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Veterinary Immunology and Immunopathology, 17 (1987) 453–464
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

RAPID DETECTION OF VIRAL-SPECIFIC ANTIBODIES BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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ABSTRACT

Winston, S., Fiscus, S., Hesterberg, L., Matsushita, T., Mildbrand, M., Porter, J. and Teramoto, Y., 1987. Rapid detection of viral-specific antibodies by ELISA. Vet. Immunol. Immunopathol., 17: 453-464.

The development of three separate rapid ELISAs for detecting antibodies in host serum to three different viruses is described. These include:

1. A direct antigen assay using enzyme labelled anti-canine Ig for detecting antibodies to canine parvovirus,
2. A competitive ELISA using a feline infectious peritonitis virus-specific monoclonal antibody labelled with enzyme, and
3. A competitive ELISA using an equine infectious anemia virus-specific monoclonal antibody and enzyme labelled antigen, p. 26.

The utility and benefits of each of the three approaches is emphasized.

INTRODUCTION

The detection of antibodies to specific pathogens has proven to be diagnostically significant for a number of viral and bacterial diseases. A variety of assays, including serum neutralization (SN), hemagglutination inhibition (HI), immunodiffusion (ID) and others have been routinely used to measure the presence of specific types of antibodies. These tests are rapidly being replaced by more sensitive and faster tests such as ELISA and Western blotting.

Since the detection of the actual virus itself can be difficult because of low quantities of virus present, the life cycle of the virus relative to the clinical symptoms, and inaccessibilty of infected tissue, the presence of viral-specific antibodies can often be used as a "fingerprint" for viral infection. A recent example of such an application is HTLVIII/LAV testing (HLVIII) which depends on monitoring infection through the presence of antibody rather than antigen. Antibody screening is also useful in determining immunity or susceptibility to viral infections and is an important component of vaccine development, testing, and use. We have developed a number of ELISAs for the

0165-2427/87/$03.50

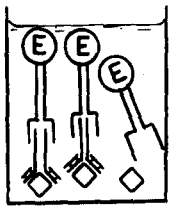
rapid detection of viral-specific antibodies associated with a number of significant animal diseases. Our approach has been to combine relatively pure antigen preparations and monoclonal antibodies, in direct and competitive assay formats. The assays have proven to be rapid, sensitive and highly specific, and can be used to monitor exposure, immunity or susceptibility to infection.

DEVELOPMENT OF RAPID ANTIBODY TESTS FOR CANINE PARVOVIRUS: DIRECT ANTIGEN ASSAYS

One of the primary causes of vaccine failure is the presence of maternal antibodies. With the development of improved vaccines that engender extremely high levels of protective antibodies this problem has been significantly increased. In the case of canine parvovirus (CPV) it has been clearly demonstrated that maternal antibodies can prevent immunization of puppies and that increased levels of maternal antibodies may prevent proper vaccination even beyond 18-20 weeks of age (Acre et al., 1983; Pollock and Carmichael, 1982). Since CPV infection is primarily a disease of younger animals it is especially important that all puppies be properly vaccinated. The presence of neutralizing antibodies (SN) to CPV in the serum is a useful measure of the susceptibility of a dog to infection (Pollock and Carmichael, 1982). In general HI titers $\geq$ 1:80 and SN titers $\geq$ 1:16 are protective. However, HI and SN results are subject to interassay procedural variations and require several days to perform (Wallace et al., 1983).

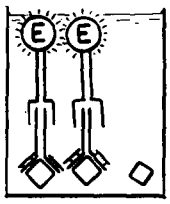
To overcome these problems of speed and variability a rapid ELISA was developed to detect the presence of antibodies to CPV (Fiscus et al., 1985a). The test components were optimized so the results closely paralleled those observed with HI and SN assays, to provide a rapid assay for immunity to CPV infection. The assays was designed as indicated in Figure 1. This assay measures the direct binding of antibody to antigen that has been bound directly to the solid phase component of the test; in this case a microtiter well. To minimize the number of individual steps the test was performed with a simultaneous incubation of canine serum and anti-canine Ig enzyme conjugate. To ensure specificity of binding, virus was purified by concentration to remove low molecular weight components in the growth medium and then successively centrifuged through 30% sucrose (w/w) and to equilibrium in cesium chloride. The virus was essentially pure as judged by SDS-PAGE and silver staining. Because of high yields and nearly identical antigenicity, the virus used was feline panleukopenia virus rather than a parvovirus strain of canine origin.

