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Utility of One Step Immunoassay in Detecting False Negativity in Routine Blood Bank Screening of Infectious Diseases

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1. Introduction

Immunoassays are chemical tests used to detect or quantify a specific substance, the analyte, in a blood or body fluid sample, using an immunological reaction. Immunoassays for antibodies produced in viral hepatitis and HIV are commonly used to identify patients with these diseases.(Bishop et al., 2001). The commonly used immunoassay methods for detection of infectious diseases are immunoprecipitation, which measures the quantity of precipitate formed after the reagent antibody (precipitin) has been incubated with the sample and reacted with its respective antigen to form an insoluble aggregate, and enzyme immunoassay in the form of enzyme-linked immunosorbent assay (ELISA). The basic principle of these assays is the specificity of the antibody-antigen reaction.(Burtis & Ashwood,2001).

Though being very specific and sensitive, immunoassays are easy to perform which has contributed to it's widespread use and tremendous success.(Henry,2001). Their high specificity results from the use of antibodies and purified antigens as reagents. An antibody is a protein (immunoglobulin) produced by B-lymphocytes (immune cells) in response to stimulation by an antigen. Immunoassays measure the formation of antibody-antigen complexes and detect them via an indicator reaction. High sensitivity is achieved by using an indicator system (e.g., enzyme label) that results in amplification of the measured product.

The purpose of an immunoassay is to measure (or, in a qualitative assay, to detect) an analyte. Immunoassay is the method of choice for measuring analytes normally present at very low concentrations that cannot be determined accurately by other less expensive tests. (Wallach,2000). Immunoassays for antibodies produced in viral hepatitis, HIV, and syphilis are commonly used to identify patients with these diseases. Although immunoassays are both highly sensitive and specific, false positive and negative results may occur. False-negative results may be caused by improper sample storage, reagent deterioration, improper washing technique or prozone effect. False-positive results have been reported for samples containing small fibrin strands that adhere to the solid phase matrix or due to substances in the blood or urine that cross-react or bind to the antibody used in the test. (Wild, 2000).

Large quantities of antigen in an immunoassay system impair antigen-antibody binding, resulting in low antigen determination. This is called the 'high dose hook effect'. The first

description of the prozone effect in the literature was made by Miles et al.,1974. Large quantities of antigen in an immunoassay system impair antigen-antibody binding, resulting in low antigen determination. This is called the prozone or high dose hook effect, which describes the inhibition of immune complex formation by excess antigen concentrations. The prozone or high-dose hook effect, documented to cause false-negative assay results >50 years ago, still remains a problem in one-step immunometric assays. (Brensing,1989; Haller et al,1992;Landsteiner,1946). To detect the prozone effect, samples are often tested undiluted and after dilution. (Saryan et al,1989). If the result on dilution is higher than for the undiluted sample, then the undiluted sample most likely exhibited the prozone effect. Unfortunately, this approach increases labour and reagent costs for assays that may only rarely encounter extremely high analyte concentrations.

2. Material and methods

The present study was performed on voluntary blood donors at our transfusion centre. Hepacard device (J Mitra Laboratory Systems-India) for detection of hepatitis B surface antigen was labelled with patient's identification number. Blood was collected by venipuncture and allowed to clot naturally and completely. Subsequently serum was separated from the clot with the help of a clot retractor. Then 70 µl of donors serum was added into the inbuilt sample well of the hepacard device containing the coated antibodies, using a calibrated dropper and allowed to react for 20 minutes. Results were read thereafter in the form of visually detectable pink control and test lines.

3. Observations

The hepacard device when read after 20 minutes showed only one distinct pink test line and no control line. Serial dilutions (1:10,1: 20) of the donors serum sample was performed in normal saline and the test was re-run with serum samples of each dilution step-wise. Serum sample with 1: 10 dilution showed a control and faint pink test line.(Figure 1). This faint pink test line intensified to a broad pink band when the test was performed with 1: 20 diluted serum sample of the donor.(Figure 2). So to overcome the prozone effect, which

