DIAGNOSIS BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Daniel J. Ellens[1)]
Central Veterinary Institute, Department of Virology,
39 Houtribweg, NL 8221 RA Lelystad, the Netherlands.

ABSTRACT

A variety of enzyme-linked immunosorbent assay (ELISA) systems are being applied in the detection and quantification of both antibodies and antigens in bacterial, viral and parasitic infections. To date, the use of ELISA in research on neonatal diarrhoea has been described for the detection of rotavirus, bovine coronavirus and enteropathogenic *Escherichia coli* infections.

Some features of the double antibody sandwich ELISA system are discussed with particular reference to those elements most important in determining the specificity and sensitivity of the test.

INTRODUCTION

About 10 years ago Van Weemen and Schuurs (1971) and Engvall and Perlmann (1971) introduced enzyme-labeled probes for immunological assay purposes. The novel approach appeared to have distinct advantages over conventional immunological tests. Its sensitivity was comparable to radioimmunoassay and yet it missed the drawbacks of the latter technique. In fact ELISA seemed to provide all the elements for an ideal immunoassay: safe, sensitive, easy to perform, inexpensive, utilizing a tracer molecule with great stability, not requiring sophisticated equipment and suited for automation.

Perhaps the major area in which enzyme-linked immunosorbent assays emerge as the best substitutes for other assay systems, is in large scale screening programmes for various antigens or antibodies, especially in those situations where only qualitative information is needed. Systems making use of microplates are particularly well suited for this purpose. In many cases, simple visual observation of the enzyme-mediated reaction is adequate. Some ELISA screening systems have been completely automated (Ruitenberg et al., 1977). In recent years ELISA has been successfully

[1)] Present address: Organon Teknika B.V., Department Membranes & Adsorption Stationsplein 1, 5281 GH Boxtel, the Netherlands.

P. W. de Leeuw et al. (eds.), *Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea*
© ECSC, EEC, EAEC, Brussels-Luxembourg 1981

employed not only in sero-epidemiology for bacterial, viral or parasitic human diseases, but also for agricultural and veterinary mass surveys (Clark and Adams, 1977; De Leeuw et al., 1980).

TYPES OF ASSAY

Two basic systems have been developed for enzyme immunoassays: blocking systems and trapping systems.

Blocking systems. Fig. 1a shows the principle of a blocking test for the detection of antigen. The antigen to be detected competes with a known quantity of enzyme-labeled (standard) antigen for a limited amount of specific antibody. The difference in optical density between control wells, containing only labeled antigen, and wells containing both standard antigen and test samples, is used to estimate the quantity of antigen present. This design of ELISA has to be used when the antigen possesses only one antigenic site (hapten). A similar system can be applied for the detection of antibody (figs. 1b and 1c). The advantage of this method for the detection of antibody is that antibodies of different species, directed against the same antigen(s), can be detected and quantitated using the same assay. For instance, bovine, porcine, human and simian sera have been examined by one blocking assay for the presence of antibodies against group-specific rotavirus antigens (Yolken et al., 1978).

The ELISA-inhibition technique, as shown in Fig. 1d, may also be listed under this heading. The test measures the ability of putative antigen-containing specimens to inhibit the binding of a limited amount of antiviral antibody to bound viral antigen. This assay does not require the use of high titered antiviral antisera in contrast with all other enzyme immunoassays for the detection of antigens. It has been described for the detection of Candida antigen in sera (Segal et al., 1979) and influenza virus in nasal washings (Berg et al., 1980).

Trapping system. The most widely used method for the detection of antibody is the indirect enzyme immunoassay (Fig. 2a). The method requires purified antigen and a conjugate that should be free of antibodies against the antigen. In principle the method permits the use of a single anti-species conjugate for the detection of antibodies against different agents. In addition, class-specific antibody activity may be measured by using appropriate conjugates. However, competition between antibodies of different classes may lead to a blocking effect: small amounts of anti-rotavirus IgA, for instance, may not be detected in the presence of a large excess of anti-rotavirus IgG.

The double antibody sandwich method (Fig. 2b) has been applied for the detection of a variety of high molecular weight antigens such as viruses and bacterial components. The method requires high titered specific antibody for trapping the antigen. The trapped antigen can be detected either directly or indirectly (Figs. 2b and 2c). The latter method requires antigen-specific antibodies prepared in two different species. However, neither of these have to be conjugated.

The indirect double antibody sandwich method can also be used for the detection of class-specific antibody (Fig. 2d). First, the specific class of antibody is trapped and incubated with antigen. Bound antigen is then detected by the appropriate conjugate. The advantage of this design is that competition between different classes of antibody does not occur.

