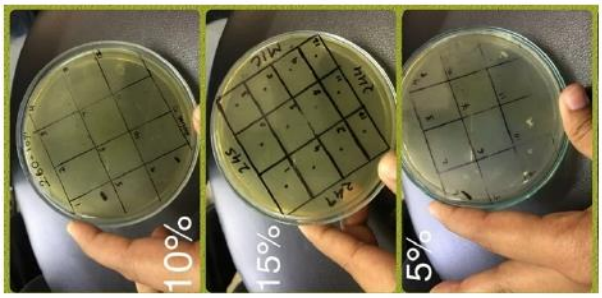
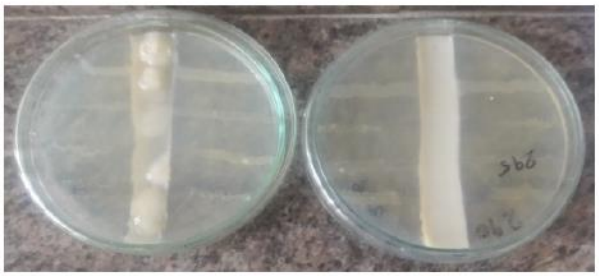
KISHINCHAND CHELLARAM COLLEGE

SOP's of Microbiology Techniques

Under DBT STAR College Scheme





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STUDY OF MOTILITY OF BACTERIA USING TETRATHIOZOLIUM DYE

Aim: To study motility of bacterial cells

Principle:

There are a variety of ways to determine motility of a bacterium—biochemical tests as well as microscopic analysis. If fresh culture of bacteria is available, microscopy is the most accurate way to determine bacterial motility and **'**Hanging drop method**'** is a commonly used microscopic technique. Motile bacteria move about with structures called flagella (a few exceptional bacteria move with the help of axial filaments, which cannot be seen in the microscope). In semi-solid agar media, motile bacteria 'swarm' and give a diffuse spreading growth. The visualization is enhanced by the addition of tetrazolium to the test medium. The tetrazolium is a colorless salt which becomes red when reduced, occurring as a result of bacterial metabolism. Motile organisms show a diffused growth thourough the medium, which can be visulaised, while non motile organism show growth only at the point of inoculation.

Requirements:

Medium: Sterile Motility agar containing tetrathiozolium dye.

Culture: 18- 24 hour given test culture, Positive control– *E.coli* and Negative control - *Staphylococcus aureus*

Miscellaneous: St. Inoculating needle

Procedure:

1. Inoculate the tube using a sterile inoculating needle and stab the centre of the medium approximately half way down the length of the tube. Do not stab thourough to the bottom of the tube.
2. Incubate at 37°C overnight.
3. Incubate an uninoculated tube as a control along with positive and negative control.

Composition of Motility agar:

Pancreatic Digest of Gelatin	10.0 g	Tetrathiozolium dye	0.05 g
Sodium Chloride	5.0 g	Distilled water	1 litre
Beef Extract	3.0 g	pH	7.3 ± 0.2
Agar	3.5 g		

Reference :

1) Aygan, Ashabil & Arikan, Burhan.; Mini Review An Overview on Bacterial Motility Detection; 2019.

STUDY ANTIBACTERIAL SPECTRUM OF INSOLUBLE SULPHA DRUG BY DITCH PLATE METHOD

Aim: To check antimicrobial activity of sulpha (partially soluble) drug.

Principle:

Ditch plate method is the method chosen to test the anti-bacterial activity of compounds. It is a preliminary method to screen the anti-microbial potential of compounds / drugs, which are insoluble or partially soluble in the aqueous phase. In this method, the test compound is seeded in an agar plate and the test organisms are streaked across to test the inhibition of the growth as a marker of anti-microbial activity. This method is suitable when a large number of organisms are to be tested against one antibiotic.

Requirements:

Medium: Sterile Mueller Hinton agar plate -1

Sterile Molten Mueller Hinton agar – 7ml -1

Culture: 18- 24 hour given test culture

Reagents: Test sulpha Drug.