To be able to detect canine Ig bound to the virus a monoclonal antibody was developed that recognized canine Ig bound to virus. Hybridoma supernatants were initially screened against canine Ig and positive clones were rescreened against canine Ig bound to virus in the microtiter well. Thus antibodies were



Diluted canine serum ( ) and enzyme-conjugated anti-canine IgG monoclonal antibody ( ) are added simultaneously to CPV (◇) coated wells and incubated for 5 min.

Wash



Substrate is added and color change is observed after 5 min.

Figure 1. Diagramatic representation of direct antigen ELISA for detecting antibodies to canine parvovirus.

selected that not only had the correct specificity but would also be useful in the predesigned assay format. Each component of the assay was optimized to provide maximum discrimination between positive and negative sera (Fiscus et al., 1985). This included optimizing the type of microtiter well, the coating and blocking buffer, the diluting buffer for sera, the enzyme conjugation method for the monoclonal antibody, the washing buffer, and the times of incubation. The results from comparing eighty-nine sera by both SN and ELISA are shown in Table 1. If a cut off of $\geq$ 1:16 is taken as the minimum level necessary for protective immunity then the ELISA has a sensitivity of 86.7% and

TABLE 1

Comparison of serum neutralization (SN) and enzyme linked immunosorbent assay (ELISA) for canine parvovirus (CPV) antibodies

	SN 1:8	SN 1:16
ELISA (-)	38	7
ELISA (+)	5	39

a specificity of 89.6%. In general, the sera with SN titers of 1:8 to 1:32 were the most discordant with the ELISA results. Since 13 separate sera repeatedly assayed either 9 or 10 times each showed 100% reproducibility in the ELISA, the discrepancies may be accounted for by the variability of SN assays one to two dilutions in either direction. Overall there was a good correlation between SN and ELISA and the ELISA can be used as a rapid screen for monitoring the level of immunity to CPV.

DEVELOPMENT OF A COMPETITIVE ELISA (CELISA) FOR FELINE INFECTIOUS PERITONITIS VIRUS USING ENZYME-LABELLED MONOCLONAL ANTIBODY

The causative agent of feline infectious peritonitis (FIP) is the coronavirus, FIPV, which is antigenically related to transmissible gastroenteritis virus of swine (TGEV), canine coronavirus (CCV), feline enteric coronavirus (FECV) and human coronavirus 229 E (Horzinek et al., 1982; Pederson et al., 1981). One of the more important elements of pathogenesis is the development of immune-complexes containing FIPV-specific antibodies. Because of this the most commonly used assay for FIPV infection is an immunofluorescence assay (IFA) using FIPV or TGEV-infected cells as substrate. Elevated titers to FIPV are indicative only of exposure and may not be correlated with active disease.

To develop a FIPV-antibody based ELISA the strategy outlined for CPV (Figure 1) was followed. Unfortunately, the results were highly irreproducible and a poor correlation with either clinical signs or IFA results was obtained. The direct antigen assay approach was abandoned and an attempt was made to utilize a series of coronavirus-specific monoclonal antibodies in a competitive ELISA format (Fiscus et al., 1985) represented schematically in Figure 2.

A series of monoclonal antibodies were developed and characterized by reactivity in ELISA (Figure 3) and immunoblotting (Figure 4). For competitive ELISAs three representative antibodies were chosen that had high ELISA titers to one of the three major viral components; COR15 for the envelope glycoprotein E1, COR13 for N, the nucleocapsid protein, and COR2, for the peplomer protein. The specificity of monoclonal antibodies offers clear advantages and disadvantages in developing competitive assays. The monoclonal nature of the antibody and its use as the "signal" in the assay ensures a highly specific result. The system can also be easily manipulated for optimal sensitivity. The major disadvantage is that only one epitope is being analyzed in a competition assay and may not represent the overall immune response of the animal to the virus. Because of this constraint, antibodies recognizing each of the three major viral components were tested.

