Experiment 5

Junqi Zhang

Department of Medical Microbiology & Parasitology
Shanghai Medical College, Fudan University

Experiment 5

1. Widal test
2. Ziehl Neelsen Stain (Acid-Fast stain)
3. Identification of Predominant isolates in the Gastrointestinal Tract
   - -Gram Stain
   - -Broth Inoculation

Widal test

- Patients' suffering from enteric fever would possess antibodies (agglutinins) in their sera
- Widal test is a tube agglutination test for the detection of agglutinins (antibodies) for H and O antigen for salmonella in patients with enteric fever
- The test is named after Georges Fernand Isidore Widal, a French physician and bacteriologist, born March 9, 1862, Algeria; died January14, 1929, Paris
- The result will be used as an indirect signal of the infection

3.Widal test: Tube Dilution Agglutination Test

Principle:

Patients' suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed, coloured Salmonella antigens in a tube agglutination test

Widal test is named after Georges Fernand Isidore Widal, a French physician and bacteriologist, born March 9, 1862, Algeria; died January14, 1929, Paris

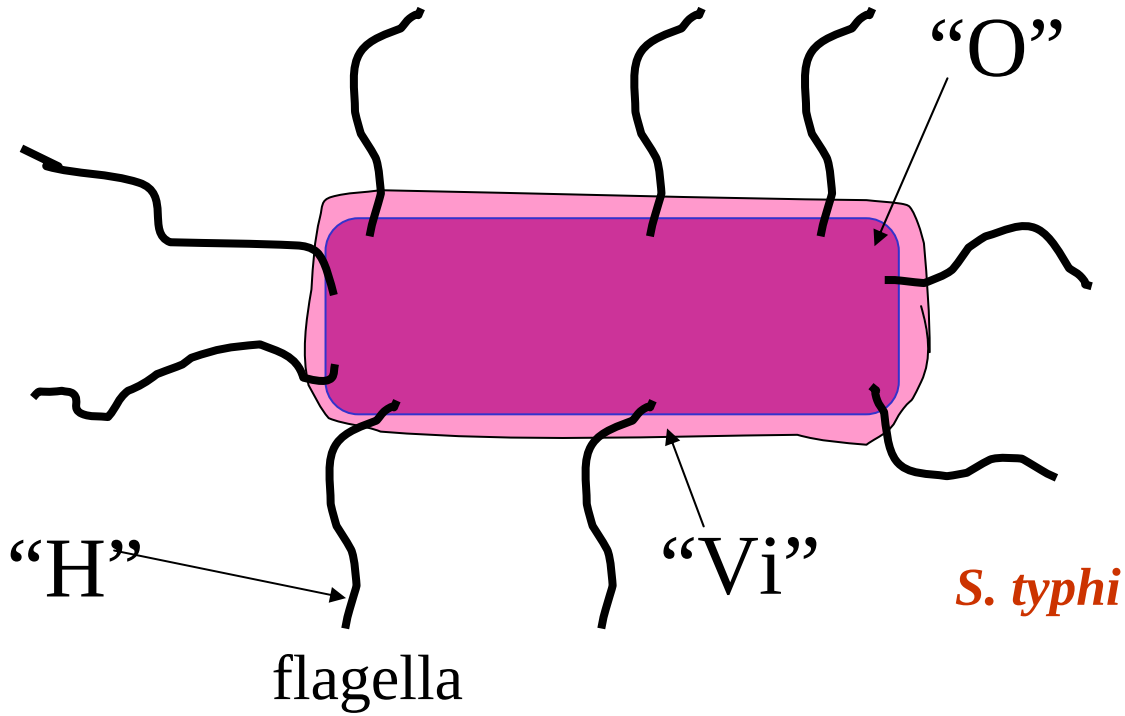
Widal test

Serum agglutinins(antibodies) rise sharply during the **second and third weeks** of *Salmonella* Typhi infection

This test demonstrates the presence of somatic (O) and flagellar (H) agglutinins(antibodies) to Salmonella typhi in the patient's serum using suspensions of O and H antigens. Antigens of S. paratyphi A, S. paratyphi B, S. paratyphi C are included in most commercial kits

The result will be used as an indirect signal of the infection

Antigenic Structure



S. typhi

Preparation of antigens

- *Salmonella* typhi is used to prepare *S.* typhi O and *S.* typhi H antigens.
- O antigens for *S.* paratyphi A and *S.*paratyphi B are not taken as they cross-react with *S.*typhi O antigen.
- H antigen suspension is prepared by treating overnight broth culture or saline suspension of Salmonella with 0.1% formalin.
- For preparing O antigen suspension, Salmonella are grown on phenol agar (1:800) to inhibit flagella. The growth is then emulsified in small volume of saline, mixed with 20 times its volume of alcohol, heated at 40 ℃ 50 ℃ for 30 minutes and centrifuged
- The antigens are treated with chloroform (preservative) and appropriate dyes are added for easy identification of antigens.

