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REVIEW PAPER

Limitations and Practical Problems in Enzyme-Linked Immunosorbent Assays

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ABSTRACT

The major factors that limit the performance of enzyme-linked immunosorbent assays (ELISAs) have been described. Different variations have been explored for specific applications. Some of the common problems that are encountered while carrying out ELISAs have been highlighted. Suggestions have been made to overcome the difficulties and to improve the reliability of the enzyme immuno assays.

1. INTRODUCTION

Rapid and accurate diagnosis of microbial infection is desirable for optimal patient management and for prevention of the spread of infection in the population. Rapid diagnosis is hampered due to the time required for the cultivation and identification of infecting microorganisms[1]. Therefore, a great deal of interest has been shown in the development of new practical techniques for the rapid diagnosis of infections. Most of these techniques involve immunoassays which rely on the specific identification of infecting agents by labelled antibodies or detection of their specific antibodies in the clinical samples (serum, CSF, etc) using labelled antigen of the concerned microorganism.

One of the immunoassays that can provide efficient diagnosis of microbial infections is enzyme-linked immunosorbent assay (ELISA). This technique involves the immobilisation of antigen or antibody on to a solid matrix and reaction of the solid phase antigen or antibody with specific labelled antibodies or antigens. The measurement of the labelled antibody or antigen bound to solid phase allows for the objective and quantitative determination of the antigen or antibody in clinical specimens[2,3]. Quantification of antigen during the course of an illness might be utilised to monitor clinical improvement or to assess the response to anti-microbial chemotherapy[4]. Although enzyme immunoassays are rapidly replacing many other methods used to detect or quantify substances with important biologic or pharmacologic properties due to simplicity, sensitivity and specificity of the test, these assays are governed by a number of factors, like selection of solid phase, antigen, antibody, enzymes, etc. The various factors that limit the performance of enzyme immunoassays have been discussed in this paper.

2. SELECTION OF SOLID PHASE

For the detection of antigen or antibody in ELISA, either antigen or antibody is attached to the solid phase. This permits the separation of immunologically reacted material from unreacted materials during the test. It is imperative that the solid phase should take up an adequate amount of the immunoreactant in a reproducible manner. Variability at this stage is probably still a major factor in determining the precision of all solid phase immunoassays[5].

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Different solid phases commonly used in ELISA are given in Table 1. Various stationary supports have been used to immobilise antigen and antibody, such as particles of cellulose, polyacrylamide, cross-linked dextran and plastic beads or balls[6-9]. All these are satisfactory solid phases, but in the washing steps they need centrifugation which is inconvenient and time consuming[16-18]. The use of preformed materials, such as tubes, discs and plastic microtitre plates (polystyrene, polyvinyl chloride, polypropylene and polycarbonate), overcomes the need for centrifugation as they can be washed easily. Disposable polystyrene microtitre plates have been the most widely used solid support in enzyme immunoassays because they can be coated easily with high reproducibility[10,11,19]. Besides they are convenient for ELISA as they permit the evaluation of a large number of clinical specimens with a minimum amount of reagents and manipulations[17,18]. It has been found that antigens are adsorbed on polystyrene plate passively in alkaline solution at an adequate concentration. However, for some purposes, where a high uptake of the immunological reagent is needed, polyvinyl chloride plates are preferred[11]. These have been found to be especially suitable for coating with immunoglobulin for use in assays for antigen detection. The limitations with polyvinyl chloride plates are that

Table 1. Solid supports used in ELISAs

Solid phase	Nature of binding	Remarks	Reference
Magnogel/ magnetic beads	Covalent binding using bifunctional agents	Rapid separation using magnet	
Cellulose particles	Physical adsorption and/or electrostatic binding	Separation by centrifugation	
Polyacrylamide	Covalent binding following treatment with bifunctional agents	Separation by centrifugation. Used also as immunosorbents	
Cross-linked dextran	-do-	-do-	8
Plastic beads	Hydrophobic interactions	Large surface area, rapidity of washing and reduced background	9
Polystyrene micro strips/ plates	-do-	Excellent for routine quantitative assays using ELISA readers	10
Polyvinyl chloride plates	-do-	Higher antigen binding capacity. Higher background values and increased noise observed	11
CNBr-activated cellulose paper	Covalent attachment	Used especially for detection of allergen specific *IgE*	12
Nitrocellulose paper stick/ strip	Hydrophobic and electrostatic interactions	Simple and cheap support for qualitative or semi quantitative assays	13
Polystyrene cuvettes	-do-	Useful while using spectrophotometers/colorimeters	14
Polystyrene 'pegs'	-do-	Very useful for screening of hybridoma culture supernatants	14
Nitrocellulose paper combs	-do-	Very useful for testing large number of samples. Differential detection is possible	15

they yield higher background because of the higher uptake of antigen and at times this poses difficulty in quantitation[9].