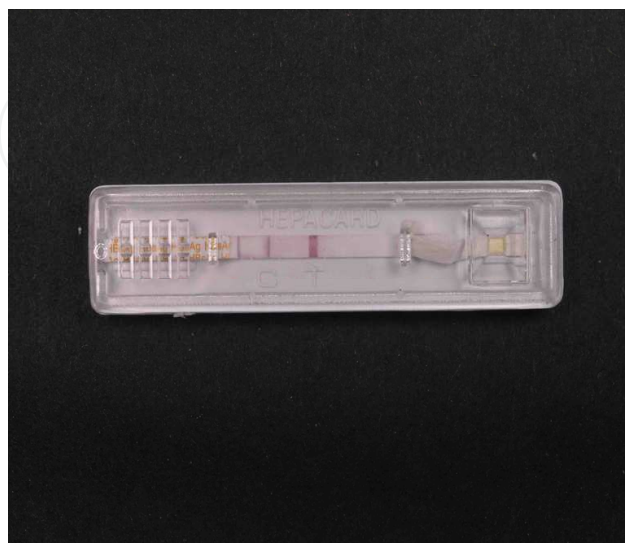


Fig. 1. Depicting faint pink test line

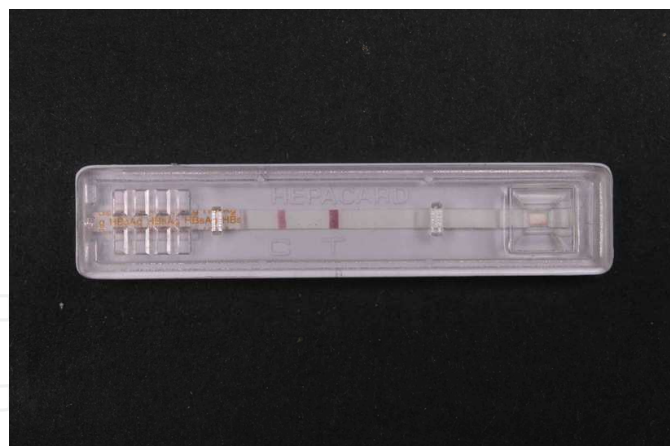


Fig. 2. Depicting broad pink test line

describes the inhibition of immune complex formation by excess antigen concentrations, the donors serum sample was serially diluted. In our case, a 1:10 dilution did not show a prominent pink line but a higher dilution of 1:20 was tried to get a broad pink band.

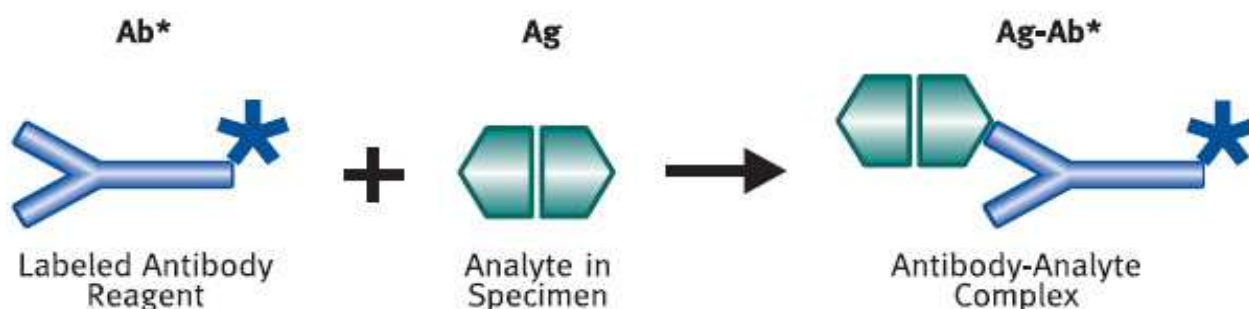


Fig. 3. Schematic diagram showing antigen-antibody reaction in the hepacard device.

4. Discussion

The intensity of an antigen-antibody interaction depends primarily on the relative proportion of the antigen and the antibody. A relative excess of either will impair adequate immune complex formation. (Stites et al, 1997). This is called the 'high dose hook effect' or the 'prozone phenomenon'. This has been classically described in serological tests for diagnosis of brucellosis. (Young ,1995). In addition to hormonal assays, the high dose hook effect has also been demonstrated in immune-based techniques used in the measurements of CA 125, IgE and prostrate specific antigen. (Wolf,1989; St-Jean et al, 1996). All immunoassays are based on antigen antibody interactions. The high dose hook effect often interferes with the assay result. The goal in the immunoassay in screening of infectious diseases should be to minimize erroneous results; so as not to endanger patient health and the blood supply. Reporting of an erroneous result can have serious medical implications, and sample pooling is a simple method for detecting falsely low concentrations attributable to the prozone effect. Although this screening approach increases reagent costs by 10% and involves additional labour to prepare and analyze pools, it is considerably more cost-effective than analyzing all samples undiluted and after dilution, which doubles reagent costs.

