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DOT-BASED ENZYME IMMUNOASSAY FOR DETECTION OF CANINE PARVOVIRUS ANTIGEN (S) IN FECAL SAMPLES OF LIVING DOGS AND ORGANS OF DEAD AND STILLBIRTH PUPPIES

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INTRODUCTION

Canine parvovirus is known as the etiological agent of haemorrhagic enteritis in dogs which sometimes ends fataly in young puppies due to severe myocarditis (Appel and Carmichael, 1978; Peru et al., 1980; and Meyer, 1980). The infection of dogs with Canine Parvovirus (CPV) has been reported to be widely distributed in many countries of the world like Holland (Osterhaus et al., 1980), Israel (Peru et al., 1980), West Germany (Klunker et al., 1983), USA (Kramer et al., 1980 and Carmichael et al., 1981), as well as Australia (Sabine et al., 1982), Costa Rica (Hernendez et al., 1984), India (Sherikar and Paranjape, 1985), and Nigeria (Kamalu, 1985). In Egypt CPV infection has been first reported to occur in police dogs only from clinical and histopathological findings (Bucci et al., 1982).

In the last 2 decades, enzyme-linked immunosorbent assay has found its way in the field of rapid diagnosis of a variety of animal diseases (Bommeli, 1983, and Charan and Guatam, 1984) bacause of its simplicty, sensitivity and specificity over most other most other serological assays for antigen and antibody detection. The successful detection of the CPV antigen (s) in feces of infected dogs has also been

achieved using the solid phase-ELISA (Milbrand et al., 1984 and Herbst et al., 1986). The dot enzyme immunoassay also proved its applicability in the diagnosis of some human and animal pathogens like visceral leishmaniasis (Pappas et al., 1983), pseudorabies virus (Afshar et al., 1986), rinderpest virus (Afshar and Myers, 1986, and Madboly et al., 1987), herpes simplex virus type 1 and 2 and herpesvirus simiae (Herberling and Kalter, 1986), bovine brucellosis (Chand et al., 1988), and peste-des-petits rumiannts (Timothy and Ojeh, 1989). It should also be emphasized that the Dot-ELISA has been used of the detection and analysis of a variety of proteins (Howe and Hershey, 1981 ; DeBlas and Cherwinski, 1983; Beutin et al., 1984 ; Davis et al., 1984; and Porter and Porter, 1984) , including immunoglobulin (Herbrink et al., 1982; and Beyer, 1984). The value of this technique and its application in clinincal diagnosis of human and animal diseases has been the subject of some recent reviews (Gordon et al., 1983 and Towbin and Gordon , 1984).

In the present paper, a dot enzyme immunoassay (Dot-ELISA) is described for the detection of CPV antigen (s) in fecal samples of infected military dogs as well as in different organs of dead and stillbirth puppies as compared with the solid-phase ELISA.

MATERIAL AND METHODS

CPV-antigen: A reference positive CPV-antigen prepared from tissue culture adapted virus was kindly supplied by the National Veterinary Diagnostic Laboratories, Ames-Iowa, U.S.A. The supplied antigenic material was used in the dot assay in undiluted form.

Refernce antisera: A specific antiserum against field strain of CPV and rabbit anti-CPV serum were also gifted by the same Laboratory in Ames-Iowa, U.S.A. A goat anti-rabbit immunoglobulin conjugated with peroxidase was purchased from Miles (U.S.A.).

Samples preparation for antigen detection: Fecal materials have been obtained from diseased as well as apparently healthy puppies. Necropsy tissues and organs (heart and pericardial fluids as well as the small intestine) were taken from recently dead and/or aborted puppies. The collected specimens were submitted as quick as possible to the Virology Laboratory, Faculty of Veterinary Medicine, Cairo University and processed to a 10% suspension in Eagle,s Minimum Essential Medium (MEM), clarified at 3000 revolution per minute (rpm) for 30 min. at 4 c,after being mixed with antibiotic (gentamycin, 500 ug/ml medium). The supernatents were then filtered through millipore membranes (0.2 um pore size, Sartorius , West Germany) and kept frozen at -70 C till use.

Dot-ELISA procedures: principally the procedures adopted by Hawkes et al., (1982) have been followed with slight modification. Briefly: nitrocellulose membrane filter (0.2 um pore size, Sartorius, West Germany) has been immersed in distilled water for 5 min., then allowed to dry at room temperature (RT) (the membrane should be handled throughout the work with special forceps). 3 to 5 ul of the prepared suspected ampels were dotted onto the center of an area in the nitrocellulose filter equivalent to that of the well opening of a microtiter plate; antigenic materials were allowed to dry on the membrane for 15 minutes at 37 C, thereafter the free protein binding sites were blocked by immersing the membrane in TBS (Tris 50 mM + 200 mM NaCl, pH 7.4) containing 10% horse serum (Hs) and 0.5% bovine serum albumin (BSA) for 60 min. at RT. The dotted antigenic materials were then incubated with the primary specific rabbit anti-CRV serum which was previously diluted 1:10 in diluting buffer (TBS, pH 7.4 + 0.25% BSA + 2.5% Lactalbumin Hydrolysate "LAH" + 0.05% tween 80) for 2 hrs. at RT over a shaker. The unreacted antibody molecules were then removed by washing the membrane filter in washing buffer (TBS, pH 7.4 + 0.05% tween 80) for 30 min. over a shaker with change of the washing buffer each 10 min. The membrane filter was then

