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Course Title: Immune & Molecular Diagnostics

Unit-III

Measurements of specific proteins in serum, CSF & Urine

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Unit III:

Measurements of specific proteins in serum, CSF & Urine- Immunoglobulins (Igs), paraproteins & cryoglobulins, investigation of complement disorders- assays for individual components (EIA, RIA), Functional assay for complement pathways- AH50, C50- detection of complement breakdown products- C3nephritic factor, Functional assays for immune complexes. Clinical significance of C- Reactive proteins- cryoglobulins in patient specimens- test methods for detection. Tests for allergy- Total serum IgE, allergen specific IgE- serology based methods-in vivo-skin prick test, in- vitro- RAST (1^{st}, 2^{nd} & 3^{rd} generation methods)- cell based methods- Allergan induced mediator release assay- histamine release, LTC4 release (Cellular antigen Stimulation Test- CAST), Flow cytometric basophil activation assay (Flow Assay Stimulation Test- FAST)- CD63 CD203c, test for hypersensitivity III- precipitating antibodies.

PRESENTATION: 1

Measurements of specific proteins in serum, CSF & Urine

- The measurement of specific proteins, particularly immunoglobulins and paraproteins, in serum, cerebrospinal fluid (CSF), and urine is crucial in diagnosing and monitoring various diseases, including autoimmune disorders, infections, and haematological malignancies.

IMMUNOGLOBULINS

1. Immunoglobulins (Ig)

Immunoglobulins, or antibodies, are glycoproteins produced by B cells that play a key role in the immune response by recognizing and binding to specific antigens.

a. Types of Immunoglobulins

1. **IgG**: The most abundant antibody in the blood and extracellular fluid; provides long-term immunity.
2. **IgA**: Found in mucosal areas, such as the respiratory and gastrointestinal tracts; provides protection on mucosal surfaces.
3. **IgM**: The first antibody produced in response to an infection; primarily found in blood and lymphatic fluid.
4. **IgE**: Involved in allergic reactions and responses to parasitic infections.
5. **IgD**: Functions primarily as a receptor on immature B cells.

b. Measurement Techniques:

1.Nephelometry and Turbidimetry:

1. Quantitative methods based on light scattering properties of antigen-antibody complexes.
2. Commonly used for measuring total immunoglobulin levels.

2.Enzyme-Linked Immunosorbent Assay (ELISA):

1. Highly sensitive method that uses enzyme-labeled antibodies to detect and quantify specific immunoglobulins.
2. Useful for specific isotype measurements (e.g., IgG, IgA, IgM).

3. Immunofixation Electrophoresis (IFE):

1. Used to identify specific types of immunoglobulins and their heavy and light chains.
2. Essential for diagnosing monoclonal gammopathies.

4 Western Blotting:

1. Detects specific proteins in a sample by separating them using gel electrophoresis and transferring them to a membrane, where they are identified using specific antibodies.

c. Clinical Significance

1.Serum Immunoglobulins:

1. Elevated levels may indicate infections, autoimmune diseases, or chronic inflammatory conditions.
2. Decreased levels can be seen in immunodeficiency disorders.

2.CSF Immunoglobulins:

1. Measurement of IgG in CSF is crucial in diagnosing multiple sclerosis (MS). The IgG index and presence of oligoclonal bands in CSF are key diagnostic markers.

3.Urine Immunoglobulins:

1. Bence-Jones proteins (free light chains of immunoglobulins) in urine are indicative of multiple myeloma or other plasma cell dyscrasias.

PARAPROTEINS

2. Paraproteins (Monoclonal Proteins)

- Paraproteins, also known as monoclonal proteins or M-proteins, are abnormal immunoglobulins or immunoglobulin fragments produced by a single clone of plasma cells. Their presence is a hallmark of monoclonal gammopathies.

a. Types of Paraproteins

1. **Monoclonal IgG, IgA, IgM, IgD, IgE**: Complete immunoglobulin molecules produced by abnormal plasma cells.
2. **Light Chains (Kappa, Lambda)**: Free light chains found in serum or urine, commonly associated with conditions like multiple myeloma.

b. Measurement Techniques

1.Serum Protein Electrophoresis (SPEP):

1. Separates proteins in the serum based on their size and charge.
2. Identifies the characteristic M-spike, a hallmark of monoclonal gammopathy.

2.Immunofixation Electrophoresis (IFE):

1. Confirms the presence and type of monoclonal protein by identifying its heavy and light chains.
2. More specific than SPEP and often used for confirmation.

3.Free Light Chain Assay:

1. Measures the concentration of free kappa and lambda light chains in the serum.
2. Useful in diagnosing and monitoring multiple myeloma and related disorders.

4.Urine Protein Electrophoresis (UPEP):

1. Detects paraproteins excreted in urine, especially Bence-Jones proteins.

c. Clinical Significance

1. Monoclonal Gammopathy of Undetermined Significance (MGUS):

1. A benign condition with the presence of monoclonal protein but no symptoms of disease.
2. Requires monitoring as it can progress to malignancies like multiple myeloma.

2. Multiple Myeloma:

1. A malignant plasma cell disorder characterized by the overproduction of monoclonal immunoglobulins or light chains.
2. Diagnosed through the presence of an M-spike in SPEP/IFE and detection of light chains in urine or serum.

3. Waldenstrom's Macroglobulinemia:

1. A type of non-Hodgkin lymphoma characterized by the production of monoclonal IgM.

4. Amyloidosis:

1. Deposition of misfolded proteins, often light chains, leading to organ dysfunction. Measurement of light chains helps in diagnosis and monitoring.

d. Diagnostic and Monitoring Uses

- **Diagnosis**: Identifying the presence of paraproteins is critical in diagnosing multiple myeloma, MGUS, and other monoclonal gammopathies.
- **Monitoring**: Regular measurement of paraprotein levels helps in assessing disease progression and response to therapy in conditions like multiple myeloma.

CRYOGLOBULINS

- Cryoglobulins (CGs) represent a possible dysfunction of the immune system.
- CGs are immunoglobulins that undergo reversible precipitation or gelling when exposed to temperatures below 37°C and re-dissolve upon rewarming.
- Most CGs (95%) consist of immune complexes (ICs) containing rheumatoid factor (RF); they are known as “mixed” CGs to differentiate them from monoclonal CGs, which do not contain RF or antigen-antibody complexes.

