Mycology

Introduction : **"Mycology"** is the branch of biology that deals with the study of fungi. The "fungus' is a latin word that means "mushroom". Raymond Sabouraud is known as father of Mycology.

Cell structure and Morphology : The cell structure of fungi is almost similar to that of any other member of super kingdom Eukaryota.

It differs from the bacteria in the following respects :

Sr. No.	Feature	Fungi	Bacteria
1	**Taxonomic status**	Eukaryotes	Prokaryotes
2	**Cell wall Composition**	Chitin, mannan, polysaccharide. Some members are capsulated	Muramic acid, teichoic acid, Lipopolysaccharide. Some members are capsulated
3	**Cytoplasmic membrane**	Contains ergosterol	Lacks ergosterol
4	**Cytoplasmic contents**	Include mitochondria, endoplasmic reticulum	Lack mitochondria, endoplasmic reticulum
5	**Nucleus**	True nucleus with nuclear membrane, paired chromosome	No nuclear membrane. Nuclear body equivalent to single chromosome
6	**Spores**	Asexual and sexual reproductive spores	These are non reproductive structures and are endospores
7	**Staining character**	These are Gram positive	These may be Gram positive or Gram negative
8	**Reproduction**	Both sexual and asexual mode of reproduction	Mode of reproduction is by binary fission.

Morphological Features : Since the fungi are eukaryotic, with range of internal membrane system, membrane bound organelles and a well-defined cell wall, which are composed largely of polysaccharides and chitin. These show considerable variation in size and shape but can be broadly divided into two main groups :

(a) Yeast
(b) Molds

(a) Yeast : The yeasts are unicellular fungal organisms, which reproduce by asexual process known as budding i.e. blastospore formation with narrow-and broad-based budding or by fission. The yeasts may produce chains of elongated cells known as pseudohyphae which resemble mycelia of molds and some of these may also produce true mycelia. Some of the yeasts are reproduced by sexual process also showing the telemorph state as seen in Cryptococcus neoformans i.e. Filobasidiella neoformans. The yeast are ubiquitous in the environment being found on fruits, vegetables and other plant materials (exogenous). Some live as normal inhabitans in and/or on the human body (endogenous). Therefore, these yeasts may be found in the clinical specimens as commensals without any medical significance.

Yeast-like : These fungi also reproduce by budding but the buds fail to get separated and elongation takes place forming pseudohyphae as seen in Candida species.

(b) Molds : The molds are composed of branching filaments known as hyphae. They grow by apical extension, forming an interwoven mass called as mycelium. The hypha is a structural unit of the mycelium. In most of the fungi the hyphae have regular cross-walls i.e. septate as seen in Aspergillus, Penicillium and many other fungal genera but in lower fungi these are usually absent i.e. non-septate or sparsely septate with wide-angle branching as seen in zygomycetes. Hyphae that grow submerged or on the surface of a culture medium are called vegetative hyphae because they are responsible for absorption of nutrients. The hyphae that project above the surface of the medium are called aerial hyphae and produce specialized structures called as conidia.

Morphological classification :

(i) Yeast : The yeasts are unicellular organism and divide by budding. Most of these are considered as non-pathogenic like Saccharomyces cerevisiae but a few are pathogenic as well i.e. Cr. Neoformans.

(ii) Yeast-like : These fungi also reproduce by budding but the buds fail to get separated and elongation takes place forming pseudohyphae as seen in Candida species.

(iii) Molds : These develop from spores which germinate to form vegetative hyphae. The hyphae may or may not have septation. Dermatophytes, Aspergillus, Penicillium and Mucor are few examples of molds.

(iv) Dimorphic fungi : These fungi have two type of morphological characters. These are pathogenic fungi which are yeast like in host tissue (at 37^0C) and mold like a artificial medium (at 25^0C).

Important structures of fungi :

Hypha & Pseudohypha are important structures of fungi. Their differences are given below :

Hypha	Pseudohypha
- It is an elongated tubular, thread-like branching filament of even width.	- A chain of elongated cells is known as pseudohypha e.g. yeast
- It is formed by apical elongation	- It is formed by budding
- Cell walls are parallel to each other	- Cell walls are constricted at septa
- Septa are straight and perpendicular	- Septa are curved
- Septum and constriction is absent at origin of branches	- Septum and constriction is present at origin of branches

Types of Hyphae

Aseptate and Septate Hyphae

When septa are present hypha is called septate hypha and when it is absent it is called aseptate.

Examples : Septate hyphae are present in Aspergillus and Penicillium, while aseptate are present in zygomycetes.

- Hyaline and Pigmented Hyphae

Dark coloured hypha is called pigmented and colourless is called hyaline.
Examples : Pigmented hyphae are present in phaeoid fungi such as Wangiella, Aureobasidium while hyaline hyphae are present in Aspergillus and Penicillium.

- Vegetative and Aerial Hyphae
- The hyphe, which is submerged in the media or is on the surface of media is called vegetative hypha, while the hypha, which projects above the medium is called aerial hyphae.
- Vegetative hyphae absorb nutrients from media for growth, hence concerned with nutritive function.
- Aerial hyphae carry spores, which are units of reproduction, hence concerned with reproductive function.
  Examples : Filamentous fungi possess vegetative as well as aerial hyphae.
- Spiral Hypha : These are spirally coiled hyphae commonly seen in Triphyton mentagrophytes.
- Racquet hyphae : There is regular enlargement of one end of each segment with the opposing end remaining thin. Seen in Epidermophyton floccosum, Trichophyton mentagrophytes.
- Nodular hyphae : This is an enlargement in the mycelium that consists of closely twisted hyphae. Often seen in Trichophyton mentagrophytes and Microsporum canis.
- Favic hyphae : These are the group of hyphae tips that collectively resemble a chandelier or the antlers of the deer (antler hyphae). They occur in Triphyton schoenleinii and Trichophyton violaceum.
- Pectinate hyphae : These are short, unilateral projections from the hyphae that resemble a broken comb. Commonly seen in Microsporum audouinii.

Mycelium

Entangled mass of hyphae is called mycelium. Mycelium are of three kinds :

1. Vegetative mycelium are those that penetrates the surface of the medium and absorbs nutrients.
2. Aerial mycelium are those that grow above the agar surface.
3. Fertile mycelium are aerial hyphae that bear reproductive structures such as conidia or sporangia.

Spore : Spores are fruiting bodies produced by fungi for asexual or sexual reproduction.

Types of spores

Asexual spores

- They are formed by budding, fission and mitosis.
- Asexual spores are of the following types :
  1. Chlamydospores – asexual spores, which are large with thick double refractile wall produced from hyphal cell. They are resistant. They store nutrients and function as spores. They may be formed in groups or occur singly. They may be terminal or intercalary.
  2. Arthrospores – asexual spores produced as a result of fragmentation of hyphae. They are cuboidal, rectangular spores formed in chains, with a slightly thick wall and are released at maturity.
  3. Blastospores- asexual spores produced as a result of budding.
  4. Sporangiospores – asexual spores present inside the sporangium.

Conidia

- They are asexual reproductive structures borne exogenously on specialized hyphae called conidiophores.
- There are two types of conidia- macroconidia and microconidia.

Sexual spores

- They are produced as a result of meiosis.
- Sexual spores are of the following types :
- Ascospores are sexual spores inside a sac-like structure called ascus e.g. Ascomycetes.

- Zygospores are sexual spores formed by sexual conjugation of two fungi e.g. zygomycetes.
- Basidiospores are sexual spores formed on basidium e.g. Basidiomycetes.

Cell Structure

1. Capsule : Some of the fungi produce an extracellular polysaccharide in the form of capsule e.g. Cryptococcus neoformans. The capsular material determines virulence and plays an important role in eliciting host immune response and provides the substance for diagnostic criteria like latex agglutination test for cryptococcosis.
2. Cell wall : The fungi possess a multilayered rigid cell wall exterior to the plasmalemma. This cell wall is structurally and biochemically complex, containing chitin, a water insoluble homopolymer of N-acetyl glucosamine, a polymer of N-acetyl-D-glucosamine in β1-4 glycosidic linkage, as its structural foundation. Mammans, glucans and other complex polysaccharides in association with polypeptides are layered on the chitin.

   In the filamentous fungi, the biosynthesis of chin occurs at the growing tip and is controlled by activity of chitin synthase. The cell wass of the pathogenic fungi is important for following reasons :

(i) They contain specific adhesive molecules that are contact point of the organism for the attachment and subsequent invasion.

(ii) It acts as a protective barrier and is an obstacle that must be considered for choosing antifungal drugs that need to enter the cell wall for their potentially effective action.

(iii) The chitin and β-glucans found in the pathogenic fungi are not present in the host, therefore, these compounds particulary the enzymes are involved in biosynthesis and degradation and safe target sites of the antifungal drugs.

3. Plasmalemma : Inner to the cell wall, membrane enclosing the complex cytosols is called the cytoplasmic membrane or plasmalemma which is composed of glycoproteins, lipid and ergosterol.
4. Cytosols : Fungal cells possess a complex cytosol. As the fungi are eukaryote organisms, the cells have at least one nucleus, nuclear membrane, mitochondria, microvesicles, microtubules, ribosomes, golgi apparatus, a double membrane endoplasmic reticulum and other cytoplasmic structures. The nucleus of the fungi are enclosed by a membrane and contain most of the cellular DNA. They have a true nucleolus rich in RNA.

Reproduction : Fungi reproduce by asexual, sexual and parasexual means.

Asexual Reproduction : Asexual reproduction are termed either spores or conidia depending on their mode of production. Asexual spores are produced following mitosis where as sexual spores are produced following meiosis.

Spores : The asexual spores of zygomycetes, which are known as sporangiospores form within sac like structure known as sporangia. The sporangospores result from the mitotic cleavage of cytoplasm in the sporangium. The sporangia are borne on special hyphae called sporangiophore. Thie endogenous process of spore formation within a sac is known as sporogenesis.