incubated with the secondary specific anti-species goat anti-rabbit immunoglobulin conjugated with peroxidase (diluted 1:2000 in diluting buffer) for further 2 hrs. at RT, the membrane was washed as mentioned above. The specific antigen-antibody reaction could be visually read by immersing the membrane in freshly prepared enzyme substrate (4-chloro - 1 - naphthol, prepared as a 3 mg/ml stock solution in pure methanol). Just before use, 1.7 ml of the stock solution were mixed with 8.3 ml of TBS and 30 ul of H202 (3%). The enzyme reaction was stopped after 10-15 min. by washing the membrane with tap water . Specific positive reactions appeared as dark blue dots at the individual sites of the dotted antigens.

Standard indirect ELISA for antigen detection: The procedures described by Anderson et al., (1982) have been followed with slight modification. Briefly: 100 ul of suspected antigenic materials were added to the individual weels of ELISA microplate (Nunc, Denmark) and incubated for 2 hrs. at 37 C, followed by overnight incubation at 4 C. Thereafter the supernatents were aspirated and then 50 ul volumes of fixative mixture (60% acetone + 25% ethanol + 15% methanol) were added for fixation for 15 min. at 4 C. Plate was allowed to air dry for further 15 min. at RT followed by addition of 200 ul volumes of phosphate-buffered saline containing 0.5% tween 80 to block free protein binding sites for 2 hrs. at RT. 100 ul volumes of reference rabbit anti-CPV (diluted 1:10 in PBS containing 10% HS + 0.3% BSA + 0.05% tween 80) were then added and incubated for 1 hr. at 37 C, followed by washing of the microplate with PBS containing 0.05% tween 80 (3 times, each 10 min.). The reacting antibodies were allowed to interact with a specific goat anti-rabbit immunoglobulin conjugated with peroxidase diluted 1:2000 in diluting buffer (100 ul/well) for further 1 hr. at 37 C, followed by washing the plate as mentioned above. Specific enzyme reaction was screened by adding the substrate 1,2 phenylene-diamine (Merck, Darmstadt, West Germany) which was

prepared according to Reggiardo et al. (1980): 10 mg 1,2 phenylenediamine were dissolved in 1 ml pure methanol and added to 22.5 ml dist. water, then 30ul of H202 (30%, Merck, Darmstadt, West Germany) were added to the substrate working solution just immediately before added to the microplate. 100 µl volumes of substrate solution were then added to individual wells and colored reactions allowed to develop at RT in a dark place for 30 min., then enzymatic reaction was stopped by adding 50 µl volumes of 2.5 N H2s04 and extinctions of the developed colors were measured in the Dynatech ELISA microreader at an optic density (OD) of 490 nm.

RESULTS

Detection of CPV-antigen: Prepared tissue homogenates were propably dotted on the nitrocellulose membrane in a semiquantitative manner, where positive reactions appeared as blue dots in varying intensities from intense blue (high positive) to nocolour or barely perceptible (negative). **Figl 1** shows clearly how far is the specificity of the antigen-antibody reactivity, where only visible blue dots were developed in antigenic materials suspected to contain CPV, which were originally collected from dead aborted and/or stillbirth puppies. The same figure shows the high density of virus antigenic material in the pericardial fluid as indicated from the developed high dot reactions. The small intestine showed itself in this test as a good target organ for virus isolation, owing to the intense blue dot reactions it gave. A weak positive reaction has also been developed by fecal antigenic material collected from living diseased dogs, if compared with the totally negative reactions given by fecal samples of the apparently healthy dogs.

Comparative evaluation of Dot-ELISA for detection CPV-antigen(s) in relation to standard indirect solid phase ELISA: When the antigen homogenates were applied on

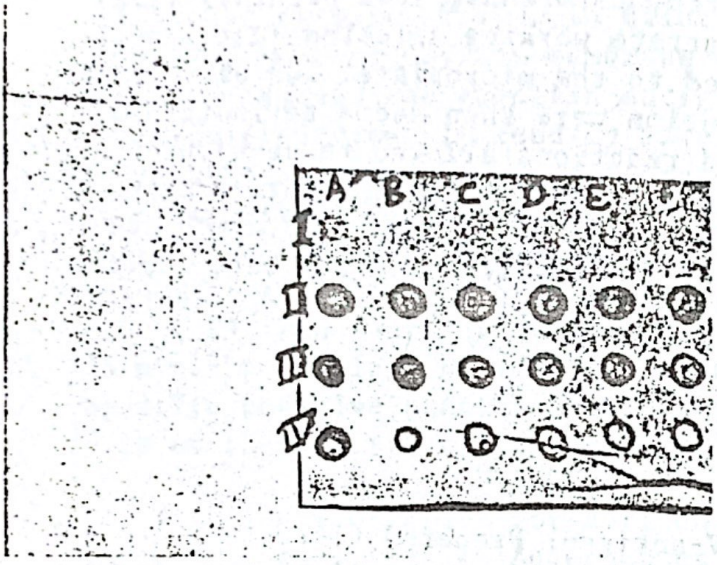

Fig.1: Shows the dark colored dots of the positive peroxidase reaction given by different organs and feces as tested by the dot-ELISA.

