Evaluation of a dot ELISA kit for measuring immunoglobulin M antibodies to canine parvovirus and distemper virus

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A dot ELISA for the detection of immunoglobulin M (IgM) antibodies to canine distemper virus (CDV) and canine parvovirus (CPV) was assessed. The titres of IgM antibodies to CDV and CPV in 100 dogs were measured by the Immunocomb ELISA kit and compared with the results derived from the immunofluorescence assay (IFA). There was a strong correlation between the results of the dot ELISA technique and the IFA ($P<0.001$). The dot ELISA kit was also used to assess the changes in the levels of immunoglobulin G (IgG) and IgM antibodies to CPV and CDV in 10 puppies vaccinated with a polyvalent vaccine. High levels of IgM antibodies to CPV were first detected seven days after they were vaccinated, and after nine days all the pups had high titres of IgG antibodies to CPV. High levels of IgM antibodies to CDV were detected after nine days and the highest average titres were recorded after 12 days. IgG antibodies to CDV were present from nine days after vaccination.

PARVOVIRUS disease and distemper are the most important contagious viral diseases of dogs and certain other carnivores worldwide, and both are often accompanied by high mortality. Distemper is caused by canine distemper virus (CDV), a morbillivirus of the paramyxovirus family, and parvovirus disease is caused by canine parvovirus (CPV) type 2 (Appel and Summers 1999).

The serodiagnosis of acute distemper is usually based on the detection of a significant rise in the titre of specific immunoglobulin G (IgG) antibody in paired serum samples taken sufficiently far apart, usually at an interval of two weeks (Blixenkrone-Moller and others 1991). However, in many fatal cases of distemper, no detectable serum-neutralising antibodies can be found (Appel 1969). Furthermore, when a significant rise in antibodies is detected, the result may already have lost its clinical significance (Barben and others 1999).

Antibodies of the immunoglobin M (IgM) class are produced early in viral infections, and their presence indicates current or recent viral multiplication. The diagnostic potential of assaying IgM antibodies has been proposed by Florent (1986), Blixenkrone-Moller and others (1991), Barben and others (1999), von Messling and others (1999) and Griot-Wenk and others (2001), but the assay of IgM antibodies to CDV and CPV is costly and time-consuming, and the techniques are not easily available to the diagnostician. The availability of a simple ELISA kit for use under clinical conditions would enable clinicians to base their diagnosis on a single serum sample, at an early stage of the disease.

Previous studies have shown the dot ELISA technique to be a reliable semi-quantitative test for the detection of IgG antibodies to CPV and CDV (Waner and others 1996, 1998). In this study a dot ELISA test kit (Immunocomb; Biogal Laboratories) has been evaluated for its potential to detect IgM antibodies to CPV and CDV semi-quantitatively.

MATERIALS AND METHODS

Sera

Two groups of serum samples were used. The first consisted of samples taken from 100 dogs from small animal clinics throughout Israel. Blood samples were collected from the cephalic vein, allowed to clot and then separated by centrifugation; the sera were stored at –20°C until tested. These samples were assayed by the immunofluorescence antibody test (IFA) and by the dot ELISA method for IgG and IgM antibodies to CPV and CDV, and the correlation between the two techniques was assessed.

The second group of samples was used to assess the IgM and IgG response of dogs after they had been vaccinated against CPV and CDV. Blood samples were taken from 10 pups from three litters: four of the pups were cavalier King Charles spaniels, four were miniature dachshunds, and two were of mixed breed. The pups were vaccinated at eight weeks of age with a commercially available polyvalent vaccine (Duramune DA_2LP+PV; Fort Dodge) containing CDV, adenovirus types 1 and 2, parainfluenza virus, CPV and *Leptospira* bacterin (*Leptospira* Icterohaemorrhagiae and *Leptospira* Canicola). Samples were taken from the pups just before they were vaccinated and two, seven, nine, 12, 16, 23 and 30 days later. These samples were assayed for IgG and IgM antibodies to CDV and CPV by the dot ELISA method only.

Serological assays

IFA The IFA tests were carried out on coded samples, using 10-well Teflon-coated, autoclaved slides (Erie Scientific). Cells infected with the virus were cultured directly on the slides in a moist tissue culture incubator, until about 20 per cent of the monolayer was infected. The slides were then rinsed with phosphate-buffered saline (PBS), air-dried, fixed in cold acetone, air-dried and stored at –85°C. For the IFA for CPV the Crandell feline kidney cell line (CrFK) was infected with virulent CPV-2b virus isolated from the faeces of an infected dog. For the IFA for CDV the Vero African green monkey kidney cell line was infected with the Onderstepoort strain of CDV.