Widal test

Procedure

1.Array forty test tubes into 4 rows, 10 tubes per row. Label the dilution ratio in every tube.

2.Add 0.5ml saline into the first tube, and 0.5ml saline into other tubes, in every row.

3.Add the diluted serum 0.5ml(the dilution is 1:5) to the first tube of first row, and beat upon three times. Then transfer 0.5ml to the second tube. Repeat these one by one till the ninth tube, and then discard 0.5 ml from the ninth tube. Only add 0.5ml saline in the tenth tube to compare.

4.Do the same in the next 3 rows. Then the dilution ratio of every row in turn is 1:10, 1:20, 1:40, 1:80, 1:160 till to 1:2560. The tenth tube is comparative tube.

Widal test

Procedure

5.Add antigen as follow:

a)add 0.5ml typhoid “H” antigen to every tube of the first row.

b)add 0.5ml typhoid “O” antigen to every tube of the second row.

c)add 0.5ml A paratyphoid antigen to every tube of the third row.

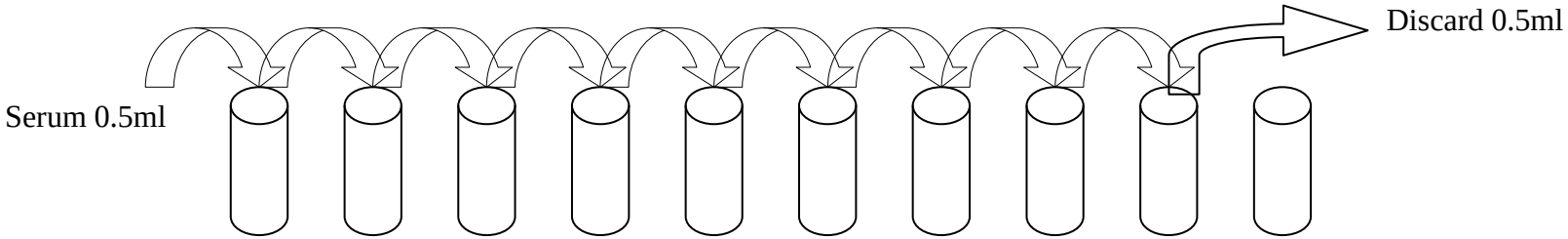
d)add 0.5ml B paratyphoid antigen to every tube of the forth row.

e)At this time, the dilution ratio of every row in turn is 1:20, 1:40, 1:80, 1:160, 1:320 till to 1:5120. The tenth tube is comparative tube .

6.Incubate at 37℃ for 18-24 hours. Then observe and record the results.

7.Firstly observe the comparative tube. There should not be any agglutination reaction. Secondly observe others tubes one by one. The highest serum dilution ratio of the tube that can be seen the obvious agglutination is the agglutination titer of this serum to specific antigen(next day).

Order	1	2	3	4	5	6	7	8	9	10
Saline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Serum dilution	1:10	1:20	1:40	180	1:160	1:320	1:640	1:1280	1:2560	



Final dilution	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	control

Reading

◆In all cases, look first at the control tubes and proceed only if they show satisfactory results. There should be no appreciable sedimentation of the bacteria.

◆Pick up the individual tubes of each row of the patient's specimens and look first at the supernatant.

- When a complete agglutination occurs, practically all the bacteria are removed from the supernatant which appears absolutely clear.
- When the reaction is negative the suspension should look as turbid as the antigen control is.
- The agglutination of O antigen appears as a "matt" or "carpet" at the bottom.
- Agglutination of H antigens appears loose, wooly or cottony. The highest dilution of serum that produces a positive agglutination is taken as titre. The titres for all the antigens are noted. The in-between reactions can be categorized into +, ++, +++.

Broth Inoculation

1. To inoculate a broth culture, hold a labeled broth tube in your left hand and your loop in your right hand using the thumb and first two fingers
2. Flame to sterilize the loop
3. Remove the cap from the broth tube with the little finger of the right hand curling against your palm
4. Flame the opening of the broth tube
5. Touch the loop to the desired colony on the plate and then stir the loop around in the broth
6. Reflame the opening of the tube, return the cap, and place the tube in a test tube rack
7. Flame sterilize the loop.
8. Incubate inoculated broth at the appropriate atmospheric conditions, temperature(37 ℃), and time(16-24hs).
9. Examine broth for any signs of growth including, turbidity with or without gas bubbles, hemolysis (in blood cultures), pellicle formation and precipitate on the bottom of the tube or bottle.

