

IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Principles and applications of Immuno-diffusion, immuno-electrophoresis, immuno-fluorescence, ELISA, Western blotting, Minimal Inhibitory Concentration (MIC), Kirby-Bauer method and Widal test

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17-May-2006 (Revised 30-Jan-2007)

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Keywords

Immuno-diffusion; immuno-electrophoresis; immuno-fluorescence; Immunoassay; Enzyme Linked Immunosorbent Assay (ELISA); Western blotting; Immunoblotting; Minimal Inhibitory Concentration (MIC); Kirby-Bauer method; Widal test.

Introduction

The basis of antigen-antibody reaction has been extensively exploited to devise techniques to qualitatively and quantitatively detect the presence of different biomolecules with high sensitivity and specificity. Any biomolecule that has a complex structure, is protein in nature and possesses high molecular mass (> 1000 Dalton) and internal complexity when injected into an animal body produces specific proteins termed as antibodies. The complex carbohydrates (bacterial LPS or Lipopolysaccharides) and nucleic acids (RNA and DNA) are also immunogenic albeit to a lesser extent. The antibodies have the inherent property to bind to the antigen used to elicit antibody response with high avidity and sensitivity. The antigens encompass the biomolecules that are part of bacteria, fungi, protozoan parasites, etc. The antigens also include smaller molecules (< 1000 Daltons) such as *p*-nitrophenol, and other purely chemical molecules that are known as haptens. Haptens are made immunogenic by their binding with carrier molecules such as Bovine Serum Albumin (BSA), Limphet Keyhole Hemocyanin (LKH) molecules to produce modified antigens. Thus theoretically the injection of an exogenous protein that is foreign to an animal (host) when injected into its tissue through a suitable route (intramuscularly, subcutaneous and intra peritoneal) elicit formation of specific antibodies. The process of injection of a foreign antigen into an animal host is called immunization. The immunization involves a series of injections of a given antigen by a suitable route periodically over a period of time. The method of immunization that generates a detectable and a sustainable immune response in the host is called primary immunization. When an antigen is injected into the host, the IgM class of antibodies are the first to develop, followed by gradual development of IgG antibodies. During a natural infection the detection of an antigen specific IgM antibody indicates a recent history of infection. However, if antigen specific IgG antibody preexisted in the host, a quite early history of a natural infection or immunization is often seen. The ability of the antigen to produce an enhanced and prolonged immune response by formation of specific antibodies (humoral immunity) is achieved by combining/ mixing a given antigen with non-specific immuno-stimulatory compounds called adjuvants. The judicious use of an adjuvant is essential to induce a strong antibody response to soluble antigens. The adjuvants include aluminum phosphate, aluminum oxide, Freund's Complete (FCA) and Freund's Incomplete (FIA) adjuvants. For developing a state of immunity in human and animals, the antigens are mixed with aluminium phosphate, aluminium hydroxide (alum); and for use in experimental animals FIA or FCA might be used. Freund's adjuvant should be used when a small amount of the immunogen is available for immunization. If large amount is available or if the compound is known to be highly immunogenic, then other adjuvants can be used. However, FIA or FCA must never be given intravenously. The use of ISCOMs (Immune Stimulating Complexes) and Quill A (sourced from *Quillaza* plant) as adjuvants has also been reported. The purpose of any immunization protocol using an antigen is to achieve a strong protective immunity. The animals such as guinea pig, rabbit, horse, mouse and rats are often used to generate specific polyclonal antibodies. However, the use of hybridoma technique developed by Kohler and Milstein (1975) can produce bulk amount of specific monoclonal antibodies *in vitro*.

The antigen-specific IgM or IgG class of antibodies can be purified in large amounts by use of affinity chromatography. Protein A, a protein of *Staphylococcus aureus* cell wall has a natural inherent ability to bind to IgG antibody of a variety of animals and humans. Protamine has natural affinity to bind IgM antibody. The ligands like Protein A or protamine when covalently (stably) attached to support matrices (dextran such as Sephadex, Sephacryl and Sepharose and Cellulose) provides an efficient tool to purify large amounts of IgG or IgM antibodies for analytical purposes. Further, use of matrix-bound antigen provides easy affinity separation/purification of antigen-specific antibodies from serum obtained from a

previously immunized animal or ascities obtained from mice injected with hybridoma producing specific antibodies. These purified monoclonal antibodies or polyclonal antibodies can be generated for use in highly specific and sensitive techniques like immuno-diffusion, immuno-electrophoresis, ELISA, Western blotting, Immuno-flourescence microscopy, Fluorescence Activated Cell Sorter (FACS) analysis, Solid Phase Immune Electron Microscopy (SPIEM) and many clinical diagnostic tests for detection of bacterial (Widal test for detection of *Salmonella thypi*/ typhoid), mycoplasma and viral infections (Hepatitis A and Hepatitis B viruses). On the other hand, presence of antigen specific IgM or IgG antibody in the body fluid/ blood suggests a recent or a previous exposure to a pathogenic bacterial or viral agent (Hepatitis C, Hepatitis E and HIV). The availability of highly specific antibodies against a range of antigens is a big advantage to detect an array of pathogenic microbes as well as cancers and tumors.

The number of electrophoretic separation methods has increased dramatically (Westermeyer 1993) since Tiselius' pioneer work for which he received the Noble Prize in 1948 (Tiselius 1937a and b). Development of these methods has progressed from paper, cellulose acetate membrane and starch gel electrophoresis to molecular sieving, disc-PAGE, SDS-PAGE, and immuno-electrophoresis and finally to Western blotting, Southern Blotting, ELISA and FACS analysis.

Immunodiffusion

For easy diffusion of relatively bigger molecules such as antigens (Ag) and antibodies (Ab; size > 150 kDa), the frictional resistance of the gel is kept negligible so that the movement of the biomolecules is not hindered and these molecules can easily diffuse towards each other when separated in space. Agarose gels with concentration of 0.7 to 1.0 % are often used in clinical laboratories for the analysis of antigens and antibodies and also for their quantitative analysis. This technique is performed at neutral pH; antigen and antibody solutions are poured into wells created in the solidified agarose on a glass slide. The wells of uniform diameter and depth are cut in the gel and filled individually with an antigen or an antibody solution. When molecules such as soluble Ag diffuse from a homogenous solution into an agarose gel, the concentration falls from a maximum at the solution/ gel interface to zero at the leading edge of the region penetrated. Thus the system rapidly adjusts to provide a complete antigen concentration gradient. Somewhere along this concentration gradient will be an antigen concentration that will give equivalence with any given concentration of Ab. Based on this concept a range of highly sophisticated immunodiffusion assays have been developed to detect and quantitate Ab and Ag.

Ag and Ab diffuses towards each other through the porous agarose gel, and on physical contact Ag and Ab if recognize each other (are specific) tend to form insoluble immune complexes (precipitates) that appear as a milky band (precipitin line/ band) that can be seen with the naked eyes. The precipitin band appears at the point of equivalence where concentration of antigen and specific antibody is equivalent. The non-precipitated proteins can be washed out. The precipitin band can be made prominent by fixing and staining (using Amido black or Coomassie Brilliant Blue R-250 dye). The method requires 24 h or more for detection of a specific antigen and antibody reaction.

Single immunodiffusion

This method involves the diffusion of Ag from a solution into an agarose gel containing Ab. Mancini (Mancini *et al.*, 1965) first described this technique known as Single Radial

Immunodiffusion (SRID). In this technique, a range of antigen concentrations is poured in wells cut in agarose gel containing corresponding antibody solution on a glass slide. The Ag diffuses radially through the porous gel out of the well resulting in the formation of a precipitin line that appears to move outwards, eventually becoming stationary at zone of equivalence where Ag complexes optimally with the Ab. The precipitin band diameter at equivalence is a function of the antigen concentration. By plotting precipitin ring diameter or circular area at equivalence against antigen concentration, a reference curve may be plotted for determining the concentration of antigen in unknown solutions. This technique is commonly used for determining the concentrations of various plasma proteins such as IgG, and IgM in patients suspected to be suffering from agamma-globulinaemia and multiple myeloma, respectively.

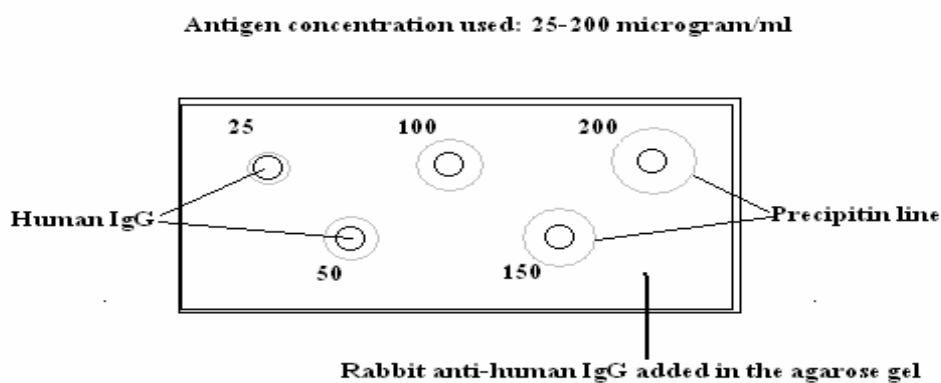


Fig. 1: Single Radial Immunodiffusion for estimation of human IgG

Double immunodiffusion in two dimensions (Ouchterlony technique)

In this technique, both Ag and Ab diffuse towards each other through the porous agarose. Each of the reactants develops a concentration gradient having highest concentration close to the periphery of the well. A 2-mm thick layer of the agarose gel is prepared on a rectangular glass-slide. The circular wells (which are 10-15 mm apart) are punched in the agarose gel with a gel-punch. A typical pattern used to compare different antigen fractions is shown in Fig. 2. The peripheral wells are filled with different concentrations of the same Ag (0-150 microgram/ml of human serum albumin), and the Ab (rabbit anti human-albumin antibody) is added in the central well. This slide containing the Ag and Ab solutions is kept in a humid chamber at 8°C for 24-48 h. The pattern of the precipitin line so formed is observed. No precipitin band is formed towards the well containing buffer alone (without antigen).

The antiserum used in the center wells contained antibodies to most of the components of the human serum, the presence of a single precipitin line between this well and the peripheral wells indicates that these well contained an apparently pure human albumin that does not

appear to be contaminated with any other human serum protein. It shall however be noted that this test does not provide information about any contaminant to which the center well contain any antibodies.