Procedure:

1. A ditch (15 mm x 70 mm) is cut into a sterile MH agar plate.
2. The test drug / compound is added to 7 ml molten MH agar butt at 40°C and this mixture is poured into the ditch and allowed to solidify.
3. The ditch should be made in level with the rest of the agar by pouring the mixture. The different bacterial cultures are streaked perpendicular to the ditch using nichourome wire loop.
4. The plate is then incubated at 37°C for 24 hours.
5. The results are observed as inhibition of bacterial growth on the ditch as well as adjacent to the ditch.

Reference :

1) W. G. Rice, A. Mercedes Lonergan, Ditch-Plate Method for Testing Bacterial Resistance to Antibiotics*, *American Journal of Clinical Pathology*, Volume 20, Issue 1_ts, January 1950, Pages 68–70,

DETERMINATION OF MIC BY AGAR DILUTION METHOD

Aim: To determine the MIC of antimicrobial agent by agar dilution method

Principle:

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against other methods, such as disk diffusion. In clinical laboratories they are used to establish the susceptibility of organisms that give equivocal results in disk tests, especially for organisms where disk tests may be unreliable, and when a more accurate result is required for clinical management. In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in microplate wells of broth (broth microdilution), containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC.

The agar dilution method for determining antimicrobial susceptibility is a well-established technique.In the agar dilution method, different concentrations of antimicrobial agent, are added to non-selective agar medium before solidification. The test microorganisms are added to the plates in different spots. There are several advantages of the agar dilution method including capacity to test different strains at once, easy detection of contamination and capacity to test opaque materials.

Requirements:

Medium:St. Mueller Hinton agar plate with different concentration of antimicrobial agent

Culture: 18- 24 hour culture suspension of the test organism

Procedure:

1. Add appropriate dilutions of antimicrobial solution to molten test agars
2. Mix the agar and antimicrobial solution thoroughly and pour into Petri dishes
3. Allow the agar to solidify at room temperature, make certain that the agar surface is dry before inoculating the plates
4. Cultures adjusted to the 0.5 McFarland standard contain approximately 1 to 2×10^8 CFU/mL with most species, and the final inoculum required is 10^4CFU per spot of 5 to 8 mm in diameter.

5. Apply an aliquot of each inoculum to the agar surface. Appropriate dilution of the inoculum suspension should be made depending on the volume of inoculum delivery so as to obtain a final concentration of 10^4 CFU/spot
6. Inoculate a growth-control plate (no antimicrobial agent) first and then, starting with the lowest concentration, inoculate the plates containing the different antimicrobial concentrations. Inoculate a second growth control plate last to ensure there was no contamination or significant antimicrobial carryover during the inoculation.
7. Streak a sample of each inoculum on a suitable nonselective agar plate and incubate overnight to detect mixed cultures
8. Allow the inoculated plates to stand at room temperature until the moisture in the inoculum spots has been absorbed into the agar, i.e., until the spots are dry, but no more than 30 minutes. Invert the plates and incubate at 35±2°C for 16 to 20 hours

For Determining Agar Dilution End Points:

1. Place the plates on a dark, non-reflecting surface to determine the end points. Record the MIC as the lowest concentration of antimicrobial agent that completely inhibits growth, disregarding a single colony or a faint haze caused by the inoculum.
2. (NOTE: If two or more colonies persist in concentrations of the agent beyond an obvious end point, or if there is no growth at lower concentrations but growth at higher concentrations, check the culture purity and repeat the test if required.)

Reference :

1) CLSI document – 'Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition' January 2012 M07-A9 Vol. 32 No. 2 Replaces M07-A8 Vol. 29 No. 2

ESTIMATE THE MINIMUM BACTERICIDAL CONCENTRATION OF VARIOUS DISINFECTANTS

Aim: To estimate the MBC of various commercial disinfectants.

Principle:

Minimum inhibitory concentrations (MICs) are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials. The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a bacterium over a fixed, somewhat extended period, such as 18 hours or 24 hours, under a specific set of conditions. It can be determined from the broth dilution of MIC tests by sub-culturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction such as ≥99.9%. The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antimicrobial agent that greatly inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent resulting in microbial death. In other words, if a MIC shows inhibition, plating the bacteria onto agar might still result in organism proliferation because the antimicrobial did not cause death. Antibacterial agents are considered bactericidal if the MBC is no more than four times the MIC.