Conidia : Conidia arise either by budding off conidiogenous hyphae or by differentiation of preformed hyphae. These develop following mitosis of a parent nucleus and are formed in any manner except involving cytoplasmic cleavage. This exogenous process is known as conidiogenesis, a process that occurs both in yeasts and moulds. Conidia are borne on specialized structure called conidiospore.

Conidia production may be blastic or thallic. In blastic development the conidium begins to enlarge and a septum is formed. Here the conidium originates from part of parent. In thallic mode of development the conidium is differentiated by a septum before its differentiation. Thus the conidium results from the conversion of entire parent cell into the conidium.

Sexual Reproduction : Sexual reproduction are produced by the fusion of two nuclei that then generally undero meiosis.

The first step in sexual methods of reproduction involves plasmogamy (cytoplasmic fusion of two cells). The second step is karyogamy (fusion of two compatible nuclei), resulting in production of diploid or zygote nucleus. This is followed by genetic recombination and meiosis. The resulting four haploid spores are said tobe sexual spores, e.g. zygospores, ascospores and basidiospores.

In a sexual spore is produced only by fusion of a nucleus of one mating type with a nucleus of another mating type, the fungus is said to be heterothallic. In contrast, homothallic moulds produce sexual spores following the fusion of two nuclei form the same strain. For sexual reproduction to occur, two compatible isolates are required.

Zygospores, which are the sexual spores of zygomycetes are round, thick walled reproductive structure that result from the union of two gamentagia. Ascomycetes produce sexual spores ascospores in a special sac like cell known as ascus. In basidiomycetes the basidiospores are released from basidium, which is the terminal cell of a hyphae.

Classification of fungi on the basis of reproduction :

Parasexual reproduction : Parasexual reproduction, first seen in Aspergillus is known to occur in basidiomycetes, ascomycetes and deuteromycetes. The process involves genetic recombination without the requirement of specific sexual structures.

Classification of fungi on the basis of Reproduction

1. Zygomycetes : Commonly known as bread moulds, these are fast growing terrestrial, largely saprophytic fungi. Hyphae are coenocytic and mostly aseptate. Asexual spores include chlamydoconidia, conidia and sporangiospores. Sporangiospores may be simple or branched. Sexual reproduction involves producing a thick-walled sexual resting spore called a zygospore.
   Medically important orders and genera include :
   (i) Entomophthorales : Conidiobolus and Basidiobolus are involved in subcutaneous zygomycosis.
   (ii) Mucorales : Rhizopus, Mucor, Rhizmucor, Absidia and Cunninghamella are involved in subcutaneous and systemic zygomycosis (formely called Mucormycosis).
2. Basidiomycetes : They exist as saprobes and parasite of plants. Hyphae are dikaryotic and can often be distinguished by the presence of clamp connections over the septa. Sexual reproduction is by the formation of exogenous basidiospores, typically four, on a basidium. Occasional species produce conidia but most are sterile.
   Genera of medical importance include :
   (i) Telemorph of Cryptococcus neoformans, which is Filobasidiella neoformans.
   (ii) Agents of basidiomycosis such as Coprinus and Schizophyllium.
   (iii) Mushroom poisoning by Aminita, Lepiota, Coprinus and Psilocybe etc.
3. Ascomycetes : They exist as saprophytes and parasites of plants. Hyphae are septate with simple septal pores. Asexual reproduction is by conidia. Sexual reproduction is by the formation of endogenous ascospores, typically eight in an ascus.
   Medically important genera include the
   (i) Telemorphs of known pathogenic fungi e.g. Arthroderma (of Trichophyton and Microsporum), Ajellomyces dermatitidis (of Blastomyces dermatitidis), Pseudallescheria boydii (of Scedosporium apiospermum)

(ii) Agents of mycetoma, like Leptosphaeria.

(iii) Agents of black piedra, like Piedraia hortae.

4. Deuteromycetes : Deuteromycetes are also known as Fungi imperfect because of absence of sexually reproducing forms (telemorph or perfect stage). As their telemorph continue to be discovered, they would be classified among the previous categories, until then this remains an artificial and heterogenous group. There are three classes of Fungi Imperfecti.

(i) Blastomycetes : These include asexual budding forms of Cryptococcus, Candida, Torulopsis and Rhodotorula. Depending on the presence of melanin in their cell walls, they may be non-dematiaceous or dematiaceous.

(ii) Hypomycetes : A class of mycelia moulds which reproduce asexually by conidia or hyphae. Hyphae are septate. This class contains the majority of medically important fungi. Dematiaceous hyphomycetes are those conidial fungi that produce dark brown, green-black, or black colonies and are the causative agents of phaeohyphomycosis. Hyaline hypomycetes include those conidial fungi, which are not darkely pigmented, colonies may be colourless or brightly coloured. These include the agents of hyalohyphomycosis, aspergillosis, dermatophytosis and dimorphic pathogens like Histoplasma capsulatum.

(iii) Coelomycetes : These produce acervuli, which are tightly bound mats of hyphae on which conidia are produced.

Laboratory Diagnosis of Fungal Infection

The following clinical samples are of help in arriving at a diagnosis depending upon the underlying clinical condition.

(A). In case of superficial mycoses and cutaneous mycoses : Affected hair, skin scrapings and nail clippings.

(B). In case of subcutaneous mycoses : The scrapings or crusts from the superficial parts of subcutaneous tissues, aspirated samples of pus and biopsy of the affected part.

(C). In case of systemic mycoses : The possible samples in case of systemic mycoses include biopsy, pus, sputum, blood, CSF, faeces and edges or scrapings from the lesions.

Transport of samples : In case of skin, hair and nail clippings, the samples are either collected in paper envelope or sterile petridishes. For rest of the samples, sterile containers are used for collection of the sample.

Following techniques are used in the direct examination

1. Wet mount :

Principle : In this method, a drop of Potassium Hydroxide (KOH) is mixed with the clinical material i.e. skin, hair or nails and KOH is allowed to act on the sample for at least 15-20 minutes. The sample is then examined under the microscope, first under the low power and then under high power to demonstrate fungal elements.

KOH helps in clearing the keratin material and thus makes the fungal elements more prominent.

Reagent :

- 70% v/v ethanol.
- 20% w/v KOH.

Procedure

(1) Cleanse the affected area with 70% v/v ethanol.

(2) Collect skin, crusts, pieces of nail or hairs on a clean piece of paper about 5 cm square (dark coloured paper)

(3) Now place a drop of KOH reagent on a clean slide.

(4) Transfer the specimen (small pieces) to drop of KOH, cover with coverslip.
(5) Keep slide in a petridish with a lid, together with a damp piece of cotton inside.
(6) Keep in incubator
For skin - 20-30 minutes.
For hairs- 10-15 minutes.
For nails- 3-6 hours.

Note : for nails, the incubation period is overnight also (By dissolving the nails scraping in very little amount of 20% KOH solution in a small test tube).

(7) Now examine the preparation under high power objective.

2. Lactophenol Cotton blue preparation (LCB) : A typical morphology and arrangement of spores etc can be demonstrated through this preparation. Fungi can be identified upto species level. It is done only on fungal growth obtained from culture.
Procedure : Place a drop of 95% alcohol on a slide-gently tease out a fragment of the culture with needles. Let the most of the alcohol evaporates. Add a drop of lactophenol cotton blue stain. Apply a coverslip, avoiding bubbles. Exert gentle pressure. Remove any excess stain with the edge of a blotting paper. After few minutes, look under microscope.

3. **Calcoflour white (CFW) stain mount :** It is a sensitive method and the CFW stained sample is examined under UV light. Fungal elements if present fluoresce brightly under UV illumination.

4. **Negative Staining :** India Ink and nigrosin are the two examples of negative stains. Either of these two stains is used to demonstrate the capsule around some yeasts e.g. *Cryptococcus neoformans*. The capsular material does not stained background.

Fungal Cultivation

Culture Methods : The most commonly used media for culturing fungi is the Saboraud's Dextrose agar (SDA). Two types of SDA are used, a) in which antibiotics are added to prevent the growth of bacteria, and b) without antibiotics.
After inoculation, the culture media is incubated both at 25^0C and 37^0 for a period varying from 48 hours to 4 weeks depending upon which fungus is suspected in the sample.

Culture Media

1. Sabouraud's dextrose agar : This is the most commonly used medium in the diagnostic mycology laboratory. This is now used by almost every mycology laboratory for primary isolation of fungi from the clinical specimens as well as subcultures of the fungal isolates. The colony forms, microscopic characteristics and other physiological properties of the fungi cultivated on Sabouraud's dextrose agar may vary considerably according to the type of peptone used in the medium. The ingredients of this medium are as follows :

Peptone	10 gm
Dextrose	40 gm
Agar	20 gm
Distilled water	1000 ml

Autoclave the ingredients at 121^0C for 15 minutes and adjust the final pH of 5.6. Sometimes, saprobic fungi grow rapidly on this medium and often overgrow obscuring the true pathogen.

2. Sabouraud's dextrose agar with antibiotics : A selective medium is required for the isolation of significant fungal pathogens and to avoid bacterial and fungal contaminants. In the Sabouraud's dextrose agar, while boiling but before autoclaving, add the following antibiotics to make the medium selective for the fungal pathogens :

Cycloheximide	500 mg
Chloramphenicol	50 mg
Gentamicin	20 mg

Dissolve cyclohexidime in 10 ml acetone, add it to the boiling medium and mix properly. Similarly, dissolve chloramphenicol/gentamicin in 10 ml of 95% alcohol and add to the boiling medium. Remove from heating and mix well. Dispense the medium in the tubes and autoclave at 121^0C for 15 minutes.

3. Corn meal agar (CMA) : The corn meal agar is used to observe the chlamydospore production in the yeasts and it has following ingredients :

Cornmeal	8 gm
Agar	4 gm
Distilled water	200 ml
Tween 80 (1%)	2 gm

Heat the cornmeal and water at 600 for 1 hour and filter through filter paper. Add distilled water to make it 200 ml and then add agar. Autoclave it at 121^0C for 15 minutes and pour in the plates. Cornmeal-Tween agar is prepared byadding Tween 80 (polysorbate 80) before autoclaving. Zein can also be use din place of cornmeal which is a protein extracted from cornmeal (Zein 40 gm/100 ml).