A number of sera from experimentally and naturally infected cats were tested using the protocol described in Figure 2. The competition for the E1

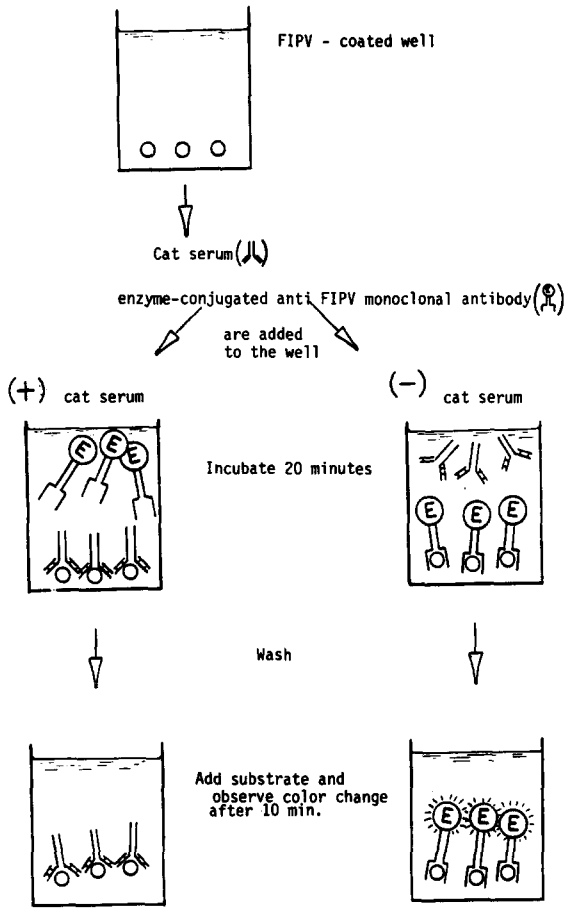


Figure 2. Diagramatic representation of competitive ELISA (CELISA) for detecting antibodies to feline infectious peritonitis virus.

glycoprotein gave the best correlation with IFA (Fiscus et al., 1985) and clinical histories of the cats. A representative CELISA profile of one experimentally infected cat and one control cat is shown in Figure 5. The initial immune response of the cat was to E1, and this response closely paralleled the IFA result. The nucleocapsid-specific monoclonal antibody was not significantly inhibited (> 50%) until 60 days following infection. As measured by the competitive ELISA, the cat did not develop a significant anti-peplomer response. The control cat remained essentially negative by both IFA and competitive ELISA. Out of 205 samples that were positive in the CELISA, 98.5% had anti-E1 antibodies, 58.5% had anti-N antibodies, and 4.8% had anti E2 antibodies. Only cats that developed antibodies to E2 had neutralizing antibodies, although some of these cats succumbed to infection. However, several developed neutralizing E2 antibodies only late in infection so it is

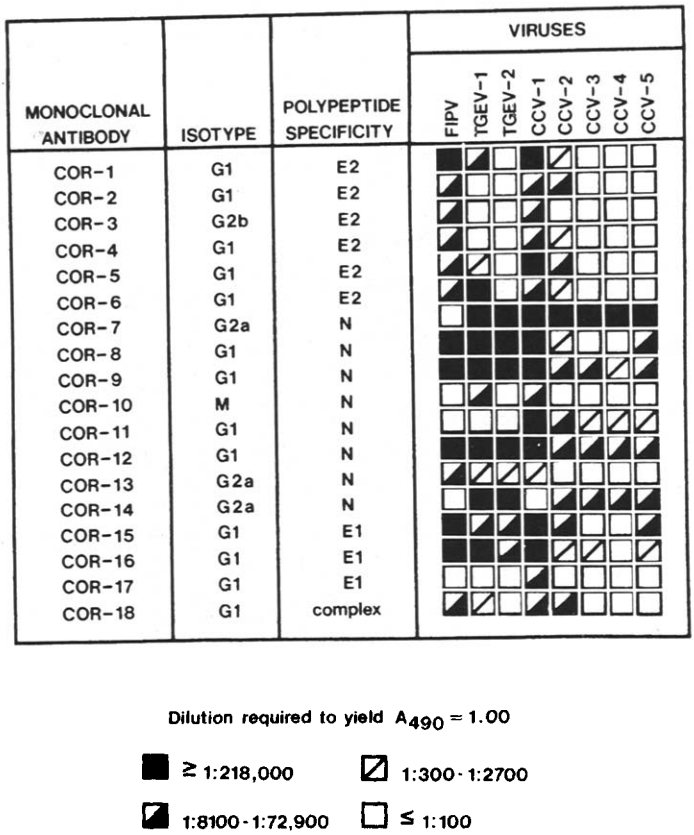


Figure 3. Specificity and ELISA titer of ascites fluid containing monoclonal antibodies to coronaviruses. FIPV (feline infectious peritonitis virus), TGEV (transmissible gastroenteritis virus), CCV (canine coronavirus). A490 = 1.00, optical density of 1.00 at 490 nm.

unclear whether or not neutralizing antibodies can play a protective role. These results may have implications in the strategy for developing vaccines for FIPV. In summary, one approach to developing competitive ELISAs using monoclonal antibodies is to first develop a bank of antibodies that recognize several or all of the major viral structural components. These antibodies can then be systematically analyzed and results compared to either existing tests such as ID, SN, HA, or IFA or to immunoblotting results. It was observed with FIPV, that monoclonal antibodies that recognized different epitopes on the same protein gave similar results in the competitive ELISAs, suggesting that there are identical sets of epitopes on individual proteins that are recognized both by the cat and the mouse. This may not be true for all viral patients.