The limited antibody binding capacity of the solid phase and the problem of antibody elution can be overcome by the use of an antibody, covalently linked to the solid phase[20,21]. An effective means of accomplishing this is to add amino groups to the solid phase by treating polystyrene with sulphuric and nitric acids. The antibody can then be covalently linked with amino groups of the solid phase with glutaraldehyde. Many of the commercially available plates (for example, NUNC immunoplate I) are already chemically treated or activated and the antigen or antibody can be taken up in the wells of microtitre plates quantitatively without any loss in the subsequent steps. Thus, the use of covalently linked, strongly bound antibodies can markedly improve the sensitivity of solid phase immunoassay[22]. Cyanogen bromide activated cellulose paper discs are also used for covalent coupling of antigens for development of sensitive assays[12].

It has been found that non-plastic supports like nitrocellulose can also be used to coat antigen or antibody[13]. These materials have binding capacities up to 500 $\mu g/cm^2$ whereas plastics[22] have binding capacity limited to about 0.5 $\mu g/cm^2$. Therefore, such supports have an edge over the plastic supports. Since higher concentrations of immunoglobulin can be bound, paper as solid matrix has the potential for greater sensitivity than the plastic ones. The main problem with these solid phases arises from their high binding capacity, namely, high degree of non-specific binding. When these solid phases are used, extensive washing is needed. After the antibody is bound, the solid phase needs to be treated for blocking the unreacted sites of the support with higher concentrations of protein solutions (for example, 1-3 per cent BSA) to prevent non-specific adsorption of labelled reagents.

Another aspect of solid phase which merits attention is its shape. It has been reported that convex surfaces such as balls can be coated better than the plates[5]. Compared to microtitre plates, a very large number of balls can be better coated in one step with minimal loss of the immunoreagent and with a high degree of reproducibility. Small variations in temperature exist between the centre and the edge of the plates which affect the reproducibility of coating the antigens or antibodies to microwells of the plates. However, plates permit a fast direct reading by the very efficient, modern photometers adapted to this specific purpose. For antibody estimation and quantitative antigen detection, the microplates technique offers an advantage because the equipment for semi or nearly complete automation are available. However, for precise quantitative assays convex objects, such as polystyrene balls, which allow a high degree of reproducibility are preferred[5]. As discussed earlier, the solid phase carriers should have high binding capacity. But it has been noticed that batch to batch variation with respect to binding capacity may occur[23]. Therefore, each batch of material should be tested prior to using it as solid phase. This testing should also include the determination of within-batch variation.

A method of testing a representative sample of a batch of beads or microtitre plates is explained below.

(a) Coating of the solid phase with 5-10 $\mu g/ml$ of the reference antigen or antibody in a suitable buffer by incubating for 2 hr at 37 °C or overnight at 4 °C.

(b) Washing of the coated solid phase to remove unabsorbed material by washing with phosphate buffered saline containing Tween-20. Blocking the sites that are not occupied with the antigen or antibody with 1 per cent bovine serum albumin or any other blocking agent (like fetal calf serum, goat serum, milk powder, gelatin, etc) by incubating for 2 hr and washing.

(c) Incubation of the plates with appropriately diluted enzyme-labelled antibody or antigen/antispecies antibody for about 1 hr at 37 °C and washing.

(d) Addition of the substrate and observation of the results after appropriate incubation and after arresting the enzyme reaction.

Such a procedure may indicate the suitability of the solid phase material. A standard deviation of 0.05 with an absorbance reading of 1.0 may be considered acceptable.

3. IMMUNOCHEMICALS FOR COATING THE SOLID PHASE

The success of enzyme immunoassays (EIA) in the detection of antigen or antibody in clinical material not only depends upon the solid phase used to coat the immunoreactants, but also upon the nature and the type

of antigen or antibody and its concentration[17,24]. The rate and extent of coating of antigen or antibody will depend on the diffusion coefficient of the adsorbing molecule, the ratio of surface area to be coated to the volume of coating solution, the time of coating, the temperature and the *pH*[25,26]. Appropriate buffer, *pH* and ionic strength are to be chosen for coating the antigens or antibodies keeping in view the stability of the components in the coating buffer. Therefore, optimal conditions for coating the solid phase must be determined by checkerboard titrations using reference reagents[9,27]. These conditions should be strictly adhered to in the subsequent tests for obtaining reproducible results. For most proteins and lipoproteins, satisfactory coating is achieved with solution at 1-10 μg/ml in carbonate buffer (*pH* 9.6). The adsorption occurs rapidly, and is generally completed within 1-2 hr at 20-25 °C. However, for convenience, overnight coating at 4 °C is often used and is found satisfactory[9,11].