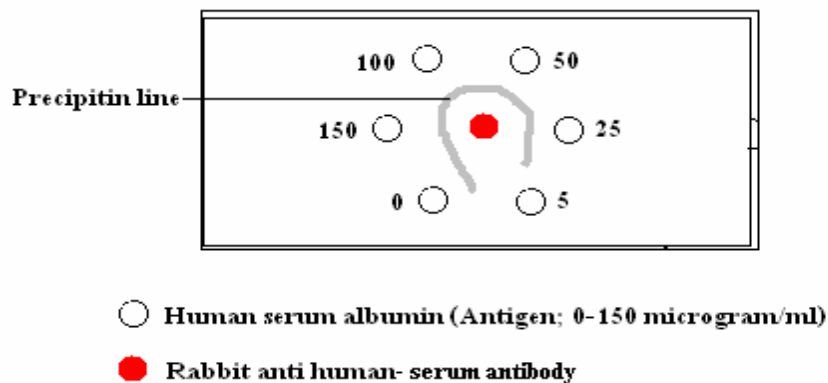


Fig. 2: Schematic diagram of double immunodiffusion in two dimensions using varying concentrations of antigen (human serum albumin) and a specific rabbit antibody (anti human-serum antibody)

A reaction of identity occurs between an Ab and Ag containing identical antigenic determinants, and produces smoothly fused precipitin band (Fig. 3a). A reaction of non-identity occurs when the antiserum contains antibodies to both antigens but the two antigens do not share a common determinant. The two-precipitin lines are formed independently with different antibody molecules and cross without interaction (Fig. 3b). A reaction of partial identity occurs when two antigens have at least one common determinant, but where the antiserum contains antibodies to a determinant in one antigen that is absent from the other (Fig. 3c).

The relative position of a precipitin line provides an estimate of antigen concentration i.e. more concentrated the antigen preparation; more shall be the distance at which precipitin line will be formed (Fig. 4). The shape of a precipitin line gives a rough estimate of the relative molecular mass of a globular protein antigen. The major antibody is usually IgG with a relative molecular mass of 15 kDa. Globular proteins with relative molecular masses significantly less than 150 kDa will diffuse through the gel more rapidly and will yield a precipitin band facing the concavity towards the low molecular mass reactant (antigen). Those with molecular weights significantly greater than 150 kDa will diffuse more slowly and produce a curved precipitin line in the opposite direction. Antigens with relative molecular masses similar to the IgG shall produce a straight precipitin band.

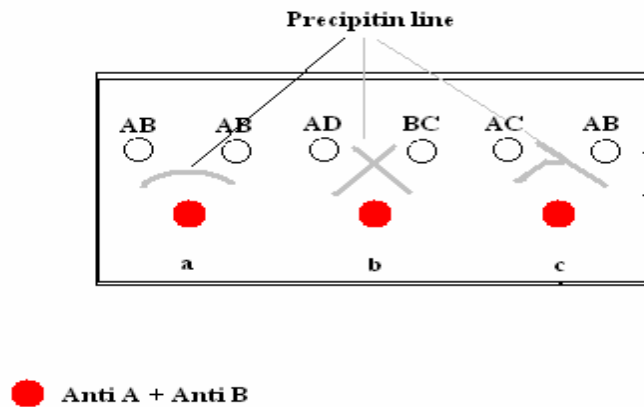


Fig. 3: Various patterns of precipitation (Ouchterlony technique) formed during double diffusion in two dimensions. a: Reaction of identity (see two lines are fused); b: Reaction of non-identity (see two precipitin lines cross); c: Reaction of partial identity (see right line spurs). A, B, C and D represent antigenic determinants. Antiserum A + Antiserum B was used in each of the depicted reactions

Thus double immunodiffusion technique is used for detection of antibody-specific antigens in the immune-sera, chromatographic fractions, cell fractions and to obtain information whether two antigens are identical, different or may share common antigenic determinants.

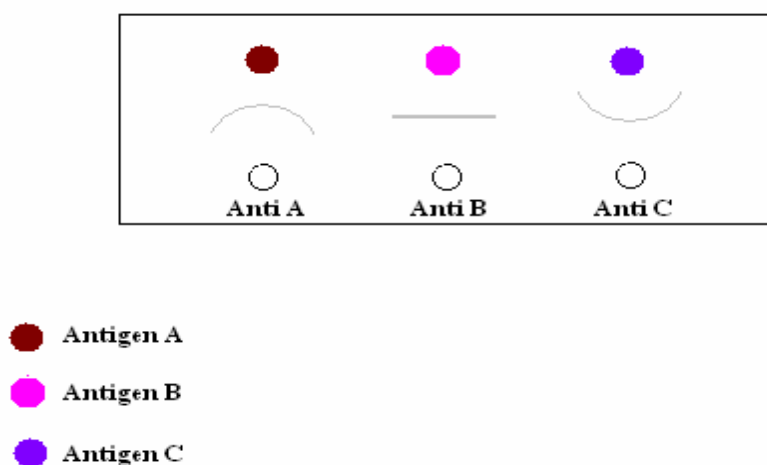


Fig. 4: The effect of relative molecular size of an antigen on the shape of the precipitin lines formed in double immunodiffusion (Ouchterlony technique)

Immunoelectrophoresis

The principle of immunoelectrophoresis is the formation of precipitate lines (insoluble immune complexes) at the point of equivalence (zone of equivalence) of the antigen and its corresponding antibody. This technique combines the specificity of immuno-precipitin reactions with the separation of molecules by electrophoresis in a molecular sieving medium. Usually the analysis is carried out in an agarose gel containing barbitone buffer on a microscopic slide. In this technique, it is important that the ratio between the quantities of Ag and Ab be correct (antibody titer). When the amount of antibody is in excess, statistically at most one Ag molecule binds to each molecule of Ab. When the amount of Ag is in excess, at most one molecule of Ab binds to each Ag molecule. However, at a specific Ag/ Ab ratio (equivalence point) optimal amount of Ag-Ab immune complexes (macromolecules) are formed. They consist of an Ag-Ab-Ag-Ab network and are immobilized in the gel matrix because of their bulky size as an immuno-precipitate. The white/ milky precipitate lines are visible in the gel and can be revealed with protein stains. The agarose gels are used in immuno-electrophoresis and the separation times are exceedingly low: about 30 minutes. The method is highly specific and the sensitivity is also very high because distinct zones are formed. Immunoelectrophoresis can be categorized into three principles.

Counter immunoelectrophoresis

In an agarose gel exhibiting high electro-osmosis, the buffer is set at a pH of about 8.6 so that Ab does not carry any charge. A suitable pattern is cut with a gel punch and 1-10 micro liters of solutions containing 1-100 micrograms of antigen are added to the wells. Thick wet filter paper wicks to the electrode buffer connect the slides, and a direct electric current of about 8 mA per slide is passed for 1-2 h. The sample and the Ab placed in opposite wells move towards each other, the negatively charged Ags migrate electrophoretically and the antibodies are carried by electro-osmotic flow (Fig. 5). The interaction of Ag with the Ab results in the formation of precipitation lines. This technique is more rapid (15-25 min) than the Ouchterlony method, which may take days to produce a clear result. Also this technique is more sensitive because all of the molecules migrate towards each other rather than diffusing radially. This method has been used and described by Estela and Heinrichs (1978). The technique is especially useful in forensic science for establishing the origin of body fluids such as blood, semen and saliva.

Zone immunoelectrophoresis

According to Grabar and Williams (1953) firstly a zone of electrophoresis is run in an agarose gel, followed by the diffusion of the Ag fraction towards the Ab which is pipetted into rectangular narrow troughs cut in the side parallel to the electrophoretic run. The charged molecules will have been separated electrophoretically but they will not be visible (Fig. 6). Immediately after the voltage supply has been disconnected, the troughs are filled with an appropriate antiserum and incubated over-night at room temperature in a humid chamber. The antigens diffuse radially and the antibodies diffuse laterally resulting in the Ag-Ab precipitation arcs. Despite the use of agarose, which acquires a smaller charge than agar, the electro-osmotic flow of water during electrophoresis moves all of the antigens towards the cathode. This results in an apparent cathodic migration of gamma globulins, including IgG antibodies. This technique may be used to determine the purity or detection of a particular antigen in the sera, culture filtrates, tissue or cell-extracts or fractions from any preparative procedure.

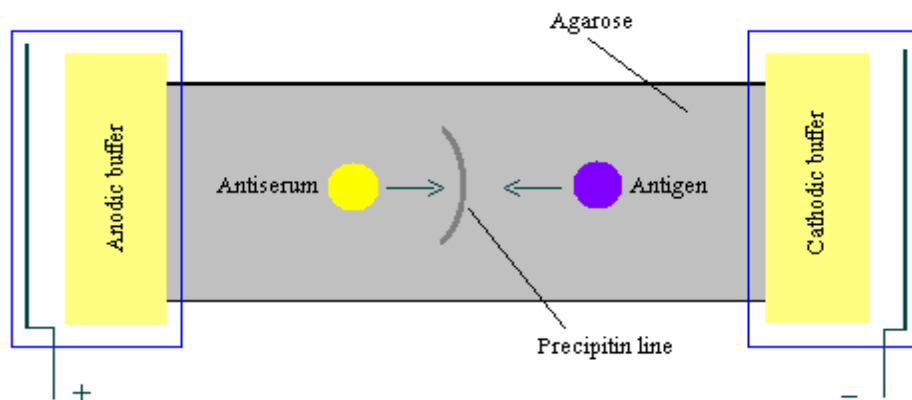
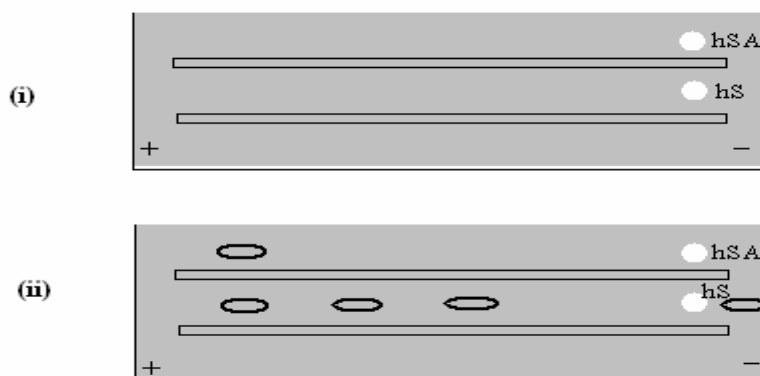


Fig. 5: The schematic diagram of counter immunoelectrophoresis

In the Laurell's rocket electrophoresis (Laurell 1966) and the related methods; Ags migrate in an agarose gel, which contains a definite concentration of Ab. As in the above method, the Abs are not charged because of the choice of the buffer. As the sample migrates one Ab will bind to one Ag until the ratio of concentrations corresponds to the equivalence point of the immunocomplex. The result is that the rocket shaped precipitin lines is formed (Fig. 7); the enclosed areas are proportional to the concentration of Ag in the sample. A series of modifications to this technique exist, including two-dimensional ones.