Requirements:

Media: St. Mueller Hinton broth (MH)- 50 ml

St Mueller Hinton Agar (MH) plates- 2

Glassware: St. Dilution tubes- 10

St. 10 ml pipette-2

St. 1ml pipette-1

Sample: Various commercial disinfectants like Sunny floor cleaner, Lizol, Dettol, Savlon etc.

Culture: Growth method or direct colony suspension, equivalent to a 0.5 McFarland standard culture suspension of *E.coli* and *S. aureus*

Procedure:

1. Add 2ml of MH broth to all tubes.
2. Add 2ml of disinfectant to the first tube. This is 1:2 dilution.
3. Transfer 2 ml from the first tube to the second tube and mix well.

4. This process is repeated to give serial dilution of the disinfectant. (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1: 256, 1:512, the dilutions can be further increased depending on pilot study).
5. Do not add disinfectant to the last tube which serves as the positive control and a tube with only disinfectant but lacking the culture will serve as negative contol.
6. Add 0.1ml of the culture suspension to all tubes and incubate at 37°C overnight.
7. After incubation, note the lowest concentration inhibiting growth of the organisms and record this as the MIC.
8. Spot inoculate all the tubes showing no visible growth (incase of opaque solutions; spot all the tubes) on MH agar plates and incubate at 37°C.
9. Post incubation check for growth. The first dilution not showing growth is taken as the MBC.

References:

1) Taylor. P.C, Schoenknecht. F. D, Sherris. J. C, Linner. E. C; Determination of minimum bactericidal concentrations of oxacillin for Staphylococcus aureus: influence and significance of technical factors; Antimicrobial Agents And Chemotherapy, p. 142-150, 1983.

ANALYSIS OF SYNERGISTIC ACTIVITY OF DRUGS

Aim: To analyse the synergistic acticity of two drugs using the paper strip method

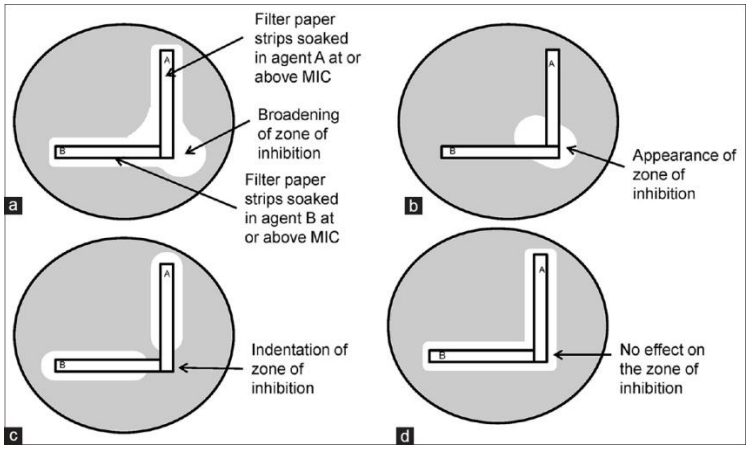
Principle:

Antimicrobial resistance seriously thoureatened human health. Combination therapy is generally an effective strategy to fight resistance in the following ways:

1) Treatment with two drugs against which the infecting bacterium is sensitive prevents outgrowth of mutant bacteria resistant to either drug.
2) Two drugs may be given because they are strongly synergistic in their bactericidal action being strongly cidal in combination though not completely cidal when used individually.

Synergy testing methods have been used to assess the interaction of antibiotic combinations in vitro. The testing may show the following results:

a) Synergistic effect- The drugs are more effective when used in combination compared to when used individually.
b) Additive effect: the sum of the individual effect is equal to the ffect observed in combination.
c) Antagonistic effect: Drugs when used in combination are less efctive compared to when used individually.
d) Indifferent effect: The overall inhibitory effect is insignificantly different from the sum of individual effect;



Requirements:

Media: St Mueller Hinton Agar (MH) plates- 2

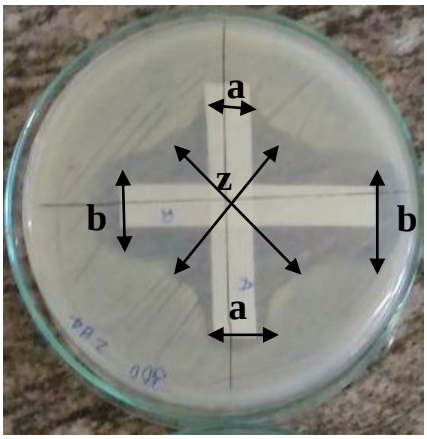
Sample: Antibiotic solution to be tested- 2