- Cryoprecipitation occurs because of the rapid formation of cold-insoluble immune complexes formed by IgM that display RF activity and are complexed to IgG

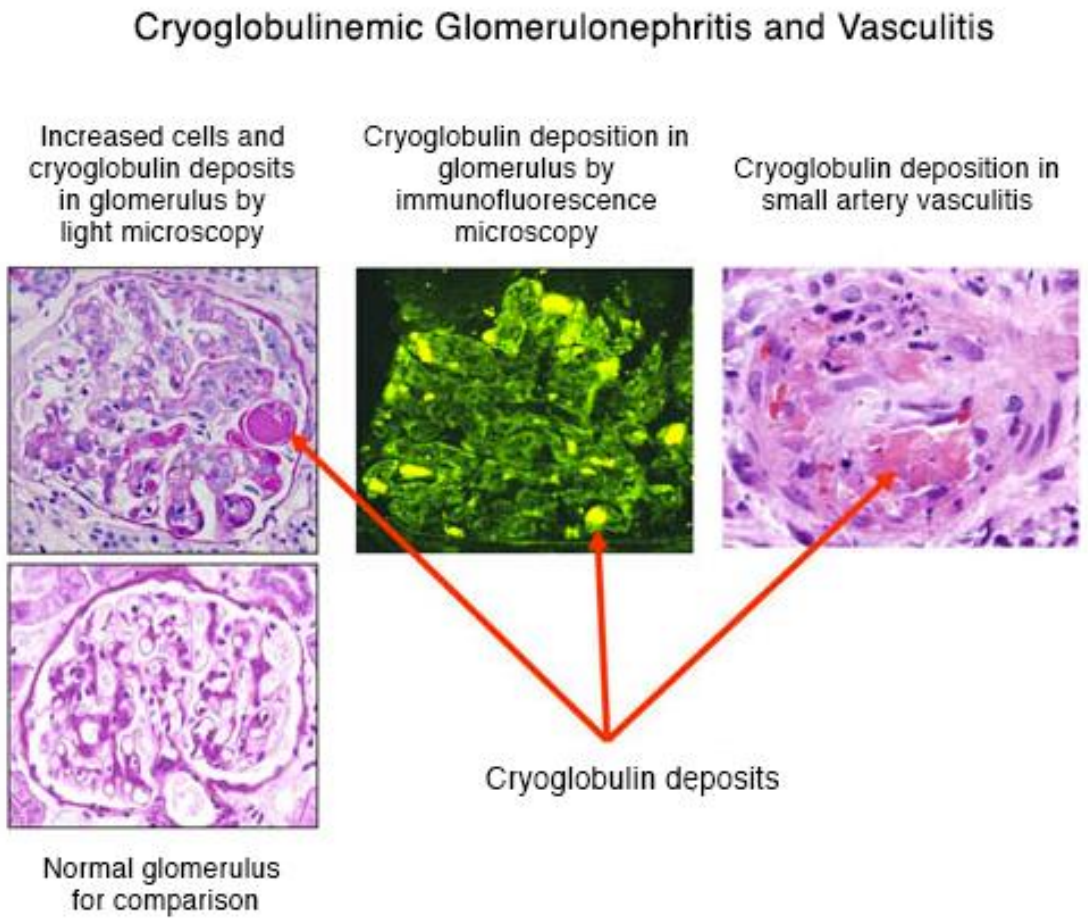


Table 1 Characteristics and classification of cryoglobulins.

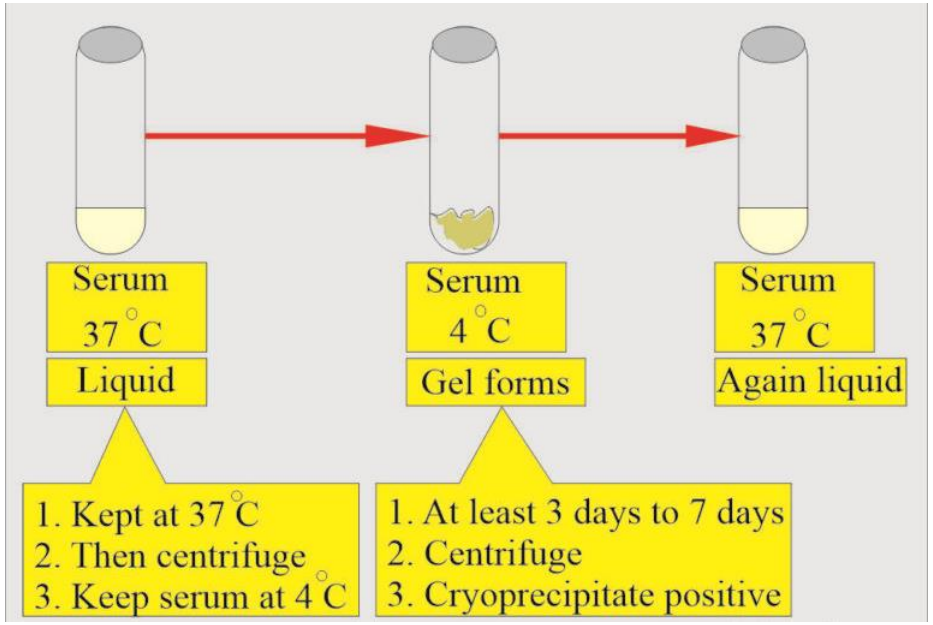
Cryoglobulinemia	Type I	Type II	Type III
Immunoglobulin classes	IgM (most abundant) IgG (IgG2, IgG3) IgA (rarely) FLCs	IgM vs IgG IgG vs IgG IgA vs IgG (rarely)	IgM-IgG IgM-IgG-IgA IgG-IgA-FLCs
Clonality	Monoclonal Ig	One or more monoclonal Igs + polyclonal Igs	Polyclonal Igs Oligoclonal Igs + polyclonal Igs (microheterogeneous)
Mixed cryoglobulinemia	No	Yes	Yes
Frequency	25–30%	25%	50%
Associated diseases	MGUS SMM Waldeström macroglobulinemia MM Other lymphoproliferative diseases	SAD: Sjögren's syndrome, SLE, RA LPD: B-cell lymphoma, NHL Solid tumors Cold-agglutinin disease *Infectious*: Chronic HCV infection Other infections (HIV, HBV) *Essential MC (unknown cause)*	SAD: LES, RA Intestinal diseases Biliary cirrhosis Solid tumors *Infectious*: HBV, HIV, Epstein-Barr, cytomegalovirus Endocarditis, spirochetes Fungal infections Parasitosys *Essential MC (unknown cause)*

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; SAD, systemic autoimmune diseases; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; LPD, lymphoproliferative disorders; NHL, non-Hodgkin lymphoma; FLC, free light chains.