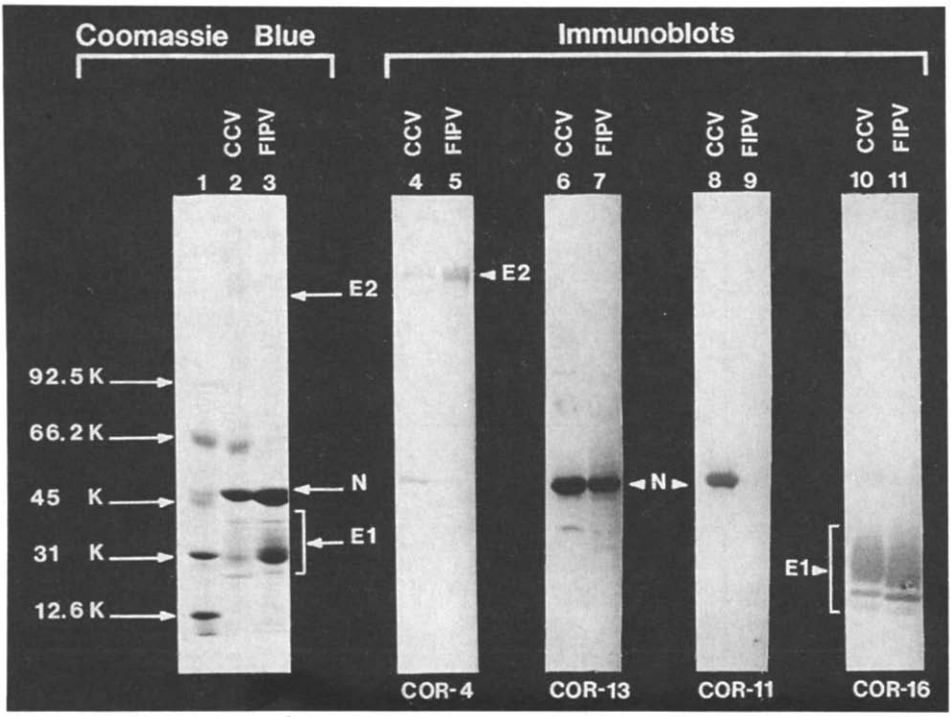


Figure 4. Commassie Blue stain of SDS-PAGE purified canine coronavirus (CCV) and feline infectious peritonitis virus (FIPV) and immunoblotting of the purified virus with four separate monoclonal antibodies.

DEVELOPMENT OF COMPETITIVE ELISA FOR ANTIBODIES TO EQUINE INFECTIOUS ANEMIA VIRUS USING ENZYME-LABELLED ANTIGEN

One of the most widely used tests for the presence of viral-specific antibodies is the equine infectious anemia agar-gel immunodiffusion test (EIA-AGID). It has proven extremely useful in controlling the spread of disease as no vaccine or therapy exists. It is an excellent example of the application of antibody testing for monitoring exposure and disease, since the chronic, acute and inapparent phases of the infection make it difficult to diagnose EIA by detection of the virus itself.

The EIA-AGID was initially seen as a prime candidate for conversion to a rapid ELISA. Although the AGID is highly sensitive and specific the test requires 24-48 hours to perform and is subject to reader error in interpreting weakly reactive samples. The equine immune response to EIA virus has been well characterized and all infected horses develop antibodies to the highly conserved and abundant viral core protein p26 (Issel and Coggins, 1979). Procedures for the growth of the virus and purification of the p26 protein have been developed (Montelaro et al., 1982).