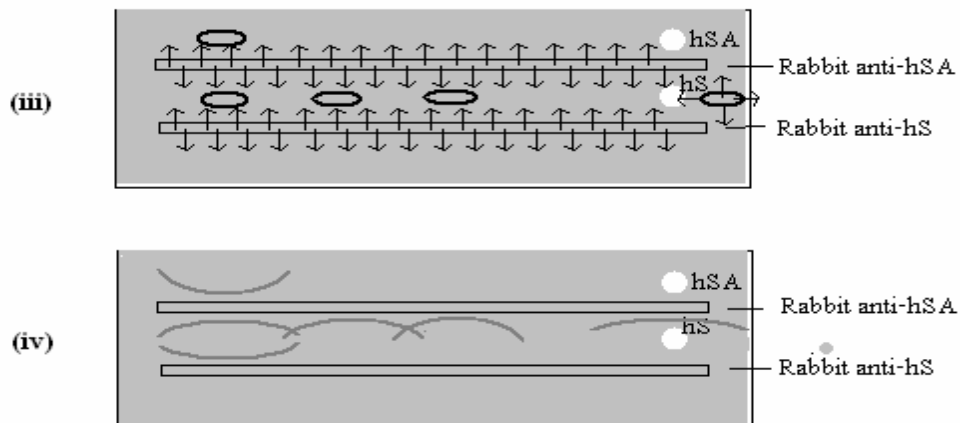


Fig. 6: Various steps in zone immunoelectrophoresis. (i) Round wells punched in the agarose are filled with human Serum Albumin (hSA) and human Serum (hS); (ii) After electrophoresis the proteins will migrate as depicted but will not be visible. Rectangular troughs are cut in the agarose and agarose is removed from troughs; (iii) Troughs are filled with the antisera, the antigens and antibodies diffuse through the porous agarose as indicated by arrows; (iv) Finally, the precipitin lines appear
Laurell's rocket electrophoresis

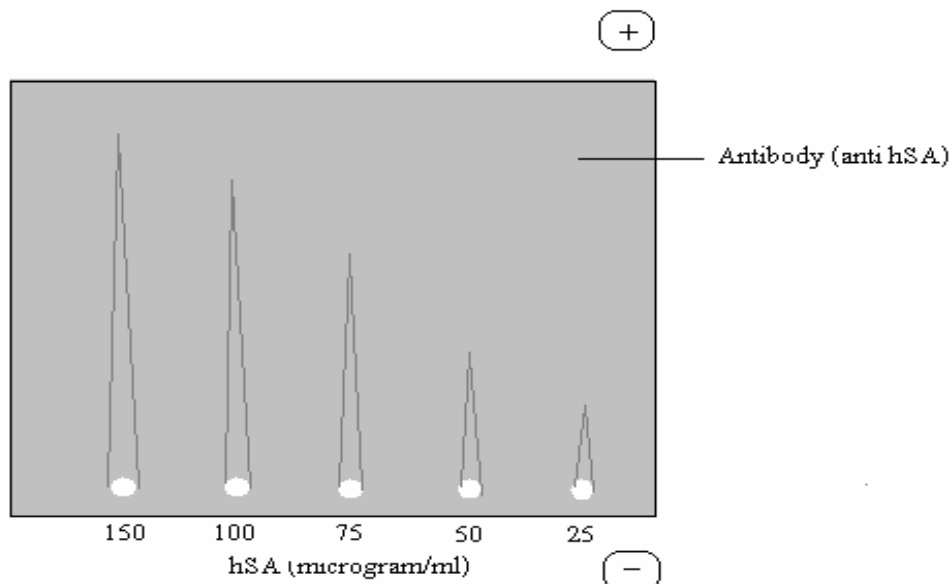


Fig. 7: A schematic diagram of Laurell's rocket immunoelectrophoresis. The agarose gel contains anti human Serum Albumin (hSA) antibody and the wells punched in the agarose contain varying concentrations of the human Serum Albumin (hSA). The electric field is applied to obtain the rocket like precipitin bands as shown above

Immuno-fluorescence

The antigens and antibodies that possess an inherent property of specificity for each other when attached/ tagged with a suitable fluorescence molecule could be used to detect or localize the cells bearing surface receptor of interest or detecting specific cell type. This type of fluorescence is called extrinsic fluorescence which can be detected with a fluorescence measuring device (fluorimeter or a UV/ fluorescence microscope). Sometimes the fluorescence molecule (propidium iodide binds to DNA in dead cells; Evans blue binds to non-fluorescence cells) may be able to bind intracellular organelle or some cell component without the use of an antibody – this is referred to as intrinsic fluorescence, and this stain is used as a counter stain. The fluorescence molecules include fluorescein, rhodamine and Texas red. The properties of these fluorochromes are summarized in Table 1. These molecules when excited with suitable wavelength absorb energy and get excited. The absorbance of energy by the fluorescence moiety occurs in less than 10^{-15} seconds. The excited molecule then fall back to ground state with release of energy in the relatively longer wavelength range in a very short time (less than 10^{-8} seconds). The release energy is detected with the help of detectors. The fluorescence can be visually seen through a UV/ fluorescence microscope. The cells bound to the antibody tagged with a fluorescence molecule appear to be distinctly colored. The same cell may be visualized to highlight different surface receptors, intracellular organelles etc by using more than one fluorescence-tagged antibodies. However, such detection is possible when the fluorescence molecules attached to different antibodies (with different specificities) are capable of excitation at the same wavelength but emit rays at different wavelengths). The cells that have been fixed on the microscope slide or suspended (live) cells or thin layer of tissue(s) can be subjected to immuno-fluorescence staining using appropriate antibodies attached to fluorescence molecules. The fluorescent cells are seen in dark to maintain high efficiency of detection as well to minimize effect of stray radiations that might influence the results.

$$E_{\text{excitation}} - E_{\text{emmission}} = \Delta E$$

$$\text{Or } h\nu_{\text{excitation}} - h\nu_{\text{emission}} = \Delta h\nu$$

$$\text{Thus } E_{\text{emmission}} < E_{\text{excitation}}$$

The energy associated with the emitted rays is always less than the energy associated with the excitation rays because the excited molecules tend to lose some of their energy ($\Delta h\nu$) by interaction with the impurities (salt ions, cellular components, particulate matter etc.) in the solution or tissue.

Labeling antibodies with fluorochromes

Antibodies can be labeled by direct coupling to fluorochromes. For example, detection of $CD4^+$ Th (T-helper cells) in a human blood film can be performed by using anti human $CD4^+$ antibody bound to fluorescein. Thus detection of target cell(s) by using a specific fluorochrome-tagged antibody is called direct fluorescence. Alternatively, at a first instance, an anti-human $CD4^+$ antibody (without fluorochrome molecule) can be used followed by an anti-human antibody tagged with a fluorochrome (say a secondary antibody, rabbit anti-human IgG-fluorescein). This approach is called as indirect fluorescence. For indirect work, suitable secondary reagents labeled with fluorochromes are available commercially. Direct labeling may be the method of choice where such conjugates are not available or where a direct label is required specifically, for example, in simultaneous visualization two antibodies

of the same class or subclass (Hansen 1967). The most commonly used fluorochromes are fluorescein and rhodamine. They can be conjugated to anti-immunoglobulin antibodies, Protein A, protein G, avidin or Streptavidin. These conjugates are available from many commercial sources or can be prepared in laboratory.

Detection of fluorochromes-labeled reagents

To detect fluorochrome-labeled reagents, a specially equipped microscope is required. The low levels of fluorescence produced in cell staining experiments means that the microscope must be equipped for epi-fluorescence in which the exciting radiation is transmitted through the objective lens onto the surface of the specimen. Absorbing radiation of the appropriate wavelength causes the electrons of the fluorochrome to be raised to a higher energy level. As these electrons return to their ground state, light of a characteristic wavelength is emitted. This emitted light forms the fluorescent image seen in the microscope. Individual fluorochromes have discrete and characteristic excitation and emission spectra. Filters are used to ensure that the specimen is irradiated only with light at the correct wavelength for excitation. By placing a second set of filters in the viewing light path that only transmit light of the wavelength emitted by the fluorochrome, images are formed only by the emitted light. This produces a black background and a high-resolution image.

Because some fluorochromes have emission spectra that do not overlap, two fluorochromes can be observed on the same sample. This allows the study of two different antigens in the same specimen even when they have identical sub-cellular distribution. However, fluorescence detection is not compatible with most histochemical stains, because the component of most of these stains autofluoresce strongly. Fluorescence detection is also not compatible with enzyme detection systems, because the deposition of insoluble compounds after enzyme detection will block the emission of light from the fluorochrome.

An extension of immuno-fluorescence technique is a Fluorescence Associated Cell Sorter (FACS), which is capable of detecting, counting or sorting various cell types on the basis of their size (30-100 micrometer size), viability (using Propidium Iodide that binds DNA of dead cells), granularity and specific surface receptors. Thus FACS uses multi-parametric analyses for detection, counting and sorting of specific type of cells of interest. The FACS is used for quantitative detection and analysis of helper T cells (Th-cells; $CD4^+$) and cytotoxic T cell (Tc-cells; $CD8^+$) to monitor the status of AIDS patients and to study the effectiveness of the anti-AIDS therapy.

Choosing the correct fluorochrome

The choice of fluorochrome is limited primarily by filter sets that are commercially available for microscope. Most filter sets are best matched to the properties of rhodamine or fluorescein. Texas red can be used with rhodamine filter sets, but these filters do not exactly match its emission spectrum. The increasing availability of phycobiliproteins, which are theoretically about 50 times brighter, is going to have substantial influence on the design of immuno-fluorescence experiments over the next few years as other filter sets become available. Fluorescein emits a yellow-green light that is detected well by the human eye and by most films. However, fluorescein is prone to rapid photo bleaching, and bleaching retardants such as DABCO or o-phenylene diamine should be added to the mounting medium. Rhodamine emits a red color and is not as prone to fading as fluorescein, but the rhodamine conjugates are more hydrophobic and therefore yield higher backgrounds than fluorescein. Texas red also emits a strong red light and its emission spectrum is different from the emissions of fluorescein than rhodamine. It is the least likely to produce problems of

fading, but it is not as widely available as either fluorescein or rhodamine. While using dual labels, fluorescein can be combined with either Texas red or rhodamine.

Table 1: Properties of fluorochromes used in cell staining (Hansen 1967)

Fluorochrome	Excitation wavelength (nm)	Emission wavelength (nm)	Color
DAPI	365	>420	Blue
Fluorescein	495	525	Green
Hoechst 33258	360	470	Blue
R-Phycocyanin	555, 618	634	Red
B-Phycoerythrin	545, 565	575	Orange, red
R-Phycoerythrin	480, 545, 565	578	Orange, red
Rhodamine	552	570	Red
Texas red	596	620	Red

Immunoassay: Enzyme Linked Immunosorbent Assay (ELISA)

Immunoassays are among the most powerful, sensitive and specific immunochemical techniques. In ELISA either antigen or antibody is initially bound to the surface provided by the wells of a microtiter plate and antibody or antigen is detected by following steps. The ELISA employs a wide range of methods to detect and quantitate antigens or antibodies (Harlow 1988). This method has been successfully exploited to detect viral, bacterial, fungal and mycoplasma by detecting the presence of surface exposed antigens on the surface of these microbes or indirectly by determining the presence of pathogen/antigen specific antibodies in the serum or other body fluids. Also ELISA has been used to detect presence of various hormones such as hCG, progesterone, FSH, LH, insulin, glycagon, thyronine, prolactin etc. besides a range of cytokines including interleukins, interferons etc. There are many variations in which immunoassay can be performed, and are classified on the basis of many different criteria. Within each type, the principle and the order of the steps are similar. However, the variable that is being tested may change. By changing certain key steps, an assay can be altered to determine either antigen or antibody level. The ELISA can be classified in three broader types.