Culture: Overnight broth culture of *E.coli, S.aureus.*

Miscellaneous: Sterile and dry Whatmann filter paper No 1 strips(8mm *6cm)- 4
Sterile cotton swab- 2

Procedure:

1. Inoculate the test culture on the MH agar plate using a sterile cotton swab.
2. Leave the plate at Room temperature for 5 mins for culture to absorb.
3. Using sterile forceps, dip one filter paper strip into one of the antimicrobial solution, leave for 10 secs and remove drawing the strip against the side of the tube to remove excess liquid.
4. Carefully place the soaked strip onto the agar surface, in trhe center of the plate.
5. Press it slightly to ensure that the whole strip is in good contact with the surface.
6. Repeat this operation with the second solution placing the strip at right angles to the first strip so as to form a cross.
7. Incubate the plate at 37°C for 24 hours.
8. Observe for sign of synergy by comparing the extent of inhibition of microbial growth surrounding the intersections of the paper strips.



References:

1) Laishouram S, Pragasam AK, Bakthavatchalam YD, Veeraraghavan B. An update on technical, interpretative and clinical relevance of antimicrobial synergy testing methodologies. Indian J Med Microbiol 2017;35:445-68
2) Doern. C. D; When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing; Journal of Clinical Microbiology; 2014; Volume 52 Number 12, p. 4124 – 4128.

BIOAUTOGRAPHY FOR THE DETECTION OF VITAMIN B12

Aim: To separate vitamin B12 from a mixture and identify it by bioautography

Principle:

Bioautography is an analytic technique in which organic compounds are separated by chromatography and identified by studying their effects on microorganisms. It was first used to identify members of the B6 group, vitamin pyridoxine etc. and later vitamin b12. The procedure depends on the chromatographic separation of a mixture of growth factors after which the position of these factors on the paper strip are revealed by the organisms capable of using one of the several of these growth factors.

Requirements:

Media: Sterile molten Harrison et.al. agar butt- 01

Culture: 18hour old culture suspension of E. coli 113-3 Davis (Vitamin B12 auxotroph) density adjusted to 0.1 O.D. at 540nm.

Reagents: vitamin mixture to be assayed, Standard vitamin B12.

Glassware: St. empty petri plate, St. 1ml pipette, chromatography chamber saturated with the solvent system(butanol- 9.4 ml, acetic acid- 17ml, 0.6 M KH_2PO_4- 13 ml, Methanol- 10 ml) saturated overnight.

Miscellaneous: Whatmann filter paper no. 6- 8cm X 6cm

Procedure:

1. Inoculate 0.3 ml of the E.coli 113- 3 D culture in the molten agar butt and pour it into an empty petri-plate.
2. Spot the standard and the assay mixture on the chromatography paper and allow it to run.
3. After removing from the chamber, mark the solvent front.
4. Cut it into appropriate size and place it on the assay medium.
5. Incubate at 37°C overnight.
6. The portion of the chromatogram containing B12 will show the growth of the test culture.
7. Mark the distance travelled by Vitamin B12 from this. Calculate the Retention factor value (The ratio of the distance from the center of the spot for a given mixture component to the distance traveled by the mobile phase, also known as the solvent front) and compare it with the standard Vitamin B1.

References:

1) Ueta. K, Nishioka. M, Yabuta.Y, Watanabe. F; TLC-Bioautography Analysis Of Vitamin B12 Compound From The Short-Necked Clam (Ruditapes Philippinarum) Extract Used As A Flavoring; Journal of Liquid Chromatography & Related Technologies ; Volume 33, Issue 7-8; 2010.

APPLICATION OF ROTARY VACUUM EVAPORATOR FOR DRYING OF AQUEOUS BEET ROOT EXTRACT

Aim: To obtain a dry extract of Beetroot using a rotary vacuum evaporator.