Interpretation of widal test:

While interpreting the test following factors must be considered

1. Timing of test is important, as antibodies begin to arise during **end of first week**. The titres increase during second, third and fourth week after which it gradually declines. **The test may be negative in early part of first week**.
2. Single test is usually of not much value. A rise in titre between two sera specimens is more meaningful than a single test. If the first sample is taken late in the disease, a rise in titre may not be demonstrable. Instead, there may be a fall in titre.
3. Baseline titre of the population must be known before attaching significance to the titres. The antibody levels of individuals in a population of a given area give the baseline titre. A titre of 100 or more for O antigen is considered significant and a titre in excess of 200 for H antigens is considered significant.

Interpretation of widal test:

4.Patients already treated with antibiotics may not show any rise in titre, instead there may be fall in titre. Patients treated with antibiotics in the early stages may not give positive results.

5.Patients who have received vaccines against Salmonella may give false positive reactions. This can be differentiated from true infection by repeating the test after a week. True untreated infection results in rise in titre whereas vaccinated individuals don’t demonstrate any rise in titre.

6.Those individuals, who had suffered from enteric fever in the past, sometimes develop anti-Salmonella antibodies during an unrelated or closely related infection. This is termed anamnestic response and can be differentiated from true infection by lack of any rise in titre on repetition after a week.

7.Antigen suspensions with fimbrial antigens may sometimes give false positive reactions due to sharing of fimbrial antigens by some Enterobacteriaceae members. Antigen suspension must be devoid of fimbrial antigens.

Report for Widal test

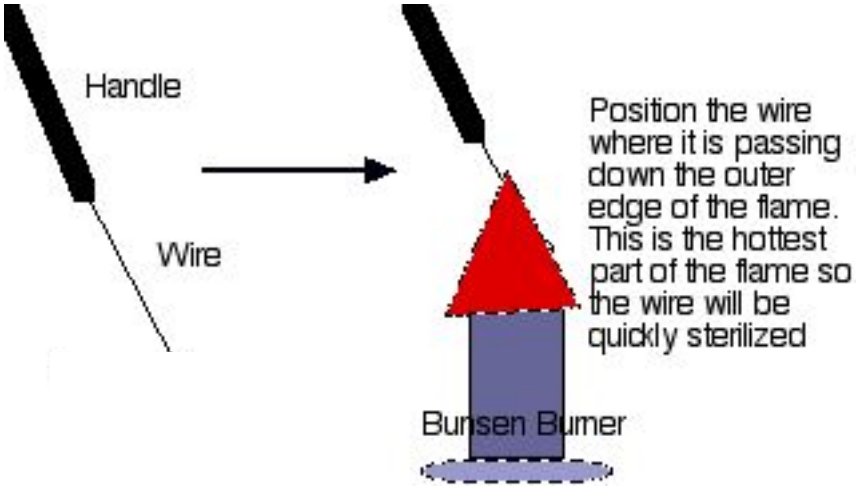
Principle
Result
Interpretation(discuss)

Design an experiment used to isolate and identify *Salmonella* typhi from feces sample

Broth Inoculation

The needle

- Needles are straight wires (no loop) used to pick up bacteria from closely packed colonies or to inoculate in a very defined area.
- We commonly used needles to inoculate semi-soft media.
- The needle is first sterilized by holding it into a flame until the wire glows red . Once the needle has had few moments to **cool**, The needle is then **touched** to the desired colony.
- Do not try to stir the needle around. The bacteria are transferred to the target media by a single stab, straight down and back up with minimal wobble



Questions:

1.Please describe the characteristics of the enteric bacteria?

2.How to use the SELECTIVE media or DIFFERENTIAL media to isolate pathogenic bacteria from the feces samples?

3.Please describe the characteristics of the salmonella antigenic structure ?

