dogs with problems of acute diarrhoea, 147 samples were tested in both assays (Fig. 1). All except two of the 79 HA-negative faecal samples were also negative in the DAS-ELISA. The two discrepant samples showed low values in the DAS-ELISA (A450 nm = 0.4). All of the 34 HA-positive samples that could be confirmed with a CPV-specific antiserum in the HI test also proved to be positive in the DAS-ELISA. Samples giving high titres in the HA generally showed high A450 nm values in the DAS-ELISA too, whereas low-titre samples also showed low A450 nm values. All of the 34 HA-positive samples that could not be confirmed to be specific with a CPV-specific antiserum in the HI test and therefore should be considered 'false positives', were indeed negative in the DAS-ELISA.

Comparison of HI test and indirect ELISA

To compare the indirect ELISA and the HI tests for the detection of CPV-specific antibodies a total of 96 serum samples were tested in both tests (Fig. 2). Of 27 HI negative samples (titre $\leqslant 10$), 10 were also negative in the indirect ELISA (titre $\leqslant 50$). Most of the remaining serum samples showed relatively low antibody titres in the indirect ELISA ($\leqslant 450$). Of the 69 HI-positive samples, all were also positive in the indirect ELISA. The overall correlation between samples found positive or negative in both assays was 82%. The correlation between antibody titres in all the sera found in both assays proved to be good (correlation coefficient 0.77). However, titres found in the ELISA were about 50 times higher than those found in the HI assay.

Comparison of HI test and competitive ELISA

To compare the competitive ELISA and the HI tests for the detection of CPV-specific antibodies, a total of 116 serum samples were tested in both tests (Fig. 3). Of 46 HI-negative samples, 42 were also negative in the competitive ELISA.

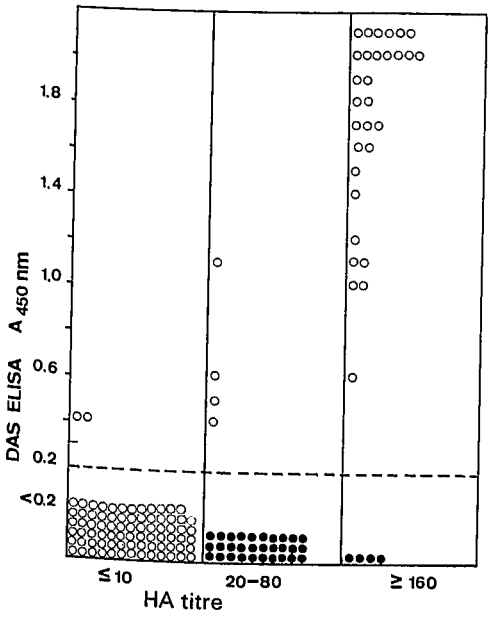

Fig. 1. Detection of CPV in dog faeces by HA and DAS-ELISA (n = 147). The specificity of the HA reactions was tested by inhibiting the haemagglutination with a CPV-specific antiserum. Faecal samples with HA titres $\geqslant 20$ that could not be confirmed in the HI test are represented by closed symbols (•). The dashed line indicates the A450 nm cut-off value for positive DAS-ELISA score.

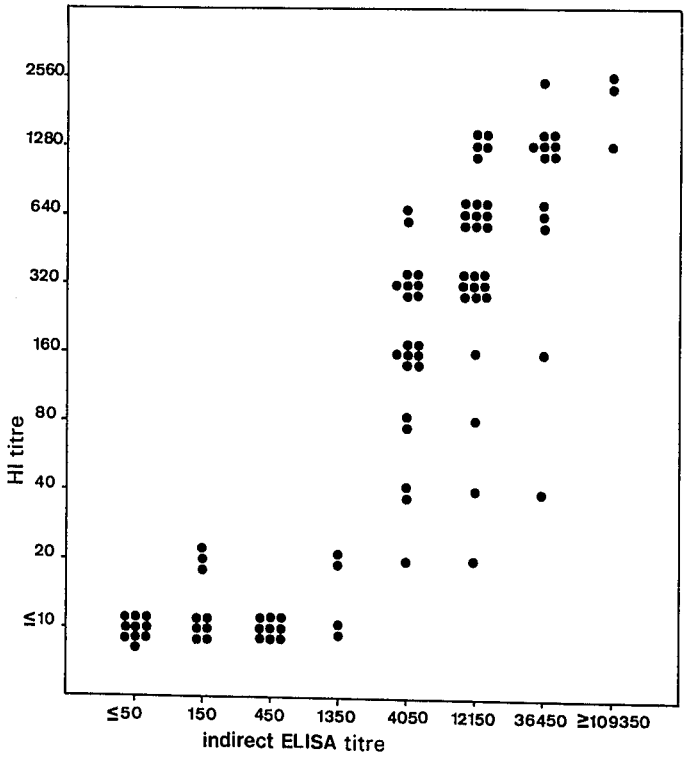

Fig. 2. CPV-specific antibody titres in dog sera measured by HI and indirect ELISA (n = 96).