- (i) Antibody capture (Competitive) ELISA
- (ii) Two antibody sandwich ELISA
- (iii) Antigen capture ELISA

All three types can be performed by direct or indirect approach. In direct ELISA, the probe antibody is directly conjugated to an enzyme, while in case of indirect approach a second anti-species antibody is conjugated to an enzyme. The enzymes, which are covalently conjugated to the antibody, possess high turnover number, are highly stable and on reaction with a suitable chromogenic substrate result in the formation of soluble or insoluble colored end product. By measuring the absorbance at particular wavelength the intensity of the color is read. Higher the absorbance more shall be the concentration of the antigen or antibody being detected. In qualitative assay, the development of color is indicative of a positive

reaction. Another variation of ELISA that uses properties of both direct and indirect detection is the biotin-streptavidin system. Here, the antigen or antibody is purified and labeled with biotin. The biotinylated reagent is detected by binding with streptavidin that has been labeled with an enzyme.

In an antibody capture assay, the antigen is attached to a solid support, and labeled antibody is allowed to bind. After washing, the assay is quantitated by measuring the amount of antibody retained on the solid support. In an antigen capture assay, the antibody is attached to a solid support and labeled antigen is allowed to bind. The unbound proteins are removed by washing, and measuring the amount of bound-antigen quantitates the assay.

Antibody-capture immunoassay

An indirect ELISA is used to measure an antigen-specific antibody level in the samples. A putative antiserum (may contain antigen-specific antibody) is reacted with specific antigen attached to a solid phase. The specific antibody molecules bind to the antigen and all other material is washed away. Exposure of the antigen-antibody complex to an enzyme-labeled anti-immunoglobulin antibody results in binding to specific antibody molecules adsorbed from the serum sample. The complex is washed and the substrate for the enzyme is added, resulting in formation of a colored end product whose concentration is directly proportional to the amount of specific antibody in the serum sample under analysis (Fig. 10). This type of ELISA is used to detect a pre-exposure to Hepatitis C or Hepatitis E virus by measuring the level of virus specific IgG-antibody in the serum of the suspected patient.

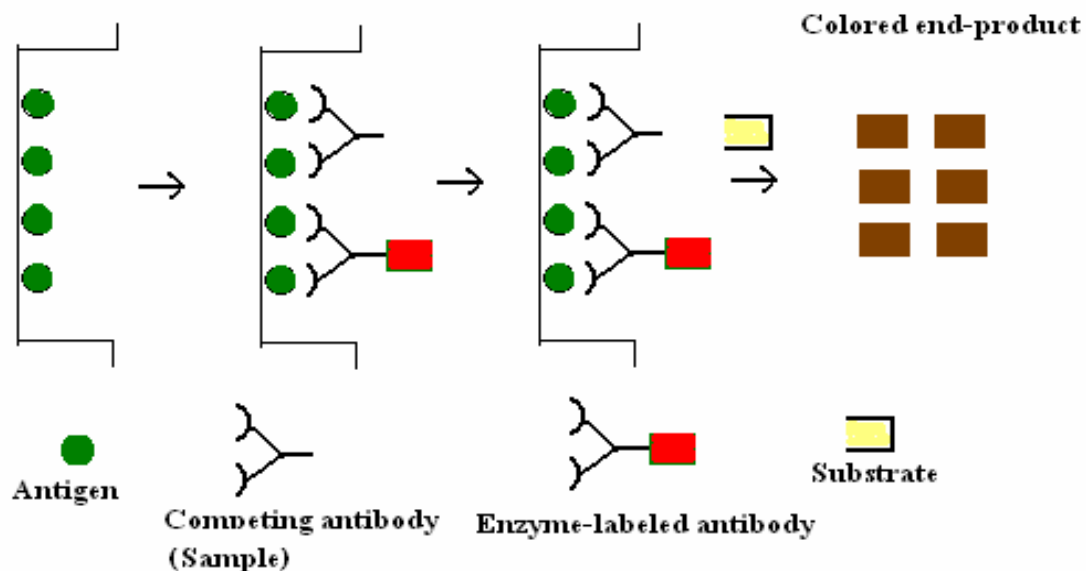


Fig. 10: Detection of antigen-specific (unlabeled competing) antibody by antibody-capture ELISA

ELISA has replaced Radio-Immuno Assay (RIA) despite the latter being extensively automated and even more sensitive. This is because ELISA is cheaper, lacks the radiological hazards of RIA and is suitable for use in small laboratories lacking radioactivity-counting facilities.

A variation of this method is called competitive ELISA. In this method, a mixture of a known amount of enzyme-labeled antigen and an unknown amount of unlabeled antigen is allowed to react with a specific antibody attached to a solid phase. After the complex has been washed with buffer, the enzyme substrate is added and the enzyme activity is measured. The difference between this value and that of a sample lacking unlabelled antigen is a measure of the concentration of unlabeled antigen. A major limitation of this technique is that each antigen may require a different method to couple it to the enzyme. This limitation is overcome in two-antibody sandwich ELISA.

Two-antibody sandwich ELISA

In the two-antibody sandwich assay, one antibody specific for a particular antigen is permitted to bind on the solid surface, and the antigen (infected sample like serum, blood, CSF, urine, saliva etc.) is allowed to bind to the 1st antibody. The second antibody that is also specific for the same antigen but conjugated to an enzyme is added. The unbound 2nd antibody is washed off with a buffer solution and the enzyme substrate is added. Measuring the amount of chromogenic end product produced by the enzyme-labeled 2nd antibody, which is also specific to the same antigen and hence binds it, makes it quantitative assay. The amount of colored product produced measured under standard conditions is directly proportional to the amount of antigen present in the sample (Fig. 11). The two antibodies specific for an antigen used in this assay are raised in two different animals. For example if 1st antibody is raised in rabbit against an antigen 'X', the 2nd antibody is produced in mouse (say a monoclonal antibody) using the same antigen ('X'). This type of ELISA is used in detection of Hepatitis A virus, and Hepatitis B virus surface antigen (HBsAg) to confirm infection. To detect and quantitate antigens, the most useful method is the two-antibody sandwich assay. These assays are quick and reliable and can be used to determine the relative levels of most protein antigens.

The sensitivity of any type of ELISA may be greatly enhanced by enzyme amplification. The primary enzyme product is used to trigger a secondary coupled enzyme system that can generate a large quantity of chromogenic product. Since the product of first enzyme need not be measured but acts catalytically only on the second system, enzymes not currently used for ELISA may become important in these systems e.g. aldolase or glucose-6-phosphatase. Enzyme amplification of a double-antibody assay in which alkaline phosphatase, the primary enzyme degrades the substrate NADP^+ to NAD can be achieved by using alcohol dehydrogenase as the amplifying enzyme and a tetrazolium dye as a redox acceptor. When alcohol dehydrogenase is triggered, the tetrazolium dye is reduced to a colored formazan product, which can be assayed spectro-photometrically, e.g. iodonitrotetrazolium violet may be reduced to a red formazan and the yellow thiazolyl blue may be reduced to a blue formazan. Under appropriate conditions 500 molecules of formazan are produced by the primary enzyme system. Enzyme amplification can be achieved as a one step process in which both enzymes and substrates are reacting at the same time; or a two-step amplification in which the first enzyme is inhibited before or during the addition of the second enzyme and substrate. Enzyme amplification immunoassays have already been developed for hormones, viruses, bacteria and tumor markers.

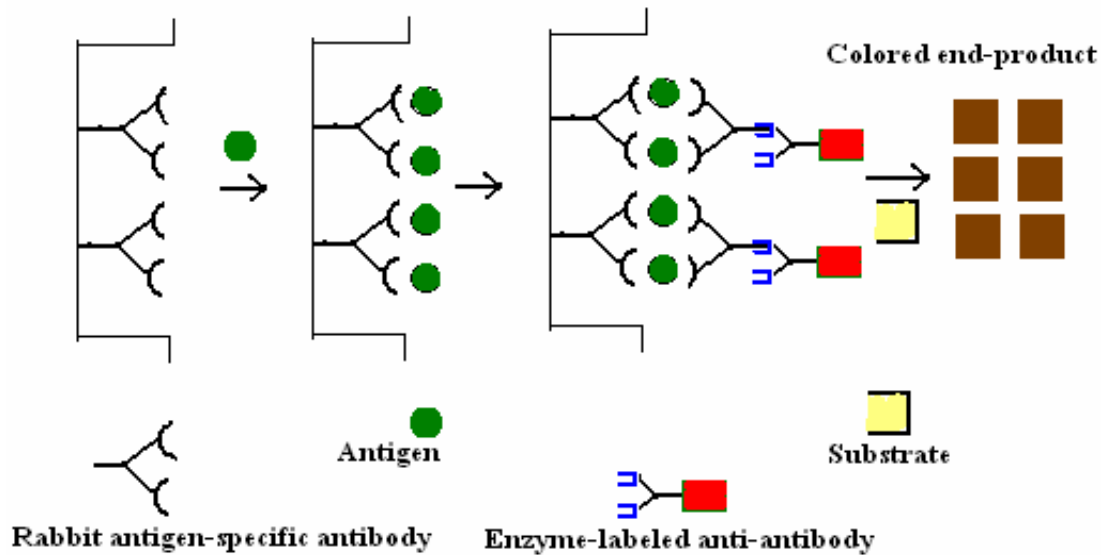


Fig. 11: Two-antibody sandwich ELISA for detection of specific antigen

Antigen capture assay

In this assay the antibody is immobilized to the solid surface. An enzyme-conjugated antibody is then added followed by extensive washings with the buffer to wash off the free enzyme-conjugated antibody. Subsequently, a suitable substrate specific for the enzyme is added and the colored end product is measured spectrophotometrically (Fig. 12). This type of assay is primarily used to detect and quantitate antigens in a given sample. The major limitation of this assay is that each of the different antigens must be individually labeled with an enzyme. This approach of making various enzyme-conjugated antigens is laborious and costly. Thus, the two-antibody sandwich assay is most commonly used to perform antigen-capture assay with very high sensitivity and specificity.

Antibody detection by indirect-ELISA

Antigen-specific antibody detection by indirect-ELISA can be used to detect and quantitate antibodies, and can be used to compare the epitopes recognized by different antibodies. When labeled antibody assays are performed with excess antigens on the solid phase (i.e. enough to saturate all the available antibody), the presence and level of antibodies in a test solution can be measured (Fig. 13). The general protocol is simple; an unlabeled antigen (purified or partially purified) is immobilized on a solid phase, and the antibody (in a body fluid sample e.g. blood, serum, cerebral spinal fluid, saliva etc.) is allowed to bind to the immobilized antigen. The antibody can be labeled directly or can be detected by using a labeled secondary

anti-antibody that will specifically recognize the antibody. The amount of antibody that is bound determines the strength of signal.