The IFA procedure was carried out as follows: five-fold serial dilutions of serum in PBS were prepared, starting at 1:10; 25 µl of each dilution was placed in an appropriate well and the slides were incubated at 37°C in a moist chamber for 30 minutes. At the end of the incubation the slides were rinsed with PBS, washed twice in PBS, rinsed with distilled water, and air-dried. For the IgG assay, 25 µl of a 1:100 dilution of a fluorescein isothiocyanate-conjugated rabbit anti-dog IgG (FITC anti-dog IgG, H+L chain; Jackson ImmunoResearch Laboratories) was used as a secondary antibody. For the IgM assay, 25 µl of a 1:50 dilution of a fluorescein isothiocyanate-conjugated goat anti-dog IgM (FITC-anti dog IgM, µ-chain specific; Bethyl Laboratories) was used as a secondary antibody. The slides were again incubated at 37°C in a moist chamber for 30 minutes and then washed as described above. The slides were examined under a fluorescent microscope. The titre of antibody was defined as the highest serum dilution in which specific fluorescence was detected in infected cells.

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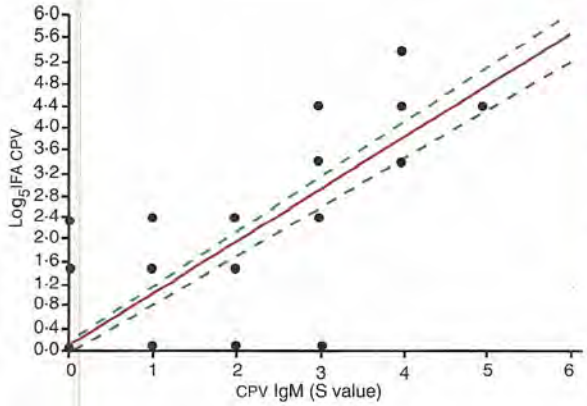

FIG 1: Correlation and 95 per cent confidence limits for the relationship between the serum immunoglobulin M (IgM) antibody titres to canine parvovirus (CPV), measured by immunofluorescence and by dot ELISA

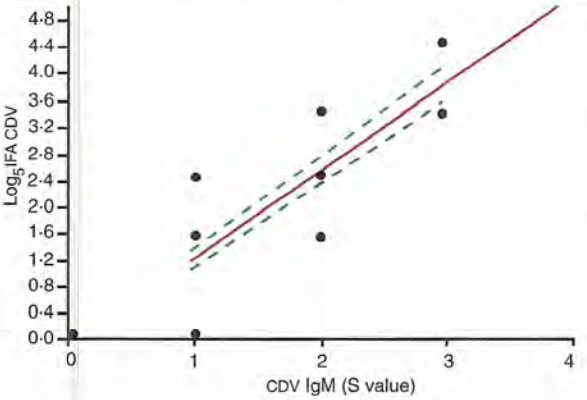

FIG 2: Correlation and 95 per cent confidence limits for the relationship between the serum immunoglobulin M (IgM) antibody titres to canine distemper virus (CDV), measured by immunofluorescence and by dot ELISA

Dot ELISA assays The assays for IgG antibodies to CPV and CDV were carried out with the Immunocomb dot ELISA (Biogal Laboratories) as described by Waner and others (1996, 1998), the same basic procedure being used for the antibodies to both viruses. Antigen for the kits was purchased commercially and consisted of complete viral antigen derived from tissue culture (VMRD). Strain f-11 of CPV-2a was cultured on CrFK cells and maintained in serum-free medium. The Rockborn strain of CDV was cultured on NBLDL cells in serum-free medium. The antigens were concentrated from cell-culture fluids and precipitated with polyethylene glycol.

In brief, for the dot ELISA IgM assay, the sera were diluted 1:36 in buffer and incubated with the antigen spots for 20 minutes. After washing to displace unbound antibodies, the combs were allowed to react for 20 minutes with goat anti-dog IgM whole molecule alkaline phosphatase conjugate (Bethyl Laboratories). After two successive washing steps of two minutes each, the bound antibodies were detected after 10 minutes' incubation with a precipitating chromogen, 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (Biosynth International). The concentration of IgM antibodies to CPV and CDV in each sample was determined by using the CombScale method with the colour scale provided with the kit. The colour reactions were converted to concentrations by comparing each spot with the colour reaction of a spot from a known positive pretitrated serum sample. The results were expressed as S units on a scale from 0 to 6.