The prozone or (high-dose) hook effect, still remains a problem in one-step immunometric assays (Pesce,1993; Vaidya et al,1988; Zweig & Csako, 1990), immunoturbidimetric assays (Jury et al,1990), and immunonephelometric assays (Van Lente,1997) for immunoglobulins. To detect the prozone effect, samples are often tested undiluted and after dilution. (Saryan et al,1989). If the result on dilution is higher than for the undiluted sample, then the undiluted sample most likely exhibited the prozone effect. Unfortunately, this approach increases labor and reagent costs for assays that may only rarely encounter extremely high analyte concentrations. An alternative approach involves pooling patient samples and measuring the pool and a 10-fold dilution of the pool. (Cole et al,1993). If one or more of the samples in the pool is falsely low because of the prozone effect, then the results from the undiluted and diluted pools (after correcting for the 10-fold dilution) will differ significantly. (Cole et al,1993). Other approaches to eliminate the prozone effect include using two-step immunoassays that have a wash step between the addition of sample and labeled antibody (Vaidya et al,1988), and the use of neural network classifier systems that analyze reaction kinetics. (Papik et al,1999).

Serum immunoglobulins can be markedly increased in patients presenting with large myeloma tumor burdens and may lead to falsely low results in nephelometric assays. (Van Lente,1997). Anthony W. Butch, 2000 combined 50- μ L aliquots from each of 10 samples used to dilute each sample 10-fold in order to eliminate any prozone effect. The concentrations of IgG, IgA, and IgM in the pool were measured using a nephelometer (BNII; Dade Behring, Inc.) and compared with the mean values when all samples in the pool were analyzed (calculated value). When the two values for an immunoglobulin differed by a specified quantity, all samples in the pool were reanalyzed after a 10-fold dilution.

Anthony W. Butch, 2000 further stated that criteria for detecting the prozone effect are based on data obtained from routine samples during a 10-day period. Measured immunoglobulin concentrations for 27 pools (10 samples per pool) were compared with the mean values of samples in the pools. The range of values for the measured serum pools and the differences between the measured pool value and the value derived from the mean of individually measured samples in the pool (calculated value) for each immunoglobulin were as follows: IgG, range 10.20-32.50 g/L, mean difference 4.6%, SD 4.1%; IgA, range 0.31-17.90 g/L, mean difference 12.6%, SD 8.6%; and IgM, range 0.27-5.96 g/L, mean difference 13.2%, SD 8.2%. The small SD indicated that none of the samples exhibited the prozone effect. A percentage difference less than the mean plus 2 SD was considered acceptable and was determined to be 15% for IgG, 30% for IgA, and 30% for IgM. Large differences were considered suggestive of a prozone effect. (Anthony W. Butch, 2000).

The ability of this approach to identify samples exhibiting the prozone effect during routine analysis was further evaluated by Anthony W. Butch, 2000 during a 6-month period. Approximately 750 samples/month were received, and 460 pools were analyzed. Ten samples from five different myeloma patients were identified as being falsely low because of the prozone effect. Four samples were from patients with IgA myeloma, and one was from a patient with IgG myeloma. The discrepancy between the measured and calculated pool was 62-88% (initial difference). When the sample generating the erroneous value was identified and the "correct" result (obtained after dilution) was used in the calculation, the difference between the measured and calculated pool was within the established limits of 30% for IgA and 15% for IgG (corrected difference). The falsely low values differed from the actual

results by as much as 11-fold for IgA and 40-fold for IgG. The prozone effect was not restricted to IgA and IgG because samples exhibiting this phenomenon were also identified, when measuring IgM. (Anthony W. Butch, 2000).

A 2% incidence (1 of 46 pools) for the prozone effect when measuring immunoglobulins may be higher at institutions not specializing in the treatment of multiple myeloma. However, the incidence of multiple myeloma over the age of 25 is 30 per 100 000 (Cooper & Lawton,1987), and most laboratories will eventually encounter a sample exhibiting the prozone effect when measuring immunoglobulins by nephelometry.(Van Lente,1997). Reporting of an erroneous result can have serious medical implications, and sample pooling is a simple method for detecting falsely low concentrations attributable to the prozone effect. Although this screening approach increases reagent costs by 10% and involves additional labor to prepare and analyze pools, it is considerably more cost-effective than analyzing all samples undiluted and after dilution, which doubles reagent costs. Furthermore, this simple prozone detection method can be adapted to other nephelometric assays with the potential for erroneous results from antigen excess. (Anthony W. Butch, 2000).