PRE-ANALYTICAL PROCEDURE

- Appropriate laboratory testing of CGs involves simple biochemical quantification, which requires strict pre-analytical protocol adherence to maintain the specimen at a **temperature of 37°C**, especially during the initial steps of the test; the lack of a stable temperature from sample collection to analysis may result in misdetection of CGs, with a detriment for the patient.
- Blood samples (at least 10mL in volume) must be immediately transported to the laboratory under conditions that maintain 37°C for at least 30min until clotting is complete.
- The sample is generally centrifuged at 2000g for 10min at 37°C; however, some authors recommend 2000g for 30min at 37°C or do not specify a time or speed. Then, serum is transferred to a thin tube containing a preservative such as **Sodium azide and stored at 4°C**

- The tube containing the serum sample is placed at 4°C and observed daily for at least 7 days. Recently, it has been proposed to extend this observation period to 15 days because of the occurrence of late-appearing cryoglobulins.
- CG cause a fine whitish or granular precipitate but can also produce a gel or crystals; redissolution is completed in a few minutes at 37°C.



Isolation and purification

- This step is generally carried out on another blood sample (10mL of serum may be necessary).
- After precipitation of the serum at 4° C, the supernatant is removed, and the cryoprecipitate is purified by means of a succession of three series of precipitation at 4°C and redissolution in washing buffer at 37°C during a time course of 3 days.

Note:

- Washing buffer - phosphate buffered saline (PBS) or 3% polyethylene glycol 6000 in PBS and it must be kept at 4°C

Quantification

- The cryoprecipitate should be dissolved by incubation at 37°C. CGs may also be treated with reducing solutions, such as 10% acetylcysteine, 1% β-mercaptoethanol or 0.5mmol/L dithiothreitol
- **Cryocrit (CRT)** that represents the measurement of total proteins, via immunonephelometric quantification of immunoglobulins or as the area under the curve of the gamma region over the course of electrophoresis of the resolubilized cryoprecipitate (performed at 37°C).

- It consists of the measured percentage ratio between the volumes of cryoprecipitate and serum obtained by centrifugation at 4°C for 15min at 1700g.
- Cryoprecipitate proteins could be quantified by a sensitive method derived from the Biuret method with reading at 485nm; the calibration range should be performed with purified immunoglobulins. The sensitivity of this method is 20mg/L.

Immunochemical typing

- The purity of the cryoprecipitate is verified by good-sensitivity electrophoresis that allows the detection of a monoclonal component by a narrow strip.
- Typing is carried out by immunofixation electrophoresis using monospecific antisera for each heavy (γ, α, μ) and light (κ, λ) chain of immunoglobulins.
- The most sensitive method is the immunoblot (Western blot).

INVESTIGATION OF COMPLEMENT DISORDERS

- The complement system is a crucial part of the innate immune response, consisting of over 30 proteins that work in a cascade to fight infections, mediate inflammation, and clear immune complexes.
- Complement disorders can result from deficiencies or dysregulation of these proteins, leading to increased susceptibility to infections, autoimmune diseases, or other immune-related conditions.
- Investigating complement disorders involves various laboratory tests to assess the functionality and levels of complement proteins.

1. Overview of the Complement System

- The complement system can be activated via three pathways:
- **Classical Pathway**: Triggered by antibodies bound to antigens.
- **Lectin Pathway**: Activated by mannose-binding lectin (MBL) binding to microbial carbohydrates.
- **Alternative Pathway**: Activated directly on microbial surfaces.
- These pathways converge at the formation of C3 convertase, leading to a common terminal pathway that results in the formation of the membrane attack complex (MAC) and cell lysis.

2. Clinical Manifestations of Complement Disorders

- **Recurrent Bacterial Infections**: Deficiency in components like C3 can lead to increased susceptibility to infections.
- **Autoimmune Diseases**: Dysregulation of the complement system can contribute to autoimmune diseases like systemic lupus erythematosus (SLE).
- **Angioedema**: Deficiency in C1 inhibitor (C1-INH) leads to hereditary angioedema (HAE).
- **Paroxysmal Nocturnal Hemoglobinuria (PNH)**: A disorder caused by complement-mediated lysis of red blood cells due to lack of regulatory proteins.

3. Diagnostic Approach to Complement Disorders

a. Initial Screening Tests

1.Total Hemolytic Complement (CH50) Assay:

1. Measures the overall functional activity of the classical pathway.
2. A reduced CH50 indicates a deficiency or dysfunction in one or more components of the classical pathway (C1-C9).

2.Alternative Pathway Hemolytic Assay (AH50):

1. Measures the functional activity of the alternative pathway.
2. A reduced AH50 suggests defects in components like Factor B, Factor D, or properdin.

3.Lectin Pathway Functional Assay:

1. Assesses the functionality of the lectin pathway.
2. Detects deficiencies in MBL, MASP-1, MASP-2, and associated proteins.

b. Complement Component Measurement

1. C3 and C4 Levels:

1. Low C3 and C4 levels are common in conditions like SLE or other immune complex diseases.
2. Isolated low C3 suggests activation of the alternative pathway, while low C4 suggests classical or lectin pathway activation.

2. C1q Level:

1. Deficiency in C1q is associated with immune complex diseases like SLE.

3. C1 Inhibitor (C1-INH) Quantitative and Functional Assay:

1. Low levels or functional defects in C1-INH are diagnostic of hereditary angioedema (HAE).

4. C5-C9 Levels:

1. Deficiencies in these components can lead to recurrent infections, particularly with Neisseria species.

c. Specialized Tests

1.Complement Factor H and I:

1. Deficiencies in these regulators can lead to atypical hemolytic uremic syndrome (aHUS) and other complement-mediated diseases.

2.Factor B and Properdin Levels:

1. Important in the alternative pathway; deficiencies can result in increased susceptibility to bacterial infections.

3.Autoantibody Detection (e.g., Anti-C1q, Anti-Factor H):

1. Detects autoantibodies that inhibit complement regulation, seen in autoimmune conditions.

d. Genetic Testing

1.Complement Gene Mutations:

1. Genetic testing can identify mutations in genes encoding complement proteins (e.g., C3, CFH, CFI) in cases of inherited complement deficiencies or aHUS.