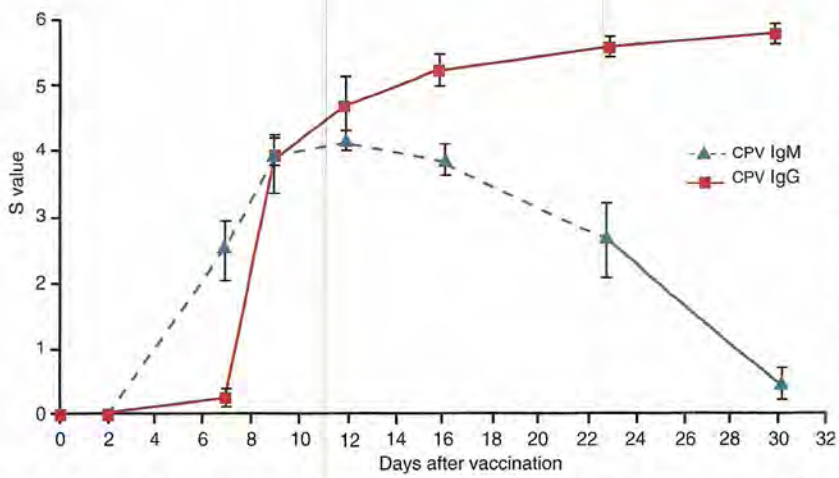

FIG 3: Changes in the mean (se) levels of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies to canine parvovirus (CPV), measured by dot ELISA, in puppies vaccinated at eight weeks of age with a polyvalent attenuated vaccine

TABLE 1: Pearson's correlation coefficients (r) for the relationships between the results of the dot ELISA and immunofluorescence antibody tests for immunoglobulin M (IgM) and immunoglobulin G (IgG) to canine parvovirus (CPV) and canine distemper virus (CDV) in 100 dogs

Parameter	r	P value (two-tailed)
CPV IgM	0·777	<0·0001
CDV IgM	0·833	<0·0001
CPV IgG	0·900	<0·0001
CDV IgG	0·822	<0·0001

Statistical tests

The correlation between the $\log_5$ IFA titre and the ELISA results, expressed in S units, was assessed by using Pearson's correlation coefficient (r).

RESULTS

Table 1 shows the Pearson's correlation coefficients for the relationships between the results of the dot ELISA and the IFA techniques for IgM and IgG of CPV and CDV, and Figs 1 and 2 show the correlations and 95 per cent confidence limits between the IFA titres (as their $\log_5$ values) and the S values for the IgM of CPV and CDV, respectively. There was a good correlation between the two techniques for the IgM and IgG titres of both CPV and CDV, and in both cases the probability values were highly significant (P<0·0001).

In the vaccination experiment, one pup did not react immunologically to the CPV and CDV components of the vaccine, and another pup did not react to the CPV component; both pups were excluded from the CPV vaccination data and one from the CDV vaccination data. IgG and IgM antibodies to both CPV and CDV were detected in all the other pups by the dot ELISA method, and the average antibody responses of the pups to CPV and CDV for 30 days after they were vaccinated are shown in Figs 3 and 4, respectively.

High levels of IgM antibodies to CPV were first detected after seven days in seven of the eight pups, at which time IgG antibodies were detected only at low levels in two pups. The highest titres of IgM were present between nine and 16 days. After 23 days, six of the eight pups still had high titres of IgM antibodies to CPV, but after 30 days only one pup had a high titre. After nine days all the pups had high titres of IgG antibodies to CPV, which persisted throughout the experiment (Fig 3).

After two days, three of the nine pups had low levels of IgM antibodies to CDV (S=1), and after seven days five of them had low titres. High titres of IgM antibodies to CDV were detected after nine days in seven of the pups and after 12 days all the pups had significant titres; after 16 days six of the pups had high titres, but after 23 days only one pup had a high titre. The highest average titre of IgM antibodies was observed 12

days after the pups were vaccinated. Six of the nine pups developed IgG antibodies to CDV after nine days and subsequently all nine pups had high titres of IgG, which persisted throughout the experiment.

DISCUSSION

The good correlation between the results of the two methods demonstrated that the dot ELISA technique could detect IgM antibodies to CPV and CDV in dogs and measure them semi-quantitatively. The correlation between the dot ELISA technique and the IFA was high for both IgM and IgG antibodies, and the results for IgG antibodies agreed with previous studies in which the dot ELISA levels of IgG antibodies to CPV and CDV were compared with the haemagglutination inhibition and serum neutralisation tests, respectively (Waner and others 1996, 1998).