The one-step sandwich immunoassay is increasingly replacing the traditional two-step immunoassay due to obvious advantages such as assay speed. However, the one-step sandwich immunoassay suffers from the 'hook' effect irrespective of the analyte characteristics. The 'hook' effect is dependent primarily on the analyte concentration. Three different model analytes, human growth hormone (hGH), the dimeric form of hGH (D-hGH, having a discrete number of repeating epitopes) and ferritin (multiple epitopes) having different immunological properties have been employed in studies of the one-step sandwich immunoassay. The characteristics of each of the model analytes offer new insights into general guidelines for assay procedures. These guidelines permit rapid optimization of assay conditions for an immunoassay without a prior knowledge of the immunological characteristics of the antibody or antigen. Both experimental and theoretical data show several instances where high capacity solid-phase antibodies can effectively shift the 'hook' to relatively higher analyte concentrations. The effect of the concentration of labeled antibody on assay response was examined theoretically. (Fernando & Wilson, 1992; Uotila et al, 1981)

Lebeouf et al, 2005 described a case of a 41-yr-old man with metastatic medullary thyroid carcinoma. Despite extensive disease in the neck as well as metastatic lesions in the liver, his serum calcitonin, measured with a commercial one-step immunoradiometric assay, was only minimally elevated (244 ng/liter). After serial dilutions, a nonlinear relationship became evident, suggesting the presence of a "hook effect." Treatment of the serum with heterophilic blocking reagent revealed no change. Calcitonin was then measured with a different immunoradiometric assay and revealed a much higher level. Similar discrepancies were found in different samples from various patients when analyzed with different calcitonin immunoassays. They concluded that clinicians following patients with cancer and using tumor markers need to be aware of the phenomena such as the hook effect, because a low calcitonin result could give false reassurance to both the patient and the clinician and could dramatically change the prognosis of the patient (Lebeouf et al, 2005 ;Quayle & Moley ,2005).

Unnikrishnan et al,2001 have reported that large quantities of antigen in an immunoassay system impair antigen-antibody binding, resulting in low antigen determination, a

phenomenon known as 'high dose hook effect' in a patient with a large macroprolactinoma. In this patient, the correct estimate of serum prolactin (PRL) was obtained only after appropriate dilution of serum. They suggested that in order to avoid the high dose hook effect, the serum PRL be estimated in appropriate dilution in all patients with large pituitary tumours. This is particularly important when the clinical suspicion of high PRL is strong, as in women with amenorrhoea-galactorrhoea and men with long standing hypogonadism. They further suggested that in order to accurately estimate PRL in patients with large pituitary tumours, PRL should be assayed in 1:100, 1:200 or even higher dilutions of serum in order to get an accurate estimate of serum PRL.

Miles et al,1974 and Miles & Hales,1968 have stated that a high dose hook effect is observed, if too much free hemoglobin that is not bound to the gold-labeled antibody reaches the test result region. In this case the antibody immobilized at the test result region becomes saturated with free hemoglobin. This prevents the binding of the hemoglobin complexed with the gold-labeled antibody, thus interfering with the formation of the test result line. The test result appears negative in spite of the presence of hemoglobin in the sample. The high dose hook effect can be avoided using the color of the sample as a guide. The visual detectable color caused by hemoglobin vanishes between 10^{-3} and 10^{-4} dilution. At this concentration range, there is no danger of a high dose hook effect. In contrast, samples that are clearly colored due to hemoglobin are likely to cause false negative results because of the high dose hook effect. Good results are obtained when the extract has a "straw" color. They suggested that if one is concerned, that a negative result is from High Dose Hook Effect, then a simple remedy is to dilute the extract and re-run the sample. (Miles et al,1974;Miles & Hales,1968). Immunoassay is an *in vitro* procedure, and is therefore not associated with complications. When blood is collected, slight bleeding into the skin and subsequent bruising may occur. The patient may become lightheaded or queasy from the sight of blood.

4.1 Immunoassays and forensic science

Forensic toxicology encompasses the determination of the presence and concentration of drugs, other xenobiotics and their metabolites in physiological fluids and organs and the interpretation of these findings as they may have impact on legal issues. These include medical examiner investigations, driving under the influence of drugs/alcohol and other transportation accident investigations, workplace pre-employment, random and for-cause drug testing and judicial monitoring of arrestees and parolees. For the most part, forensic toxicologists use commercial immunoassays directed primarily towards abused drugs. Commercial immunoassays developed for therapeutic monitoring of other drugs, veterinary drugs and pesticides, as well as immunoassays developed in research laboratories for specialized studies, may find a role in the forensic toxicology laboratory for specialized cases. While most commercial immunoassays have been developed for a urine matrix, they have been applied by forensic toxicologists to other matrices, including blood, hair, saliva, sweat, tissue homogenates, blood stains and most other physiological samples that may be of value in the investigation. The non urine matrix usually is much more complex in its composition. Sample pretreatments that range from simple deproteinations to multistep extractions to remove matrix components and/or concentrate the sample are often required. The heterogeneous RIAs and ELISAs usually require less rigorous, if any, pretreatments. (Bell, 2006; Moody, 2006).