Instead of using specific antibody, the solid phase can also be coated with specific receptors (Figs. 2e and 2f). For instance, the ganglioside GM 1 has been used by Svennerholm and Holmgren (1978) to trap heat-labile enterotoxin.

THE DOUBLE ANTIBODY SANDWICH TECHNIQUE

The detection and quantification of antigen by the double antibody sandwich technique is usually done in four steps:

1. coating of the solid phase with antibody,
2. incubation of the coated surface with putative antigen containing samples,
3. incubation with specific antibody enzyme conjugate, and
4. estimation of bound conjugate activity.

Some points of importance to obtain optimal results with the double antibody sandwich ELISA are discussed below.

Preparation of specific antisera. Specificity and sensitivity of this type of assay are highly dependent on the availability of high titered antisera possessing high specificity. Usually such sera are prepared by hyperimmunization of rabbits, guinea pigs or goats with purified antigen. Prerequisites are that the animals have very low pre-immunization antibody levels, that the antigen preparation used for hyperimmunization is free of contaminating proteins and that the appropriate antigenic determinants are present. With rotavirus, for instance, purification procedures using CsCl centrifugation may damage the outer capside antigenic determinants (Kalica et al., 1977). Similar problems are well-known in the purification of coronaviruses. However, loss of coronavirus peplomers can be prevented

by glutardialdehyde fixation during sucrose gradient rate zonal centrifugation of the virus (Dr. D. van Zaane, pers. comm.).

Due to the presence of immune complexes, antigens isolated from faeces are often contaminated with antibody. Use of these antigen preparations for hyperimmunization may lead to antiglobulin antibodies which interfere in the ELISA. This problem may be circumvented by hyperimmunizing orally infected newborn animals with antigen prepared from their own faeces (Ellens et al., 1978). Sera prepared by inoculation of rabbits with immune precipitates obtained by cross-immunoelectrophoresis of purified rotavirus against rabbit-anti-rotavirus serum are also suited for use in ELISA (Grauballe, these proceedings).

Coating of the solid phase. In general proteins adsorb to solid phases by either polar or apolar interactions. Polar interactions dominate the adsorption process at hydrophilic surfaces. Such interactions are sensitive to changes in environmental conditions so that proteins often can be removed from hydrophilic surfaces by exposure to extreme pH, high ionic strength or by extensive rinsing. At hydrophobic surfaces the adsorption process is dominated by apolar interactions. Hydrophobic regions of the protein surface are attached to the sorbent. The affinity between a given protein and a sorbent surface increases with increasing hydrophobicity of that surface. Desorption from hydrophobic surfaces usually does not occur. Protein adsorption isotherms generally develop well established plateau values that range between, say, 1 and 5 mg/m^2, corresponding to closely packed monolayers (Norde, 1980). For ELISA purposes, generally a tenfold lower adsorbed protein concentration is used. Too high a concentration of protein during coating may give rise to high background values or prozone phenomena. If the protein concentration used for coating is too low, the sensitivity of the ELISA will decrease. It is therefore essential to establish the optimal concentration for coating in each antibody-antigen system.

In view of the facts mentioned above, hydrophobic surfaces should be used as solid phase in ELISA, in spite of the given that hydrophilic surfaces absorb some proteins more efficiently at low concentrations.

Optimal adsorption at hydrophobic surfaces occurs around the isoelectric point of the protein, although for some proteins, e.g. - globulins, the optimal pH range is rather broad. Thus, when total serum is used

for coating a solid phase with antibody, a pH of 9.5 is advantagous, because at this pH most contaminating serum proteins are adsorbed with low efficiency, whereas the coating efficiency of IgG is only slightly lower than at pH 7.2. However, to prevent non-specific reactions the use of purified reagents for coating is to be preferred. Neither the time nor the temperature of incubation appears to be very critical. Several hours at 4-37°C are used generally. When, during incubation, the tubes or plates are rotated at an angle, a larger surface area is coated. Coated plates or tubes have a long shelf life (more than one year) when stored between -20°C and +4°C.

Washing procedure. Several procedures can be used for washing plates or tubes between successive incubation steps. Generally the individual wells or tubes are washed with a 0.05% Tween solution by three cycles of filling and sucking-off. Reproducible results have also been obtained by rinsing microplates by immersing them in a washing solution (Grauballe, these proceedings) and by the use of a 96-channel shower device (Ellens, these proceedings).

Incubation with antigen. Depending on the antigen, incubation may be done at 4°C or at 37°C in order to reduce the reaction time. In the presence of excess antigen the antigen-antibody reaction in ELISA is virtually completed within half an hour. At more diluted antigen concentrations several hours of incubation may be needed to obtain an appropriate degree of antigen binding. It must, however, be kept in mind that detachment phenomena may occur.