4. Rice Starch agar : The rice starch agar is used to produce the chlamydospores of Candida albicans faster as compared to the cornmeal agar. The ingredients of rice starch agar are :

Cream of rice	4 gm
Tween 80	2 gm
Agar	4 gm
Distilled water	200 ml

Add cream of rice to the boiling water and continue boiling for 30 seconds. Filter through cotton tampon and add water to make up one litre. Add agar and Tween 80 and dissolve while heating. These ingredients are autoclaved at 121^0C for a period of 15 minutes. Pour into a cylinder and leave overnight in waterbath at 60^0C. Decant clear portion and re-filter through cotton and again autoclave at 121^0C for 15 minutes. Adjust the pH at 6.2.

Other important and selective culture medium :

1. Brain-heart infusion agar : The brain-heart infusion agar is used for growing fastidious pathogenic fungi such as Histoplasma capsulatum and Blastomyces dermatitids.
2. Biphasic medium : This medium is used for isolating fungi from blood culture.
3. Cysteine-Heart and Haemoglobin agar : Cysteine-heart and haemoglobin agar may also be used in place of brain-heart infusion agar.
4. Bird seed agar : Bird seed agar or niger seed agar or Staib agar with antibiotic is an important medium used in the diagnosis for primary isolation of Cryptococcus neoformans.

Inoculation onto media : Specimens for the isolation of fungi are inoculated on to slants or agar plates. When slants are used, the culture tube should be large enough (2 x 15 cm) to provide a wide surface area for growth. The screw caps should be left loosened during incubation to permit an adequate supply of oxygen.

Incubation : The optimum temperature for the recovery of most pathogenic fungi from clinical specimens is 25-30^{0}C. Fungi grow optimally at this temperature, but bacteria have a slower growth rate. If the etiologic agent suspected is a dimorphic fungus, culture should also be incubated at 37^{0}C. Cultures are generally maintained for 4-6 weeks and should be examined weekly or twice a week for growth. Growth of Candida, Aspergillus, Mucor and Rhizopus species appear within 24-72 hours. Therefore, culture should be examined for growth daily for the first week.

Examination the Incubation : Once an organism has grown, it is examined for characteristic gross and microscopic structures, so that identification can be made. Pigment on the reverse side of the colony or in aerial mycelium is noted. For microscopic examination slide mounts should be made in lactophenol cotton blue. A slide culture may be prepared occasionally, when the initial isolate fails to show conidial morphology. Characteristics that should be observed are septate versus non-septate hyphae, hyaline or dematiaceous hyphae and the types, size, shape and arrangement of conidia.

Cultivation of Candida :

Specimen : Samples are collected appropriately depending on the site involved e.g. swabs from local lesions and urine from UTI patients. Collection is done by a standard procedure.

Culture :

Media : SDA with antibiotics- two slants are inoculated, one incubated at room temperature and another at 370C. Culture examined daily for first week and twice a week after that. Corn-meal agar, tetrazolium reduction medium and CHROM agar help in species identification.

Colony characters :

- On SDA medium, colony are smooth, yeasty and cream colonies.
- LCB shows presence of yeast and pseudohyphae.
- Tetrazolium reduction medium and CHROM agar form coloured colonies.

Candida species form different types of features on Corn-meal agar given as below :

Features of Candida species on Corn-meal agar :

C.albicans : Pseudohyphae in clusters, large thick-walled, terminal chlamydospores.

C.tropicalis : Formation of blastospores in groups, pseudohyphae.

C. kefyr : Abudant pseudohyphae, elongated cells lie parallel to each other like a log in stream, few blastospores.

C. krusei : Elongated cells have tree-like arrangement or cross-match tick appearance, elongated blastospores in few strains.

C. parapsilosis : Blastospores singly or in small clusters along with pseudohyphae, large mycelia elements called giant cells.

C. guilliermondii : Fine small pseudohyphae with small cells with chains of ovoid blastospores.

Species of Candida and colony colour on TRM medium and CHROM medium

Species	Colour on TRM medium	Colour on CHROM medium
C.Albicans	Pale pink	Light green
C.tropicalis	Orange pink	Blue with pink halo
C.krusei	Pink and dry	Pink
C.parapsilosis	Rose pink	Cream

C.guilliermondii	Pink and pasty	
A. Kefyr	Salmon pink	

Cultivation of Dermatophytes :

Specimen : Skin scrapings, hairpieces and nail pieces.

Culture :

- SDA with cycloheximide and chloramphenicol is used for routine cultivation of fungi
- Two slants are inoculated- one incubated at 25^0C and another at 37^0C.
- Spot inoculation is preferred on slants to differentiate growth from contaminants.
- Slants observed daily for first weekand on alternate days thereafter for growth.
- Rate and nature of growth and LCB picture help in identification of the agent.

Colony and LCB picture of common dermatophytes are given below :

- T. mentagrophytes :
  Growth rate : 2 weeks
  Colony : Observe – Buff to pink, flat fluffu. Reverse- Buff to brown
  On LCB picture macroconidia may be seen. Microconidia many and there is spiral hyphae.
- T. rubrum
  Growth rate : 1 week
  Colony : Observe : White cottony and reverse is red.
  On LCB picture, macroconidia may be seen and microconidia are many.
- T. schoenleinii
  Growth rate : 1-2 weeks
  Colony observe : Gray brown, folded and reverse is gray brown.
  On LCB picture, Macroconidia and microconidia are not seen. There is Antler hyphae .
- T. verrucosum
  Growth rate : 2-3 weeks
  Colony observe : Buff, folded and reverse is white or buff.
  On LCB picture, Macroconidia and microconidia are rare and many chlamydospores.
- T. violaceum
  Growth rate : 1-2 week
  Colony observe : Purple, waxy and reverse is deep purple.
  On LCB picture, macroconidia and microconidia are not seen. There is chylamydospores and thick hyphae
- M. canis
  Growth rate : 1-2 week
  Colony observe : white and silky and reverse is deep yellow.
  On LCB picture, macroconidia many with septa and microconidia small and few. There is racquet hyphae.
- M. gypseum
  Growth rate : 1 week
  Colony observe : Pale brown powdery and reverse is buff to yellow brown.
  On LCB picture, macroconidia are many and microconidia are few or absent.
- M. audouinii
  Growth rate : 1-2 weeks
  Colony observe : Buff pale orange and reverse is pale orange.

On LCB picture, macroconidia and microconidia are not seen. There is pectinate and thick hyphae and chlamydospores.

- E. floccosum
  Growth rate : 1 week
  Colony observe is gray yellow folded and reverse is yellow brown.
  On LCB picture, macroconidia are present and microconidia are not seen. There is many chlamydospores and distorted thick hypae.

- Dermatophyte test medium can also be used when SDA is not available.
- It is used to isolate and differentiate dermatophytes from bacterial contaminants.
- Dermatophytes turn the medium red by raising the pH of medium through metabolic activity, while most bacteria and other fungi do not.
- Malt extract agar is used to study sporulation of fungi.

Cultivation of Aspergillus
Specimen : Sputum, bronchoalveolar lavage, transbronchial biopsy, pus, skin biopsy.
Culture :

- SDA with antibiotics-two slants are inoculated, one incubated at room temperature and another at 37^0C. Culture is examined daily for first week and twice a week after that.
- Other media used are – Czapek-Dox agar, potato dextrose agar, and malt extract agar.

Colony characters and LCB picture of Aspergillus species

- A.flavus
  Colony oberve is velvety, yellow to green or brown and reverse is golden to red brown.
  On LCB picture, colonies are rough, pitted, spiny conidiospores, uniseriate phialides cover entire vesicle, point ou in directions, conidia are yellow-green
- A. fumigates
  Colony oberve is velvety or powdery, smoky green and reverse is white to tan.
  On LCB picture, colonies are smooth condidiophore, uniseriate phialides cover upper half of vesicle parallel to axis of stalk, conidia are blue-green.
- A.niger
  Colony observe is wooly, initially white to yellow, becomes brown-black and reverse is white to yellow.
  On LCB picture, Conidiophore of variable length biseriate phialides cover entire vesicle forming radiate head, conidia are black.

Laboratory Contaminants :The description of colony morphology is based on the growth observed on the commonly used laboratory media like Sabouraud dextrose agar. The common fungal laboratory contaminants are given below :

(a) Zygomycetous fungi
(b) Hyaline hyphomycetes
(c) Phaeoid fungi
(d) Yeast and Yeast-like fungi
(e) Look-alike fungi

The zygomyceteous fungi have broad, non-septate or sparsely septate hyphae. The hyaline hyphomycetes have septate hyphae without distinct melanin pigment in their cell wall neither in culture nor in tissue section. The phaeoid hyphomycetes are those that generally have melanin in their cell wall in culture and in tissue section when

the organisms are involved in an infective process. The yeasts and yeast-like fungi are round, elliptical or irregular shaped depending on fungal species involved.

Penicillium species : The hyphae are white, septate and multibranched. The growth is rapid to develop colony and conidiospores arise in various forms producing phialides singly or in groups or from branched metulae giving brush-like appearance. The conidia are unicellular and in chain with youngest as base. They are colorless and may have dark-green or blue-green pigmentations. Sometimes, conidiophores are aggregated to stalk forming synnemata which are called as coremia. Morphologically, the colonies of Penicillium are usually green and the conidiogenous cells are cylindrical to bottle-shaped and aggregated in compact penicillin as compared to Paecilomyces where the conidiogenous cells are flask-shaped.

Rhizopus : The colonies of Rhizopus are fast spreading white to light grey in the beginning but become darker as sporangia mature. The hyphae are broad non-septate with thin walls submerged or aerial stolon with brown root-like short branches i.e. rhizoids, under the points where sporangiospore develops. The sporangiophores are hyaline to brown, erect, single or in groups of 2-3. Producing sporangia with columellae at the tip. The sporangia are globose, hyaline at first, turning dark brown, grey or black as the sporangiospores mature.