Fig. 4. Rapid trace robotic workstation used in forensic lab.

4.2 Types of Immunoassay

4.2.1 Enzyme Immunoassay (EIA)

Enzymes occur naturally and catalyze biochemical reactions. Enzymes are cheap, readily available, have a long shelf life, easily adaptable to automation and automation is relatively inexpensive. The techniques pose no health hazards, little reagent enzyme necessary, can be used for qualitative or quantitative assays. The test tubes are filled with the antigen solution (e.g., blood/serum) to be assayed. Any antigen molecules present bind to the immobilized antibody molecules. The antibody-enzyme conjugate is added to the reaction mixture. The antibody part of the conjugate binds to any antigen molecules that were bound previously, creating an antibody-antigen-antibody "sandwich". After washing away any unbound conjugate, the substrate solution is added. After a set interval, the reaction is stopped (e.g., by adding 1 N NaOH) and the concentration of colored product formed is measured in a spectrophotometer. The intensity of color is proportional to the concentration of bound antigen. (Bosch et al, 1975; Engvall & Perlmann,1971; Schuurs & Van Weemen, 1980; Van Weemen & Schuurs,1971).

4.2.2 Rapid Immunoassays

Membrane based cassettes are rapid, easy to perform and give reproducible results. Membrane coated with antigen or antibody produces color reaction. Designed to be of single use and are disposable. Different types of rapid tests are membrane based enzyme immuno-assay, particle agglutination assay and immunochromatography

4.2.3 Immunochromatography

Test sample is applied to the sample well, from where it migrates forward. Sample dissolves labeled antigen or antibody to which it binds, and migrates further towards detection zone, where it will bind to immobilized antigen or antibody. Finally color change occurs.



Fig. 5. Workstation of enzyme Immunoassay.