To prepare test samples from faecal material, simple homogenization in several volumes of phosphate buffered saline usually suffices. Clarification or filtration of the homogenates may remove antigen aggregates.

Arklone extraction has been used to disrupt immune complexes in the faeces (Ellens et al., 1978). Reproducibility may be improved by adjusting the pH of the extract using phenol-red as an indicator (Middleton et al., 1977).

Conjugate. The enzymes Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (AP) are most widely used in the preparation of ELISA conjugates. HRP is relatively cheap and is coupled quite efficiently to antibodies by the method of Wilson and Nakane (1978). Orthophenylenediamine (OPD) and 5-aminosalicylic acid (5-AS) are both suitable HRP substrates, although 5-AS has some advantages over OPD (Gielkens, these proceedings). Paranitro-phenylphosphate is generally used as AP substrate. There is no

significant difference between the sensitivity of ELISAs using AP or HRP. Recently, it has been reported that use of fluorescent or tritiated AP substrates increases the sensitivity of ELISA 100 to 1,000-fold. However, these substrates do not allow visual reading (Harris et al., 1979).
At high conjugate concentrations the binding is rapid and may be completed within half an hour. However, the use of high conjugate concentrations is expensive and may lead to high background values. Therefore the minimum concentration of a given conjugate needed for optimal results has to be determined by checkerboard titration. At the optimal conjugate concentration, incubation for 2-4 hours is usually sufficient.

Reading. Most systems in use for determining enzyme activity utilize a substrate that is converted to a coloured product which can be estimated photometrically (Gielkens, these proceedings). To determine the absolute concentration of antigen, a reference, in which the antigen content has been determined with another assay, is needed. For routine antigen determination a relative quantification of the antigen concentration in terms of ELISA units is often more convenient.

Design of confirmation reactions. In general there are two different systems to confirm the specificity of a double antibody sandwich test: i. coating the solid phase with a pre-immune serum in parallel with hyperimmune serum (Fig. 3a) and ii. blocking the detection reaction (conjugate binding) by prior incubation with a known positive serum, preferably a convalescent serum of an experimentally infected gnotobiotic animal (Fig. 3b). By coating the solid phase with a pre-immune serum, the *physical specificity* of the reaction is investigated; i.e. non-specific adsorption can be excluded. The *immunological specificity* of the reaction is not confirmed since the hyperimmune serum may contain antibodies directed against other antigens (Fig. 3a). For this purpose the blocking test, as mentioned previously, has to be performed (Fig. 3b). The physical specificity of the reaction can also be confirmed by the use of pre-immune serum as blocking serum. Mentioned pre-immune serum can also be added directly to the conjugate as a carrier protein.

REFERENCES

Berg, R.A., Rennard, S.I., Murphy, B.R., Yolken, R.H., Dolin, R. and Straus, S.E.: New enzyme immunoassays for measurement of influenza A/Victoria/3/75/ virus in nasal washes. The Lancet, vol. I: 851-853, 1980.

Clark, M.F. and Adams, A.N.: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34: 475-483, 1977.

De Leeuw, P.W., Ellens, D.J., Straver, P.J., Van Balken, J.A.M., Moerman, A. and Baanvinger, Th.: Rotavirus infection in calves in dairy herds. Res.Vet.Sci. 29: 135-141, 1980.

Ellens, D.J., De Leeuw, P.W., Straver, P.J. and Van Balken, J.A.M.: Comparison of five diagnostic methods for the detection of rotavirus antigens in calf faeces. Med.Microbiol.Immunol. 166: 157-163, 1978.

Engvall, E. and Perlman, P.: Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8: 871, 1971.

Harris, C.C., Yolken, R.H., Krokan, H. and Hsu, I.C.: Ultrasensitive enzymatic radioimmunoassay: Application to detection of cholera toxin and rotavirus. Proc. Natl. Acad. Sci. U.S.A. 76: 5336-5339, 1979.

Kalica, A.R., Purcell, R.H., Sereno, M.M., Wyatt, R.G., Kim, H.W., Channock, R.M. and Kapikian, A.J.: A microtiter solid phase radioimmunoassay for the detection of the human reovirus-like agent in stools. J.Immun. 118: 1275-1279, 1977.

Norde, W.: Adsorption of proteins at solid surfaces. In: Adhesion and adsorption of polymers. Polymer science and technology, vol. 12B, Plenum Press, N.Y., Lieng Huang Lee ed., p. 801, 1980.