There is no satisfactory way to predict the suitability of an antigen for use in the enzyme immunoassay. The best way is to test the material at various dilutions using reference sera and reference conjugates. Because antigens are suitable for other serological tests does not mean that they will give acceptable results in enzyme immunoassays. It is essential to determine whether the crude antigen extracts from different microbes react non-specifically with the test sera. For this purpose it may be necessary to prepare and use negative controls (for example, sera from uninfected animals). Values given by these control materials should be subtracted from those given by the antigen or antibody preparations[9]. Preferably pure antigens and reagents should be used to develop sensitive and specific assays.

4. SEPARATION STEPS AND INCUBATION

In heterogeneous immunoassays, a series of addition and separation steps followed by lengthy incubation periods and repeated washings are involved[9]. The washing steps must be carried out in such a manner that there is no carry-over from one step to the next. The microplate format is very easy for the washing steps since 96 wells can be washed simultaneously, while the sequential washing of individual tubes, beads or discs is time consuming and requires more reagents as well[16,17]. However, if magnetic beads are used for coating, then the separation can simply be achieved by the use of a magnet. A modification that appears to be simpler is the double determinant assay[22]. In this assay, antibody directed at one bacterial or viral determinant is used to coat the solid phase, and labelled antibody directed at another determinant is added to the liquid phase. The specimen and label can be added simultaneously to the solid phase so that competition between solid phase and liquid phase antibody may not occur. After incubation, the solid phase is simply washed and the substrate is added. These assays are easy, rapid, require only a single washing step, and can readily be automated.

In the 'peg' modification of the microtitre plate assays, lids to plates have an aligned set of pegs (coated with antigen or antibody) that reach into the wells. The solid phase reaction occurs on the pegs but the soluble substrate reaction usually takes place in the wells which are measured in the conventional plate assays. These assays are particularly suitable for screening of the hybridoma culture supernatants[14].

Dipsticks or nitrocellulose strips are very useful solid supports, used either as a single strip[28] or in a comb format[15]. These are useful in qualitative or semi-quantitative assays. Dot-blot or slot-blot systems are also available for screening large number of samples. Slot-blot assays can give quantitative information when used with a densitometric scanner[29]. With proper use of the nitrocellulose paper strips even differential detection of various antigens is possible. This is very much useful in the detection of Western blots, i.e., electrophoretic transfer of proteins separated previously by electrophoresis to nitrocellulose paper[30].

5. SELECTION OF ENZYME FOR CONJUGATE PREPARATION

The selection of an enzyme is directly related to the amplification effect imparted by the enzyme moiety (the formation of many product molecules per test antigen molecules)[11]. Amongst the huge number of enzymes of the living world, only a few ones have found widespread applications[3,11]. Availability, purity, cost, stability at room temperature and storage at 4 °C, production of conveniently measurable signal on reaction with available safe and cheap substrates, and the higher turnover number, are some of the factors that make the enzymes suitable for coupling with antigens or antibodies. The enzymes that satisfy these criteria and most often employed in heterogeneous ELISAs include alkaline phosphatase[3], horseradish peroxidase[23,31],

β-D- galactosidase[9,11], urease[14] and penicillinase[14]. Horseradish peroxidase is now a clear favourite for most workers because of its low cost, easy conjugation procedures and availability of wide variety of substrates[9]. *β-D*-galactosidase is an inducible enzyme of bacterial origin and can easily be produced. It has also got some fluorimetric substrates for sensitive enzyme assays. Penicillinase and urease are the recent addition to the inventory of immunochemicals. These two enzymes are not present in the mammalian tissues, and hence would not pose the problem of endogenous activity increasing the background noise. Moreover, they have simple, stable and safe substrates that can give clear end points and are very useful in qualitative and semi-quantitative assays. The salient features of some of the commonly used enzymes are summarised in Table 2.