I. Negative (feces of apparently healthy dogs).
II. High positive (pericardial fluid).
III. High positive (small intestine).
IV. Weak positive (feces of diseased dogs).

Table (1): Detection of CPV antigen in faeces and organs with dot-ELISA as compared with solid phase ELISA.

Status	Samples	ELISA reading							Dot-ELISA
Living cases (AH)	Faeces	No	54	55	56	57	58	59	Negative
		R	0.0	0.0	0.0	0.0	0.0	0.0	
Dead aborted (still birth	Heart	No	5	2	7	10	3	17	High positive
		R	0.96	0.93	0.93	0.91	0.86	0.84	
	Intestine	No	7	5	11	10	4	1	High positive
		R	0.91	0.86	0.76	0.73	0.69	0.63	
Living cases (diseased)	Faeces	No	29	30	32	33	34	0.36	Weak positive
		R	0.52	0.49	0.43	0.32	0.29	0.23	

AH : Apparently healthy
NO : Number of sample
R : ELISA reading

the solid plastic matrix of the ELISA microplate, the indirect reaction with specific anti-CPV and anti-species conjugate proved again the high density of virus antigens in the heart (pericardial fluid) and the small intestine with reading extinctions ranging from 0.84 to 0.96 and from 0.63 to 0.91 respectively, runnunig in parallel consistency with the reactions given in the dot-ELISA with lower reactions given by the fecal material of living diseased dogs with reading extinctions ranging from 0.23 to 0.52. Again the feces collected from living apparently healthy dogs gave negative reactions (extinctions of 0.00) as clearly depicted in **Table, 1.**

DISCUSSION

Since haemorrhagic enteritis constitutes a serious problem among young military puppies in Egypt, Bucci et al. (1982) carried out their clinical and histopathological studies on affected dogs and suggested the disease to be CPV infection. Recently, Abd El-Ghany (1988) succeded in isolating the CPV as an etiological agent of the disease by injecting young healthy puppies with the collected suspected materials obtained from dead and stillbirth puppies. In the present work, the collected processed materials were tested for the presence of CPV antigen using the developed Dot-ELISA technique and compared with the conventional indirect solid phase ELISA. The data presented in **Table 1** and **Figure 1** suggest that the heart and small intestine are very good targets for detection of CPV in dead and stillbirth puppies. In other work (manuscript in preparation), the solid phase ELISA has been used for screening the presence of CPV in different organs of affected puppies, where the heart was the organ of choice and gave a 100% positivity in both dead and stillbirth puppies followed by the small intestine, spleen and kidneys. The liver gave the least reactions and lungs seemed to be free from viral antigen (s). The results obtained by Klunker (1982) and Frost and Klunker (1984) using the immunofluorescence

test are in consistence with those presented in this work, where they succeeded to detect CPV antigen (s) in the fifferent organs of 58 examined dogs and provedthat the small intestine is the organ of choice , although these authors did not examine the heart for the presence of the CPV antigen. Herbst et al. (1986) pointed out that electron microscopy is somewhat more sensitive in detecting CPV FROM FECAL SAMPLES THAN ELISA, yet they stated that ELISA is simpler and quicker and could be used by others as a reference test (Senda et al., 1986).

By focusing some light on the readings presented in **Table,1** and **Figure 1**, is can be concluded that the Dot-ELISA gives comparable results matched with those given in the solid phase ELISA. With both techniques, it has been proved that fecal samples can occasionally be used for detection of CPV antigen (s) in spite of the relatively weak reactions obtained **(Figure, 1)** . However, the applciation of the Dot-ELISA on fecal samples was of great importance in rapid diagnosis of the disease specially in living cases. Due to its rapidity, simplicity and higher sensitivity, Dot - ELISA could be recommended as a reliable test for detection of CPV antigen (s).

SUMMARY

A dot-enzyme immunoassay has been developed for the direct detection of canine parvovirus (CPV) in feces and organs of diseased as well as of dead aborted and/or still birth puppies. The assay has been proved to be reproducible, simple to perform and sensitive when compared with conventional standard solid-phase ELISA, where the heart and small intestine found to be the organs of choice for the detection of CPV antigen (s). Inspite of the weak reaction given by fecal materials, yet the application of the dot-ELISA showed great importance in fast diagnosis of CPV in living

diseased dogs. The positive reactivities given by the dot-ELISA gave a 100% correlation with those of the solid-phase ELISA, therefore, the dot-ELISA could be recommended as a fast reliable technique for the diagnosis of CPV infection.

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