1. Identification of Predominant isolates in the Gastrointestinal Tract

Objective:

Help to further understand the characteristics of the Gram-Negative Enteric Bacteria

Table 15-2.Rapid,Presumptive Identification of Gram-Negative Enteric Bacteria

Lactose fermented rapidly
E coli: metallic sheen on differential media; motile; flat, nonviscous colonies
Enterbacter aerogenes: raised colonies, no metallic sheen; often motile; more viscous growth
Klebsiella pneumoniae: very viscous, mucoid growth; nomotile
Lactose fermented slowly
Edwardsiella, *Serratia*, *Citrobacter*, *Arizona*, Providencia, *Erwinia*
Lactose Not fermented
Shigella species: nonmotile; no gas from dextrose
Salmonella species: motile; acid and usually gas from dextrose
Proteus species: ”swarming” on agar; urea rapidly hydrolyzed (smell of ammonia)
Pseudomonas species: soluble pigments, blue-green and fluorescing; sweetish smell

Selective Medium: culture medium that allows the growth of certain types of organisms, while inhibiting the growth of other organisms

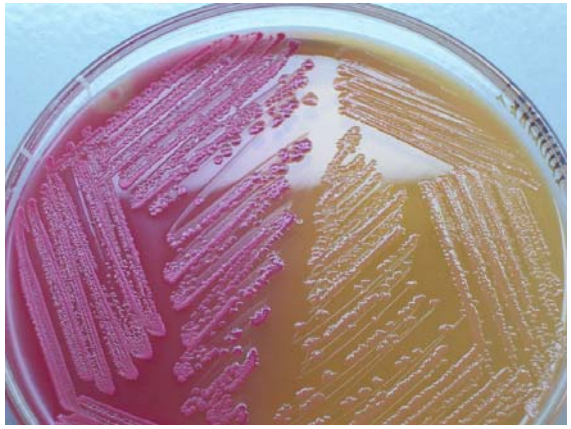
- dyes in the medium (e.g.: crystal violet in MacConkey's) or high salt concentration in the medium (e.g.: 7% salt in MSA) inhibit the growth of unwanted microorganisms

Differential Medium: culture medium that allows one to distinguish between or among different microorganisms based on a difference in colony appearance (color, shape, or growth pattern) on the medium

- dyes in the medium (e.g.: eosin/methylene blue in EMB) or pH **indicators** change the color of the medium as sugars in the medium (e.g.: lactose in EMB & MacConkey's and mannitol in MSA) are fermented to produce acid products

Differential media

- contain special dyes and carbohydrates
  - Lactose fermenting: colored
  - Nonlactose fermenting: nonpigmented



MacConkey's agar

MacConkey's Agar

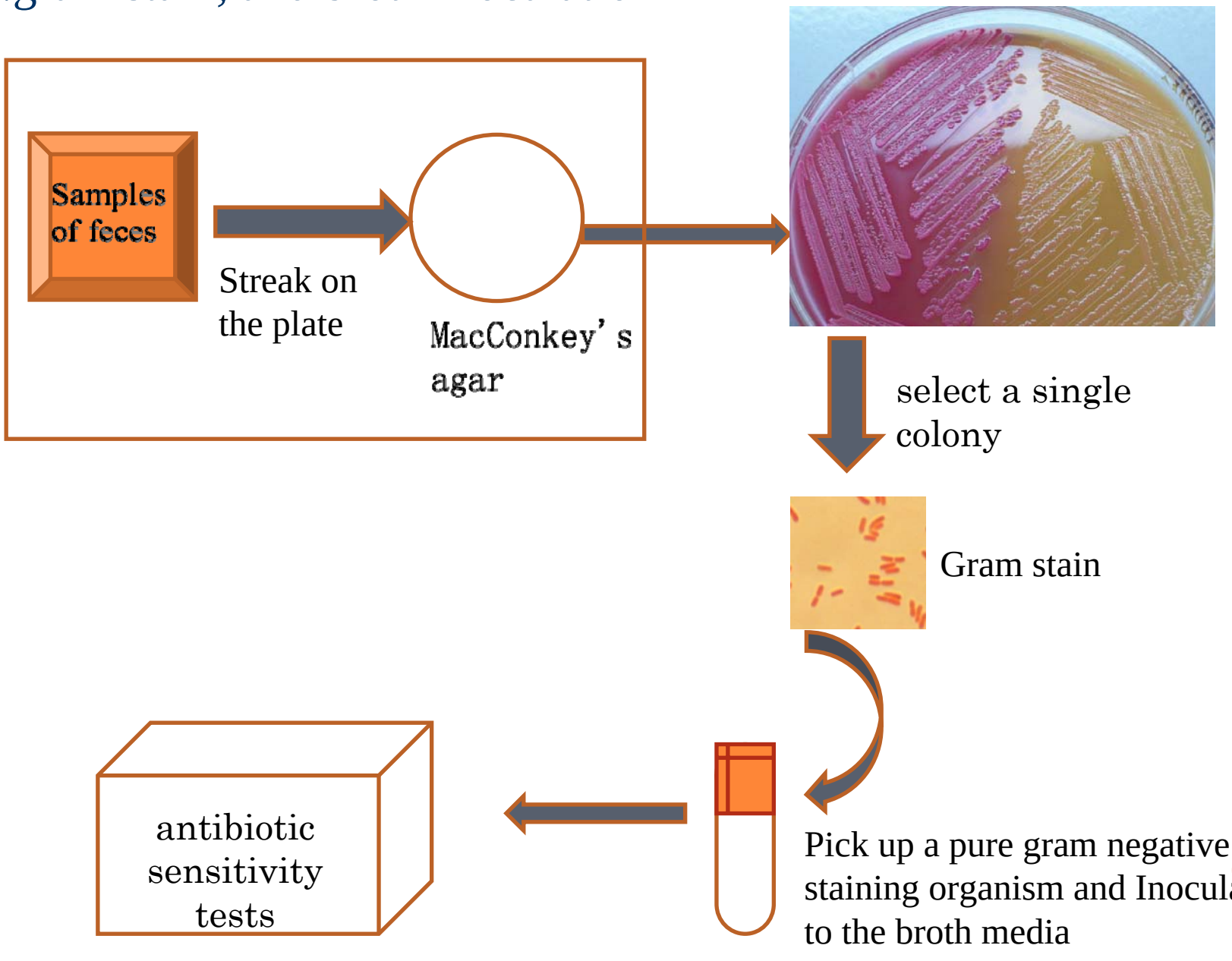
selective for: **gram-negative bacteria**