6. SUBSTRATES

The chief requirement of a substrate is to provide a sensitive detection method for the enzyme in the conjugate[1]. Substrates should be cheap, safe, easy to use, and should produce an easily observable signal, once catalysed by the enzyme[9]. Most of the workers have used chromogenic substrates which are initially colourless and which on catalysis give a strong colour. Ideally the substrate should give completely soluble products with a high extinction coefficient (i.e., dense colour per unit degraded) in most of the quantitative ELISAs. Enzymes can give raise to soluble products or the products that are insoluble. In the case of immunohistochemical staining for the localisation of antigens in the tissue sections or visualising the immunochemical reaction on nitrocellulose matrix, precipitates at the reaction sites are preferred. Some of the substrates widely used in ELISA are given in Table 3. The end point of an enzymic reaction can either be measured as rate (change in appearance or disappearance of colour per unit time) or measuring the intensity of the colour developed, stopping the reaction after stipulated time. Caution should be exercised in using the correct ionic strength and *pH* of the buffer for solubilising various substrates and for arresting the reaction with appropriate stop solution. For quantitative measurements correct wavelengths should be selected. To avoid health hazards, due care needs to be taken while handling the carcinogenic substrates. To reduce the non-specific background noise in case of photosensitive substrates, they should be protected from light. In case of peroxidase system, the

Table 2. Salient features of commonly used enzymes in heterogeneous ELISA

Enzyme	M. wt	Turn over No.	Remarks
Alkaline phosphatase		60,000	First enzyme used in EIA. Sensitive soluble and insoluble substrates available. Can be used both in plate EIA and for immunohistochemical staining. Endogenous activity has to be blocked.
Horseradish peroxidase	40,000	21,000	Cheap enzyme with variety of soluble and insoluble substrates. Sensitive substrates are light-sensitive and carcinogenic. Endogenous activity has to be blocked in histochemical localisation.
β-D-galactosidase	540,000	12,500	It can be prepared in bulk from bacteria-sensitive substrates available.
Penicillinase	31,000	160,000	It is not present in eucaryotes. No non-specific interference of endogenous activity. Clear and simple end point of decolorisation for qualitative and semi-quantitative assays.
Urease	480,000	52,000	Not present in mammalian tissues. Less problem of endogenous signal generation Clear cut and sharp end point. Can be used in electrode based enzyme immunoassays and also in competitive binding immunoassays.

Table 3. Salient features of commonly used substrates in enzyme immunoassays

Enzyme	Substrate	Co-substrate (or) chromogen	Buffer	Colour*	End product	Stop solution	Wavelength stopped (nm)
Alkaline phosphatase	*p*-nitrophenyl phosphate (PNPP)	–	Diethanolamine	Yellow	Soluble	*NaOH*	410
	5-bromo-4-chloro 3-indolyl phosphate (BCIP)	Nitro blue tetrazolium (NBT)	Tris	Purple	Insoluble	DW wash	
	Naphthol AS-MX phosphate	Fast red BB	Tris	Red	Insoluble	DW wash	
		Fast blue BB	Tris	Blue	Insoluble	DW wash	
Horseradish peroxidase	Hydrogen peroxide	*o*-phenylene diamine (OPD)	Citrate-phosphate	Orange	Soluble	3M *HCl*	492
		5-amino salicylic acid (5AS)	Hot Water	Orange	Soluble	3M *NaOH*	550
		o-dianisidine (ODD)	Citrate-phosphate	Brown	Soluble/ Insoluble	5M *HCl*	530
		3,3′,5,5′-tetra methyl benzidine (TMB)	Citrate-phosphate	Blue	Soluble	2M H_2SO_4	450
		2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulphonic Acid) (ABTS)	Citrate-phosphate	Green	Soluble	1% SDS	405
		3,3′-diamino-benzidine (DAB)	Tris	Brown	Insoluble	DW wash	
		4-chloro-1-naphthol (4C1N)	Tris	Blue	Insoluble	DW wash	
		3-amino-9-ethyl carbazole (AEC)	Acetate	Red	Insoluble	DW wash	
β-D-galactosidase	*o*-nitrophenyl galactopyronoside (ONPG)		Phosphate	Orange	Soluble	2M Na_2CO_3	420
	Chlorophenol-*β-D*-galactopyranoside		HEPES	Red-violet	Soluble		574
	Resorufin-*β-D*-galactopyranoside		Phosphate	Red	Soluble		572
	5-bromo-4-chloro-3-indolyl-*β-D*-galacto-pyranoside (X-gal)		Phosphate	Blue	Insoluble		
Penicillinase	Benzyl penicillin	Iodine/ starch	Phosphate	Blue to clear	Soluble	12N *HCl*	
Urease	Urea	Bromo cresol purple (BP)	*NaOH*	Yellow to purple	Soluble	2M $MgCl_2$	588

substrate H_2O_2 should be prepared freshly every time and added to the cosubstrate to visualise the colour production.

