Laboratory Guidance

MICROBIOLOGY DIAGNOSIS
MIC 320

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1st Lab: Human as habitats

Microorganisms that inhabits our body make up our normal microbiota also known as normal flora. The normal microbiota do not harm us, but also in some cases can actually benefit us. Some normal biota protect us against the disease by preventing the over growth of harmful microbes, while other produce useful substance such as vitamin K and some B vitamins. On the other hand, under some circumstances normal microbiota can make us sick or infect people we contact. For example, when some normal microbiota leave their habitat they can cause disease.

Any sites in the body that is accessible to microbes as long as the site has sufficient moisture, and provides nutrients can serve as an excellent habitat for a wide variety of microorganisms. The skin is a prime example and it has a several distinctive habitats for microorganisms. The outer layers of the skin, the epidermis, is too dry for most microorganism. However, microorganisms are commonly found associated with apocrine glands (in underarms, genital regions, nipples, and umbilicus) and sebaceous glands (hair follicles). These areas of our body provide plenty of moisture and nutrients.

Another factor that affected the niche occupied by microbes indigenous to human is their oxygen requirement. It is clear that the large intestine is the home to a large number of anaerobic microbes, but anaerobs are also important members of the normal microbiota of the mouth and skin. One must not forget that certain areas in the mouth and skin are anaerobic.

In this exercise, you will characterize an isolate from the skin in terms of its cellular morphology and tolerance of certain environmental conditions.

Objective

- To learn about and observe microorganisms that make up our normal biota
- To isolate and characterize bacteria from different place on our skin

Materials

Sterile swabs
Tubes with steril water
Nutrient agar plates
Nutrient agar plates with 5% NaCl, 10% NaCl, and 15 % NaCl
Yeast extract glucose broth tubes at pH 3.0, 5.0, 7.0 and 9.0
Thioglycollate tubes
Gram stains reagents
Microscope slides
Incubators at 30°C, 37°C, and 40°C

Procedure

1. Choose two areas of the skin that differ in terms of moisture and degree of exposure to the outside environment.
2. Swab these areas and isolate microorganisms from each site by streaking onto nutrient agar plates. Note: The swabs can be moistened in sterile water.
3. Incubate the plates in incubator at 30℃ for 24 hours.
4. After 24 hours,Stain the bacteria, inoculate the bacterial colony on to nutrient agar with various salt concentration, yeast extract glucose broth tubes and thioglycollate place the plate in incubator at 30℃ for 24 hours.
4. Stain the Inoculate the bacterial colony on nutrient agar plate and incubate in incubator at 37℃ and 40℃ for 24 hours.
5. Observe the characteristic of the bacteria: morphology, gram stain, environmental influences (pH and temperature level) to bacterial growth.

Reference

Hudson BK. Sherwood LK. 1997. Exploration in Microbiology a discovery based approach. Prentice hall: USA.
Tortora GJ. Funke BR. Case CL. 2007. Microbiology an Introduction ninth edition. Pearson Benjamin Cummings: San Fransisco.

2nd Lab : Pathogens of the Urinary tract

The urinary system is composed of organs that regulate the chemical composition and volume of the blood and as a result excrete mostly nitrogenous waste products and water. Since it provides an opening to the outside environment contacts it allows other microorganisms to occupied the urinary system. The urinary system are moist and compared to skin, more supportive of bacterial growth.

Normal urine is sterile, but it may become contaminated with microbiota of the skin near the end of its passage through the urethra. Thus, when we collected urine directly from the urinary bladder has fewer microbial contaminants than voided urine.

The resident flora of the system are those microorganisms that live in close proximity to the urethra. Sometimes these organisms, especially the fecal bacteria, ascend the urinary tract and cause infection. A UTI (Urinary Tract Infection) is primarily one of two types; a cystitis (infection of the bladder) or a pyelonephiritis (infection of the kidneys). More than 90% of UTIs are caused by normal intestinal tract bacteria.

UTIs are casually caused by one predominant bacterium (Table 1). It will colonize the urinary tract and dominate all other potential pathogen, Most urinary tract infection are caused by members by the family Enterobacteriaceae (Table 1.2). From this family, *Eschericia coli* is the predominate pathogen in both uncomplicated and complicated infections (such as patients with structural or neurological abnormalities)

Table 1. Agents of Uncomplicated Urinary Tract Infection

Eschericia coli
Proteus mirabilis
Klebsiella pneumonia
Enterobacter sp.
Enterococcus sp.
Staphylococcus saphrophyticus
Other *Enterobacteriaceae*