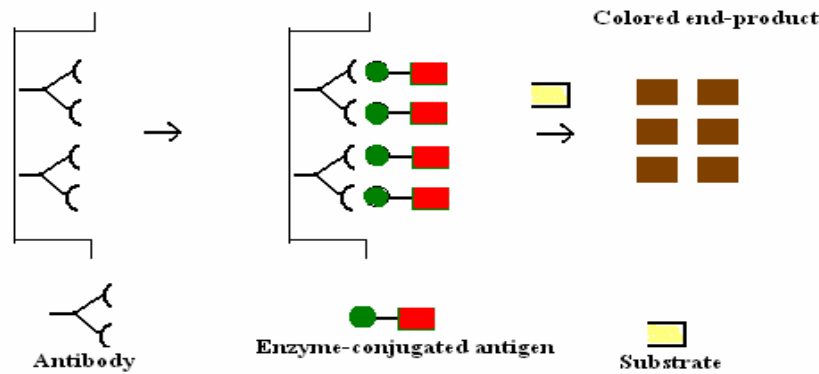


Fig. 12: Concept of antigen-capture immunoassay

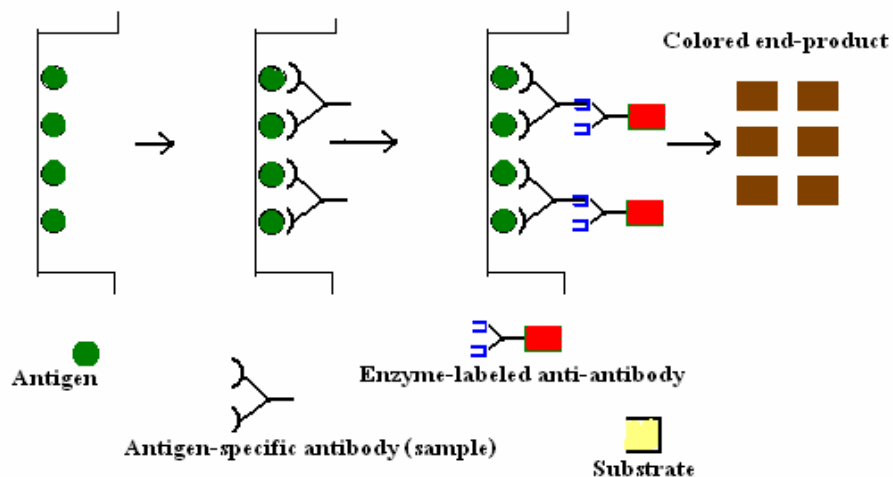


Fig. 13: Detection of specific antibody by indirect-ELISA

Detection

All immunoassays rely on labeled antigens, antibodies, or secondary reagents for detection. Three factors that will affect the sensitivity of a labeled antibody assay are (1) the amount of antigen that is bound to the solid phase, (2) the avidity of labeled moieties used to label the antibody, and (3) the type and number of labeled moieties used to label the antibody. Antibodies usually are labeled with enzymes or biotin. These can also be labeled with radioactive compound and fluorochromes. Of the methods used to label, radioactivity has

biological hazards. The fluorochromes have few applications for immunoassays. Although they can be very sensitive, they require expensive equipment to use and, unlike radiometric or enzymatic detection, no alternative methods can be used to locate and quantitate positives. Various enzymes can be used to label antigens or antibodies such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). These enzymes act on suitable substrates to yield soluble or insoluble end-product (Table 2). Some chromogenic substrates may be carcinogenic and shall be handled with utmost care.

Table 2: Various combinations of enzyme and chromogens

Enzyme	Substrate	End-product
Horseradish peroxidase (HRP)	<i>o</i> -Phenylenediamine (OPD)	Soluble
	Tetramethylbenzidine (TMB)	Soluble
	4-Chloro-1-naphthol (CN)	Insoluble
	3-Amino-9-ethylcarbazole (AEC)	Insoluble
	3, 3', 4, 4'-tetraaminobiphenyl (DAB)	Insoluble
Alkaline phosphatase (AP)	<i>p</i> -Nitrophenyl phosphate (PNPP)	Soluble
	Bromochloroindolyl phosphate-nitro blue tetrazolium (BCIP/NBT)	Insoluble

Western blotting or Immunoblotting

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. Immunoblotting can be used to determine a number of important characteristics of protein antigens, the presence and quantity of an antigen, the relative molecular mass of the polypeptide chain, and the efficiency of extraction of the antigen. It is particularly useful when dealing with antigens that are insoluble, difficult to label or easily degraded, and thus less amenable to analysis by immuno-precipitation. Immunoblotting can be combined with immuno-precipitation to permit very sensitive detection of minor antigens and to study specific interaction between antigens. It is also a particularly powerful technique for assaying the presence, quantity, and specificity of antibodies from different samples of polyclonal sera. Moreover, it can be used to purify specific antibodies from polyclonal sera. Because the antigen is not labeled, only the steady-state level can be determined and further analysis of its biochemical properties, modifications, or half-life is not possible.

The immunoblotting procedure can be divided into following six steps:

- i. Preparation of antigen sample
- ii. Resolution of the sample by SDS-PAGE
- iii. Transfer of the separated polypeptides to a membrane support
- iv. Blocking non-specific sites on the membrane
- v. Addition of the probe (enzyme, radioactivity or fluorochrome labeled) antibody
- vi. Detection

First, an unlabelled solution of proteins, for example an extract of cells or tissue homogenate is prepared in a gel electrophoresis sample buffer. The proteins are separated by gel

electrophoresis, and transferred to a nitrocellulose membrane that binds the proteins non-specifically. Transfer usually is achieved by placing the membrane in direct contact with the gel and then placing the sandwich in an electric field to electrophoretically move the proteins from the porous gel onto the membrane (Fig. 14 and 15). The remaining binding sites on the membrane are blocked to eliminate any further reaction with the membrane (Fig. 16). Finally, the location of specific antigens is determined using a labeled primary antibody or an unlabelled primary antibody, followed by a labeled secondary antibody. The antibody is often labeled with alkaline phosphatase or horseradish peroxidase. Addition of a suitable chromogen and the substrate specific for the enzyme conjugated to antibody results in the formation of colored insoluble precipitates (end-product) on the membrane indicating presence of a particular antigen that is being recognized by the labeled antibody.

Transfer of proteins from the gel to nitrocellulose membrane can be achieved in one of the two ways. In capillary blotting, the gel is placed on a wet pad of buffer-soaked filter paper and a sheet of membrane placed on the gel. Buffer is drawn through the gel by a heavy weight on top of the membrane sheet. Passage of buffer by capillary action through the gel transfers the separated proteins on to the membrane, to which they bind irreversibly by hydrophobic interaction. The process of capillary blotting is often carried out for 16-24 h that permits transfer of proteins from gel onto the membrane. Thus a relatively small amount (10-20%) of each protein in the gel is transferred on to the membrane. A quicker and more efficient method involves application of electric field to hasten the transfer of proteins from the gel on to the membrane, and is referred to as electroblotting (Fig. 14).

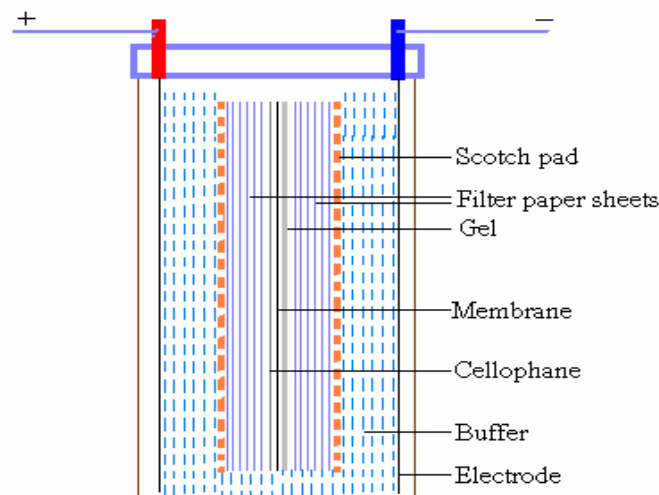


Fig. 14: Diagrammatic representation of a vertical electroblotting set up. This technique is also known as wet-electroblotting. The proteins already resolved on polyacrylamide gel are transferred onto the membrane under the influence of electric field, and their passage through the pores of the membrane is avoided by using a cellophane membrane (molecular cut-off limit of 10 kDa) underneath the nitrocellulose membrane

A sandwich of gel and nitrocellulose membrane soaked in transfer buffer is underlaid and overlaid with blotting sheets cut to the size of gel. This sandwich is compressed in a cassette and immersed in the buffer, between two parallel electrodes. The current is passed at right angle to the gel that causes the electrophoretically resolved proteins to migrate out of the

body of the gel and get transferred (blotted) on the membrane. The membrane with the transferred proteins can be examined further by specific antibody as mentioned above.

In a variation of the wet electroblotting, a semi-dry electroblotting system is preferred that uses only a limited volume of the transfer-buffer, in which a couple of sheets of filter paper are soaked is necessary, and hence is economical (Fig. 15). A cassette comprising gel and nitrocellulose membrane soaked in transfer buffer is under laid and overlaid with filter paper sheets cut to the size of gel. This cassette is placed on a wet graphite-anode. The uppermost layer of the cassette comprising buffer-soaked blotting sheets is contacted with a wet graphite-cathode. Graphite is the best material for electrodes in semi-dry blotting because it conducts well, does not overheat and does not catalyze oxidation products. A current not higher than 0.8 to 1 mA per cm² of blotting surface is recommended. The gel can overheat if higher currents are used and proteins can precipitate. The protective casing is closed and current is applied across the pair of electrodes. The proteins in the gel under the influence of applied electric field migrate out of the gel and get electroblotted on the membrane underneath in 60-90 minutes. The larger proteins take more time to transfer out of the gel than the smaller ones. Thus transfer time is required to be optimized for different proteins and for the gels of varying thickness. The membrane is subsequently examined by use of specific probe antibody (Fig. 16).

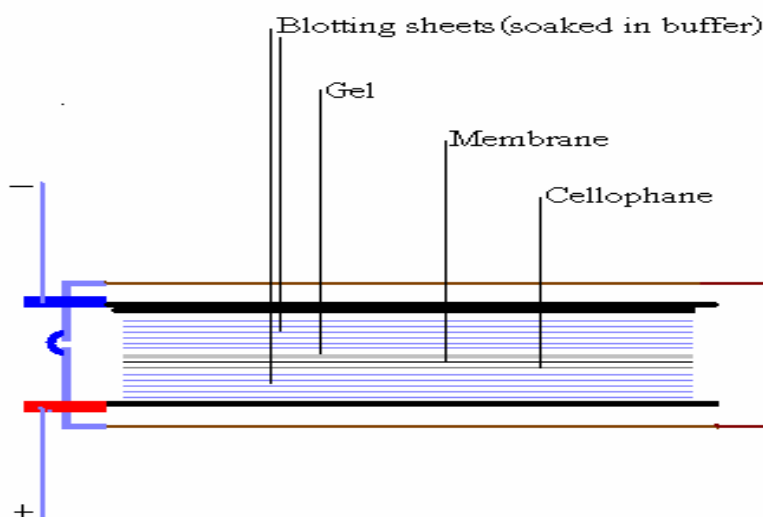


Fig. 15: Diagrammatical representation of a semi-dry horizontal blotting system

Major limitations

The major factor that will determine the success of an immunoblotting procedure is the nature of the epitopes recognized by the antibodies. The gel electrophoresis technique involves denaturation of the antigen sample, so only antibodies that recognize denaturation-resistant epitopes will bind the antigen. Most polyclonal sera contain at least some antibodies of this type, but many monoclonal antibodies will not react with denatured antigens.