7. PREPARATION OF ENZYME-ANTIGEN OR ANTIBODY CONJUGATE

Either antigens or antibodies may be labelled with the enzyme. It is essential for the competitive assays that the antigen used for labelling should be as pure as possible[11]. It is preferable to use affinity purified or partially purified antibodies of high affinity and avidity for preparing good enzyme antibody conjugates.

The objective of conjugation is to link the enzyme with antibody or antigen in such a way that each retains the maximum amount of its reactivity[9]. This is achieved by the use of cross-linking agents with at least two active groups[11,32]. The cross-linking can be accomplished in a one-step procedure (where the two components to be covalently attached are mixed together with the cross-linking agent) or a two-step procedure (where the enzyme is reacted with cross-linking agent; then the activated enzyme is reacted with antigen or antibody). Glutaraldehyde has been the most frequently used cross-linking agent, for coupling enzymes to protein antigens and antibodies in both one- and two-step procedures[9,31].

The chemical nature of the antigen, antibody and the enzyme determines the procedure to be adopted for conjugation. Various methods that have been used for the preparation of the immunoconjugates are mentioned in Table 4. Peroxidase and antibodies were coupled using glutaraldehyde, a homobifunctional agent by the interaction of amino groups present in both the enzyme and the antibodies. The two-step procedure is a modified version to get predominantly homogeneous enzyme-antibody conjugates. Periodate method has been used to couple peroxidase antibody through its carbohydrate moieties[33]. Conjugation of the sulfhydryl groups of *β-D*-galactosidase with antibody has been achieved using *N,N'-O*-phenylene-dimaleimide with high efficiency. Various reactive functional groups of the antigens can be used for labelling with enzymes.

High molecular weight polymers of conjugates often exhibit more non-specific binding to the solid phase and reactivity with rheumatoid factors present in serum[38,39]. It is, therefore, necessary that the antibody and enzyme preparations used to form conjugate have a high specific activity to compensate for the loss of activity on conjugation. This problem can be solved by labelling antibodies with low molecular weight cofactors like biotin system instead of enzymes[40]. In this system,

Table 4. Conjugation methods for labelling antibodies/antigens

Enzyme	Conjugation method	Reference
Horseradish peroxidase	Two-step glutaraldehyde	32
	Sodium *m*-periodate	33
	One-step glutaraldehyde	34
	N-Succinimidyl-3-(2-pyridyl-dithio) propionate (SPDP)	35
Alkaline phosphatase	One-step glutaraldehyde	34
Penicillinase	One-step glutaraldehyde	34
β-D-galactosidase	*m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester (MBHS)	36
Urease	One-step glutaraldehyde	37

biotin-labelled antibody is reacted with solid phase antigens and following the removal of unreacted biotinylated antibody, enzyme labelled avidin complex is added[41]. Biotin-avidin system enhances the sensitivity of the test[42]. After conjugation, the antibody-enzyme/antigen-enzyme conjugate should be cleaned up by gel filtration or dialysis and concentrated by ultrafiltration. However, appropriate coupling procedures have to be employed depending upon the nature of the enzyme and antigen or antibody.