In the vaccinated dogs there were initial increases in the titres of IgM and IgG antibodies to CPV and CDV, but the IgM antibodies to both viruses were short-lived, whereas the IgG antibodies persisted. The dogs were vaccinated at eight weeks of age, when their serum maternal antibody titres to both viruses were less than 1 on the Immunocomb scale. In previous studies, using the haemagglutination inhibition and serum neutralisation techniques to detect IgG antibodies to CPV and CDV, respectively, a majority of the dogs had titres of less than 1:80 at this age (Waner and others 1996, 1998). It was therefore expected that the pups would develop active immune responses to the vaccine, without the interfering effects of maternal antibodies (Carmichael and Olin 1983); however, one of them did not respond to the CPV and CDV components of the vaccine and one did not respond to the CPV component.

Although no studies with virulent viruses were carried out, the authors consider that the dogs' IgM and IgG antibody responses to vaccination should provide a basis for evaluating the immune response in naturally infected dogs. The development of the IgM response to CDV was similar to that observed in an evaluation of an ELISA technique, using complete CDV antigen; after dogs had been infected experimentally with virulent CDV, IgM antibodies were detected for the first time after seven days (Noon and others 1980). In the present experiment, moderate levels of IgM antibodies to CDV were first detected after nine days, although five of the nine pups had weakly positive levels after seven days. In the experimental infection study by Noon and others (1980), the maximum titres of IgM to CDV were detected approximately 21 days after infection, whereas in the present vaccination study high titres were observed after 12 days.

In three other studies, the detection of IgM antibodies to CDV was tested by using a recombinant nucleocapsid protein-based ELISA (Barben and others 1999, von Messling and others 1999, Griot-Wenk and others 2001). In the vaccination studies by von Messling and others (1999) and Griot-Wenk and others (2001), the CDV vaccination induced only a brief single IgM titre to the recombinant nucleoprotein two weeks after vaccination. In the vaccination study by Barben and others (1999), a weak reaction was detected after seven days, but only after 14 days had all the dogs developed IgM antibodies to CDV nucleoprotein. In the same study, experimentally infected dogs gave similar results, with 10 of 12 developing IgM antibodies against CDV nucleoprotein 14 days after infection (Barben and others 1999). The results of the present study, in which IgM antibodies to CDV were detected against the complete antigen, appeared to be more sensitive than the studies in which only IgM antibodies to nucleocapsid CDV were assayed; five of the nine dogs had measurable IgM antibodies after seven days, and seven had them after nine days.

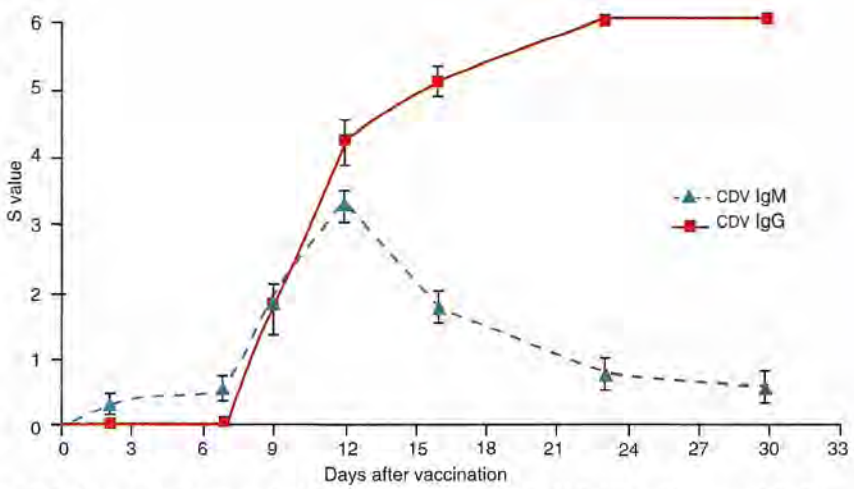

FIG 4: Changes in the mean (se) levels of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies to canine distemper virus (CDV) measured by dot ELISA, in puppies vaccinated at eight weeks of age with a polyvalent attenuated vaccine

The authors were able to find data from only one study of the IgM response of dogs to CPV. Florent (1986) first detected IgM antibodies to CPV 10 days after dogs had been infected with virulent virus, and found that the specific IgM antibodies decayed to undetectable levels within 25 days after the infection. The dot ELISA results appeared to be more sensitive, and detected IgM antibodies seven days after the dogs had been vaccinated.

To the best of the authors' knowledge, the dot ELISA data for the dogs' IgM and IgG responses to vaccination with CPV are the first to study the development and decay of these antibodies after vaccination with a polyvalent vaccine. In the infection study by Florent (1986), only three data points were examined, preinfection and 10 and 25 days after infection. In the dot ELISA study, the levels of IgM and IgG antibodies to CPV were assessed on eight occasions, from prevaccination to 30 days after vaccination.