- growth of gram-positive bacteria (e.g.: *Staphylococcus aureus* in the image below) is inhibited by the crystal violet dye and bile salts in the media

differential for: **lactose fermentation**

- neutral red pH indicator turns red in the presence of acid by-products of lactose fermentation
- gram-negative bacteria *Proteus vulgaris* and *Salmonella typhimurium* grow on MacConkey's agar, but do not ferment lactose (media appears yellow to light pink in color & colonies are colorless; swarming of *Proteus* is inhibited)

2.gram stain, and broth inoculation



2.gram stain, and broth inoculation

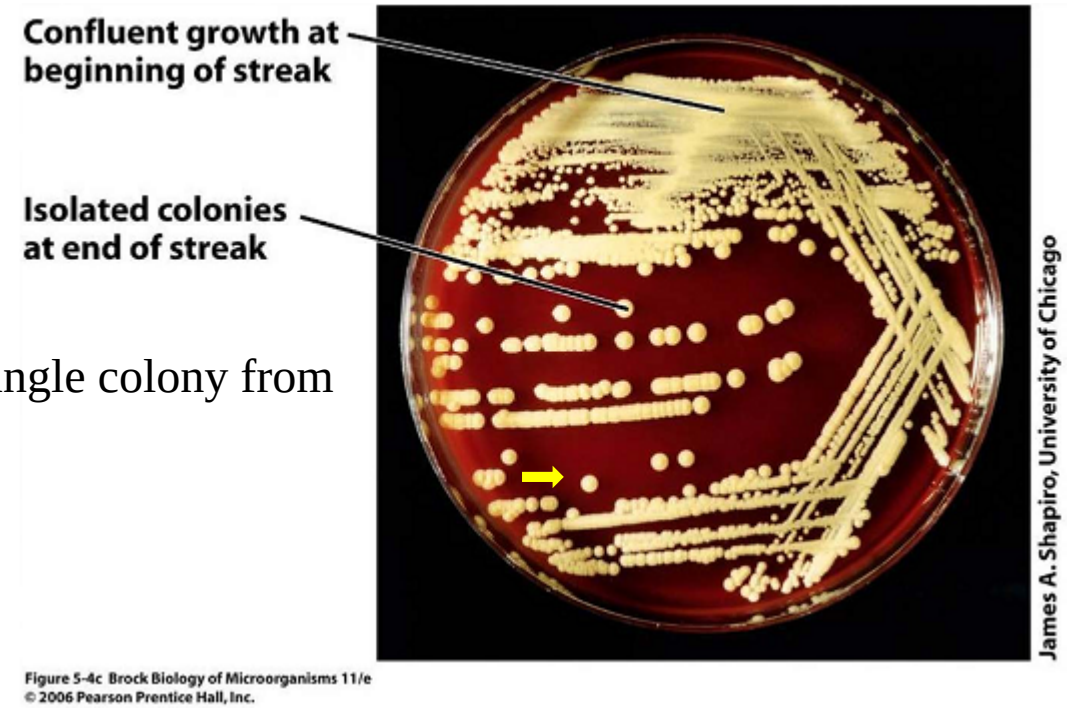


Figure 5-4c Brock Biology of Microorganisms 11/e
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Pick up a single colony from the plate