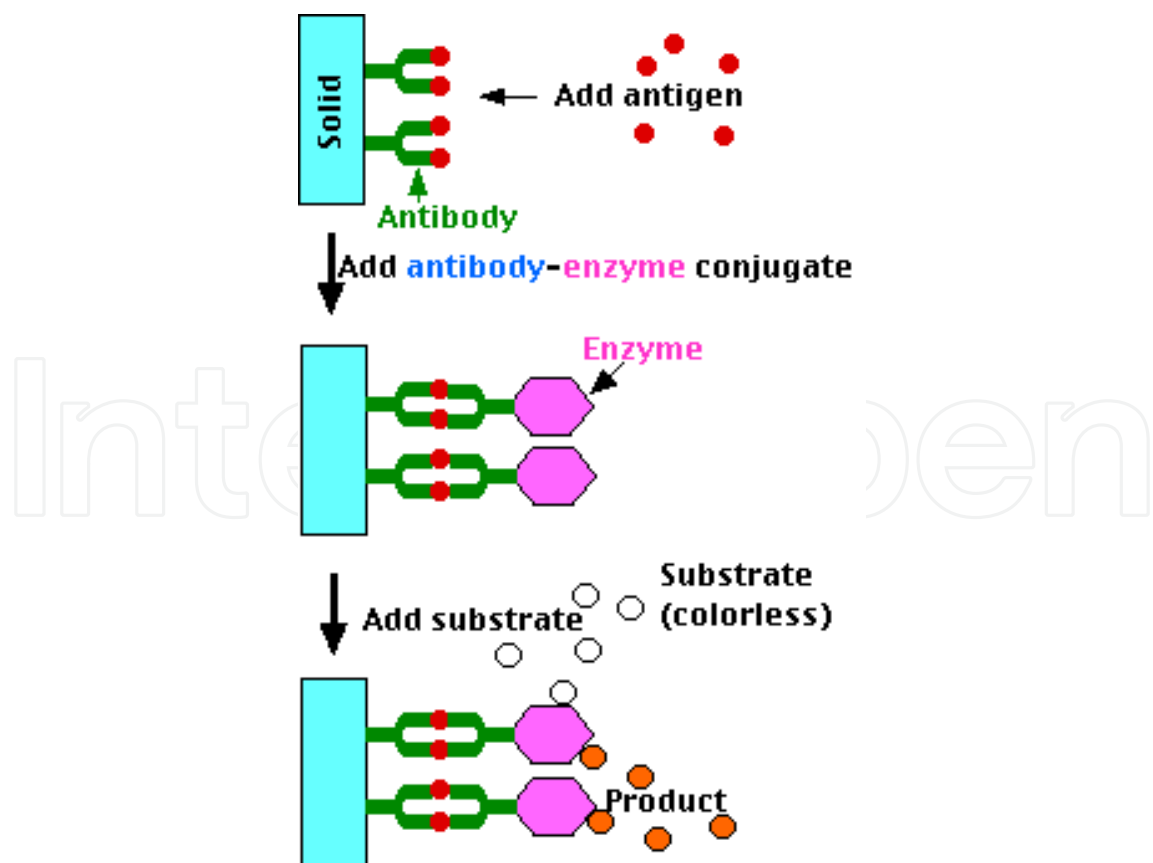


Fig. 6. Schematic diagram of EIA



Fig. 7. Depicting Immunochromatography cassette

4.2.4 Chemiluminescent Immunoassays

The process of chemiluminescence occurs when energy in the form of light is released from matter during a chemical reaction. Large number of molecules capable of chemiluminescence are luminal, acridium esters, ruthenium derivatives, and nitrophenyl oxalates. Uses sodium hydroxide as a catalyst. Light emission ranges from quick burst or flash to light which remains for a longer time. Different types of instruments are required based on emission.

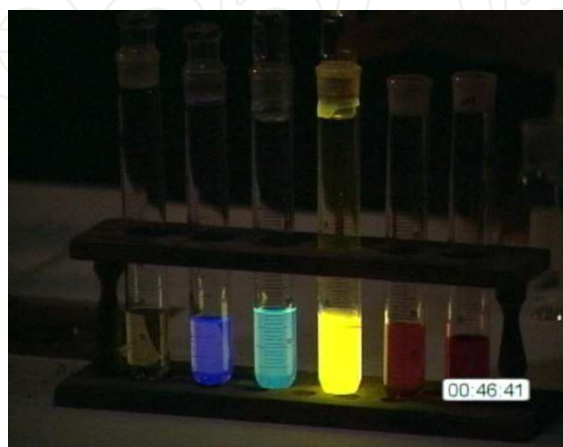


Fig. 8. Test colors in different samples

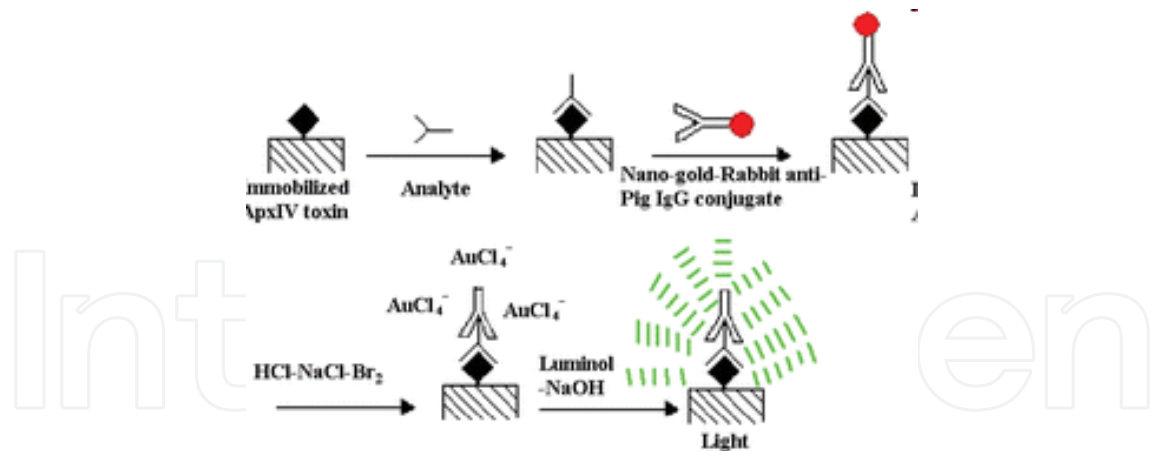


Fig. 9. Schematic diagram of chemi-luminescent Immunoassay.