Mucor : This is a rapidly growing zygomycete, light to dull grey or brownish, with abundant aerial and substrate mycelia with rhizoids or stolons being absent. The hyphae are broad with thin walls, mostly non-septate. Sporangiophores are branched, bearing spherical sporangia at the tip of each branch ; the sporangia are thin-walled with columellae becoming dark as the sporangiospores mature. When sporangia rupture, fragments of wall remain at base of columellae. The sporangiospores are globose, oval or cylindrical with smooth walls.

Germ Tube test for C. albicans :
A germ tube is a filamentous outgrowth from a yeast cell. The filament lacks a constriction at the point of origin which differentiate it from the pseudohyphae.
Procedure :

1. Put 0.5 ml of serum in a test tube.
2. Transfer yeast colonyfrom grown culture by using inoculation loop.
3. Incubate it for 35-37^{0}C for 2-3 years.
4. Now transfer a drop of mixture on a glass slides and cover it with coverslip.
5. Examine under low power and high power objective.

Observation :
Presence of sporulating yeast cells - +ve
No sporulating yeast cells - -ve

Some important infection caused by fungal agents

Superficial Mycoses

1. **Tinea nigra :** It is a superficial fungal infection of skin causing brown to black deeply pigmented lesion of skin of the palm and occasionally of the soles. Usually there is no scaling, erythema, induration or pruitus. The causative organism is *hortae wernickii*, a black yeast.

Laboratory Diagnosis : Skin scrapings from the affected site are collected. 1) Microscopic examination : On KOH preparation, typical brown, septate, branching hyphae and budding yeast like cells are diagnostic of Tinea nigra. 2) The fungus can also be cultured on Sabouraud's dextrose agar (SDA)

2. **Piedra :** It is a superficial infection of hair. It is of two types :
   (a) Black Piedra
   (b) White Piedra

Black Piedra : It is caused by fungus *Piedra hortae*. In it discrete, hard, brown black nodules of the size of about 1 mm are formed in the hair shaft. The hair become brittle and break easily. Usually the scalp hair are affected. Itching is conspicuously absent.

Laboratory diagnosis : Specimen : Infected hair along with the nodules are collected.

Microscopic examination : Dark colored septate hyphae containing asci with ascospores which have whip like appendages at both ends can be demonstrated.

Culture : The typical morphology of fungus can be demonstrated on culture.

3. **Dermatophytosis/Tinea/Ring worm :** It is the most common type of superficial mycoses seen in human beings. It affects keratinized tissues of skin, hair and nails but does not penetrate the living tissue.

Etiological/causative agents : In human beings, three genera cause dermatophytosis, i.e.1) *Trichophyton* which affects all the three, i.e. skin, hair and nails, 2) *Microsporum*, which affects skin and hair but not the nails and 3) *Epidermophyton*, which affects skin and nails but not the hair.

Clinical features : Clinically, the lesions are circular with erythematous border with scaling and inflammation.
According to the site of infection, various terminologies are used, e.g.

1) **Tinea capitis :** If hair shaft in the head area is involved.
2) **Tinea barbae :** If the beard area is involved.
3) **Tinea corporis :** If the body is involved.
4) **Tinea mannum :** If the hands are involved.
5) **Tinea cruris :** If the groin region is involved.
6) **Tinea unguium :** If the nail plate is involved.

Laboratory diagnosis : Skin scrappings, nail clippings or affected hair are collected and processed.

1) **Microscopic examination :** The sample is put in a drop of 10-20% KOH and preparation examined under 40^X under the microscope. The fungal elements if present appear as branching septate hyphae or chain of arthrospores.
2) The sample is inoculated onto SDA and an attempt is made to isolate the fungus.

On culture, the three genera causing dermatophytoses can be differentiated on the basis of the colony morphology, pigment production and microscopic features. In case of *Trichophyton*, there are abundant pencil shaped macroconidia. In case of *Microsporum*, there are abundant spindle shaped macroconidia. In case of *Epidermophyton*, typical club shaped macroconidia are seen with abundant microconidia.

Sporotrichosis

Sporotrichosis (Rose gardener's disease)**:** It is subcutaneous mycoses caused by *Sporothrix schenckii*, a dimorphic fungus. It is ulcerative disease of skin and subcutaneous tissues. It usually follows thornprick injury.

Laboratory diagnosis :

1) **Microscopic examination :** LCB preparation: Mycelial form shows sterigmata bearing flower like clusters of small conidia whearas yeast form (37°C) shows cigar shaped cells.
2) **Culture :** Definitive diagnosis is achieved if fungus can be isolated and dimorphism can be demonstrated.

Histoplasmosis

Histoplasmosis : It is a systemic mycoses caused by dimorphic fung, "*Histoplasma capsulatum*." The cases are seen mostly in America, Africa and some parts of southern Asia. From India, about 30 authentic cases of histoplasmosis have been reported. The disease usually affects lungs, liver, spleen, lymph nodes, kidney and skin.
The infection is usually asymptomatic or a mild self limiting disease.

Laboratory diagnosis : Specimen : Sputum, biopsy from skin ulcer and lymph node are the specimens of choice.
Microscopic examination : *Histoplasma capsulatum* is present in reticuloendothelial cells where it is present in the yeast form.
1)**Microscopic examination :** Mycelial form shows spherical spores with finger like projections whearas yeast form (37°C) shows small (2-4 µm) yeast cells within cytoplasm of macrophages.
2) Culture : Definitive diagnosis is achieved if fungus can be isolated and dimorphism can be demonstrated.
3) Serological tests : Latex particles agglutination, precipitation and complement fixation tests.

Candidiasis

Candidiasis : It is the commonest fungal infection of skin, mucosa and internal organs. It is caused by yeast like fungus, "*Candida albicans*". It is a normal flora of skin, mucous membrane and gastrointestinal tract.

Morphology: It is oval to spherical, measuring about 4-8 □m in diameter. On Gram staining, it is Gram positive in character.
Clinical Features: It causes infection in the immunocompromised patients like AIDS, diabetes, burns, neutropenia, steroid usage, prolonged usage of antimicrobial agents etc.
In such patients it causes following infections :

1) **Mucocutaneous infection :** Oral lesions e.g. thrush, stomatitis and glossitis; GIT, e.g. Oesophagitis, gastritis, Vulvovaginitis, balanitis; Ocular candidiasis.
2) **Cutaneous infection :** It causes skin and nail infection at moist areas like axilla, groin, in between the fingers (intertrigo). In infants, it can cause diaper rash.
3) **Systemic :** Endocarditis, meningitis, Pulmonary candidiasis, urinary tract infection, arthritis, disseminated candidiasis, endophthalmitis.

Laboratory diagnosis : Specimen : Depending upon the site of involvement, the sample e.g. Scrapings from the skin and mucous lesion and biopsy from deeper lesions, is collected.

1. **Microscopy :** On direct wet mount examination of Gram staining or KOH preparation of the sample, oval to spherical budding yeast cells, sometimes also with psudohyphae are seen. The presence of pseudohyphae indicates colonization of the tissue.
   '**GERM TUBE TEST'** is done for identification of *C. albicans*. It forms germ tubes (i.e. long, thin projections from budding cells without any constriction at its base) when incubated for two hours at 37°C with pooled human serum.
2. **Culture :** Typical whitish pasty colonies/growth can be demonstrated on SDA.
3. **Serological tests :** Serological tests like PCR, ELISA, RIA, Counter immune electrophoresis.

Cryptococcosis

Cryptococcosis : It is a fungal disease caused by encapsulated yeast, "*Cryptococcus neoformans*". The fungus is normally present in the soil with high nitrogen content and in the faeces of birds like pigeon.

Morphology : Cryptococcus neoformans is a true yeast, round in shape measuring about 5-20 □m in diameter with a large capsule around it.

Mode of infection : It enters the human body through respiratory tract by inhalation.

Clinical features : It causes the disease in immunocompromised patients like AIDS, prolonged use of corticosteroids, antibiotics and immunosuppressive drugs.

In such patients, it causes :

1) Pulmonary cryptococcosis,
2) CNS : meningoencephalitis
3) Visceral cryptococcosis : Any organ or tissue can be invaded by this fungus
4) Osseous : Bones are involved,
5) Cutaneous cryptococcosis.

Laboratory diagnosis : Specimen : Depending upon the site of involvement, sputum, CSF, blood or pus may act as the sample of choice.

1) Microscopy : Direct microscopy with India ink shows, round budding yeast cells with a clear halo around them. Gram staining shows gram positive yeast cells.

2) On culture, on SDA it produces typical cream to buff colored, highly mucoid colonies.

3) Serological tests : Latex agglutination, Indirect fluorescent antibody test, complement fixation test

4) Animal pathogenicity test : Swiss albino mice is used as the experimental animal for inoculation of specimen.

Aspergillosis

Aspergillosis : It is a systemic fungal disease caused by species of Aspergillus. Of these *A. fumigatus* is the most common. Others are *A. niger, A.flavus.*

Source of infection : Infection is acquired by inhalation of the spores.

Clinical features : It usually affects the immunocompromised individuals although immunocompetent persons can also acquire infection.

It causes :

1) Pulmonary disease e.g. Allergic aspergillosis (asthma like condition), allergic bronchopulmonary aspergillosis (ABPA), fungal ball (aspergilloma) and invasive aspergillosis.
2) CNS aspergillosis,
3) PNS (paranasal sinuses) aspergillosis,
4) Endocarditis.

Laboratory diagnosis :

Specimen : Depending upon the site of infection, sputum, lung biopsy, CSF etc. may be collected.

Direct microscopy : On KOH mount, hyaline septate hyphae showing dichotomous branching (i.e. branching at 45 degree angle) are seen. On culture, typical colony morphology/growth of *Aspergillus* species can be demonstrated. LCB preparation shows asexual conidia borne on sterigmata in form of chains at ends of conidiophores and branching, septate hyphae.

Immunodiagnosis : ELISA, RIA, Latex agglutination, **Immunodiffusion test :** Molecular methods : PCR

Mucormycosis /Zygomycosis

Mucormycosis /Zygomycosis : It is a fungal infection caused by saprophytic fungi of species *Mucor, Rhizopus and Absidia.*

Habitat : These are common saprophytes of soil and decaying material.