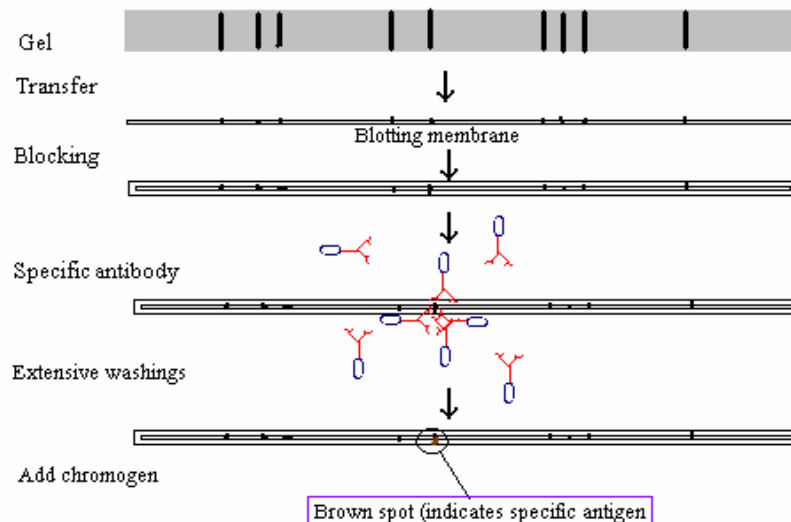


Fig. 16: Important steps in localization of specific antigen(s) during Western-blotting

The partial or incomplete transfer of the electrophoretically resolved proteins from the gel onto the membrane also limits the detection of antigens by the probe antibody. Generally, the high molecular weight proteins/antigens take more time to transfer than the relatively low molecular antigens. Thus the transfer time for optimal electroblotting of antigens onto the membrane shall be optimized for different antigen mixtures.

The antigen density immobilized at the surface of the membrane also favors the retention of low-affinity antibodies on the membrane by increasing the frequency with which antibodies leaving the membrane bind to adjacent sites. The sensitivity of immunoblotting is also determined by the detection method. For most detection systems, this limit will be about 20 femtomoles. For a 50-kDa protein it shall be approximately 1 ng. The loading capacity of the gels is limited and an antigen usually cannot be detected when its concentration falls below 1 ng/sample. In case of a typical SDS-PAGE, the capacity of a lane is approximately 150 µg. Distortion is seen if large amounts are used. The detection limit of most immunoblots is 1-10 ng.

Choice of probe antibody

Three types of antibody preparations can be used for immunoblotting, which include polyclonal antibodies, monoclonal antibodies and pooled monoclonal antibodies.

Polyclonal antibodies: Polyclonal antibodies probably are the most widely used type of antibody preparation for Immunoblotting. Many sera will also contain antibodies that will bind to a number of denaturation-resistant epitopes on the antigen. This will result in multiple antibody molecules binding to a given polypeptide (antigen) and will generate a relatively stronger signal than a monoclonal antibody. Also a polyclonal serum will usually contain high concentration of specific antibodies, they can often be diluted extensively. Thus, diluted antibodies can be used to reduce non-specific background problems without reducing sensitivity. The polyclonal antibodies normally are used as a whole serum that will contain

entire repertoire of circulating antibodies found in the immunized animal at the time blood was withdrawn to obtain serum. Therefore, the serum may contain high titered antibodies that specifically recognize spurious antigens. This is particularly troublesome in the analysis of microbial antigens. Serum may contain antibodies against bacteria, a virus or a fungus that has infected the host recently.

Use of monoclonal antibodies: The main advantage of employing monoclonal antibodies for immunoblotting is the specificity of its interaction. Monoclonal antibodies have an inherent specificity to bind with high affinity to a single epitope; it provides an elegant tool for identifying a particular region of the antigen. Monoclonal antibodies that function well in immunoblotting usually interact with epitopes that are defined by short stretches of the protein's primary sequence. As such, they can be used to examine small regions of the antigen. The major limitation in using monoclonal antibodies in immunoblotting is that many will not recognize epitopes that are destroyed by the denaturing reagents used in the preparation of the sample. However, some monoclonal antibodies work well and can give extremely clean and sensitive results.

Use of pooled monoclonal antibodies: Use of pooled monoclonal antibodies provide advantage of the best properties of both polyclonal and individual monoclonal antibodies, combining specificity and sensitivity. The pool must be composed of monoclonal antibodies that react with distinct denaturation-resistant epitopes and that do not show spurious cross-reactions. A few antigens have been studied in detail. However, wherever possible, a well-chosen pool of monoclonal antibodies should be used for immunoblotting (Table 3).

Table 3: Use of monoclonal or polyclonal antibodies in immunoblotting

S. No.	Property	Polyclonal antibodies	Monoclonal antibodies	Pooled monoclonal antibodies
1	Signal strength	Usually good	Variable	Excellent
2	Specificity	Good, some background staining	Excellent but some cross-reaction	Excellent
3	Good features	Generally recognize denatured antigen	Highly specific and unlimited supply	High signal strength, specificity and unlimited supply
4	Bad features	Nonrenewable, background staining and need for titration	Many do not recognize denatured antigens	Non-availability

Choice of membranes

Nitrocellulose, activated paper, and activated nylon have all been used successfully to bind the transferred proteins (Towbin *et al.*, 1979; Renart *et al.*, 1979; Gershoni and Palade 1982, 1983). Nitrocellulose is the most commonly used, but it has certain disadvantages. The proteins are not covalently bound to it, and nitrocellulose is brittle, especially when dry. However, nitrocellulose is still the most commonly used membrane. It is available in pore sizes from 0.05 μm to 0.45 μm . The pore size is a measure of the specific surface: smaller the pores, the higher the binding capacity. Proteins adsorbed on nitrocellulose can be reversibly

stained so that the total protein can be estimated before specific detection (Salinovich and Montelaro 1986). Activated paper (diaz groups) can be purchased commercially and shall bind proteins covalently. It has good mechanical strength but unfortunately the coupling method is incompatible with many gel electrophoresis systems. Nylon itself can bind only a small amount of protein and is not suitable for most applications. However, it has excellent mechanical strength and does not change size during washing or drying. Activated (positively charged) nylon solves some of these problems but has higher backgrounds. Because of these obvious limitations, the best compromise for most situations is nitrocellulose membranes. Unfortunately a blotting membrane, which binds 100% of the molecules, does not exist yet.

Widal test: Serological detection of antibodies to *Salmonella typhi* and *S. paratyphi*

Typhoid fever is a life threatening systemic infection all over the globe and continues to be a major health problem in many developing countries of the world. There are 16 million new cases of typhoid occurring globally (Ivanoff 1995). The annual incidence of typhoid fever has been reported as more than 13 million cases in Asia and causing more than 6 lakhs deaths worldwide annually (Ivanoff *et al.*, 1994). India is the second most populous country of the world with majority inhabiting the rural areas with restricted access to modern diagnostic techniques. Typhoid continues to be one of the leading causes of morbidity and mortality in India. The Widal test is used to demonstrate rising titers of antibodies to flagellar (H) and somatic (O) antigens in typhoid and paratyphoid fever. Tests for the presence of *Salmonella*-specific antibodies in the patient's serum may be of value in the diagnosis of enteric fever, but are of little help in that of salmonella food-poisoning. The patients' serum is tested by tube-agglutination for presence of antibodies against H antigen, O antigen and Vi suspensions of enteric fever bacteria *e.g.* *S. typhi* and *S. paratyphi*, which are commonly associated with typhoid in most of the countries including India. These suspensions may be prepared from suitable stock laboratory cultures by the various methods for suspensions for tube agglutination tests, but commercially prepared suspensions are generally used. Antibodies to *S. typhi* and *S. paratyphi* have classically been measured by agglutination of patient's sera with suspensions of *S. typhi*, a process first described by Widal in 1896. Since high titers of antibodies could be detected early in the clinical course of the disease, and because cultures of blood were often more difficult to obtain and probably not of optimum sensitivity, the Widal test assumed the stature of a definitive diagnostic marker for typhoid fever. The test was used diagnostically in two ways; either a single high titer of antibodies during the 1st week of illness, or a greater than fourfold titer rise in sera taken 1 to 2 weeks apart, was considered diagnostic. Both the O and H antibody titers are found to be useful diagnostically, although the O is considered more reliable. It should be noted that some patients with known immunological disorders, such as rheumatoid arthritis, give false positive tests. The H or O antibody levels may rise non-specifically sometimes due to cross-reaction with other enterobacteria. Where adequate bacteriologic facilities are available, the preferred method of diagnosis is clearly by culture.

The patient's serum is tested in two-fold serial dilutions against each of the different salmonella antigen suspensions. The agglutination test is performed for each of the selected salmonella antigen in small serum tubes (7 X 1 cm); six of these are used for six serum dilutions (1: 2 to 1: 128) and in the 7th tube saline is used in stead of human serum (non-serum) as a negative control.

Interpretation

The titer of the patient's serum for each salmonella antigenic suspension is read as the highest dilution of serum giving visible agglutination, e.g. if this dilution is 1 in 240, the titer is 240. A positive reaction is first detected about the 7th to 10th day of the illness in enteric fever, so that a negative result at an early stage is inconclusive. The strength of the reaction for the infecting serotype increases progressively to a maximum titer about the end of the 3rd week. Thus the demonstration of the rising titer e.g. four-fold or greater rise, between tests made in the 1st and 3rd week is highly significant. An increasing O antibody level signifies acute infection, whilst a raised H antibody level may indicate the serotype of the infecting organism (Sherwal *et al.*, 2004).

Positive results in a single test by no means prove the presence of enteric fever nor negative results its absence. From an extensive study of sera collected from patients during and after the outbreak of typhoid fever in Aberdeen in 1964, Brodie (1977) concluded that the value of the Widal reaction as an aid to diagnosis was very limited. In contradiction of generally held opinion, he found that the H-antibody titer was a more reliable indicator of infection than the O-antibody titer. However, another study conducted in Malaysia (Pang and Puthucheary 1983) demonstrated the diagnostic value of the Widal test in an area where typhoid was endemic. The interpretation of the serology thus requires consideration of factors such as previous immunization, the stage of illness and the effect of any antibiotic treatment, etc. Widal's test and particularly its interpretation require an expert. While interpreting the results of a Widal test, the following points must be considered.

- i. The serum of some normal (uninfected) persons agglutinate salmonella-antigen suspensions at dilutions up to about 1 in 50, so that titers cannot be taken as significant unless they are greater than about 1 in 100.
- ii. Persons who have received Typhoid vaccine may show high titers to each of the salmonellae in the vaccine and only if a marked rise of titer to one serotype is observed can the result be regarded as diagnostically relevant. H-agglutinins tend to persist for many months after vaccination but O-agglutinins tend to disappear sooner, generally within six months.
- iii. For determining the serotype of the infecting organism, the H-reaction is more reliable than O-reaction because the different serotypes have some O-antigens in common.
- iv. Non-specific antigens, such as fimbrial antigens, may be present in the test suspensions and they tend to give false-positive results by reacting with a homologous agglutinin present in the serum of some uninfected individuals.
- v. The Widal reaction is positive in many healthy carriers, and such a carrier may suffer a pyrexial illness due to a different, non-salmonella infection. Although a negative reaction does not exclude the carrier state, a positive reaction, particularly a Vi titer of 10 or greater, is often said to be helpful for its recognition (Colle *et al.*, 1989).