GRAM STAIN

- Procedure

1. Prepare bacterial smears
2. Gram stain
3. Observe by microscope

PREPARATION OF SMEAR

Bacteria from colonies

- Clean a glass slide and place a small mark slightly off center using a grease pencil
- Using your loop, transfer one small drop of water(about two loop fulls) to the center of the slide, being careful to be close to but not overlapping the grease pencil mark. Do not transfer too much water because these drops will have to air dry
- Sterilize your loop and **touch** a single colony and transfer the bacteria to the water droplet on the slide and mix well. DO NOT scoop up a whole colony; you'll have vastly too much bacteria. If while mixing you see an opaque slurry of bacteria on the slide you have too many bacteria for effective staining

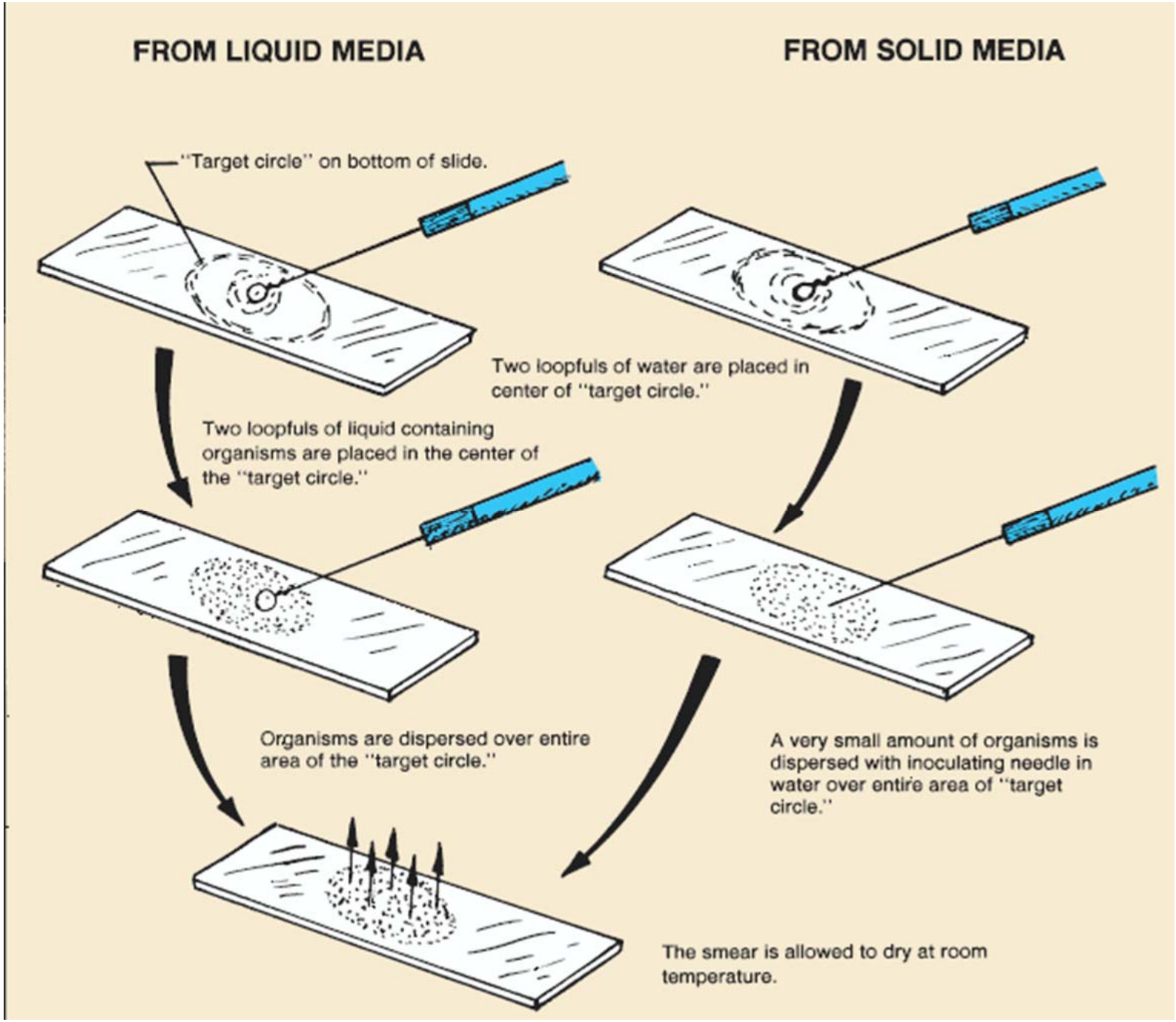
PREPARATION OF SMEAR

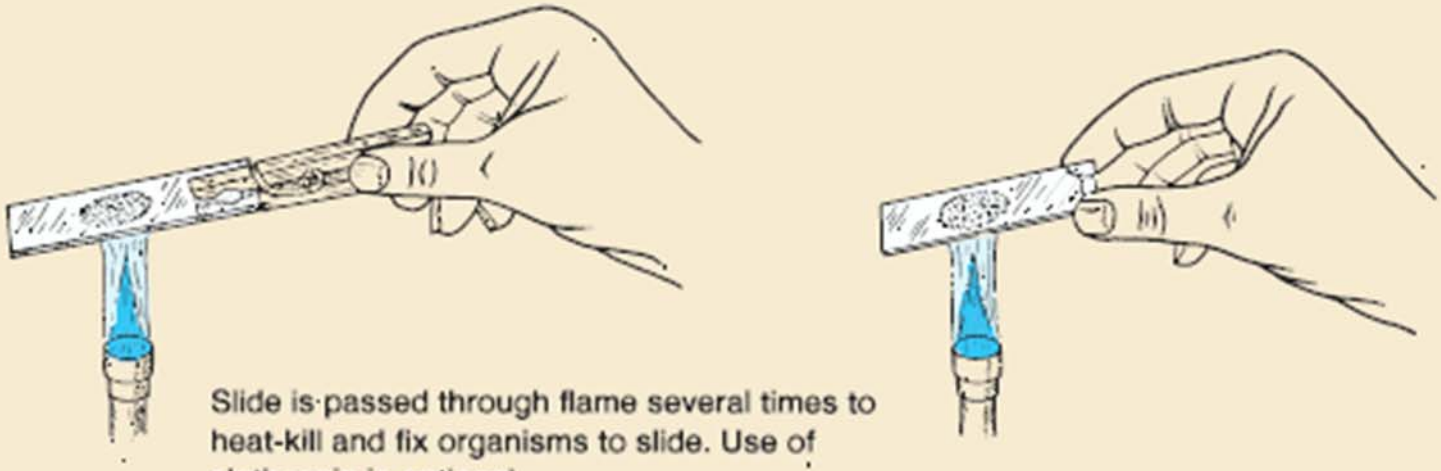
Drying

- Allow the smear to air dry. You CAN NOT heat the sample nor blow on it to hasten drying time because that could force bacteria into the air leading to contamination and possible infection

Heat fixation.

- Holding the slide by one edge, pass it slowly through a bunsen burner flame. Do not move so slowly that the edge of the slide you're holding heats up to uncomfortable levels. This heat fixation step denatures bacterial proteins causing the cells to stick to the slide while also killing the bacteria making them safe for the following steps


FROM LIQUID MEDIA
FROM SOLID MEDIA
"Target circle" on bottom of slide.
Two loopfuls of water are placed in center of "target circle."
Two loopfuls of liquid containing organisms are placed in the center of the "target circle."
Organisms are dispersed over entire area of the "target circle."
A very small amount of organisms is dispersed with inoculating needle in water over entire area of "target circle."
The smear is allowed to dry at room temperature.



Slide is passed through flame several times to heat-kill and fix organisms to slide. Use of clothespin is optional.