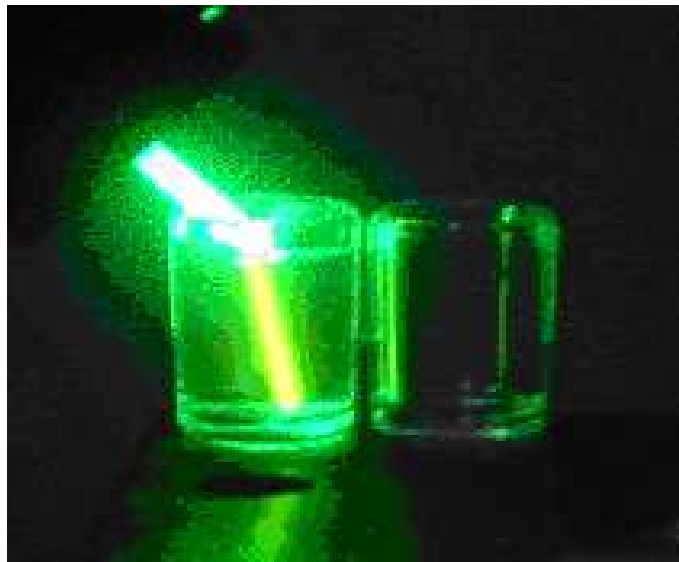


Fig. 10. Showing effect of fluorescein.

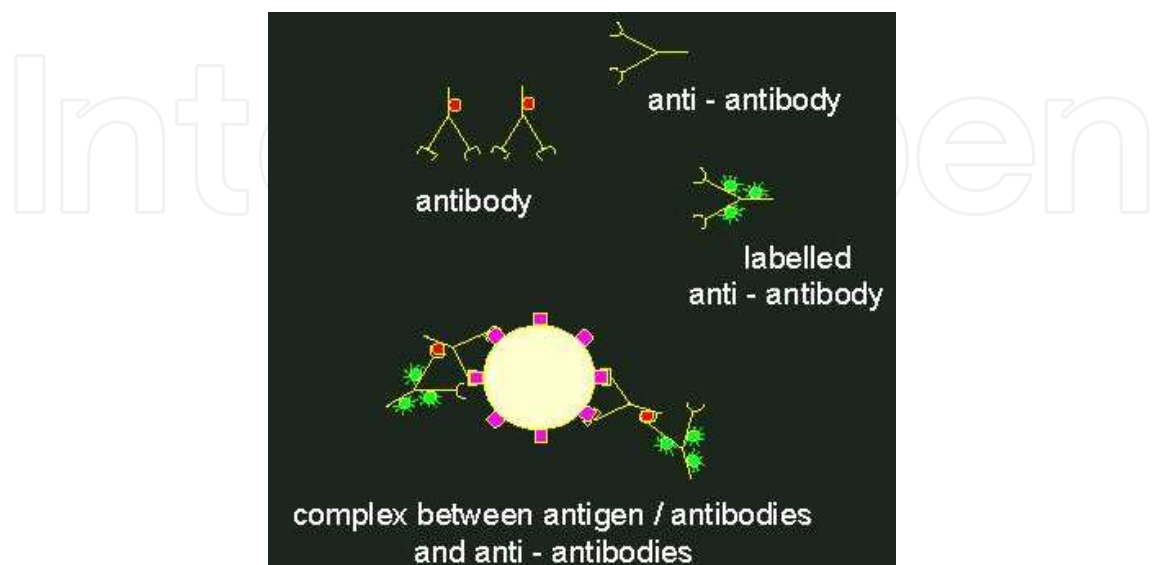


Fig. 11. Depicting antigen-antibody complex

4.2.5 Fluorescent Immunoassay

Two most commonly used markers that have ability to absorb energy and emit light are fluorescein - green and tetramethylrhodamine - red. In direct immunofluorescence, tagged antibody added to unknown antigen are fixed to the slide. If patient's antigen is present, then fluorescence is seen. Complex must form for fluorescence to occur. (Avrameas & Uriel, 1966).

4.2.6 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) involves the separation of a protein (from a mixture) using the specificity of antibody - antigen binding and quantitation using radioactivity. RIA was first described in 1960 for measurement of endogenous plasma insulin by Solomon Berson and Rosalyn Yalow of the Veterans Administration Hospital in New York (1). It is a sensitive technique used to measure small concentrations of antigens which is an example of competitive binding. Uses radioactive Iodine 125 (I^{125}) as label which competes with patient for sites. High radioactivity with small amount of patient's sample is required. Radioimmunoassay is widely-used because of its great sensitivity. Using antibodies of high affinity, it is possible to detect a few picograms (10^{-12} g) of antigen in the tube. (Catt & Tregear, 1967; Wide & Porath, 1966).