Ruitenberg, E.J., Van Amstel, J.A., Brosi, B.J.M. and Steerenberg, P.A.: Mechanization of the enzyme-linked immunosorbent assay (ELISA) for large scale screening of sera. J.Immunol.Meth. 16: 351-359, 1977.

Segal, E., Berg, R.A., Pizzo, P. and Bennett, J.E.: Detection of candida antigen in sera of patients with candiasis by an enzyme-linked immunosorbent assay-inhibition technique. J.Clin.Microbiol. 10: 116-119, 1979.

Svennerholm, A.M. and Holmgren, J.: Identification of *E. coli* heat-labile enterotoxin (LT) by means of a ganglioside immunosorbent assay (Gm1-ELISA). Current Microbiology, 1: 19-24, 1978.

Van Weemen, B.K. and Schuurs, A.H.W.M.: Immunoassay using antigen-enzyme conjugates. FEBS Lett. 15: 232-236, 1971.

Wilson, B. and Nakane, P.K.: Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Immunofluorescence and related staining techniques. Elsevier/North-Holland Biomedical Press. W. Knapp, K. Holubar and G. Wicks, eds., p. 215, 1978.

Yolken, R.H., Barbour, B., Wyatt, R.G., Kalica, A.R., Kapikian, A.Z. and Channock, R.M.: Enzyme-linked immunosorbent assay for identification of rotaviruses from different animal species. Science 201: 259-262, 1978.

LEGEND TO THE FIGURES

1. Schematic representation of blocking methods for the detection of antibody or antigen.

a. competition between labelled and unlabelled antigen
b, c. competition between labelled and unlabelled antibody
d. inhibition method for the detection of antigen (antibody combining methods).

Asterisk denotes antigen or antibody to be detected.

2. Schematic representation of trapping methods for the detection of antibody or antigen.

a. indirect method for detection of antibody
b. double antibody sandwich method for the detection of antigen
c, d. indirect double antibody sandwich method for the detection of antigen and antibody, respectively
e, f. alternative sandwich methods.

Asterisk denotes antigen or antibody to be detected.

3. Schematic representation of confirmation methods.

a. coating with pre-immune serum (⊣)
b. blocking of the conjugate binding with a specific convalescent phase serum.

—≺ denotes contaminating antibody present in immune sera. Asterisk denotes antigen to be detected.