Principle:

Natural products generally consists of secondary metabolites which could have numerous applications, hence obtaining the dried extract is of great significance. After the extraction procedure, a quick and effective separation of substances thorough evaporation is essential. The Rotary Evaporator is a tool which puts the separable substance under vacuum and heats evenly thourough a spinning motion, causing one component to evaporate and leaving the other component behind. A typical rotary evaporator has a water bath that can be heated in either a metal container or crystallization dish. The solvent is removed under vacuum, is trapped by a condenser and is collected for easy reuse or disposal. The fact that a vacuum is usually applied to the assembly means that the boiling points of the solvents are going to be significantly lower than at ambient pressure. Since the flask is rotated during the evaporation process, the surface area is larger than normal which increases the evaporation rate significantly. The solvent is collected in a flask and can properly be disposed off afterwards.

Aqueous Beet root extract was prepared by maceration technique wherein chopped pieces of 100 gm beet root were soaked in 500 ml water for 48 hours. The extract obtained was filtered and used further for drying by using rotary evaporator.

Procedure:

1. Remove the round bottom flask from the base of the condenser, and place directly above the hot bath. Inspect the flask to ensure it is clean.
2. Load your sample into the cleaned round bottom flask. Attach the round bottom flask to the condenser. If need be, use vacuum grease to create a vacuum seal between the condenser and the round bottom flask. Be sure to use a clamp to secure the round bottom flask to the condenser.
3. Inspect the collection flask which is generally located to the left of the hot bath, and below the condenser. Make sure the flask is clean before use. Make sure the tubing is securely connected to the back of the condenser.

4. The condenser should be filled with water, if not already full. Make sure the vacuum pump located next to the rotary evaporator is connected to the condenser and the release valve at the top of the condenser is turned to the closed position. Fill the hot water bath upto $3/4^{th}$ with water, for the round bottom flask to fit in.
5. The dial on the motor is used for speed control of the flask rotation. A typical rotavap uses a variable speed induction motor that spins at 0-220 rpm and provides high constant torque.
6. The aspirator vacuum is turned on. On most models, the vacuum on/off control is managed by turning a stopcock at the top of the condenser. This stopcock is later also used to vent the setup after the solvent is removed.
7. The flask is lowered into the water bath (or the water bath is raised to immerse the flask in the warm water). In most models, a convenient handle (with height locking mechanism) moves the entire condenser/motor/flask assembly up and down. Often the tilt of the condenser assembly can also be adjusted. The water bath temperature should not exceed the boiling point of the solvent. For small amounts of common solvents the bath heater is not needed.
8. The solvent should start collecting on the condenser and drip into the receiving flask. Some solvents (such as diethyl ether or dichloromethane) are so volatile that they will also evaporate from the receiving flask and be discharged down the drain. To prevent this, a cooling bath on the receiver or (in some models) a dry-ice condenser can be used. In addition, an additional trap (with dry-ice or liquid nitrogen) can be placed between the vacuum source and the condenser unit. This is particularly important if a membrane pump is used as vacuum source.
9. If the separation was successful, the higher boiling point substance will be left in the original round bottom flask, and the lower boiling point substance will be collected in the collection flask.
10. Once all the solvent evaporated (or whatever is desired at this point), the vacuum is released. The flask is raised out of the water bath and the spinning is discontinued. Remove your product from the round bottom flask and clean the flask.

    - ❖ Please note – There could be variation in the set up depending on the model, above is a general description.
    - ❖ Do not leave the rotary evaporator unattended until the pressure has equilibrated inside the flask.

Reference:

1) Standard Operating Procedure Rotary Evaporator in the P.O.W.E.R. Laboratory Elliot Sepos, April 2012.

USE OF UV-VISIBLE SPECTROPHOTOMETER TO DETECT THE PRESENCE OF NANOPARTICLES

Aim: To Use UV-visible spectrophotometer and detect the presence of nanoparticles

Principle:

The characterization of Nano materials is important for understanding their properties and applications. Nanoparticles can be characterized by UV - Visible Spectroscopy. Ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometer (UV- Vis) involves the spectroscopy of photons in the UV-Visible region. It uses light in the visible and adjacent near ultraviolet (UV) and near infrared (NIR) ranges. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. UV-Vis Spectrophotometers are mainly used to measure transmission or absorption in liquids and transparent or opaque solids. It does so by sending a beam of light thourough the sample and then monitoring the remaining light in a detector. In the case of a UV-Vis spectrophotometer the light is in the wavelength of 800 - 200nm, probing electronic transitions in the sample. A spectrophotometer thus records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum is presented as a graph of absorbance versus wavelength. Nanoparticles of different chemical nature absorb light at characteristic wavelengths which can be used to determine the presence of nanoparticles in the prepared extracts. For example silver nanoparticles specially show a peak between 380-420nm which confirms the presence of nanoparticles in the prepared extracts using UV-Visible spectroscopy.