2.Next-Generation Sequencing (NGS):

1. Allows for comprehensive analysis of multiple complement-related genes, facilitating diagnosis of complex or rare complement disorders.

4. Clinical Applications of Complement Investigations

a. Diagnosing Autoimmune Diseases

- **Systemic Lupus Erythematosus (SLE)**: Low C3 and C4 levels are used to assess disease activity and monitor response to treatment.

b. Identifying Immunodeficiencies

- Recurrent infections, particularly with encapsulated bacteria like Neisseria meningitidis, suggest complement component deficiencies (e.g., C3, C5-C9).

c. Diagnosing Hereditary Angioedema (HAE)

- Measurement of C1-INH levels and function is critical in diagnosing HAE, a condition characterized by recurrent episodes of severe swelling.

d. Monitoring Complement-Mediated Hemolysis

- **Paroxysmal Nocturnal Hemoglobinuria (PNH)**: Flow cytometry for CD55 and CD59 on red blood cells helps diagnose PNH, which is due to complement-mediated hemolysis.

e. Managing Atypical Hemolytic Uremic Syndrome (aHUS)

- Assessing complement regulators like Factor H, Factor I, and MCP (CD46) helps in diagnosing and managing aHUS, a condition involving thrombotic microangiopathy.

5. Therapeutic Implications

1.Complement Inhibitors:

1. **Eculizumab**: A monoclonal antibody that inhibits C5, preventing the formation of the terminal complement complex, used in PNH and aHUS.
2. **Berotralstat**: A kallikrein inhibitor used in hereditary angioedema to prevent attacks.

2.Replacement Therapy:

1. For certain complement deficiencies, replacement therapy with fresh frozen plasma (FFP) can provide missing complement components.

3.Gene Therapy:

1. Emerging therapies aim to correct genetic defects in complement proteins, offering potential long-term solutions for complement deficiencies.

Assays for Individual Components- EIA, RIA

- When investigating complement disorders, precise measurement of individual complement components (e.g., C3, C4, C1q, Factor H) is crucial.
- Two common methods used for this purpose are **Enzyme Immunoassay (EIA)** and **Radioimmunoassay (RIA)**. Both are highly sensitive techniques that allow quantification of specific proteins in biological samples.

1. Enzyme Immunoassay (EIA)

a. Overview

- Enzyme Immunoassay (EIA), also known as Enzyme-Linked Immunosorbent Assay (ELISA), is a widely used method to detect and quantify specific antigens or antibodies. In the context of complement components, EIA can measure levels of proteins such as C3, C4, and regulatory factors.

b. Principle

- The EIA relies on the specific binding of an antibody to its antigen.
- An enzyme is conjugated to an antibody or antigen. Upon binding to the target, a substrate is added, leading to a colorimetric, fluorometric, or chemiluminescent signal that is proportional to the amount of antigen present.

c. Procedure

1. **Coating**: A plate is coated with an antibody specific to the complement component of interest.
2. **Sample Addition**: The sample (serum, plasma, or CSF) is added to the plate, allowing the complement component to bind to the immobilized antibody.
3. **Detection Antibody**: A second antibody, linked to an enzyme, binds to the captured component.
4. **Substrate Addition**: A substrate for the enzyme is added, producing a measurable signal (color change or light emission).
5. **Quantification**: The intensity of the signal is measured using a spectrophotometer, and the concentration of the complement component is determined by comparison to a standard curve.

d. Types of EIA

- **Direct EIA**: Involves a labeled antibody directly binding to the target antigen.
- **Indirect EIA**: Uses an unlabeled primary antibody and a labeled secondary antibody for detection.
- **Sandwich EIA**: Requires two antibodies, one for capturing the antigen and the other for detection, providing high specificity.

e. Advantages

- High sensitivity and specificity.
- Can be used to measure a wide range of complement components.
- Amenable to high-throughput screening.

f. Clinical Applications

- **Measurement of C3 and C4 Levels**: Useful in diagnosing and monitoring autoimmune diseases like systemic lupus erythematosus (SLE).
- **Detection of Complement Regulatory Proteins**: Measurement of Factor H and I to diagnose conditions like atypical hemolytic uremic syndrome (aHUS).

2. Radioimmunoassay (RIA)

a. Overview

- Radioimmunoassay (RIA) is one of the most sensitive techniques for measuring low concentrations of antigens, including complement components, in a sample. It utilizes radioactively labeled substances to detect the presence and quantity of a specific protein.

b. Principle

- RIA is based on the competitive binding of radiolabeled and non-labeled (sample) antigens to a specific antibody.
- The bound and free antigens are separated, and the radioactivity of the bound fraction is measured. The amount of radioactivity inversely correlates with the concentration of the antigen in the sample.

c. Procedure

1. **Preparation**: A known quantity of radiolabeled antigen is mixed with a specific antibody.
2. **Sample Addition**: The biological sample containing the complement component (unlabeled antigen) is added, competing with the labeled antigen for antibody binding.
3. **Incubation**: The mixture is incubated to allow antigen-antibody binding.
4. **Separation**: Bound antigen-antibody complexes are separated from the free antigen.
5. **Measurement**: The radioactivity of the bound complexes is measured using a gamma counter.
6. **Quantification**: The concentration of the complement component is determined based on a standard curve.

d. Advantages

- Extremely sensitive, capable of detecting very low concentrations of complement components.
- High specificity due to the use of antibodies.

e. Limitations

- Involves the use of radioactive materials, requiring special handling, disposal, and regulatory compliance.
- More labor-intensive and time-consuming compared to EIA.

f. Clinical Applications

- **Measurement of Rare Complement Components**: RIA can be used to measure complement components present in very low concentrations that may not be detectable by other methods.
- **Research Applications**: Often used in research settings for studying complement regulation and pathology in detail.