Determination of minimum inhibitory concentration (MIC) of antibiotic(s)

The clinical microbiology laboratory assists the clinician in the choice of drugs/ antibiotics for the treatment of infections. The purpose of performing antibiotic sensitivity test or to determine minimum inhibitory concentration of a given antibiotic towards relevant pathogens in exudates and body fluids (cerebrospinal fluid, blood, urine, throat and vaginal swabs etc.) from the patient is to select appropriate antibiotic(s) to maximally kill/inactivate the target microorganism(s) *in vivo*. Thus the antibiotic(s) sensitivity tests are performed to (i) determine the degree of sensitivity or resistance of the pathogen isolated from the patient to

an appropriate range of antimicrobial drugs, and (ii) to determine the concentration of the selected drug that will be administered to the patient to eliminate the infecting pathogen in the blood of the patient. To maintain an appropriate more or less uniform concentration of the drug in blood or cerebrospinal fluid of the patient is it important to control the schedule of dosage of chosen drug over a period of time. The clinician's purpose in recommending an antimicrobial drug is to produce at the site of infection or in blood a concentration of it high enough to kill or inhibit the growth of the infecting pathogen, without exerting a significant toxic effect on the patient's tissues. The precise identity of the microbe causing an infection is seldom implicit in the clinical presentation; the laboratory helps by distinguishing relevant from irrelevant microbe in cultures from the patient and by identifying the species of the former. Strains of most pathogenic species differ from one another in their sensitivity, so that it is necessary for the sensitivity pattern of the particular strain isolated from the patient to be determined by sensitivity tests conducted in the laboratory.

The accumulated results of sensitivity tests on all isolates of a pathogenic species from patients in a hospital or local community in case of an outbreak of infection (cholera, scrub typhus, food poisoning etc.) thus provide empirical treatment to be administered to the patients suffering from serious infections. This is the first step to treat the patients before the results of sensitivity tests using a set of antibiotics are available to the clinician. The antibiotics sensitivity test generally requires 48-72 h when the results are finally available. Thus by studying changes in the resistance patterns of local pathogens, these accumulated results often indicate the urgency for change of antibiotic prescription. Besides pharmacokinetic data, sensitivity tests are the main means of determining the potential value of new antimicrobial drugs before they are introduced for clinical trials.

Assays of the concentration of an antibiotic in the blood, serum or other body fluid of a patient under treatment are done mainly when the antibiotics, such as aminoglycosides may have toxic effects on the body at concentrations required for effective antimicrobial effect. The assays are done on specimens collected before and after the administration of a dose that are expected to show the minimum and maximum concentrations achieved and the results help the clinician to recommend appropriate dosage that the drug concentration would be non-toxic but effective *i.e.* about four times the minimum inhibitory concentration (MIC) for the infecting organism. The MIC is that minimum concentration of a given antibiotic at which no visible growth of the pathogenic microorganism occurs *in vitro*. The results of sensitivity tests are generally reported as: Microorganism 'A' isolated from the given sample (say blood) is sensitive (S) or resistant (R) to antibiotic B, C, D and E. Such a report implies that the organism found in the blood sample of the patient is responsible for infection in the patient. This organism could be killed *in vivo* by administering four times the MIC of the chosen antibiotic(s) for which the pathogenic organism is sensitive. If the microorganism is reported as sensitive, the MIC is less than a half or a quarter the concentration of antibiotic likely to be found in the infected tissues of a patient given the usual schedule of doses *i.e.* the infection is treatable. A larger margin of safety may be essential in some infections. Ideally the bacteriologist shall report the physician the observed MIC of each antibiotics tested for the patient's infecting organism and leave it to the physician to decide, from his experience of the pharmacokinetics of each drug, the likely site of infection within the patient's body and the concentration of the drug likely to be achieved in the site of infection with the recommended dose and route of administration, whether the infection is likely to be sensitive or resistant to therapy. The MIC of a given drug may be assayed by any of the following three methods.

Tests with serial two-fold dilutions

The microorganism is exposed to a series of fixed antibiotic concentrations in separate cultures in agar-medium or broth. Various concentrations of the selected antibiotics are produced by dilution, usually serially two-fold, of a stock solution prepared from antibiotic powder of a known stated potency/ concentration commercially available. They should not be prepared from the therapeutic preparations, as the potency of these is not standardized with sufficient precision. The dilution steps must be followed to prepare concentration fraction or multiple of 1 microgram/ml. Such a range of antibiotic concentrations may be prepared by placing commercially available paper discs containing known amounts of antibiotic in the bottom of petri dishes before adding appropriate volumes of sterile molten agar. Alternatively, the commercially supplied antibiotic tablets may be added to appropriate volumes of sterile broth taken in test tubes.

The MIC is generally considered as the lowest concentration of a given antibiotic that prevents development of visible growth of the test organism. The test may be extended to measure the Minimum Bactericidal Concentration (MBC) of the antibiotic by preparing subcultures from each test culture and noting the smallest concentration of antibiotic from which no growth is obtained. Sometimes, a concentration of the antibiotic/drug that decreases the visible growth of an organism in a growth medium/ or broth by 50% or 90% in comparison to a control (containing no antibiotics) is also determined and referred as MIC₅₀ and MIC₉₀, respectively. The growth of bacteria is measured as turbidity or cloudiness in the broth (using a nephelometer) in comparison to a control containing no antibiotic. Although the abbreviation MBC for minimum bactericidal concentration is in general use, it is unsatisfactory because it could stand for minimum bacteriostatic concentration and also because it is inapplicable to values for non-bacterial pathogens such as fungi and mycoplasma. MCC, which refers to minimal cidal concentration, is a much appropriate term that should be used for non-bacterial pathogens preferentially. This method of two-fold serial dilution of an antibiotic is laborious and expensive but highly accurate in determining MIC value of an antibiotic (Fig. 17).

Tests with break-point concentrations

This is a modification of the above method. In this approach the test organism along with control strains of known MIC, is exposed to only two or three critical 'break-point' concentrations of the antibiotic in agar, broth or other specially formulated solid-medium (LJ medium for *Mycobacterium* spp.). The concentrations of the antibiotic are chosen so as to reflect achievable levels of the antibiotic in body fluids usually blood or urine (Waterworth 1981, 1983). If the organism is inhibited by the lower concentration it is reported as sensitive, if by the intermediate concentration, as of intermediate resistance and if not by the highest selected concentration as resistant.

Tests with diffusion gradients of concentration

In this approach, the test microorganism is uniformly seeded over an agar-based growth medium and exposed to continuous concentration gradient of antibiotic diffusing from a paper disk (disk diffusion test). A bacterium sensitive to the antibiotic is inhibited from growing in a circular zone around the paper disk: the lower the MIC of the antibiotic for the chosen organism, the larger the diameter of the zone. A comparison of the zone size with that produced in a parallel test with a control strain gives a measure of the MIC.

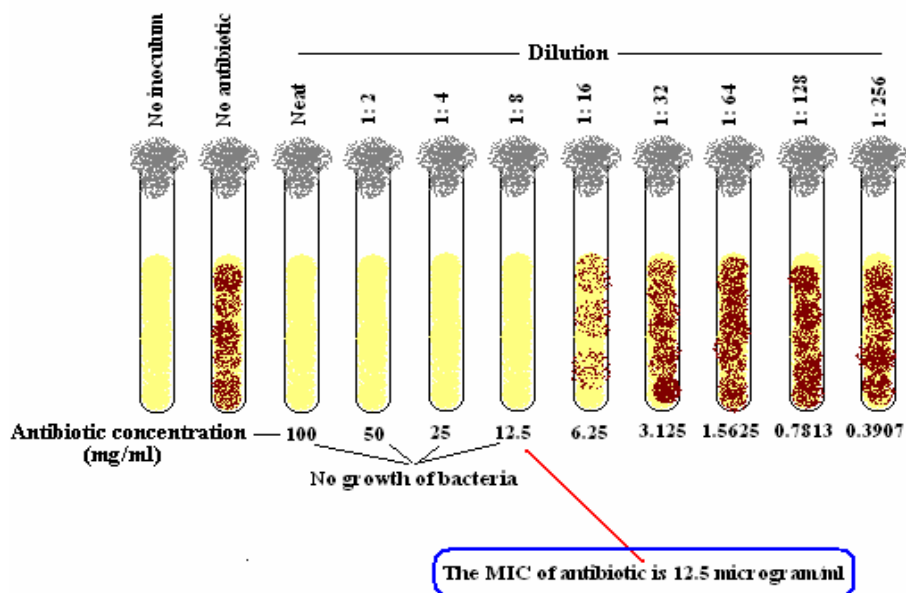


Fig. 17: The serial two-fold dilutions of a given antibiotic were made (with a starting concentration of 100 micrograms/ml) in sterile broth. One of the test-tube containing broths is kept as a control (no inoculum), and all other test tubes containing broth are inoculated with same amount of inoculum. The appearance of turbidity is observed in the test tubes after 24 h of incubation at 37°C. The highest dilution of the antibiotic that showed no visible growth of the bacteria is considered as the MIC

None of these *in vitro* designed tests are ideally satisfactory. They provide only an approximation, and sometime a poor one. The anaerobic or micro-aerophilic pathogenic organisms or other fastidious microbes that are difficult to culture *in vitro* or under strict anaerobic conditions are difficult to be assayed for MIC using chosen antibiotics. The accuracy and reproducibility of the above tests are affected by many variables such as nature, composition and pH of the test medium, the weight/amount of the bacterial inoculum, the conditions and time of incubation, the antibiotic concentration, and the organism's ability to produce an antibiotic-inactivating enzyme. However, knowing these limitations, the results of antibiotic sensitivity assay are of great importance for successful management of the patient by administration of selected dosage of chosen drugs and in the evaluation of new antibiotics (Phillips 1986).

Disk diffusion tests

The disk diffusion test is the most widely used technique for the routine testing of isolates from patients. The method is highly useful and reproducible for the pathogenic organisms that grow overnight. The antibiotic sensitivity profile for the chosen antibiotics can be determined for the microbes that include common aerobes and facultative anaerobes, rapidly growing anaerobes such as *Clostridium perfringens* and *Bacteroides fragilis*, and rapidly growing fungi such as *Candida albicans*. The test however, is unsuitable for slow growing organisms such as *Mycobacterium* spp. as the antibiotics would become too diffused before growth of bacterium has become visible. A method of culture on fixed discontinuous concentrations should be preferred for slowly growing organisms, also for measuring

accurately the sulphonamide sensitivity of meningococci (Fallon 1978) and the gonococci (Jephcott and Eggleston 1985) and the colistin sensitivity of Gram-negative bacteria (Williams and Leung 1978). Among several forms of disk diffusion tests, which vary in their methods of standardization, reading and control, the Kirby-Bauer method (Bauer *et al.*, 1966) is the most common method used to determine antibiotic sensitivity of pathogenic microbes.