Clinical features : These are usually non pathogenic to man but in conditions like diabetes mellitus, diabetic ketoacidosis, leukaemia, lymphoma and other immunosuppressive conditions, they become pathogenic.

It causes :

1)Rhinocerebral zygomycosis,
2)Pulmonary zygomycosis,
3)Cutaneous zygomycosis,
4)Gastrointestinal zygomycosis,
5) Disseminated zygomycosis.

A special feature of this fungus is that it causes invasion and thrombosis of blood vessels, resulting in ischaemia and necrosis of the affected part.

Laboratory diagnosis :

Specimen : Depending upon the site of involvement, scraping from the lesion, nasal discharge, pus, sputum, or biopsy material is collected.

1) **Direct Microscopy :** On examination of KOH mount, irregular, broad, aseptate ribbon like hyphae with wide or right angle branching at irregular intervals can be seen.
2) **On culture :** Typical colony morphology of the fungus depending upon the genera and the species can be demonstrated.
3) **Serological test :** No satisfactory serological tests are available.

Penicillosis

Penicillosis: It is caused by *Penicillium spp*. P. marneffei is a leading opportunistic pathogen in HIV patients. However, *P. notatum* was spp. from which Penicillin, an antibiotic was developed.

Clinical features: Keratitis, ear infections, penicillosis and deep infections.

Laboratory diagnosis: Depending upon the site of involvement, sample is collected.

1) **LCB preparation:** a typical brush like arrangement of conidia is seen emerging from phialides which are borne on septate hyphae.
2) **On culture :** Typical colony morphology of the fungus depending upon the genera and the species can be demonstrated.
3) **Serological test:** No satisfactory serological tests are available.

Immunology

Introduction

Immunology is the branch of science which deals with study of body's response to antigenic challenge. Immunological mechanism are involved in the protection of body against infectious agent but periodically they can also cause damage. Immunology has blood biological role involving concept of recognition, specificity and memory.

Historical events :

Jenner (1878) used nonvirulent cowpox vaccine against smallpox infection. Metchnikoff (1883) suggested the role of phagocytes in immunity. Von Behring (1890) recognized antibodies in serum against diphtheria toxin. Denys and Leclef (1895) suggested the phagocytosis is enhanced by immunization. Ehrlich (1897) put forward side chain receptor theory of antibody synthesis. Bordet (1899) found that lysis of cells by antibody requires cooperation of serum factor now collectively known as complement.

Landsteiner (1900) declared human ABO groups and natural isohemagglutinin. Richest and Portier (1902) proposed the term anaphylaxis which is opposite of prophylaxis.

Immunity : Immunity

It is the natural or acquired resistance of body to disease caused by microbes. In other words, we can say that power of body to fight against infections.

Antigens (Immunogen) : A substance (usually a protein and non-self) that causes formation of an antibody and reacts specifically with that antibody.

Antibody (Agglutinin): An immunoglobulin (Protein in nature) produced by lymphoid tissue in response to a foreign antigen (bacteria, viruses or other antigenic substance).

Immunity against infectious diseases is of following types :

a) Innate Immunity

(i) Non specific
(ii) Specific

→ Species
→ Racial
→ Individual

b) Acquired (Adoptive) Immunity

(i) Active
(ii) Passive

→ Natural
→ Artificial

Innate or Native Immunity: It is the resistance to infections which an individual possesses due to his/her genetic or constitutional make up. It may be non specific when it indicates degree of resistance to infections in general or specific when resistance to particular pathogen is concerned.

Species Immunity: It implies total or relative resistance to a pathogen shown by all members of a species. For example, human beings don't suffer from most of animal and plant pathogens. This is perhaps due to physical and biochemical differences between tissues of different host species.
Racial Immunity: Within a species there may be marked differences in resistance to infection pattern. Algerian sheep is highly resistance to anthrax as compared to European sheep. In USA, negroes are more susceptible to tuberculosis than whites.

Individual Immunity: Different individual in a given race differ in their resistance to microbial infections. It is known to be genetic in origin e.g. a large number of persons are exposed to a particular pathogen or organism but all don't develop infection. Instead, some people develop mild infection and others may develop serious infection.

Mechanics of Innate Immunity:

a) Mechanical barriers and surface secretions :

- **The intact skin:** It forms a protective layer for the internal organs. Bacteria can not penetrate the intact skin. The fatty acids secreted by the sebaceous glands in the skin and high salt concentration of sweat are effective protective barriers.
- **The mucous membrane:** It is a specialized epithelial lining capable of secreting mucous (a sticky substance) is also a very effective protective barrier.
- **The epithelial lining of respiratory tract:** It contains ciliated cells and mucus which traps the dust particles and other organisms and move them upwards.

b) **The gastrointestinal tract** also has an inherent mechanism of protection in the form of thick mucous layer lining the stomach and intestine. The highly acidic environment of stomach can kill most of the pathogens entering stomach.
c) **Phagocytic cells :** Human body contains a variety of phagocytic cells such as neutrophils, eosinophils, macrophages etc. which are present in various organs of the body as well as in blood. These cells destroy harmful microbes by engulfing them.
d) **Many non specific microbiocidal substances** are present in tissues and body fluids e.g. lysozyme, complement proteins, interferons etc.
e) **Fever :** A rise in body temperature is a natural defence mechanism. It inhibits or kills infecting organisms.
f) **Inflammation :** It is defined as cellular and vascular response to any injury e.g. Infection or physical injury. The signs of inflammation are redness, swelling, heat, pain and disturbed or altered function.

Acquired Immunity: The resistance that an individual acquired during his life span is known as acquired immunity.

It is of two types:

Active immunity and Passive immunity

1. **Active Immunity:** Active immunity may be natural or artificial.
   a) **Natural active immunity** results from either a sub- clinical or an in apparent infection by a microbe, e.g. a person who has recovered from an attack of measles or mumps or diphtheria develops natural active immunity.
   b) **Artificial active immunity** is the resistance induced by vaccines.

Vaccines

Vaccines are the preparations of attenuated live or killed micro-organisms or their products used for immunization.

Examples of Vaccines are as follows:

(A). Bacterial Vaccines :

a) **Live:** BCG for tuberculosis, Ty21a for typhoid.
b) **Killed:** Tab vaccine for enteric fever, cholera vaccine.

(B). Bacterial Products: Tetanus toxoid, Diphtheria toxoid.

(C). Viral Vaccines :

a) **Live:** Oral polio vaccine, MMR, Varicella zoster.
b) **Killed :** Salk vaccine (Injectable polio vaccine), Influenza vaccine.

2. **Passive Immunity:** The immunity that is transferred to a recipient in a ready made form is called as passive immunity. In this type of immunity, the patient's immune system plays no role. The immunity is effective immediately after passive immunization i.e. there is no lag phase or latent phase. It confers only transient or a short lived immunity.

It is of two types:

a) Natural passive immunity: It is the resistance against diseases passively transferred from mother to baby in the form of IgG antibodies which cross placental barrier to reach fetus. After birth, IgA is passed to new born baby through breast milk.

b) Artificial Passive Immunity : In this type of immunity resistance is passively transferred to recipient by administration of antibodies.

It is given in the form of :

a) Hyperimmune sera of animal or human origin.

b) Pooled human gammaglobulin e.g. Anti Tetanus serum (animal origin), Tetanus Immunoglobulin (human origin), anti snake venom.

Differences between Active and Passive Immunity

Active Immunity	**Passive Immunity**
1) Produced by host immune system as a result of antigenic stimulation.	**1)** Received passively by host.No participation of host immune system.
2) Induced by infection or as a result of contact with antigens.	**2)** Conferred by administration of antibodies.
3) Immunity is long lasting.	**3)** Immunity is short lived.
4) Immunity effective only after a lag phase i.e. time required for generation of antibodies and immunocompetent cells.	**4)** Immunity is effective immediately.
5) Immunological memory is present i.e. next response is more effective.	**5)** No immunological memory.
6) More effective and confers better protection.	**6)** Less effective and confers less protection.
7) Not applicable in case of immunodeficient individuals.	**7)** Applicable in immunodeficient individuals.

Mechanism of Acquired Immunity :Thia mechanism defends the body against specific foreign component. It is based on the principle "self" and "non-self". Any foreign particle is known as "non-self" by the body and the immune system begins its functions.

1. Cells of the immune system :

(a) Lymphocytes : The basis of the immune system is the lymphocytes. There are two types of lymphocytes : T-lymphocytes and B lymphocytes. After maturation, some lymphocytes come into circulation where as others remains confined to specific organs like spleen and lymph node.

(b) Antigen Presenting cells (APCs) : The specialized cells which include macrophages, (monocytes as blood macrophages and histocytes as tissue macrophages), B-lymphocytes, and dendritic cells e.g. Langerhans cells of epidermis of skin.

2. Types of Immune system : Two types of immune response operate in the body depending upon the type of antigen :

(a) Humoral or antibody mediated immunity : This system defends the body when antigens are present in body fluids like lymph, blood. There is active production of antibodies against these antigens by B-lymphocytes with the help of T-lymphocytes. The antibodies bind to antigen forming Ag-Ab complex & thus Ag molecules or particles are clumped or lysed, their toxins may be neutralized & readily removed after phagocytosis by neutrophils or macrophages.

(b) Cell mediated immunity : This system defends the body when antigen do not come in body fluids, rather they are confined to some cells. T-lymphocytes specifically migrate to the site of infection. Specific T-lymphocytes act against specific antigens. Different types of T-cells are as follows :

(i) Cytotoxic or killer T-cells : These are meant to kill the infected cells. They produce a protein, perforin, which makes holes in the membrane of the affected cell.

(ii) Helper T-cells : They help the killer T-cells in their action. They also help the B-cells to produce antibodies.

(iii) Suppressor T-cells : After the infection is controlled, they suppress the immune system.

(iv) Memory T-cells : This group of cells remembers the previous contact with the same antigen and helps to speed up the action in future.

3. Types of Immune responses :

(a) Primary immune response : When body comes in contact with an antigen for the first time, the response shown by it is called as primary immune response.