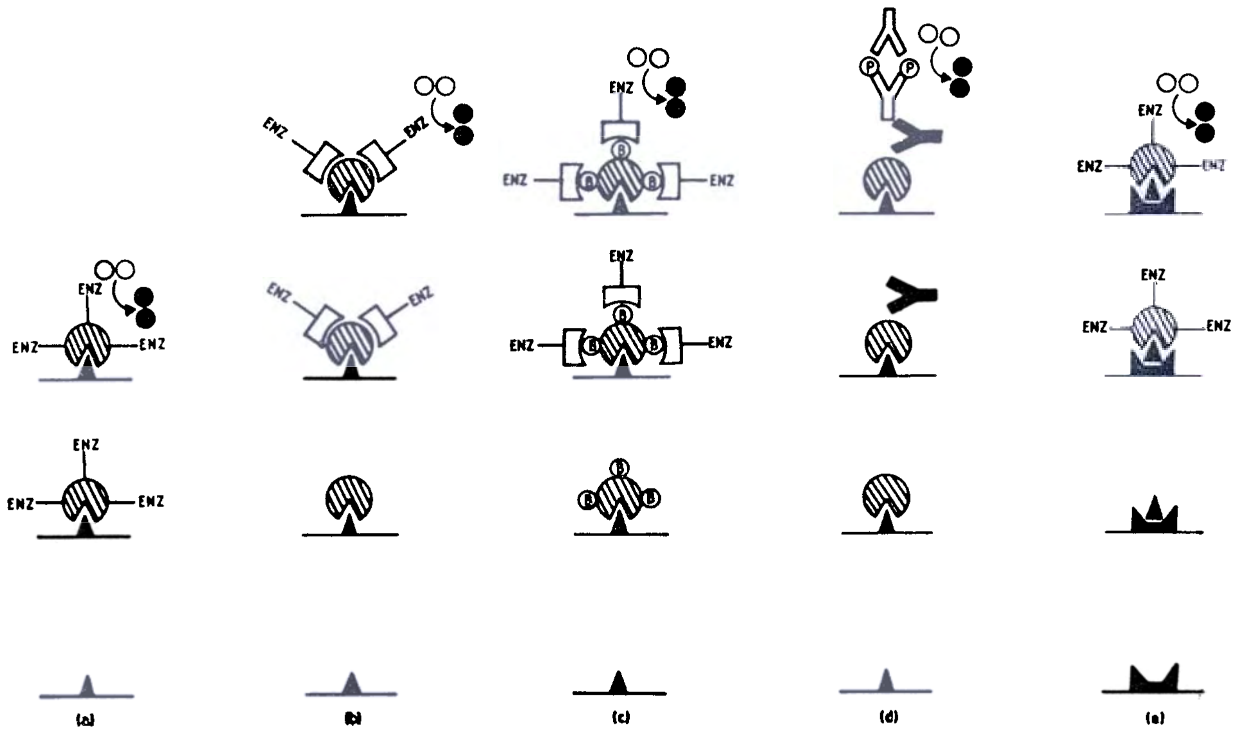
8. ASSAY DESIGN

Different assay formats generally used in the ELISA are diagramatically represented in Fig. 1. The most commonly used format for antigen detection in test sample is the direct assay[43]. In this format the specific antibodies to antigen are bound to solid phase. After incubation and washings, the test sample is added and incubated followed by the addition of antigen-specific enzyme-labelled antibody. Finally, the substrate is added. The intensity of colour generated is measured. The major limitation with direct assay is that it requires a separate labelled antibody for each antigen which is practically not possible for a clinical laboratory. Although direct assay can be performed with ease and speed, antigen in question should have atleast two binding sites to allow for binding to the solid phase and to the labelled antibody, and hence it limits the scope of this method.

The indirect assay format is preferred to overcome the major limitation of direct format of antigen detection[44]. In this format, unlabelled second antibody is substituted for the labelled antibody in the direct system. The widespread availability of the labelled anti-globulins eliminates the need for preparing labelled reagents directed against specific microorganisms.

9. SENSITIVITY AND SPECIFICITY

The capabilities of an EIA may be described in terms of sensitivity and precision. The sensitivity is the smallest amount which is measurable with a specified level of confidence[45]. The most important determinants of sensitivity and specificity in an EIA system are the immunoreagents. The sensitivity of EIA depends on antibody concentration, binding affinity between antigen and antibody, and level of detection of the enzyme employed as label[5]. Because antibody concentration is relatively constant for many polyclonal antisera, the main determinant of assay sensitivity will be antibody avidity. Since solid phase assay involves multiple incubation and washing steps, they are especially dependent on high antibody avidity. The affinity between antigen and antibody limits the sensitivity of EIA, and therefore any loss of sensitivity in ELISA may be ascribed to the decrease in the



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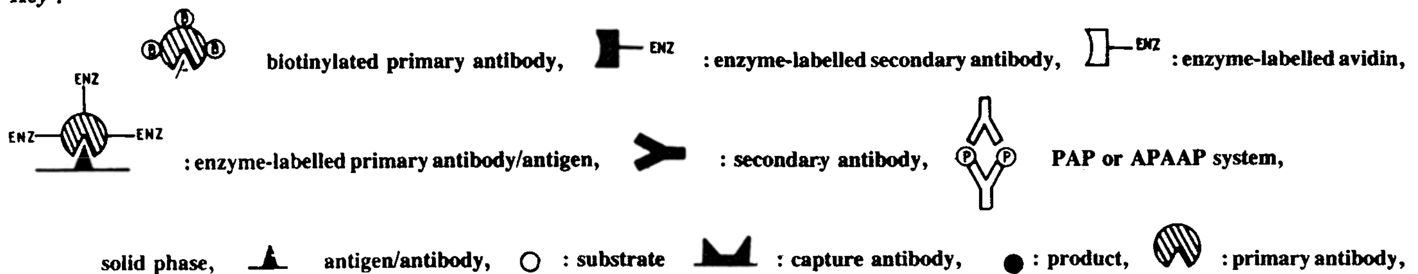

Figure 1 Commonly used enzyme immunoassay formats (a) direct ELISA for measurement of antigen/antibody; (b) indirect ELISA for measurement of antigen/antibody; (c) avidin biotin ELISA for measurement of antigen; (d) peroxidase anti peroxidase or alkaline phosphatase anti alkaline; (e) phosphatase system for detection of antigen; and (f) double antibody sandwich ELISA for detection of antigen.

antigen-antibody binding affinity[11]. The decreased affinity may result from structural changes accompanying the coupling of enzyme to antigen or antibody, and/or it may be caused by molecular distortion in the immobilised antigen or antibody.