Requirements:

Reagents: Nanoparticle preparation (Silver nanoparticles)

Miscellaneous: UV-visible spectrophotometer

Clean and dry cuvettes

Filter paper

Procedure:

1. Turn on the instrument and let it warm up for at least 5-10 minutes.

2. Select the wavelength with the dial next to the sample compartment.

3. Adjust the filter wheel so that the filter corresponding to the wavelength selected is in place.

4. Adjust the mode to display Absorbance and % Transmittance simultaneously. Adjust the mode by pressing the mode button on the face of the instrument.

5. With the sample compartment closed and empty, adjust the % Transmittance (zero percent transmission of light) to read 0% T using the left front dial.

6. Place a clean (no fingerprints), dry cuvette filled approximately 3/4 full of the blank sample (solvent only) in the sample compartment. Close the sample compartment. Adjust the % Transmittance to read 100% T (100 percent transmission of light) using the right front dial.

7. Remove the blank cuvette and place the cuvette containing the silver nanoparticle sample in the sample compartment. Close the sample compartment. Read and record the value registered on the meter.

8. Plot a graph of absorbance versus wavelength and determine absorption maxima which would be characteristic of the nanoparticles prepared.

Note: Every time the wavelength of light is changed, the instrument must be recalibrated to read 0% T and 100% T with the blank. Adjust the filter wheel if necessary. Repeat steps 2-7.

References:

1) Princy. G; A review on Green Synthesis of Silver Nanoparticles; Asian Journal of Pharmaceutical and Clinical Research: Vol 6, Suppl 1, 2013.
2) Zhang. X.F, Liu. Z.G, Shen. W, Gurunathan. S; Silver Nanoparticles: Synthesis, Characterization, Properties, Applications, and Therapeutic Approaches; International Journal of Molecular Sciences; 17, 1534; 2016.

BLOOD TRANSFUSION COMPATIBILITY TEST

Aim: To perform the compatibility test (cross matching) by saline tube method.

Principle:

The final decision of whether donor blood is safe to transfuse to a patient does not depend solely on the ABO grouping and Rho (D) typing but it depends on whether the two bloods are compatible i.e. whether there is hemolysis or agglutination when they are mixed together.

Under most circumstances, if grouping and typing tests have been performed accurately and if the donor blood of the same group and type has been selected for transfusion, they will be compatible. However, there are occasions when (1) the donor may have antibodies in his serum or (2) the patient may have antibodies in his serum or (3) there may have been a mistake in performing, reading, recording of the blood grouping and Rho (D) typing results. Considering all these possibilities, a compatible test is essential before all transfusions.

Test for compatibility is performed in the blood bank laboratory. If there is any incompatibility, the transfusion can be prevented. The procedure used to determine compatibility of donor and recipient's blood is called the cross match. The procedure is performed in two parts:

1) Major cross match i.e. the donor's cells are mixed with the patient's serum
2) Minor cross match i.e. the patient's cells are mixed with the donor's serum

If there is any hemolysis or agglutination when the cross match is performed, the bloods of the donor and the recipient are not compatible. If no agglutination or hemolysis occurs then the two are called compatible. The donor blood can be transfused to the patient.

Requirements:

Glassware: Small test tubes (Kahn Tubes)- 2

Pasteur pipette

Miscellaneous: Donor 5% RBC suspension and Serum

Patient's 5% RBC suspension and serum.

Procedure:

1. Take two Kahn tubes and label one of them as P and the other as D.
   Major cross match: In tube P add two drops of patient's serum and one drop of Donor's RBC suspension.
   Minor cross match: In tube D add two drops of Donor's serum and one drop of patient's RBC suspension.
2. Mix the contents of both the tubes gently and keep at room temperature for 30 minutes.
3. Centrifuge at 1500rpm for one minute.
4. Examine for agglutination both macroscopically and microscopically.