(b) Secondary immune response : It occurs when body comes in contact with the same antigen again. Because of the memory cells, response in quicker this time.

Antigen

A substance (usually a protein and non-self) that causes formation of an antibody and reacts specifically with that antibody e.g.

- Dead or living microorganisms
- Vegetable proteins
- Egg albumin
- Serum
- Plant or animal tissue
- Bacterial toxins
- Red blood cells
- Snake venom
- Milk

Types of Antigen and their properties :

According to ability to induce antibody formation (immunogenicity) are of two types :

1. Complete antigen (Immunogen) :
   An antigen which is able to induce antibody and react with it specifically. They are high molecular weight protein (more than 10000 daltons) but some are polysaccharides in nature.
2. **Incomplete antigen or hapten** : These are the substances (usually non-protein) which is unable to induce antibody itself but can react specifically with antibody known as hapten. These are incomplete antigens, which become complete antigens when they react with carrier molecule or schlepper. Haptens are generally

low molecular weight lipids and carbohydrates e.g. capsular polysaccharide of pneumococci, cardiolipin, polysaccharide 'C' of beta-haemolytic streptococci. A hapten may be of following two types : Simple and complex.

(a) Simple : These are simple chemical substances and monovalent. They can react with antibody but unable to precipitate the reaction.

(b) Complex : These are relatively large molecules and polyvalent. They react specific antibody and able to precipitate the reaction e.g. capsular polysaccharide of pneumococci.

Antigenic determinant and its properties

The smallest unit of chemical grouping on antigen molecule that determines specific immune response and react specifically with antibody is known as antigenic determinant or epitope.

An antigen possesses several epitopes and each epitope specific antibody formation.

Properties of epitopes are

- Their size is 25 to 35 A
- Molecular weight : 400-1000
- The determinant group on
  - (i) Protein Ag – penta or hexapeptide
  - (ii) Polysaccharide Ag – hexasaccharide

Properties of Antigens (Factors affecting immunogenicity)

1. Foreignness : The list and the most important factor for immunogenicity is the foreignness of antigen. Immune system has the ability of distinguish between self(own & non-self) foreign antigens. Only antigens which are foreign to the individual induce an antibodies formation.
2. Size : Large molecules such as haemocyanin having molecular weight 6.75 million are highly antigenic. In general, substances having molecular weight less than 10,000 are weakly antigenic or nonantigenic.
3. Chemical nature :

- Antigens are proteins, polysaccharides, lipids or nucleic acids.
- Protein antigens are more effective than polysaccharide antigens.
- Lipids and nucleic acids are less antigenic and their antigenicity can be enhanced bycoupling them with proteins.
- However, gelatine, histones and protamines are nonantigenic because of absence of an aromatic radical such as tyrosine, which is must for antigenicity.

4. Susceptibility to tissue enzymes : These are only those substances which can be metabolized & can be converted to soluble form by the action of tissue enzymes act as good antigens.
5. Antigenic specificity : The reaction between antigen and antibody is highly specific. An antigen reacts with its corresponding antibody only. It depends on the antigenic determinants on the Ag molecules.

(a) Species specificity : Tissues of all members in a particular species possess species specific antigen. Thus human blood proteins can be differentiated from animal proteins by specific Ag-Ab reaction. These immunological relationship between species specific antigens are useful in identifying the species of the organism to which blood and seminal stains belong (forensic application).

(b) Isospecificity : Isoantigens or alloantigens are antigens found in some but not all members of a species e.g. Human blood group antigens & Rh antigens on the basis of which all humans are classified into different blood groups (A, B, AB & O), Rh-positive & Rh-negative. These antigens are genetically determined and are important in

- Blood transfusion

- Isoimmunisation during pregnancy
- Providing valuable, evidence in disputed paternity.

(c) Auto-specificity :The autologous or self antigen are generally non-immunogenic but under certain circumstances, lens proteins, thyroglobulins etc. Act as auto antigens. The various circumstances include injury of lens, damage to thyroid gland or testis. These become foreign to one's own body & induce production of antibodies (auto Ab's) against themselves and the result in production of autoimmune disease.

(d) Organ specificity : The organ specific antigens are confined to particular oran or tissue.,Such antigens present in an organ or tissue of different species are called organ-specific antigens. For example, brain tissue antigen of man, shares antigenicity with brain tissue antigen of sheep.

(e) Heterogenetic or Heterophile antigen : Certain antigens of similar nature (if not identical)present in different tissues of different biological species, classes & kingdom are called heterophile antigens. The example are :

- The Forssman antigen (Forssman 1911) which is widely distributed in man, animals, birds, plants and bacteria.
- A heterophile antigen of Rickettsiae causing typhus fever is shared by certain strains of Proteus (OX 19, OX 2 and OX K).
- Epstein-Barr virus causing infectious mononucleosis shares antigenicity with sheep and Ox RBCs (Paul-Bunnel test)

Antibodies (Immunoglobulins)

Antibody (Agglutinin)

An immunoglobulin (Protein in nature) produced by lymphoid tissue in response to a foreign antigen (bacteria, viruses or other antigenic substance).

Properties of Antibodies :

1. These are globulins and therefore, also known as immunoglobulin and they contain sugar residues and hence are glycoproteins.
2. They constitute 20-25% of the total serum proteins and when separated electrophoretically, most of them migrate in gamma region, hence they are also termed gamma globulins.
3. Most of them have molecular weight of 150,000-180,000 and sedimentation coefficient 7S-8S except for IgM. IgM has molecular weight of 900,000, sedimentation coefficient 19S and termed M or macroglobulin.
4. They are thermolabile and denatured on heating at 70^0C for 1 hour and their activity is also effected by pH of the medium and other factors affecting proteins.
5. They are not denatured by ethyl alcohol at low temp. (0-4^0C) but alcohol at higher temp. Denatures them and all antibodies are immunoglobulin but all immunoglobulins may not be antibodies.
6. Natural salts such as magnesium sulphate, ammonium sulphate and sodium sulphate can precipitate but do not denature them.

Structure of Antibodies

These are "Y" shaped molecule made up of two identical heavy & two identical light polypeptide chains held together by disulphide (S-S) bonds. The longer chains are called heavy (H) chains and the shorter chains as light (L) chains. Each L chain is attached to H chain by S-S bond. The two symmetrical H chains are held together by 1-5 disulphide bonds depending upon the type of immunoglobulin. Porter & colleagues split IgG molecule by papain digestion into 2 identical Fab & Fc fragment.

Fab fragment (Fragment antigen binding) : These two identical Fab fragments contain a single antigen binding site each. The portion of heavy chain in Fab fragment is called Fb region. Each Fab fragment contains a light chain & a part of heavy chain.

Fc fragment (Fragment crystallizable) : It lacks the ability to bind antigen. Each Fc fragment contains parts of both H chains. It is the site of complement fixation, macrophagic fixation, skin fixation & regulation of catabolism.

Types of Antibodies

1. **Immunoglobulin G (IgG)** : It is the major immunoglobulin in normal serum about 70-80% of the total immunoglobulin. It is equally distributed between the intravascular and extravascular compartments. It is a monomer consisting two H and two L chains. It is the major immunoglobulin synthesized during secondary response.

   General properties of IgG :

- The heavy chain in IgG is Gamma. Its molecular weight is 150,000. Its sedimentation coefficient is 7S and half-life is 23 days. Carbohydrate content of IgG is 3% and valency is 2. Normal serum conc. Of IgG is 8-16 mg/ml. Subclasses of IgG is IgG1, IgG2, IgG3, IgG4.

  Biological activities

- It has the ability to cross the placenta and reach the foetal circulation in pregnant women and provide a major line of defense against infection in the newborn for the first few weeks.
- It neutralizes viruses, activates complement and enhances phagocytosis.
- It is able to activate complement and thus helps to attract polymorphonuclear leucocytes (phagocytic cells) by chemotactic mechanism and stimulates ingestion and killing of microorganisms.

2. **Immunoglobulin A :** It is the second most abundant immunoglobulin constituting 10-13% of the total immunoglobulins. It exists in two forms :

(i) Serum IgA : It is a monomer, with molecular weight of 1,60,000 & sedimentation constant of 7S.

(ii) Secretory IgA : IgA found on mucosal surfaces & in secretions which is also called secretary IgA. Its molecular weight is 3,85,000Da & sedimentation constant of 11S. It is a dimer formed by two units joined together by glocoprotein called J-chains.

IgA is fast moving globulin found in high conc.in colostrum, saliva, tears, nasal fluid, sweat, milk & in secretions of respiratory, intestinal & genital tracts. It protects the exposed mucous membranes from invasion by microorganisms.

General properties of IgA

- The heavy chain in IgA is Alpha and molecular weight is 160,000 (385,000). Its sedimentation coefficient is 7S (11S). Half-life of IgA is 6-8 days. Its carbohydrate content is 11% and its valency is 2 (4). Normal serum conc. Is 0.6-4.2 mg/ml. Subclasses of IgA is IgA1 and IgA2.

3. **Immunoglobulin M (IgM)** : It is the first antibody formed in every response. (Appears early 7-8 days). It constitutes 5-8% of serum immunoglobulins. It is a pentamer containing 10 H and 10 L chains – five subunits of monomer joined together by J-chain (joining chain). IgM remaisn largely confined to blood stream (80%) & is not usually found extravascularly in secretions or body cavities. Its presence in serum indicates recent infection. IgM cannot cross the placenta due to its larger size, its presence in serum of fetus or new born indicates intrauterine infection. IgM is most efficient antibody in agglutination, complement fixation, cytolytic & cytotoxic reactions.

General properties of IgM :

- The heavy chain in IgM is Mu and molecular weight is 970,000. Its sedimentation coefficient is 19S. Half-life of IgM is 5 days and carbohydrate content is 10 %. Its valency is 10 and normal serum conc. Is 0.5-2 mg/ml. It has two subclasses : IgM1 and IgM2.

4. **Immunoglobulin D or Delta globulin** : It occurs in low concentration less than 1% of the total immunoglobulins. It is structurally similar to IgG. It is a monomer containing two H and two L chains. IgD is present on the surface of B lymphocytes & serve as recognition receptor for antigens.