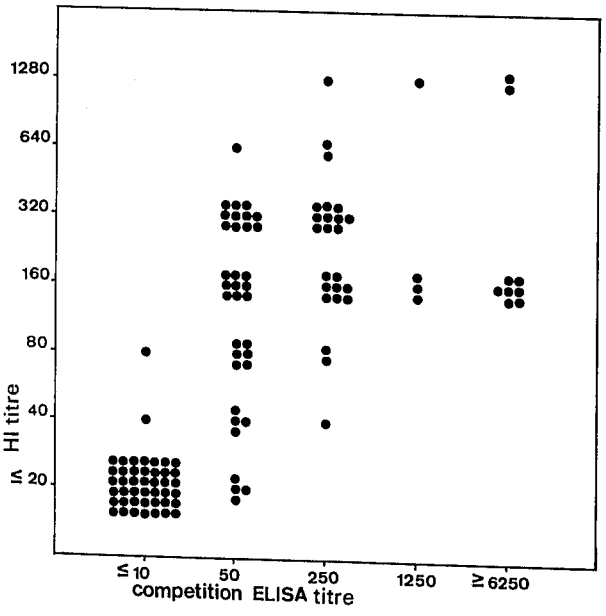

Fig. 3. CPV-specific antibody titres in dog sera measured by HI and competitive ELISA (n = 116).

The four remaining samples showed relatively low competitive ELISA antibody titres ($\leqslant 50$). Of the 70 HI-positive samples, 68 were also positive in the competitive ELISA. The two discrepant samples showed the relatively low titres of 40 and 80 in the HI, respectively. The overall correlation between positive and negative results in both assays was 95%.

DISCUSSION

For the diagnosis of CPV infections in dogs, the demonstration of CPV or CPV antigen in faeces is the most commonly used method. Besides virus isolation and negative contrast electron microscopical procedures, which are generally too expensive and time-consuming to perform, the HA method is usually considered the method of choice for this purpose. The major disadvantages of this test are its low specificity, which necessitates the parallel use of a confirmative HI test, and the fact that the test is laborious, time-consuming and difficult to incorporate in automated screening systems. The DAS-ELISA does not suffer from these disadvantages, and, especially since a combination of anti-CPV MoAbs was used, a high specificity was likely. Also, the risk of false-negative test results caused by naturally occurring variants of the virus (11) not recognised by the MoAbs used is minimised in this assay, since two MoAbs directed against two different well-conserved epitopes (12) were used in the assay.

From the present study it cannot be concluded whether the two samples showing discrepant results (out of 147) were false negative in the HA or false positive in the DAS-ELISA. It is interesting to note that the values found with these samples were the lowest found in the DAS-ELISA. Other confirmatory tests like EM and virus isolation were not used to confirm their specificity, since these have been shown to exhibit little correlation with HA and ELISA (13).

Although the value of CPV serology for diagnostic purposes is limited, it may be of special value in determining the level of maternally derived CPV-specific antibodies in pups and the optimal moment for vaccination, since the presence of maternal antibodies seems to be a major problem in the vaccination strategy against CPV. For this purpose the HI test has been most commonly used. However, this test suffers in principle from the same disadvantages mentioned for the HA assay. Therefore, we compared this technique with two different ELISA systems, employing CPV-specific MoAbs. Both these tests, but especially the indirect ELISA, proved to be more sensitive than the HI test, whereas these tests also might have had a higher specificity because of the use of the CPV-specific MoAbs.

Because it was shown with the samples exhibiting titres $\geqslant 4050$ in the indirect ELISA that the sensitivity of this test was about 50 times higher than the sensitivity of the HI test, it could be expected that low-titre sera in the indirect ELISA would score negatively in the HI. This was indeed the case: 15 samples with indirect ELISA titres $\leqslant 450$ scored $\leqslant 10$ in the HI test. The four samples with indirect ELISA titres of 1350 had HI titres of 10 or 20. A third test to confirm the specificity of these discrepant samples, such as immunoblotting or IFA, was not considered, since these tests are not more sensitive than the HI test (unpublished observation).

Perhaps the major advantage of the ELISA systems used is that they may be incorporated in automated screening systems or in test-kit procedures. This may prove to be of particular interest for direct diagnostic use, but also for the development of rapid on-site tests for the detection of maternal antibodies in pups to determine the optimal moment of vaccination.
Thus both the DAS-ELISA for the detection of viral antigen in faeces and the ELISA's for the detection of CPV-specific antibodies in serum may prove valuable and reliable tools for diagnostic purposes and be helpful in the management of CPV infections in kennel situations.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. C. Kruyssen and Ms. M. Eskens for preparing the manuscript.

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