ASSAYS FOR COMPLEMENT PATHWAY: CH50 & AH50 ASSAY

INTRODUCTION

- The complement system is a crucial component of the innate immune system.
- Responsible for enhancing the ability of antibodies and phagocytic cells to clear microbes and damaged cells, promoting inflammation, and attacking the pathogen's cell membrane.
- It consists of a series of small proteins, mostly synthesized by the liver, that circulate in the blood in an inactive form. Upon recognition of pathogens or other danger signals, these proteins are activated through one of three (3) distinct pathways:

1. The Classical Pathway (CH50),
2. The Alternative Pathway (AH50), and
3. The Lectin Pathway.

COMPLEMENT ACTIVATION PATHWAYS

There are 3 Complement Activation Pathways i.e.,

1. Classic Pathways (CH50 Assay):

- The classical pathway is primarily initiated by antibodies bound to the surface of pathogens. Specifically, IgG or IgM antibodies recognize antigens on the surface of the target cell, forming immune complexes.
- These immune complexes activate the first complement protein, C1, which leads to a cascade of enzymatic reactions. This cascade includes the activation of C4, C2, and eventually C3, which is the most abundant complement protein.
- Formation of the membrane attack complex (MAC), which causes lysis of the target cell.

2. Alternative Pathways (AH50 Assay):

- The alternative pathway, unlike the classical pathway, does not rely on antibodies for activation.
- It is initiated spontaneously by the hydrolysis of C3 in the presence of pathogen surfaces. This pathway is crucial for recognizing pathogens in the absence of specific antibodies.
- The alternative pathway operates as a surveillance system, recognizing pathogen-associated molecular patterns (PAMPs) on microbial surfaces.
- Once initiated, it triggers a similar cascade of reactions, terminating the formation of the MAC and cell lysis.

3. Lectin Pathways:

- The lectin pathway is activated by mannose-binding lectin (MBL), which bind to carbohydrates present on the surface of pathogens.
- Like the classical and alternative pathways, it leads to the formation of the MAC, contributing to the destruction of pathogens.

CH50 ASSAY

- The CH50 (Total Hemolytic Complement) assay is a functional assay used to measure the integrity and functionality of the classical complement pathway.
- It measures the amount of serum required to lyse 50% of antibody-sensitized sheep erythrocytes (red blood cells).

CH50 ASSAY: PROCEDURE

1. **Preparation of Sensitized Erythrocytes:** Sheep erythrocytes are sensitized with rabbit anti-sheep antibodies. These antibody-coated erythrocytes mimic the immune complexes that activate the classical complement pathway.
2. **Sample Preparation:** Serum from the test subject is diluted in a buffer, and varying amounts are added to the sensitized erythrocytes.
3. **Incubation:** The mixture of diluted serum and sensitized erythrocytes is incubated at 37°C for a set period, usually around 60 minutes. During this time, the complement system, if functional, will be activated, and MAC formation will lead to lysis of the erythrocytes.
4. **Measurement of Hemolysis:** After incubation, the level of hemolysis is determined by measuring the release of hemoglobin from lysed erythrocytes. The hemoglobin is released into the supernatant and can be measured by absorbance at 412 nm. The degree of hemolysis correlates with the complement activity in the serum. The CH50 value is defined as the dilution of serum required to lyse 50% of the erythrocytes in the assay.

CLINICAL SIGNIFICANCE

- **Reduced CH50 Values:** Low CH50 values may indicate a deficiency in any of the complement components involved in the classical pathway. It can be associated with immune disorders like SLE, Glomerulonephritis, or other conditions where the complement system is dysfunctional.
- **Elevated CH50 Values:** High CH50 values are rare but may occur in conditions where there is an acute phase response, often reflecting a hyperactive immune response or ongoing inflammation.

AH50 ASSAY

- The AH50 assay is designed to measure the functionality of the alternative pathway.
- This pathway does not require antibodies for initiation and can be activated directly by pathogens or other foreign surfaces.

AH50 Assay: Procedure

1. **Erythrocyte Selection:** Erythrocytes from rabbits or guinea pigs are commonly used because they spontaneously activate the alternative pathway.
2. **Sample Preparation:** Serum is diluted and added to the erythrocytes, but unlike the CH50 assay, no antibodies are required to sensitize the erythrocytes.
3. **Incubation:** The mixture of serum and erythrocytes is incubated at 37°C. If the alternative pathway is functional, complement proteins will become activated, and cell lysis will occur.
4. **Measurement of Hemolysis:** Hemolysis is measured by determining the amount of hemoglobin released into the supernatant, just like in the CH50 assay. The AH50 value represents the serum dilution required to lyse 50% of the erythrocytes.

CLINICAL SIGNIFICANCE

- **Reduced AH50 Values:** A low AH50 value suggests a deficiency in any of the proteins involved in the alternative pathway, such as C3, factor B, or factor D. Deficiencies in these components can increase susceptibility to infections, especially by Neisseria species, and are sometimes seen in patients with autoimmune diseases.
- **Elevated AH50 Values:** High AH50 values, although rare, may indicate an overactive alternative pathway, potentially contributing to conditions of chronic inflammation or tissue damage.

DETECTION OF COMPLEMET BREAKDOWN PRODUCTS- C3 NEPHRITIC FACTOR

- **C3 Nephritic Factor (C3NeF)** is an autoantibody that stabilizes the alternative pathway C3 convertase (C3bBb), leading to continuous activation of the complement system and excessive breakdown of C3.
- It plays a critical role in certain kidney diseases, most notably **C3 glomerulopathy** (C3G), which includes dense deposit disease (DDD) and C3 glomerulonephritis (C3GN).

1. Role of C3NeF in Complement System Dysregulation

- C3NeF binds to the alternative pathway C3 convertase (C3bBb) and prevents its inactivation by complement regulatory proteins such as Factor H. This stabilization leads to:
- Persistent cleavage of C3 into C3a and C3b.
- Depletion of serum C3 due to its continuous consumption.
- Deposition of C3 breakdown products in tissues, particularly the kidneys, causing inflammation and damage.

2. Diseases Associated with C3NeF

a. C3 Glomerulopathy (C3G)

- A spectrum of kidney diseases characterized by glomerular deposition of C3 with little or no immunoglobulin.
- Subtypes include:
  - **Dense Deposit Disease (DDD)**: Dense, ribbon-like deposits in the glomerular basement membrane.
  - **C3 Glomerulonephritis (C3GN)**: More variable deposits in the glomeruli.

Both are associated with C3NeF, which contributes to persistent complement activation and glomerular damage.

b. Atypical Hemolytic Uremic Syndrome (aHUS)

- A rare, life-threatening condition characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury.
- C3NeF may be present in some cases, leading to uncontrolled complement activation.