General properties of IgD :

- The heavy chain in IgD is Delta and its molecular weight is 184,000. Its sedimentation coefficient is 7S. and half life is 2-8 days. Carbohydrate content of IgD is 13% and valency is 2. Normal serum conc. Of IgD is 0-0.04 mg/ml. It has two subclasses – IgD1 and IgD2.

5. **Immunoglobulin E :** It occurs in very low concentration but the level is greatly elevated in atopic conditions such as asthma, hay fever and eczema. It is structurally similar to IgG-monomer contains two H and two L chains. It is mostly distributed extravascularly & is produced by a very small proportion of plasma cells living in intestinal & respiratory tract.

Biological Properties of IgE

- The heavy chain of IgE is Epsilon and its molecular weight is 188,000. Its sedimentation coefficient is 8S and its half-life is 2-3 days. Carbohydrate content of IgE is 12% and valency is 2. Its normal serum conc. Is 0.00003.

Antigen-Antibody reactions

Definition : When an antigen and antibody molecules are mixed together in solution, an antigen reacts with its specific antibody form a comples. This complex formation in an observable manner due to combination of specific Ag with specific Ab is called Ag-Ab reaction. Such reactions are useful in laboratory diagnosis of various diseases & in identification of infectious agents in epidemiological survey. The Ag-Ab reactions in vitro are known as serological reaction.

Characteristic features of Ag-Ab reactions :

1. The reaction is highly specific.
2. The Ag-Ab may combine in varying proportions.
3. The combination is firm but reversible.
4. The entire molecules of Ag & Ab interact during combination.
5. The combination is firm but reversible. It is affected by affinity and avidity.

- Affinity is the intensity of attraction between antigen and antibody.
- Avidity is the binding strength of the individual antibody with its specific antigenic determinant.

6. Both antigen and antibody participate in the formation of agglutinates or precipitates.

Precipitation and Flocculation :

Principle : When a soluble antigen reacts with its specific antibody in the presence of electrolytes at a suitable temperature (37^0C) and suitable pH (7.4) then they form Ag-Ab complex as an insoluble precipitate. This process is known as precipitation.

Precipitinoge (Soluble Ag)	+	Precipitin (Ab)	-	Insoluble precipitate

Flocculation : This is a precipitation reaction in which the precipitation of Ag-Ab complex remain suspected as floccules instead of sedimentation is known as flocculation.

Mechanism of Precipitation :

Lattice Hypothesis : As per lattice theory the precipitate formed as result of random, reversible reaction . According to this, an Ab molecule, which is divalent, forms a bridge between two Ag molecules. Ag being multivalent can combine with a number of Ab molecules. This combination results in the formation of multimolecular lattice, which makes the reaction visible. It is known as lattice hypothesis. This hypothesis requires that

- The Ab should be divalent (valency -2 at least)
- Ag and Ab should be in optimum proportion.

Applications :

1. Identification of bacteria e.g. detection of group specific polysaccharides substance in streptococci in Lancefield grouping, etc.
2. Identification of antigenic component of bacteria in infected animal tissue e.g. Bacillus anthracis.
3. Standardization of toxin and antitoxins.
4. Demonstration of antibody in serum e.g. VDRL for the diagnosis of syphilis.
5. Medico legal identification of human blood or seminal fluid.

Techniques of Precipitation and Flocculation reaction

1. Ring test : This is very simple for detection of antigen. When an antigen solution is layered over antiserum, a white ring of precipitate forms at the junction of two fluids. E.g. CRP, Ascoli test, Lancefield grouping of streptococci.
2. Slide flocculation test : When a drop of antigen solution and patient's serum are placed on a slide and mixed by shaking, the reaction appears in the form of floccules e.g. VDRL test for diagnosis of syphilis.
3. Tube flocculation test : An antigen and serum are placed in a tube and mixed by shaking- the result is formation of floccules e.g. Kahn test for diagnosis of syphilis.
4. Immunodiffusion test : In this technique, precipitation occurs in a gel which is more sensitive and specific than precipitation in a liquid medium. Main advantage of this technique are

- Precipitation is easily visible.
- Each antigen-antibody reaction results in formation of a distinct band of precipitation, hence the different antigens in the reacting mixture can be observed and detected.
- It also indicates identity, cross-reaction and non-identity between different antigens.

Types of Immunodiffusion

1. Single diffusion in one dimension : The antibody is incorporated in agar gel and the antigen solution is layered over it. The antigen diffuses downward, reacts with antibody and forms precipitation band. The number of bands indicates the number of Ags in a mixture. This is known as Oudin technique.
2. Double diffusion in one dimension (Oakley-Fulthorpe technique) : In this test, Ag and Ab move towards ach other through the column of plain agar and form a band of precipitate. Its main use is to determine the number of antigen in a mixture when they meet at optimum proportion.
3. Single diffusion in two dimension : In this technique, antibody is incorporated in agar gel poured on slide. The antigen is added to well cut on the surface of gel. Ring shaped bands of precipitate are formed around

wells. The diameter of ring gives an estimate of concentration of antigen. Main use of this technique are for the quantitation of soluble Ags in body fluids and for the quantitation of immunoglobulins, proteins and complement components in serum and other body fluids.

4. Double diffusion in two dimensions (Ouchterlony Procedure) : It is also known as agar gel diffusion. In this test, wells are poured in agar gel. The antiserum is placed in the central welland different Ags are placed in the surrounding wells. The result is formation of precipitation bands where they meet in optimum proportion. If two adjacent Ags are identical, the lines of precipitate will be formed by them. It is used to detect an Ag, to compare two Ag, Ab systems and in the diagnosis of bacterial, viral, fungal and parasitic diseases. E.g. Elek test for toxigenicity testing in diphtheria.
5. Immunoelectrophoresis (IEP) : It is the joint venture of electrophoresis and agar gel diffusion. This is done in two steps :
   (a) During Ist step, the antigen is subjected to electrophoretic separation in agar gel.
   (b) During IInd step, a trough is cut parallel to and slightly away from the path of electrophoretic separation and is filled with antibodies and allowed to diffuse.
   Then the antigen and antibody diffuse toward to each other to react and formation of precipitation bands are takes place. The main use of this method are to detect abnormalities in human sera in disease e.g. myeloma.
6. Counter current Immunoelectrophoresis (CIEP) : In this method, two separate wells are cut out of agar gel, one filled with Ag & other with Ab. Electric current is passed through gel. The Ag carrying a negative charge moves towards positive pole & positive charged Ab moves towards negative pole & vice versa. Line of precipitation appears at the junction where Ag & Ab meet in 30 to 60 minutes. The test is faster & much sensitive. Its main use is detection of various Ags in various body fluids e.g. Hep. B Ag & antibodies.
7. Rocket Electrophoresis (Dimensional single electroimmunodiffusion) : Antigen is placed in a well cut out of agar gel containing antibody. Antigen reacts with Ab in gel on electrophoresis. The result is formation of rocket shaped bands. Based on the height of rocket, the concentration of Ag is quantitative.
   Its main use is quantitation of proteins and other antigens in various clinical specimens.
8. Two dimensional Immunoelectrophoresis (Laurell's tech.)
   This test is performed in two steps :

(i) During first step Ag is separated by gel electrophoresis in different parts.

(ii) During 2nd step, electrophoresis is carried out at right angle to the first antibody containing gel. Then migration of antibodies into stationary antibodies carried out and formation of cones of precipitate take place as result. Each cone indicate one antigen. Its main use is detected and quantitate human serum proteins and detection of abnormal proteins present in various body fluids.

Agglutination :

Principle : When a particulate antigen (Bacteria, RBC's) is mixed with its corresponding Ab in presence of electrolytes (NaCl) at a suitable temperature (37^0C) & pH (7.4), the particles are clumped or agglutinated. Igm Ab's agglutinate better than IgG antibody.

It is more sensitive for detection of antibodies.

$$\text{Agglutinogen} + \text{Agglutinin} \xrightarrow{\text{NaCl}} \text{Ag-Ab complex (Agglutination)}$$

Mechanism : Same as precipitation.

Uses :

1. Identification of bacteria, e.g. serotyping of salmonella and shigella with known antisera.
2. Serological diagnosis of infection e.g. Widal test for typhoid etc.
3. Hemagglutination test e.g. Rose Waaler's Paul Bunnel.

Types of Agglutination test

1. Direct agglutination test

(a) Slide agglutination/Microagglutination : On a clean glass slide, mix the antiserum and antigen and agglutination occurs e.g. Blood grouping.

(b) Tube agglutination/Macro agglutination : It is a quantitative test used to determine or estimate the titer of Ab & to confirm the result of slide agglutination test. It is routinely used for serological diagnosis of typhoid, paratyphoid, brucellosis & typhus fevers.

2. Indirect or Passive agglutination test : When a precipitation reaction can be converted into agglutination by coating of the soluble Ag onto the surface of carrier molecule such as latex, bectonite or RBCs. This type of test is known as passive agglutination test.

Reverse Passive agglutination : For detection of antibody, when antibody absorbed on the surface of carrier molecule instead of antigen then it is called reverse passive agglutination test.

Passive agglutination test includes

- Latex agglutination test e.g. RA factor, HCG test, CRP test, ASO test
- Haemagglutination test e.g. Treponema Pallidum, Haemagglutination test.
- Test u sing carbon particles e.g. carbon particles mixed with cardiolipin antigen are used in Rapid Plasma regain (RPR) card test.

Complement Fixation test

In CFT, the complement is fixed to an antigen-antibody complex without any observable biological activity. Then to detect the fixation or non-fixation of complement a indicator system is introduced. In the antigen or antibody not present, then complement will not be fixed. This is mainly used for the detection of antibodies in patient sample.

Principle : The antigen have ability to react with its specific antibody and for antigen-antibody complex, this complex have ability to fixe the complement but this reaction is not visualized. Now to detect the fixation of complement we have to introduce an indicator i.e. sheep red cell coated with amboceptor (rabbit antibody to sheep red cells). Complement can lyse the red cells of sheep which is coated with amboceptor (antibody). If antigen-antibdoy complex formed, then the complement will fix and no visual activity seen means not lysis of sheep red cells occurs and the test is positive. But if there are no antigen-antibody complex form then the complement will not fixed and this free complement react with coated sheep red cells and agglutinate them and the test is negative.