The method involves use of disks of a single antibiotic (usually high content of antibiotic). These are placed on an inoculum of strictly standardized density on Mueller-Hinton agar. Three grades of sensitivity are recognized – sensitive (S), intermediate (IM) and resistant (R); by comparing the diameter of the inhibition zones with critical zone diameters in published tables. The clearance zone diameters are separated into four categories of sensitivity, two of them intermediate, by reference to published regression lines that plot the MIC for many strains of different species against the zone diameter produced by disk of the strength used in the test. However, the validity of this test for estimation of MIC of selected drug has been a subject of ambiguity and controversy (Krasemann and Hildenbrand 1980; Arvidson *et al.*, 1981). The comparative method is widely used in British laboratories. The diameter of the inhibition zone of the test organism is compared with that of a control organism of standard sensitivity tested under identical conditions on a separate plate or, preferably on the same plate. Disks of low and high antibiotic content may be used, the latter to test organisms from urine, in which many antibiotics become highly concentrated. Three grades of sensitivity that are recognized are S, IM and R. Testing both the test and control strains on the same plate (Stokes and Ridgeway 1980) eliminates many of the variables likely to affect accuracy, but not those of the inoculum or speed of growth between the two strains.

In Kirby-Bauer method of determining antibiotic sensitivity, consistent and accurate results are most likely to be obtained if the following points are addressed.

- i. One of the several suitable commercially available ‘sensitivity test’ media is used.
- ii. The medium must be prepared according to the manufacturer’s instructions.
- iii. The medium is poured to a depth of 4 mm in the flat-bottom 8.5 cm diameter Petri dish on a leveled surface.
- iv. Once set, the plates may be stored for up to a week at 4°C; their surfaces should be dried with their lids ajar before use.
- v. Accurate inoculation of the Petri dishes by swabbing is convenient.
- vi. The full surface of each disc must make firm contact with the seeded agar.
- vii. The disks shall be applied on the seed medium within a short and standard time of the seeding of plates.

Four control strains are usually recommended for use with the comparative method: *Escherichia coli* NCTC 10418 for tests of coliform from urinary tract; *Pseudomonas aeruginosa* NCTC 10662 for tests of this species, especially against aminoglycosides; *Clostridium perfringens* NCTC 11229 for tests of clostridia against metronidazole and other anti-clostridia antibiotics; and *Staphylococcus aureus* NCTC 6571, the “Oxford *Staphylococcus*”, for tests with all other antibiotics except polymixins. The working cultures of control strains should be grown weekly or monthly from stock cultures that are freeze-dried or frozen at –20°C. They should be maintained on slants of a suitable medium and sub-cultured as seldom as possible.

Antibiotic disks

Commercially prepared disks (Fig. 18) of 6 mm diameter should be used (*e.g.* Oxoid, Mast, HiMedia). Their content of antibiotic should not vary more than the limits set by the World Health Organization Expert Committee on Biological Standardization (1977) and should be coded as recommended by that committee. Disks and disk dispensers should be stored in sealed containers with a desiccant at less than 8°C. Metronidazole may deteriorate when exposed to light and disks of it should be kept in the dark as much as possible.

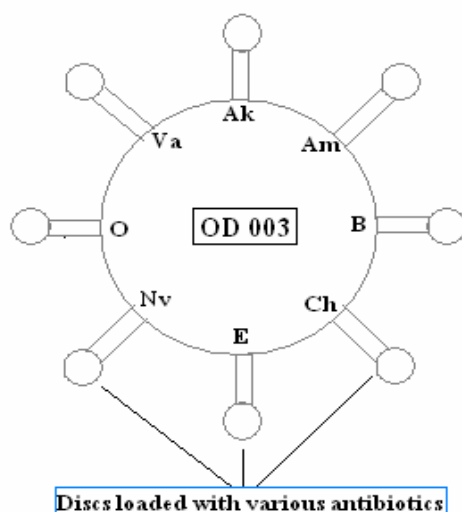


Fig. 18: An Octodisc of a commercial supplier (G-III-plus; HiMedia Laboratories Ltd., Mumbai) containing eight different antibiotics loaded on a common core disc. The antibiotics used are Amikacin [Ak, 10 µg], Amoxycilin [Am, 10 µg], Bacitracin [B, 10 units], Cephalothin [Ch, 30 µg], Erythromycin [E, 15 µg], Novobiocin [Nv, 30 µg], Oxytetracycline [O, 30 µg] and Vancomycin [Va, 30 µg]

The National Committee for Clinical Laboratory Standards (NCCLS), USA documents are used worldwide to use standard protocols for reproducible results of antibiotic sensitivity assays. To ensure compliance with current standards, laboratories should refer to the latest editions of NCCLS susceptibility testing documents. The standards are revised every 3-5 years, and the associated supplemental tables are updated annually (www.nccls.org). The following updated standards (Table 4) pertaining to *Streptococcus pneumoniae* were published in January 2003.

The NCCLS was renamed as Clinical and Laboratory Standards Institute (CLSI) on Jan 1, 2005. This institute located at Pennsylvania, USA (www.nccls.org) now provides standard protocols and documents to all affiliated stakeholders/organization in various countries. These protocols/standards are referenced in government regulations and international standards for maintaining high standards of quality controls as well as reproducibility of results of antibiotics sensitivity protocols (Table 5).

Table 4: NCCLS standards for antimicrobial susceptibility testing of *S. pneumoniae* by Disk diffusion method

Method	Standard conditions
Disk diffusion method	• Use Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. • Place no more than 9 disks on a 150 mm plate or no more than 4 disks on a 100 mm plate. • Incubate plates at 35°C in an atmosphere of 5% CO₂ for 20-24 h.
Broth dilution method	• Use cation-adjusted Mueller-Hinton broth supplemented with 2%- 5% lysed horse blood. • Incubate trays in ambient air at 35°C for 20-24 h.
Disk diffusion and Broth dilution methods	• Use the direct colony suspension procedure to prepare the inoculum. That is, take colonies directly from an 18-to 20-h sheep blood agar culture plate and, using either Mueller-Hinton broth or 9% saline, prepare a suspension equivalent to the 0.5 McFarland standard. • Inoculate plates or broth within 15 minutes of preparing the suspension. • Test only drugs which have been approved for the method used. Approved antimicrobial agents appear in the <i>Supplemental Tables</i>. • Use the criteria specific for <i>S. pneumoniae</i> to interpret results. The interpretive criteria appear in the <i>Supplemental Tables</i>. • For quality control, use the recommended organism strain and associated interpretive criteria.

E-test for MIC determination of antibiotic(s)

E-test is another concept for MIC determinations for anti-microbial agents that is based on a predefined antibiotic gradient on a plastic strip calibrated with a continuous logarithmic MIC scale a range of two-fold dilutions. E-test is based on a combination of concepts of dilution and diffusion tests (Hoffner *et al.*, 1994). Like dilution methods, E-test directly quantifies anti-microbial susceptibility in terms of discrete MIC values. As E-test consists of a predefined and continuous concentration gradient, the MIC values obtained can be more precise than values from conventional procedures based on discontinuous two-fold serial dilutions. Although processes like the disc diffusion test, the preformed and stable concentration gradient in E-test differentiates the two methods clearly. Unlike disc diffusion, E-test MICs are unaffected by drug properties such as molecular weight, aqueous solubility and diffusion characteristics or by varying growth rates of different microbes.

E-test consists of a thin, inert and non-porous plastic strip (5 x 60 mm; Fig. 19). One side of the strip is calibrated with MIC values in mg/ml and a two-letter code designates the identity of the drug. A predefined and exponential gradient of the dried and stabilized anti-microbial

agent is immobilized on the other surface of the strip with a specific concentration. When applied onto an inoculated agar plate, there is an immediate release of the antibiotic from the plastic surface into the agar matrix.

Table 5: Recommended antibiotic contents of disks for use in diffusion sensitivity tests

Drug	Content (μg)
Amikacin	30
Ampicillin	2
Benzyl penicillin	1.2
Carbenicillin	100
Cefotaxime	30
Cephazolin	30
Chloramphenicol	2.5 or 5
Ciprofloxacin	5
Clindamycin	2
Cloxacillin	5
Colistin	10 units
Cotrimoxazole	25
Erythromycin	5
Fusidate	10

Drug	Content (μg)
Gentamicin	10
Kanamycin	10
Lincomycin	2
Mithicillin	5
Metronidazole	5
Mupirocin	5
Nalidixic acid	30
Neomycin	30
Nitrofurantoin	100
Norfloxacin	10
Rifampicin	2
Streptomycin	25
Sulphafurazole	100
Tetracycline	10

A predefined, continuous and stable gradient of the drug concentrations is created directly underneath the strip. After incubation, whereby growth becomes visible, a symmetrical inhibition ellipse centered along the strip is seen. The zone edge intersects the strip at the MIC value (Fig. 20).



Fig. 19: A commercially prepared strip creates a gradient of antibiotic concentration on an agar plate

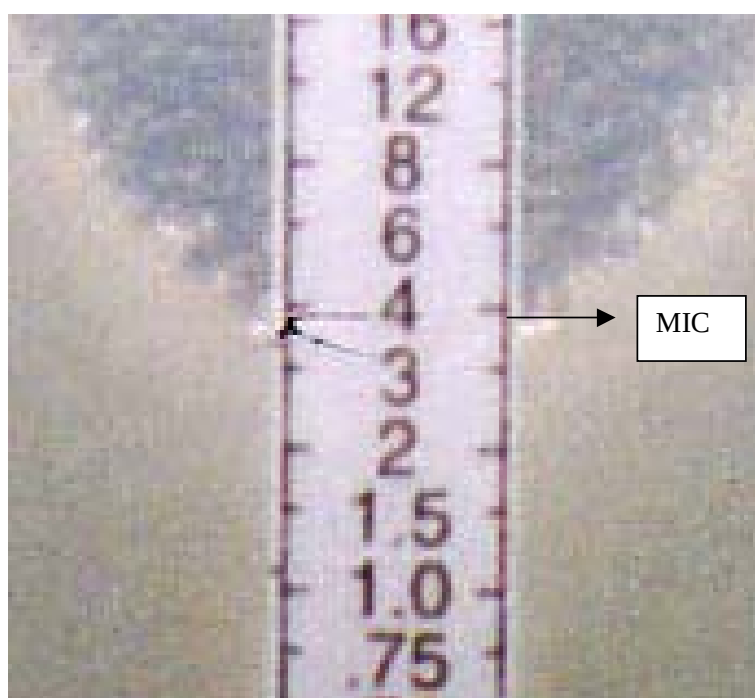


Fig. 20: The MIC corresponds to the point where the bacterial growth crosses the numbered strip

The MIC is read directly from the scale printed on the E-test strip at the point where the ellipse of the growth inhibition intercepts the strip. An E-test MIC value that falls between the conventional two-fold dilutions is rounded up to the next two-fold dilution value before interpretation.

Suggested Reading

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