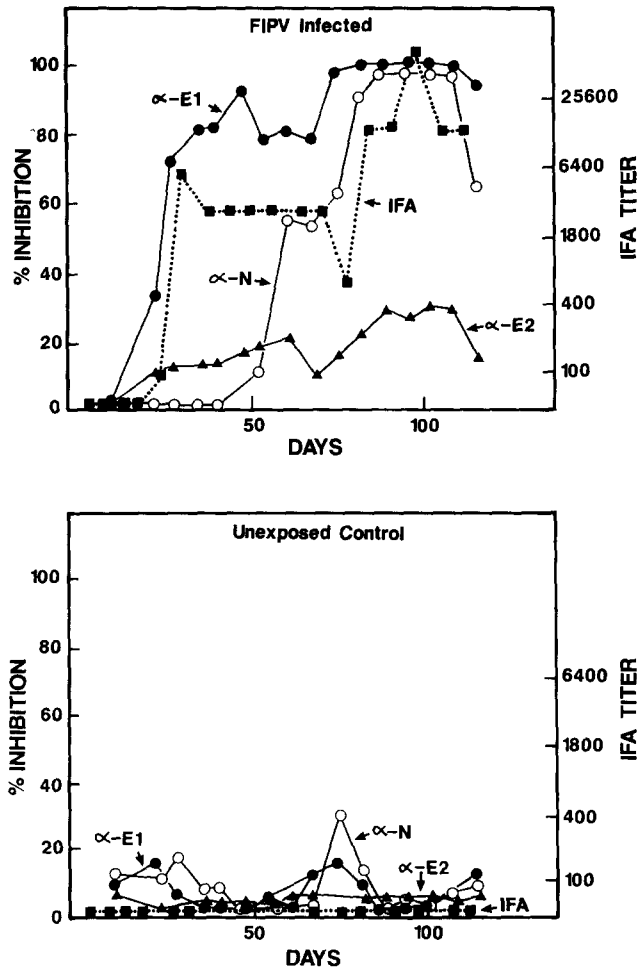


Figure 5. FIP-CELISA and IFA results from sequential serum samples taken from an experimentally infected cat and an unexposed control cat.

Because of the availability of large quantities of purified p26 the development of a direct antigen ELISA as depicted in Figure 1 was attempted. Enzyme conjugated, equine virus-specific polyclonal or monoclonal antibody was used as the detecting molecule. It was observed that a small percentage of AGID negative horse sera gave consistently positive results in the direct antigen ELISA. All of these horse sera reacted strongly to contaminating bovine serum albumin (BSA) in the AGID and it was hypothesized that these sera were recognizing the small amount of BSA (less than 0.001%) remaining in the purified antigen preparation (Matsushita et al., 1984).

To circumvent this problem a bank of p26-specific monoclonal antibodies was prepared. These antibodies were conjugated to horseradish peroxidase and used

to develop a competitive ELISA similar to that previously described for FIP (Figure 2). Although this approach proved superior to the direct antigen ELISA, several of the horse sera still gave false positive results using AGID as the standard. Again, it was hypothesized that BSA-specific equine antibodies binding to BSA in the microtiter well were interfering with the binding of the enzyme-labelled monoclonal antibody, resulting in false competition.

To circumvent this problem, an assay combining the specificity of monoclonal antibodies and the ability to purify and enzyme-label the p26 protein was developed (Figure 6). Monoclonal antibodies were specifically selected for their ability to bind to the microtiter well and "capture" enzyme-labelled p26. The enzyme-labelling of p26 was optimized so that little if any of the