Neutralization test : This is based on the principle that specific antibodies are able to neutralize the biological effect of viruses, toxins and enzymes.

Viruss neutralization test : It inclues

(i) Neutralization of cytopathic effect.
(ii) Haemagglutination inhibition test.
(iii) Toxin neutralization test e.g. Nagler reaction
(iv) Enzyme inhibition test e.g. Antistreptolysin test.

Common Serological Test

Veneral Disease Research Laboratory test (VDRL)

This test is done for diagnosis of syphilis.

Principle : This test is based on flocculation and it is non-specific test. Patient's serum is mixed with cardiolipin-lecithin cholesesterol antigen on a glass slide. The mixture is rotated for 4 minutes and observe for flocculation. The flocculation can be detected under low power objective. Antibodies appears in the serum (Reagin) reacts with VDRL antigen and form floccules.

Procedure :

Qualitative test

1. Heat the serum in waterbath at 56^0C for 30 minutes to inactivate the complement.
2. This test is carried on glass slide (7.5 x 5 cm) each with 12 paraffin rings. Pipette 50 μl inactivated patient serum into paraffin ring on the glass slide.
3. Put 50 ml each of positive and negative control sera into other paraffin rings.
4. Add one drop of working antigen suspension to each of these paraffin rings.
5. Mix with wooden sticks and rotate the slide for 4 minutes with hand on a flat surface in a circular manner in a diameter of about 5 cm or on a mechanical VDRL rotator set at about 180 r.p.m.
6. Read the test results immediately under a low power objective of a microscope.

Observation : The antigen particles appeared as small fusiform needles which remains more or less evenly dispersed in case of non-reactive. If grouping of these particles appear as clumps, it indicates reactive.

Medium and large clumps	-	Reactive
Small clumps	-	Weekly reactive
No clumps	-	Non-reactive

Quantitative test :

1. Pace 6 test tubes in a rack.
2. Pipette 200 ml of normal saline in each tube.
3. Pipette 200 ml of the patient serum in first tube and mix well. The dilution will be 1:2.
4. Transfer 200 ml from the first up to sixth tube successively. The dilution will now be 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 respectively.
5. Add 50ml of each dilution to the respective paraffin rings. Include controls as in qualitative test.
6. Add 1 drop of VDRL antigen (1/60 ml approx.) to each ring.
7. Rotate the slides on VDRL rotator adjusted to 180 r.p.m. for 4 minutes.

Note : A titre of 1:16 and above for reaginic antibody is significant for syphilis.

Widal test (By slide method)

This test is done for the diagnosis of Enteric fever. In patient's serum antibodies against S. typhi, S. paratyphi A and B are detected. Four antigenic suspensions are used for this test i.e. TO, TH, AH, BH

Principle : This is based on agglutination reaction.

Procedure :

1. On agglutination tile, place one drop (0.02ml) of patient's serum into eight wells, one well for each antigen.
2. Add one drop of appropriate antigen to each well. Two wells, for a positive control and a saline control, should be included for each antigen suspension.
3. Using separate wooden applicator sticks, mix the contents of each well, spreading over the entire well surface.

4. Rotate the tile for three minutes on a rotator or rotate manually.
5. Any agglutination observed with patient's serum indicate +ve result.

By Tube Method :

1. Arrange four rows each containing 7 clean and dry Widal tubes in a Widal rack.
2. Mix 100 ml of the patient serum with 1.4 ml of the physiological saline to obtain a 1 in 15 dilution (masseter dilution).
3. Add 0.4 ml of saline from second to seventh tube with the help of a pipette.
4. Add 0.4 ml of freshly prepared 1 in 15 dilution of serum into the first two tubes of each row.
5. After mixing, transfer 0.4 ml from second tube onwards up to sixth tube and from sixth tube discard 0.4 ml.
6. Add 0.4 ml of respective antigen namely TO, TH, AH and BH in each row from first to seventh tube.
7. The final dilution would be 1:30, 1:60, 1:120, 1:240, 1:480 and 1:960 in the first six tubes respectively while the last tube would serve as negative control.
8. Incubate the tubes at 370C in a waterbath for 18-24 hours.

Reading of result :

- Positive H agglutination show as loose and cotton-wolly clumps.
- Positive 'O' agglutination show as disc like granular deposit at the bottom of the tube.
- The highest dilution of serum which show agglutination, indicates the titre of antibody.
- Generally, a titre of 1:100 or more for 'O' agglutinins and 1:200 or more for 'H' agglutinins are significant.
- Persons who had post enteric infection or who have vaccinated may show high titre of 'H' agglutinin is due to anamnestic response. This is due to H antigen of Salmonella shared by some unrelated organisms. The anamnestric response show only a transient rise in titre. But in case of enteric fever, the titre remains sustained.
- Antibodies usually appear after 7-10 days of illness in enteric fever. The titre increases till the 3rd or 4th week and then declines gradually.

C-Reactive Protein (CRP)

Introduction : C-reactive protein is an abnormal protein that precipitates C carbohydrate of cell wall. It appears in acute phase sera of cases of pneumonia but disappears during convalescence. It also occurs in some other pathological conditions. It is not an antibody formed in response to pneumococcal infection.
Principle : It is based on passive agglutination. It is done by using latex particles coated with anti CRP antibody.
Procedure :

1. Bring serum sample and reagents to room temperature.
2. Dilute patient serum 1:5 with normal saline.
3. Place one drop of diluted patient serum and positive and negative control sera in the respective zone of the test plate.
4. Then add one drop each of latex CRP reagent to each of these sera.
5. Mix well with disposable applicator sticks and observe for agglutination within 2 minutes.

Interpretation :
Marked agglutination : > 5 μg/ml CRP in serum.

Rheumatoid factor (RA factor test)

Introduction : It is an inflammatory disease of joints and connective tissue commonly associated with serositis, myocarditis, and vasculitis and other disseminated lesions. The synovial membranes of affected joints are swollen and oedematous with dense infiltration of lymphocytes and plasma cells. It is common in adult female. RA factor test is used for diagnosis but it is not specific as RF is also found in other diseases, including the connective tissue disorders.

Principle : It is based on passive agglutination. It is done by using IgG coated latex particles resulting in agglutination.

Procedure :

1. Bring the reagent and samples to room temp.
2. Dilute patient serum 1:5 with normal saline.
3. Add one drop of diluted patient serum and one drop each of negative and positive control on the respective zones of a test plate.
4. Add one drop of latex rheumatoid reagent to each of these drops.
5. Mix well with stirring rods.
6. Look for agglutination within 2 minutes.

Interpretation

Marked agglutination - > 20 IU/ml RF conc. In serum

Antistreptolysin O test (ASO)

Introduction : This test is used to investigate post streptococcal disease. ASO antibody rise is found in 80-85% patient of Rheumatic fever.

Principle : This is based on agglutination test. The reagent contains an aqueous suspension of polystyrene latex particles with sensitized with streptolysin O. If ASO present in patient serum, then it agglutinate.

Procedure :

1. Bring reagents and samples to room temp.
2. Dilute patient serum 1:5 with normal saline.
3. Place one drop each of diluted patient serum and positive and negative control sera on the respective zones of a test tube.
4. Add one drop of latex ASO reagent to each of these drops.
5. Mix well with stirring rod and look for the agglutination for 2 minutes.

Intrepretation

Marked agglutination - > 200 Todd units/ml

Weil-Felix test

Introduction : It is useful in serodiagnosis of rickettsial infection e.g. epidemic typhus, spotted fever and scrub typhus.

Principle : It is based on agglutination test. Antigenic suspension of proteins (OX19, OX2 and OXK strain) are used.

Procedure :

1. Arrange 3 rows each containing 7 clean and dry tubes in a rack (total 21 tubes).
2. Make 1:10 dilution by mixing of 0.1 ml of the patient serum with 0.9 ml of saline.

3. Add 0.4 ml of physiological saline from second to seventh tube with the help of a pipette.
4. Add 0.4 ml of freshly prepared 1:10 dilution of serum into first 2 tubes of each row.
5. After mixing well, go on transferring 0.4 ml from second tube onwards up to sixth tube and from sixth discard 0.4 ml.
6. The final dilution would be 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 from the first to sixth tube respectively, while the last tube would serve as a negative control.
7. Incubate the tubest at 50^0C in an incubator for nearly 4 hours. Then keep at 37^0C overnight.
8. Read the test results next day and observe for granular clumps (agglutination).

Interpretation : A titre more than 1:160 is considered significant.

Immunofluorescence

Certain dyes which absorb rays of oene particular wavelength (U.V. light) and emit rays with a different wavelength (visible light) is known as Fluorescene. Fluorescent dye can be conjugated to antibodies and these labeled antibodies can be used to detect in tissues. Commonly used fluorescent dyes are fluorescein, isothiocyanate and lissamine rhodamin which exhibiting blue green and orange red fluorescence respectively.

Immunofluorescence test is of two types :

1. Direct IFA test
2. Indirect IFA test

1. Direct IFA test :

Principle : This is used for detection of unknown antigen in specimens. The specific antibodies tagged with fluroscent dye (labeled antibodies). If antigen is present then it react with labeled antibodies and fluorescence can be observed under U.V. light of fluorescent microscope.

Specimen

Labelled antibodies

Fluorescent present	Fluorescent absent
Test +ve, antigen present	Test –ve, Antigen not present.

Application :

(1) Detection of antigen of bacteria, virus or other antigen in blood, CSF, urine and tissue.
(2) Diagnosis of rabies virus antigen.

2. Indirect Immunofluorescence test

Principle : This method is used to detect antibodies in specimen. A known antigen is fixed on slide. The unknown antibody (serum) is applied on the slide. The known antigen attaches to antibody (if present), then fluorescin-tagged antibody added to human globulin. Then check the reaction under fluorescent microscope.

Applications :

Detection of antibody to any antigen in serum.