3. Detection of C3NeF

a. Clinical Indications for Testing

- Patients with low serum C3 levels and normal C4 levels, suggesting activation of the alternative pathway.
- Suspected cases of C3 glomerulopathy or aHUS.
- Persistent proteinuria, hematuria, or declining renal function with unknown etiology.

b. Laboratory Techniques for Detection

1. Hemolytic Assay

1. Measures the stabilization of the C3 convertase (C3bBb) in patient serum.
2. Involves mixing patient serum with normal human serum and testing for increased hemolytic activity due to continuous complement activation.

2. ELISA (Enzyme-Linked Immunosorbent Assay)

1. A more specific and sensitive method that detects the presence of C3NeF by measuring its binding to and stabilization of C3 convertase in vitro.

3. Western Blotting

1. Used in research settings to detect the presence of C3NeF by visualizing its interaction with complement components.

4. Flow Cytometry

1. Can be employed to assess the functional activity of C3NeF by detecting its effect on complement activation on cell surfaces.

c. Interpretation of Results

- **Positive C3NeF**: Indicates the presence of the autoantibody, which can lead to complement dysregulation and associated kidney pathology.
- **Low Serum C3**: Accompanies positive C3NeF due to continuous consumption of C3.

• 4. Clinical Implications of C3NeF Detection

- **a. Diagnostic Role**
- **C3 Glomerulopathy**: Confirming the presence of C3NeF helps in diagnosing C3G and differentiating it from other glomerular diseases.
- **Atypical Hemolytic Uremic Syndrome (aHUS)**: Detecting C3NeF can support the diagnosis in cases of aHUS with complement dysregulation.

- **b. Prognostic and Monitoring Role**
- Persistent C3NeF activity correlates with disease severity and progression in C3G and aHUS.
- Monitoring C3NeF levels can guide treatment decisions, such as the use of complement inhibitors.
- **c. Therapeutic Implications**
- **Eculizumab**: A monoclonal antibody that inhibits C5, preventing the formation of the membrane attack complex, is effective in conditions with complement dysregulation, including C3G and aHUS.
- **Supportive Treatments**: Includes management of renal disease and controlling blood pressure, proteinuria, and inflammation.

5. Challenges in C3NeF Testing

- **Heterogeneity of C3NeF**: Variability in the binding and functional activity of C3NeF among different patients complicates standardization of testing.
- **False Negatives**: Some forms of C3NeF may not be detected by conventional assays, necessitating the use of multiple complementary tests.

FUNCTIONAL ASSAYS FOR IMMUNE COMPLEXES

INTRODUCTION

- Immune complexes (IC) are one of the by-products of an active immune system and are formed by the interaction of either an antigen with its reactive antibody, an autologous antibody with a regulatory anti-antibody, or an antigen-antibody complex reacting with a anti-antibody.
- Often these immune aggregates incorporate complement, which provides pathways for the IC to interact with several different cell types and body systems.

WHY ARE IMMUNE COMPLEXES IMPORTANT?

- Immune complexes are important for several reasons related to how our immune system functions and maintains balance:
- Pathogen Clearance: Immune complexes are formed when antibodies bind to antigens, such as those from pathogens like bacteria or viruses. These complexes are then recognized and cleared by immune cells, such as macrophages, which help to eliminate the pathogens from the body.
- Immune Response Regulation: The formation of immune complexes can help regulate immune responses. For example, they can activate certain immune cells and promote the production of other immune molecules, thus helping to coordinate a more effective immune response.

- **Inflammation:** While immune complexes help in clearing pathogens, their accumulation or deposition in tissues can lead to inflammation. This happens when immune complexes are not properly cleared and become deposited in tissues, where they can trigger inflammatory responses and contribute to diseases like Systemic Lupus Erythematosus (SLE) or rheumatoid arthritis.
- **Diagnostic Indicators:** The presence and levels of certain immune complexes in the blood can be indicative of various diseases or conditions. For instance, high levels of immune complexes can be associated with autoimmune diseases or infections, providing valuable diagnostic information.
- In summary, immune complexes are crucial for both protecting the body from infections and maintaining immune system balance, but their accumulation in tissues can also contribute to inflammatory and autoimmune conditions.

Variety of Immune Complexes in Human Disease

- Antigen (excess)/antibody
- Antigen antibody (equivalence)
- Antigen antibody (excess)
- Antibody/anti-Fc (rheumatoid factor)
- Antibody/anti-F(Ab)’2 (serum agglutinator)
- Antibody/anti-FAb (allotype)
- Antibody/anti-FAb (idiotype)
- Antigen/antibody/ant-antibody

Techniques for Detection of Circulating Immune Complexes

1. Physicochemical techniques
2. Complement techniques
3. Antiglobulin techniques
4. Techniques involving cell receptors

PHYSICOCHEMICAL TECHNIQUES

Gel Electrophoresis:

- This technique separates proteins or complexes based on size and charge.
- Techniques like SDS-PAGE and native PAGE can be used to analyze immune complexes, providing information on their molecular weight and composition.

Western Blotting:

- The detection of specific protein using blotting technique.
- Also called as immunoblotting technique.(use of antibodies as probe)

Nephelometry:

- From the Greek nephelo: cloud
- It is an analytical chemistry technique used to measure the amount of turbidity or cloudiness in a solution caused by the presence of suspended insoluble particles.

Surface Plasmon Resonance (SPR):

- This technique measures the binding kinetics and affinity of antigen-antibody interactions in real-time.
- It's valuable for studying the formation and dissociation of immune complexes.

Isothermal Titration Calorimetry (ITC):

- It is a label-free method for measuring binding of any two molecules that release or absorb heat upon binding.
- ITC can be used to measure the thermodynamic parameters of biomolecular interactions, including affinity ($\mathbf{K_A}$), enthalpy ($\mathbf{\Delta H}$), entropy ($\mathbf{\Delta S}$), and stoichiometry ($\mathbf{n}$).

Mass Spectrometry:

- An analytical technique used in quantitative and qualitative analysis of molecules.

COMPLEMENT TECHNIQUES

Complement Fixation Test

Purpose:

- To measure the presence of specific antibodies or antigens by their ability to fix complement.

Procedure:

- The test involves mixing a sample containing antigens or antibodies with complement proteins and then adding a specific antigen or antibody.
- If the specific antigen-antibody reaction occurs, complement is fixed and no longer available to lyse red blood cells added later.

Complement Component Analysis

- **Purpose**: To measure the levels and activity of specific complement components.
- **Procedure**: Techniques such as radial immunodiffusion or nephelometry are used to quantify complement proteins like C3, C4, or C1q. The activity of the complement system can also be assessed through functional assays.