The results of using the dot ELISA kit to detect IgM antibodies to CDV and CPV have important practical implications. From a clinical point of view, any measurable titre of IgM can be considered as specific evidence of exposure to the virus under test. Furthermore, measuring the titres of IgM and IgG antibodies over a few days could indicate the approximate time of infection. The significance of a weak reaction (S=1) is difficult to interpret but may be the earliest indication that CPV or CDV may be involved, and a test a few days later would confirm or refute this suspicion. The early clinical signs of CPV and CDV often overlap, making the diagnosis difficult, but the detection of specific IgM antibodies to either or both viruses should help to differentiate and confirm them.

It has been shown that in certain cases of fulminant acute distemper infections, and in chronic demyelinating disease induced by CDV, there is a poor immune response, and sometimes no virus-neutralising antibodies can be detected (Appel 1969, Krakowka and others 1975). There is little information about the appearance of IgM antibodies to CDV in naturally infected cases. In one study with an ELISA using complete antigen, sera from 90 per cent of dogs and mink with acute clinical infections were IgM-positive (Blixenkrone-Moller and others 1991). In another study of dogs naturally infected with CDV, nucleocapsid-specific antibodies of the IgM class were detected in nine of 15 dogs with signs of acute distemper (von Messling and others 1999). It has been shown that IgM antibodies persist in dogs with distemper for five weeks to three months, depending on the strain of virus (Appel and Summers 1999). More experience needs to be gained in the diagnosis of acute distemper by using ELISA

measurements of IgM to draw more definite conclusions about their value.

Significant titres of IgM antibodies to CPV appeared about two days before IgG antibodies. There are no reported studies of the development of IgM or IgG antibodies during acute CPV infections. If the results of this vaccination study give any indication about what actually happens in a natural infection, the early detection of IgM antibodies should be a useful indicator.

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Effects of anaesthesia and manual restraint on the plasma concentrations of pituitary and adrenocortical hormones in ferrets

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Two experiments were carried out to investigate the effect of sampling techniques on the plasma concentrations of pituitary and adrenocortical hormones in ferrets (*Mustela putorius furo*). In the first experiment blood was collected on two occasions from 29 ferrets which were either manually restrained or anaesthetised with isoflurane. In the second experiment eight intact ferrets were fitted with jugular catheters and blood was collected on four occasions, just before and as soon as possible after they had been manually restrained or anaesthetised with medetomidine or isoflurane; blood was also collected 10 and 30 minutes after the induction of anaesthesia. Medetomidine anaesthesia had no effect on the plasma concentrations of pituitary and adrenocortical hormones. Isoflurane anaesthesia resulted in a significant increase in the plasma concentration of alpha-melanocyte-stimulating hormone (α-MSH) directly after the induction of anaesthesia. Manual restraint resulted in a significant increase in the plasma concentrations of cortisol and adrenocorticotrophic hormone (ACTH) and a decrease in the plasma concentration of α-MSH.

HYPERADRENOCORTICISM is a common disease in pet ferrets. The disease differs from that in human beings and dogs in that there are increases in the basal plasma concentrations of the adrenal androgens 17α-hydroxyprogesterone, androstenedione, and to a smaller extent dehydroepiandrosterone, rather than in cortisol (Rosenthal and Peterson 1996). In some cases, the plasma concentration of oestradiol is also increased (Lipman and others 1993, Wagner and Dorn 1994, Rosenthal and Peterson 1996). The signs of hyperadrenocorticism in ferrets, which initially occur only during the breeding season, are dominated by the effects of the excessive production of these steroids, for example, symmetrical alopecia, vulvar swelling in neutered jills and the recurrence of sexual behaviour after neutering (Lipman and others 1993, Rosenthal and others 1993, Rosenthal and Peterson 1996, Weiss and Scott 1997, Schoemaker and others 2000).

To elucidate the pathophysiology of hyperadrenocorticism in ferrets, it is important to measure the plasma concentrations of cortisol, adrenocorticotrophic hormone (ACTH), alpha-melanocyte-stimulating hormone (α-MSH), androstenedione and 17α-hydroxyprogesterone (Rosenthal and Peterson 1996, Schoemaker and others 2002). However, the influence of sampling technique on the plasma concentrations of these hormones has not been studied.

For the collection of blood from ferrets, four veins are commonly used: the jugular, cephalic, lateral saphenous and anterior caval veins (Quesenberry 1996). The lateral saphenous and cephalic veins can be used to collect only small