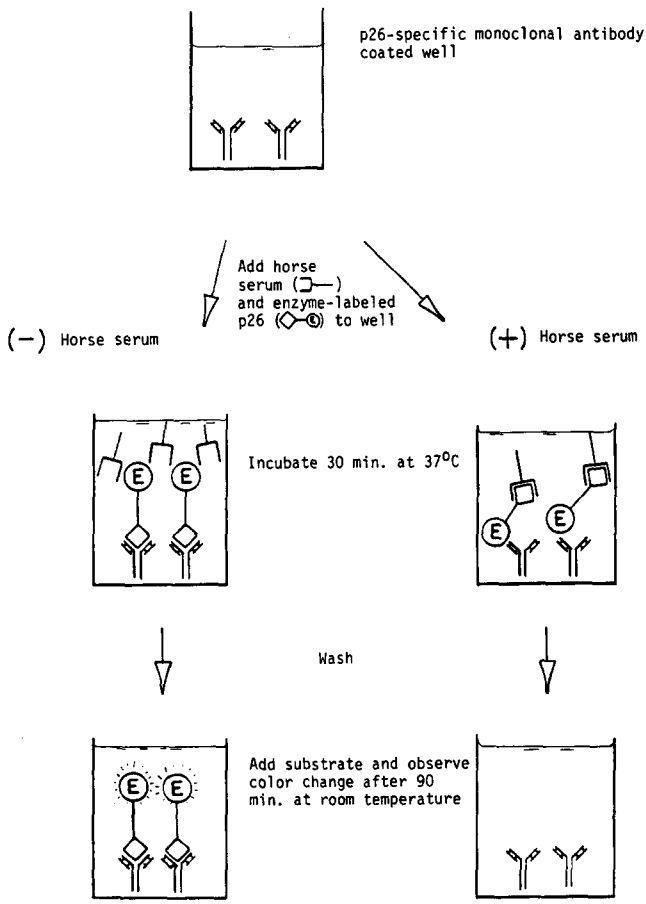


Figure 6. Diagrammatic representation of competitive ELISA (CELISA) for detecting antibodies to equine infectious anemia.

TABLE 2

Competitive enzyme linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) results obtained by testing a set of horse sera.

Equine sera	AGID	ELISA	Visual[1] Result
Control (+)	+	.52	+
Control (-)	-	1.13	-
Sample # 1	-	1.05	-
2	-	1.15	-
3	+	.12	+
4	+	.04	+
5	+	N.T.[2]	N.T.
6	+	.06	+
7	+	.06	+
8	+	.10	+
9	-	1.07	-
10	+	.04	+
11	-	1.01	-
12	+	.07	+
13	+	.09	+
14	+	.03	+
15	+	.08	+
16	-	1.08	-
17	+	.03	+
18	-	.06	+
19	+	N.T.	N.T.
20	-	1.10	-
21	+	.04	+
22	+	.14	+
23	+	.04	+
24	-	.92	-
25	-	1.00	-
26	+	.09	+
27	+	.06	+
28	+	.17	+
29	+	.08	+
30	+	.05	+

[1]Results +/- determination of ELISA results by the unaided eye.
[2]N.T., not tested.

contaminating proteins were labelled. One advantage of using monoclonal antibodies is their generally lower affinity. Thus, in a simultaneous competition for binding to antigen the horse serum will bind more efficiently in solution to the labelled antigen and competition will be readily discernible.

The results of testing a series of field samples in the EIA CELISA are shown in Table 2. The average $O.D._{405}$ of the AGID negative samples was 1.05 ± 0.07 while the average of the AGID positive samples was 0.073 ± 0.038. The CELISA

is approximately 5 to 10 times more sensitive than the AGID based on comparing end point dilution in the AGID with end point dilution giving 50% competition in the CELISA. The results are also easily visualized.

One interesting facet of the EIA CELISA is that the assay can also be used to measure EIA antigen. If virus is present in the sera, it can be bound by the monoclonal antibody and compete with the enzyme-labelled p26. Titration studies have shown that less than 16 ng of virus can be detected in the assay. Studies are underway to examine whether the initial stages of infection and viremia that occur prior to the onset of immune response can be detected.

DISCUSSION

Three approaches to the development of ELISA's for viral specific antibodies have been described. Although each differs greatly in the actual assay format they all share several basic components. First, the use of monoclonal antibodies provides excellent specificity and sensitivity. The key to selecting the appropriate monoclonal antibody in each case was in designing screening procedures that stimulated the actual utility of the antibody in the particular assay format. This allows one to rapidly select and optimize the right antibody from the large numbers of monoclonal antibodies usually generated against immunogenic molecules. Second, the purity and biochemical characterization of the virus played a major role in the development of the ELISAs. Although fairly simple in structure, each of the three viruses described here are antigenically complex. In the case of FIP, for example, it was important to first identify the protein(s) recognized by the feline immune system. Although this was already known for EIAV, the purity of the p26 protein was paramount to the successful development of the EIA-CELISA. Clearly, it is important to develop the viral purification scheme to maximize the yield of diagnostically important antigens.

Third, each component of each ELISA must be optimized. Key parameters include solid phase coating procedures and the solid phase itself, enzyme conjugation methods, sample diluent buffers, wash buffers, and time and temperature of incubation. Although each individual parameter may only slightly improve the performance of the test, in combination it may well prove quite significant.

Finally, it is apparent that no one assay format will be suitable for a wide range of viral-specific antibody tests. The type and structure, as well as the life cycle, of viruses vary greatly. Thus, it is wise to assume that the strategies used to develop such assays will be to be as clever and diverse as the virus themselves.

ACKNOWLEDGEMENTS

We wish to thank our collaborators, Dr. Jim Collins, Louis Newman, Charles Issel, and Neils Pederson for their valuable contributions in various phases of our work.

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