Fig 1

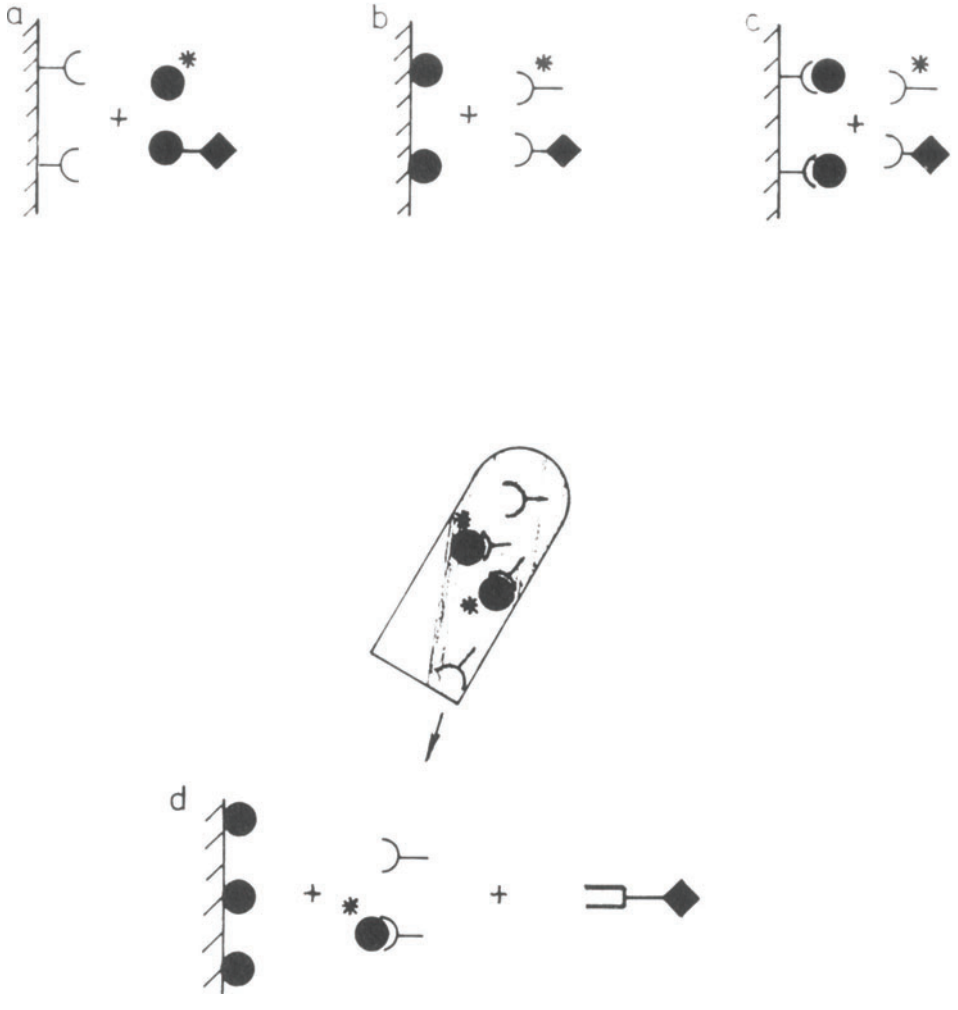

Fig 2

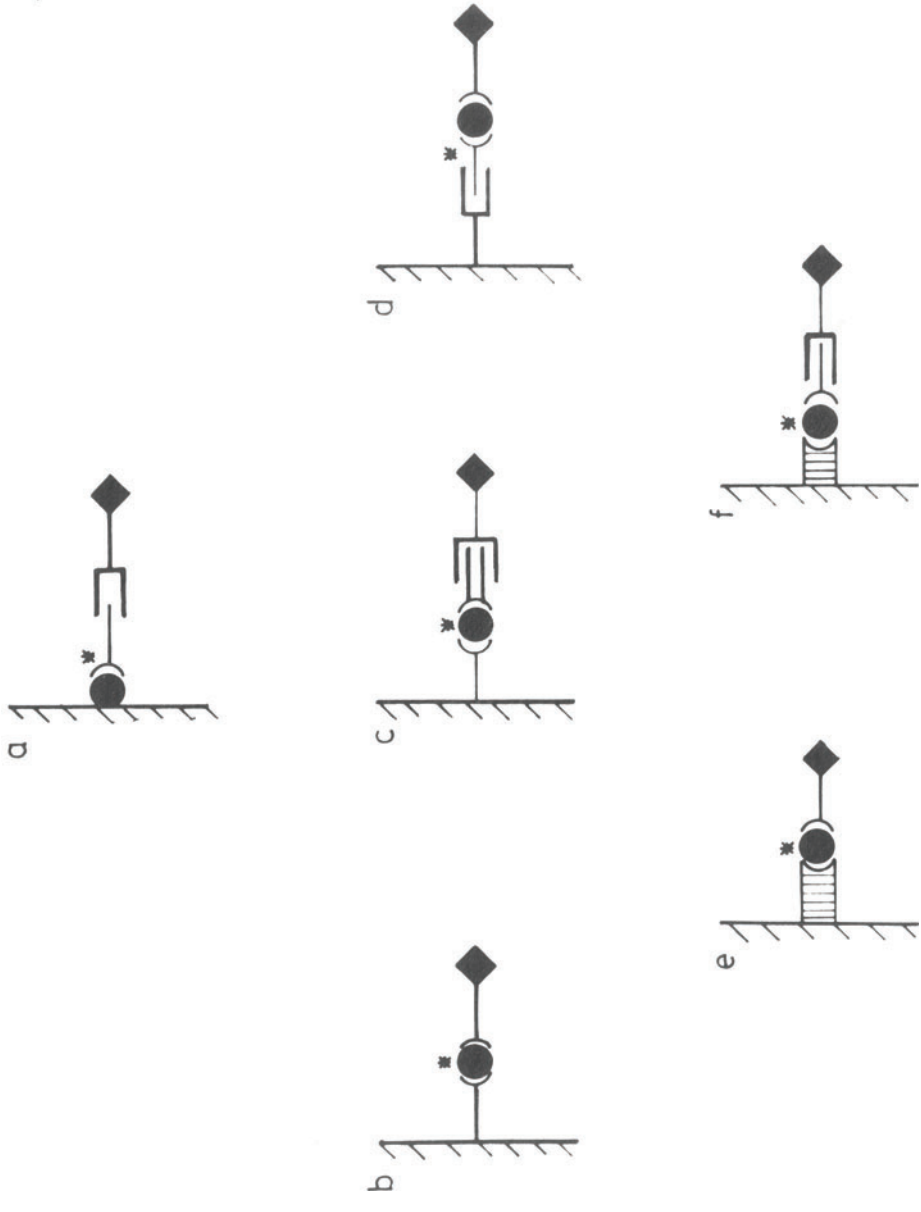

Fig 3