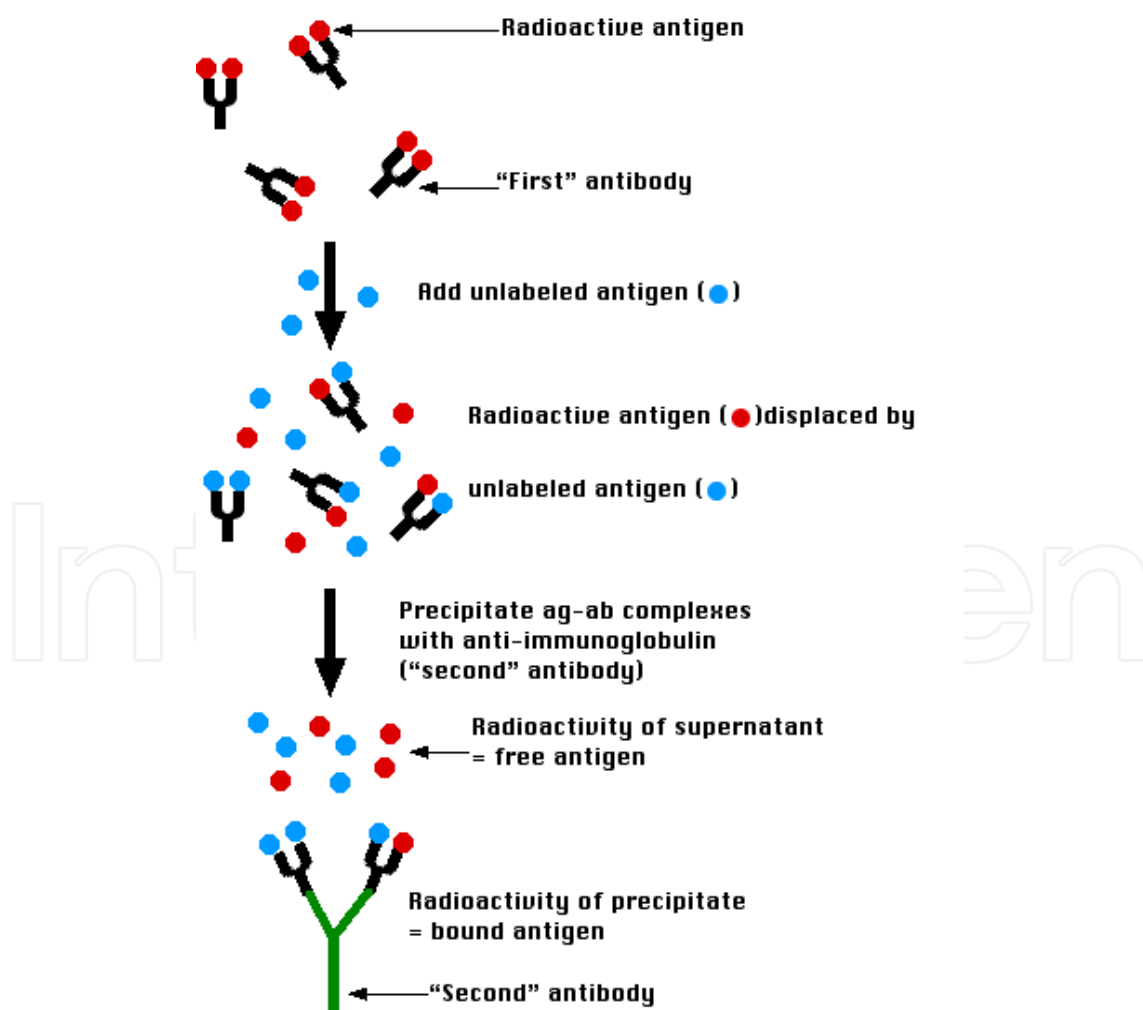


Fig. 12. Flow diagram of the process of radioimmunoassay.

The future of immunoassay lies in human toxicology, like hepatotoxicity, neurotoxicity and chemical carcinogenesis, drug discovery and food and water microbiology.

5. Conclusion

Transfusion practitioners following voluntary blood donors need to be aware of phenomena such as the 'high dose hook effect' or prozone effect, because it could give false reassurance to both the patient and the doctor. Future developments of serological assays should include monoclonal antibodies that recognize epidemiologically relevant surface antigen mutants with nucleic acid amplification techniques as an alternative to screening of blood donors, so as to reduce the false-negative results in commercial assays.

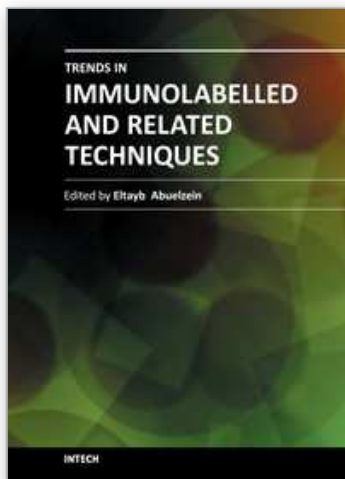
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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

How to reference

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