The principal determinant of specificity in immunoassays (in general) and ELISA techniques (in particular) is the antibody. Specificity of antibody preparations can be enhanced by using immunosorbents to remove non-specific or interfering antibodies. Even then, an antibody may cross-react with antigens similar in structure to the immunising antigen and thus limiting the specificity. Final and equally critical factor is the purity of the antigen used as immunogen and assay standard.

One major cause of decreased specificity in ELISA method is the non-specific adsorption of enzyme-labelled antigen or antibody to the solid phase[17]. This non-specific adsorption of enzyme activity can be minimised by inclusion of neutral detergent that does not interfere significantly with the antigen-antibody reaction[46].

Besides non-specific adsorption of enzyme label to the solid phase, another important factor affecting specificity is the completeness of separation of the adsorbed enzyme-labelled antigen-antibody complex from the solution containing free enzyme-labelled antigen or antibody. Most plastics display a negative electric potential at their surfaces. This may be modified by adsorption of protein. The residual electric field at the solid-solution boundary gives rise to a diffused double layer of ions at the interface. Depending on the plastic surface architecture, this double layer, containing free enzyme-labelled antigen or antibody, may be difficult to displace except by vigorous or prolonged washing. Variation in the extent of removal of non-adsorbed enzyme label present in the diffused double layer also may be a factor affecting the precision of assay[11].

10. PROBLEMS OF ENZYME IMMUNOASSAYS

The success of EIA in the detection of antigen or antibody in clinical samples not only depends upon the solid phase and immuno-reactants (type of antigen or antibody and conjugate), but also upon the absence of non-specific reactions which pose problems and influence the sensitivity and specificity of the assay. Following are some important factors which create problem in the detection of antigen in clinical samples.

10.1 Antigen Diversity

Although the antigenic composition of different strains of certain organisms such as *Staphylococcus aureus* may be similar, thus permitting the development of assays that detect antigens of most strains[47], type-specific antigens occur in many organisms. For example, assays for meningococcal, pneumococcal and *Legionella* antigens are serotype-specific; assay for one serotype may not detect antigens of other serotypes[48]. Thus, antigen assays may not be practical screening tests. If antigen common to multiple organisms, such as peptidoglycan backbone of gram positive cocci and the core lipopolysaccharide of gram negative bacilli, could be detected in blood or urine of infected patients, assays for those antigens might be useful as screening tests.

10.2 Non-specific Inhibitors

Non-specific serum factors may inhibit antigen detection. Heat-labile serum inhibitors like early complement components appear to be involved[49]. It has been noticed that Cl_q may attach to the solid phase immunoglobulin or directly to the solid phase itself thus competing with and preventing the attachment of antigen to their specific antibodies. These inhibitors can be inactivated by heating at 56 °C, by heparin and zymosan, thus improving the sensitivity of assay system.

Besides, a far more serious problem in the application of competitive ELISA is the incubation of enzyme-labelled antigens or antibodies with test solutions containing serum, urine or tissue extracts. These solutions may contain protein modifying enzymes such as proteases, and non-competitive 'enzyme inhibitors (dehydrogenases), all of which may substantially alter the activity of the enzyme label in the subsequent incubation with enzyme substrate. This problem is avoided in the non-competitive ELISA techniques where the incubation with test antigen is separate from the incubation with enzyme-labelled antigen or antibody[13].

10.3 Specific Inhibitors (Pre-Existing Antibodies)

Pre-existing antibodies bind to microbial antigens and form immune complexes, thus inhibiting antigen detection[50]. Antibodies (serum) to a variety of staphylococcal antigens occur in normal individuals,

presumably because of prior infection or colonization with *Staphylococci* or organisms containing similar antigens[51]. This inhibitory effect of serum, present in the isolated *IgG* fraction, was stable at 56 °C for 30 min, but could be inactivated by heating at higher temperatures. Similar inhibition in *Haemophilus influenzae*[47], pneumococcal and fungal infections have been reported[52]. In pneumococcal pneumonia, the sensitivity for antigen detection in blood increased from 38 to 57 per cent after the dissociation of immune complex. Thus, pre-existing antibodies impair the detection of circulating antigens in a variety of infections, limiting the usefulness of antigen assay for rapid diagnosis of infection.