Gram stain procedure

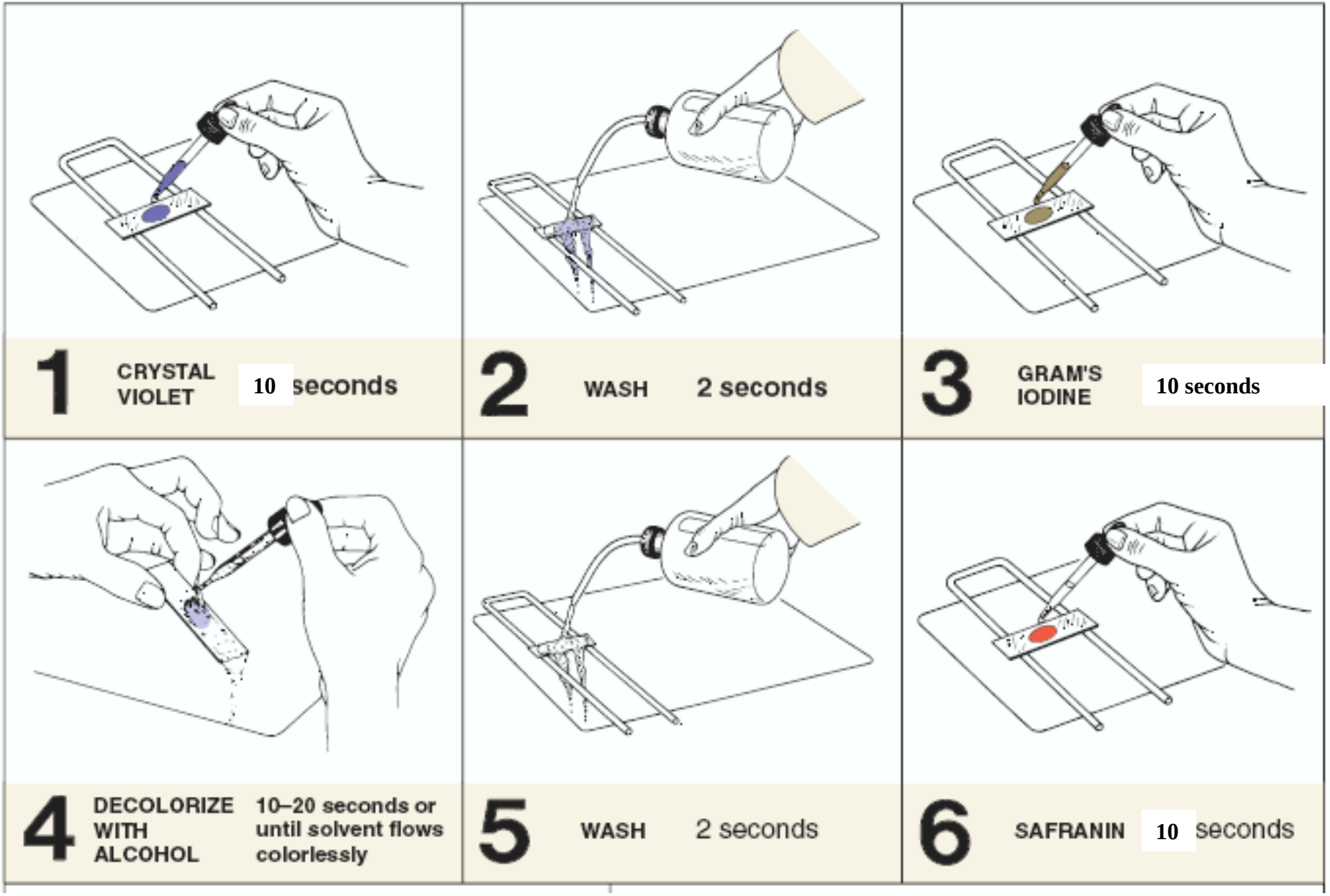
Primary stain : Cover the smear with **crystal violet** and incubate for **10 seconds.** Rinse the dye off with distilled water (dH2O), Drain off excess water

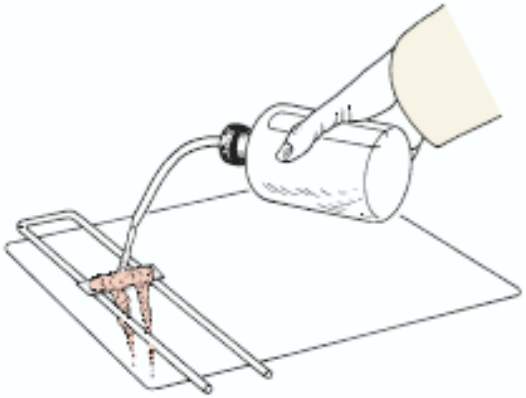
Mordant: Cover the smear with **Gram's iodine** for 10 seconds, rinse the slide with dH2O

Decolorization: Rinse the stain with 95% ethanol for ***10 to 20 seconds,*** Stop action of the alcohol by rinsing the slide with water for a *few seconds*

Counterstain: Cover the bacteria with safrinin for ***10*** seconds. Rinse with dH2O and blot the slide dry with bibulous paper

Observation: Examine the slide under oil immersion


1
CRYSTAL VIOLET
10 seconds
2
WASH
2 seconds
3
GRAM'S IODINE
10 seconds
4
DECOLORIZE WITH ALCOHOL
10–20 seconds or until solvent flows colorlessly
5
WASH
2 seconds
6
SAFRANIN
10 seconds



7 **WASH** 2 seconds

8 **BLOT DRY**