C1Q BINDING ASSAY

- **Purpose**: To assess the formation of immune complexes and their ability to activate the complement system.
- **Procedure**: The assay measures the binding of C1q (the first component of the classical complement pathway) to immune complexes, which indicates the activation of the classical pathway.

Clinical Significance of Immune Complex Detection

1. Allergy
2. Autoimmunity
3. Cancer
4. Cardiology

ELISA

- Enzyme-linked immunosorbent assay also called as enzyme linked immuno assay (EIA).
- The term ELISA was first used by Engvall & Perlma in 1971.
- It is a biochemical technique to detect the presence of specific compounds like antigens,antibody’s and hormones.
- The intensity of colour developed is directly proportional to amount of compound present in that sample.
- The colour change is caused by enzyme-linked antibody.
- It is used to diagnose HIV, Rota virus, syphilis etc.
- The ELISA test, or the enzyme immunoassay (EIA), was the first screening test commonly employed for HIV. It has a high sensitivity

REQUIREMENTS

- Microtiter plate (96 well) consists of many wells coated with polystyrene which facilitates the adsorption of compound to the plate.
- The compound to be detected which may be antigen, antibody,or hormones.

➢Primary antibody: It specifically bind to the compound.

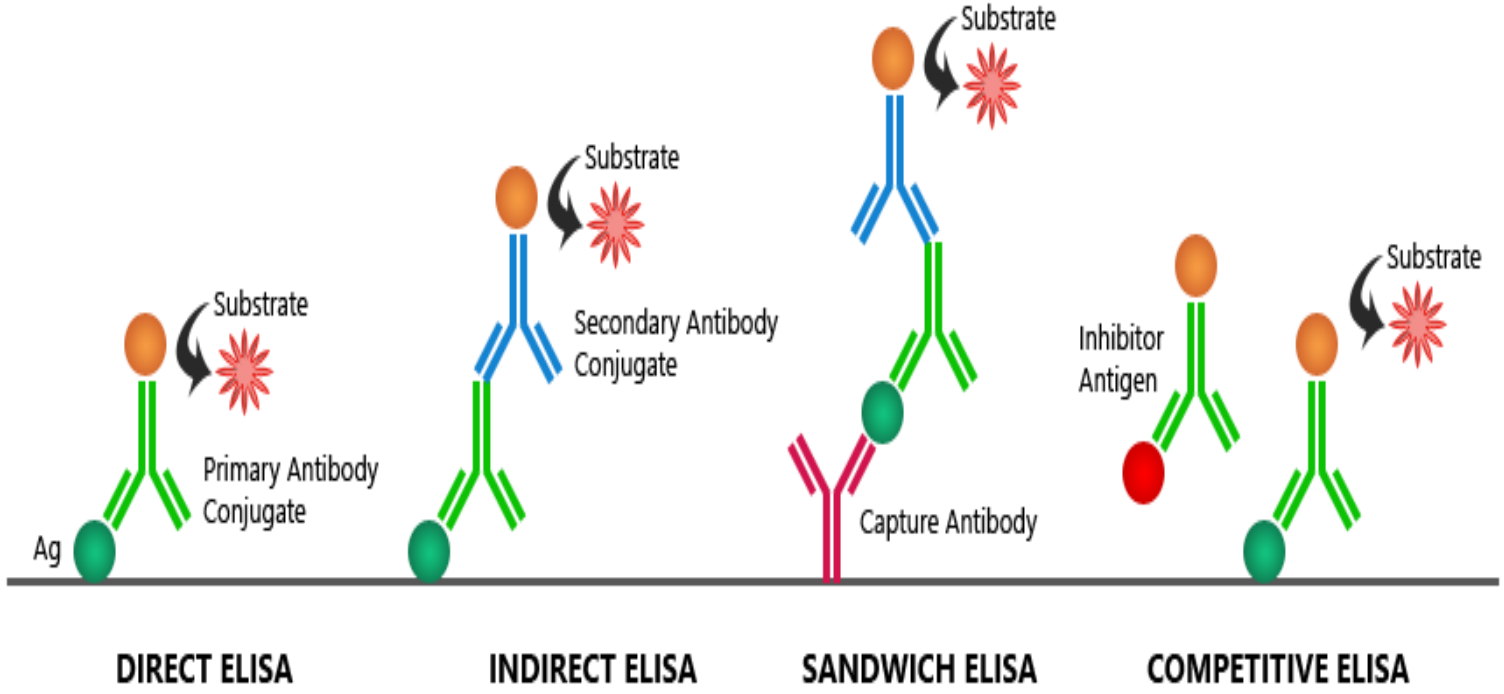
➢Enzyme-linked secondary antibody

Bind to the primary antibody and it also converts colourless substrate to coloured product and it is quantified by colorimeter.

TYPES OF ELISA

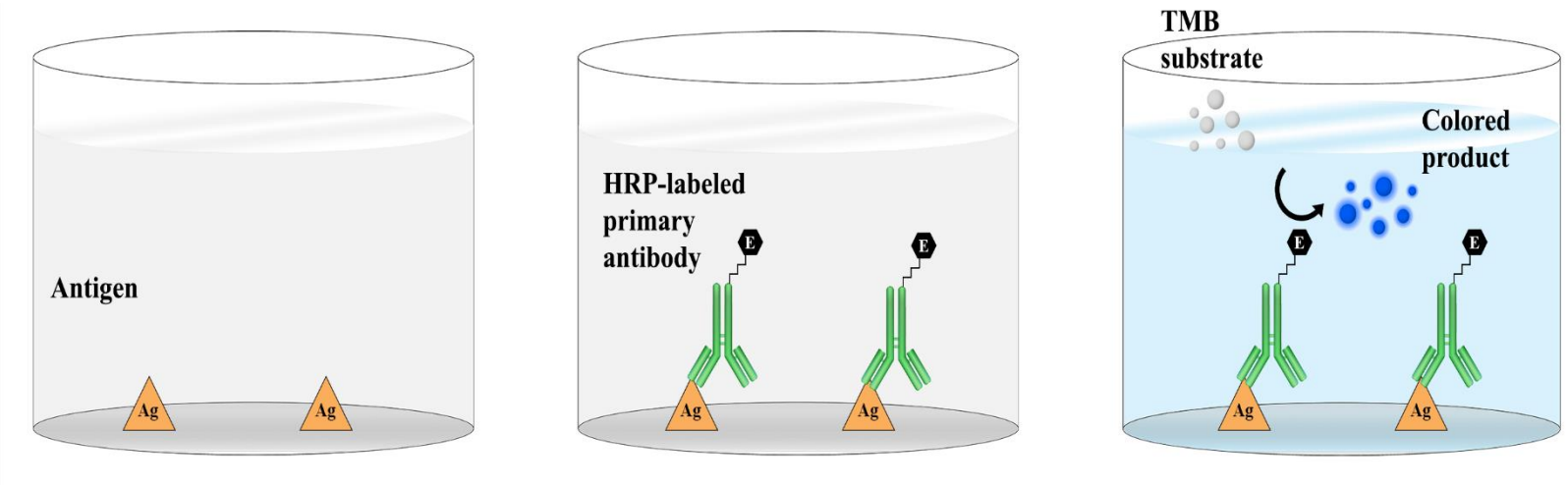
Four types of ELISA:

- Direct ELISA
- Indirect ELISA
- Sandwich ELISA
- Competitive ELISA



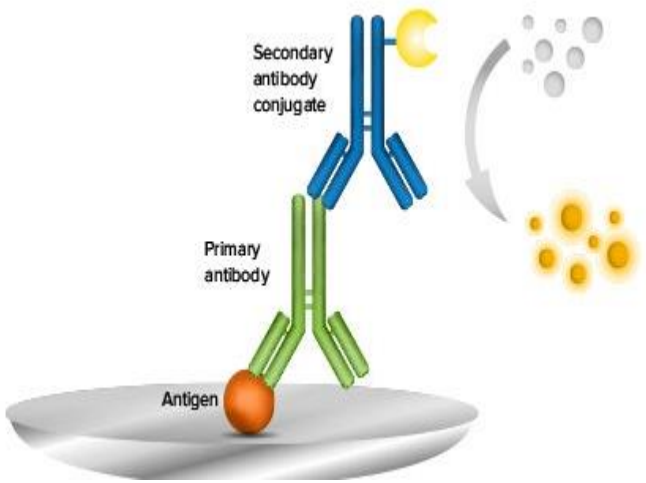
DIRECT ELISA

- The compound is attached to the polystyrene plate.
- Enzyme-linked antibody is added which bind to the compound.
- A wash is given to remove the unbound antibody.
- New substrate is added which is converted into coloured product by enzyme-linked antibody , which is then measured by colorimeter.



INDIRECT ELISA

- The compound is attached To the plate and primary antibody is added which bind to the compound. A wash is given to remove unbounded antibody.
- Secondary antibody which is linked to the enzyme is added and it binds to the primary antibody. Again a quick wash is given.
- Now, substrate is added which is converted into coloured product and measured by colorimeter.



SANDWICH ELISA

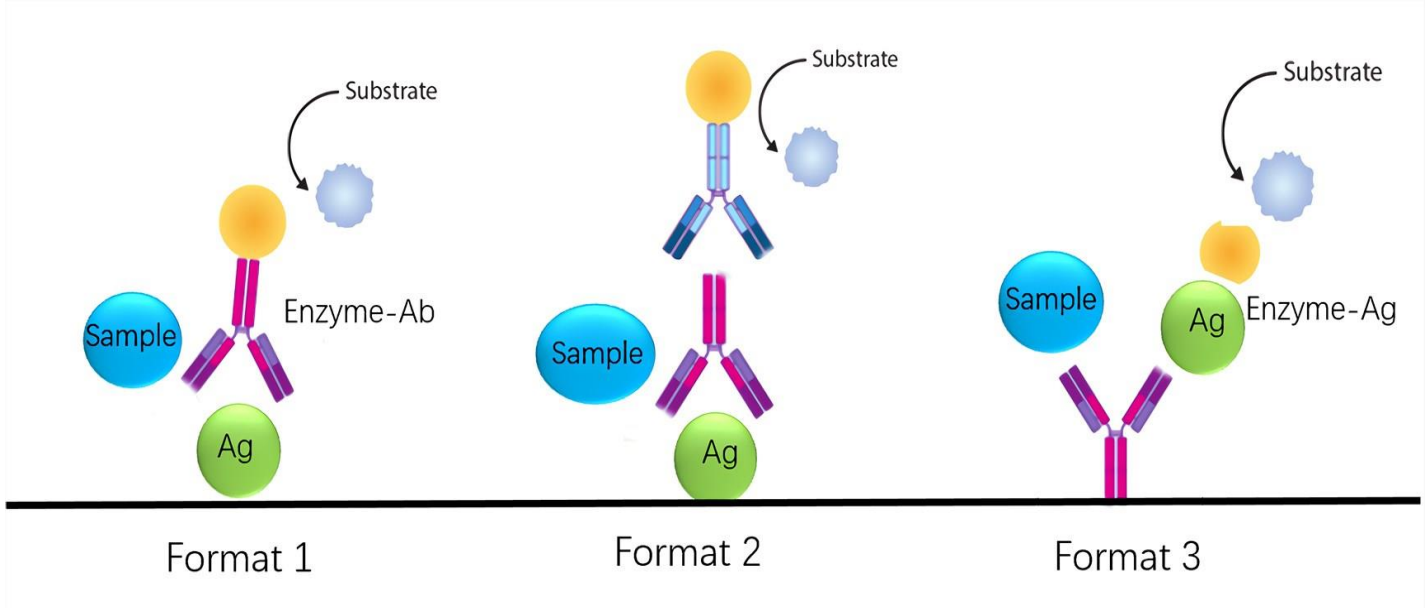
- Instead of compound, antibody is fixed to the plate, now the compound is added.
- If the antibody specific compound is present, it will bind to the antibody.
- Now, the primary antibody is added bind to the compound. A wash is given.
- Enzyme- linked secondary antibody is added which bind to the primary antibody. again a wash is given.
- Now, the substrate is added which is converted into coloured product and detected by the colorimeter.

COMPETITIVE ELISA

- First the target antibody is fixed to the plate and add the specific antigen, that we are trying to detect in the sample.
- Then add the same antigen linked with enzyme.
- Both sample antigen and enzyme-linked antigen undergo competition to bind the target antibody.
- If the sample contains more antigen than it will bind to the target antibody. A wash is given.
- Then the substrate is added; sample antigen lack of enzyme and that cannot convert coloured product.

RESULT

- Presence of antigen-colourless product
- Absence of antigen – coloured product



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