The antigens and antibodies are held together in these complexes by ionic and hydrophobic forces. These immune complexes are dissociated either by heating above 70 °C for 30-45 min or by acidification to *pH* 3.5 or lower. Heating above 70 °C dissociates the complexes and progressively denatures the freed antibodies, thus preventing their reassociation with antigen[47,49]. However, undiluted serum coagulates when heated above 65 °C. These problems can be solved if the immunoglobulins and immune complexes are first precipitated in 50 per cent ammonium sulphate solution for 15 min at 4 °C and then resuspended in distilled water. The dissociation of immune complexes by acidification involves the treatment of diluted serum with equal volume of citrate buffer at *pH* 2.7 followed by heat treatment at 96 °C for 20 min. The heated, acidified sample was then clarified by centrifugation, and the supernatant is neutralised with equal volume of phosphate buffer. The sample can be concentrated before the test by evaporation or lyophilisation.

10.4 Rheumatoid Factor

Rheumatoid factor or rheumatoid factor-like activity, may cause false positive results in sandwich ELISAs[1,47]. Solid phase *IgG* and *IgG*-enzyme conjugates are apparently recognised as 'aggregated'. Rheumatoid factor can bridge the solid phase and conjugated *IgG*, producing false positive results. Non-specific reactions have been noticed in an ELISA employed for *Legionella pneumophila* antigen detection[53]. Such non-specific binding can be overcome by using only the $F(ab)_2$ portion of *IgG* molecules, because rheumatoid factors bind to the F_{c} portion of *IgG*. The false positivity due to rheumatoid factors can also be removed by treating it with 2-mercaptoethanol[54], heating at 100 °C for 2 min and adsorption with *IgG* coated latex particles[55].

10.5 Protein A Contamination

Contamination of specimens with protein A containing *Staphylococci* is another potential source of false positive reaction[22]. Such specimens should become negative when adsorbed with non-specific *IgG*.

10.6 Impure Antigens

Another major problem in the detection of viral antigens is the presence of antibodies to host components, derived from the animal models/tissue culture system used to propagate the virus. Antibodies present in the body fluids of human beings, capable of reacting with non-viral antigens lead to false positive results[22]. A more general approach to this problem consists of absorbing an antibody with host tissue to remove cross-reacting antibodies. The absorption improves the specificity of a reagent and also eliminates non-specific reaction up to some extent. Use of purified viral antigens or synthetic antigenic determinants in turn will eliminate the non-specific false positive interactions.

Another method for the assurance of high specificity is the use of monoclonal antibodies. Using hybridoma techniques[56], the antibody directed against a single determinant of the microorganism can be prepared, decreasing the chance of having antibodies that can react with other antigens. In addition, because each molecule of antibody is directed at a specific epitope of the antigen to be measured, monoclonal antibodies may also increase the sensitivity of ELISA. At times, one problem encountered with monoclonal antibodies is that they fail to recognise common determinants of viral or bacterial antigens in clinical specimens and thus do not detect the presence of the organisms in some infected patients[22]. However, judicious use of more than one monoclonal antibody in the form of a cocktail can not only be used for highly specific identification, but can also improve the sensitivity of the assay considerably.

11. SUMMARY

Enzyme-linked immunosorbent assay is one of the modern tools available for the diagnosis in microbiology. The sensitivity of this technique is

comparable with the most sensitive radio immunoassay (RIA). ELISA does not suffer from the hazards of isotopes associated with RIA and hence, it is the preferred test system in diagnostic microbiology. Unlike the conventional immunoassays like indirect haemagglutination, complement fixation test, immunodiffusion and immunoelectrophoresis, ELISA is a very sensitive and specific test system[57]. The shortcomings and difficulties associated with ELISA are discussed in this article. Various suggestions have been outlined in choosing the appropriate test design that is best suited to specific occasions. The inherent drawbacks of individual steps in the performance of the assays have been brought out and the possible ways of circumventing them discussed. Common problems encountered in ELISA and few of the solutions to overcome them are explained. Limitations and problems associated with ELISA have been analysed with a view to exercise caution in the scientific workers involved in the use of ELISA. This will enable them not only to effectively use this excellent technique for the rapid diagnosis, but also to modify the method to be applicable in field conditions.

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