Table 1.2 Selected biochemical Reaction of Some Enterobacteriaceae

Organism	Lactose fermentation	Sucrose fermentation	H_2S	Motility	Indole	Ornithine	Citrate	Urea
Eschericia coli	+	+	-	+	+	+	-	-
Klebsiella pneumonia	+	+	-	-	-	-	+	+
Enterobacter aeogenes	+	+	-	+	-	+	+	-
Proteus mirabilis	-	-	+	+	-	+	+/-	+
Salmonella	-	-	+	+	-	+	+	-

most serotypes								
Yersinia enterolitica	-	+	-	-	+/-	+	-	-
Shigella seroproups A,B, &C	-	-	-	-	+/-	-	-	-

Objectives

- To learn the important pathogens of the urinary tract
- To learn the important pathogenic Enterobacteriaceae

Materials

Culture of

Escherichia coli

Klebsiella pneumoniae

MacConkey agar plates (MAC)
Motility-indole-ornithine tubes (MIO)
Urea agar slants
API 20E strips (bioMérieux Vitek, Inc)
Demonstration tubes of all media and bacteria listed in the table
Triple sugar iron (TSI) agar slants
Citrate agar slants
API 20E code book (bioMérieux Vitek, Inc)

Procedure

1. Two unknown bacteria will be given on MacConkey agar plates. Notes: Normally an oxidase test would be performed to verify the Gram-negative rod is an enterobacteriaceae. On the other hand, both of your unknown are from this family of bacteria and an oxidase test cannot be performaed with a colony from MacConkey plate because interfering substances (i.e., crystal violet) may cause false positive reaction
2. Proceed with the identification of each of these pathogens using the traditional tubed media (TSI, MIO, Citrate and Urea) and the API strip.
3. Observe the demonstration of tubes of all the bacteria set up by the lecturer..
4. Record your dailly ohservation in the result sheet.
5. Refer to the API 20E Codebook for identification.

Reference

Hudson BK. Sherwood LK. 1997. Exploration in Microbiology a discovery based approach. Prentice hall: USA.
Tortora GJ. Funke BR. Case CL. 2007. Microbiology an Introduction ninth edition. Pearson Benjamin Cummings: San Fransisco.

2nd Lab RESULT

Student name : _____
ID Number : _____
Date : _____

Day 1 Observation of MAC plate, inoculation of tube media and on API strip

Day 2 Observation of result
TSI:

MIO:

Citrate:

Urea:

Questions :
1. Is the bacteria is an urinary infectious agent ? explain

2. If it is not, why ? explain

3rd Lab : Isolation and Identification of Bacteria from the urinary tract

The urinary tract is normally sterile, but in some case the urine released from it can become contaminated by bacteria that inhabit the distal end of the urethra and the external genitalia. Even so, the number of bacteria in urine is typically low (i.e., ranging from 0 to 10,000 bacteria/ml). This range considered normal.

A gram stain is done on an isolate from urine that numbers in excess of 100,000 bacteria/ ml. If a gram-positive coccus is found, a catalase test wil determine whether is a staphylococcus (positive) positive or streptococcus/Enterococcus (negative). If the culture is catalase negative, bile esculin agar (BEA) will confirm the group D streptococci. This is the only bacteria can tolerate the high bile content of this agar while hydrolyzing esculin, this reaction will yields a dark brown colour.

A gram-negative rod can be tested for oxidase. A positive reaction may indicate *Pseudomonas aeruginosa*. A negative oxidase test is indicative of the enteric bacteria such as *Proteus vulgaris*, *Escherichia coli*, and *Enterobacter aerogenes*. These bacteria can be differentiated by triple sugar iron (TSI) agar, the methyl red test and the indole test.

Objectives

- To know how to isolate bacteria from the urinary tract
- To understand in general how to identify bacteria of the urinary tract

Materials

Tryptic Soy Agar Plates
Inoculating loops
Slides
Hydrogen peroxide (for catalase test)
Gram-stain reagents
Oxidase reagent
Urine sample container
Disposable gloves
Facemask
Towelette
Incubator 35°C

Procedure

First Session: Collection and Inoculation of Urine

1. Wash your hands. Use an anticeptic towelette to clean around the opening of the urethra.
2. Collect a midstream sample of urine in a clean plastic container. Put lid on, and close tightly. Place the container in a plastic bag. Store in the refrigerator if the urine will not be cultured within 1-2 hours.
   Caution : This step should be done wearing gloves under a safetyhood or behind a plastic shield placed on the countertop.

3. When ready to culture, mix the urine, and then dip a 5 µl inoculating loop into the fluid. Streak the 5 µl of urine obtained onto a tryptic soy agar (TSA) plate. Repeat this process for a second plate. Label the plate number 1 and 2. Discard the remaining urine in the restroom, and then deposit gloves, urine container, bag, and loop in a biohazard bag or similar waste container.
4. Place both plates in a 35℃ incubator.

Second session : Isolation and Identification of Urinary Tract Bacteria

1. After 48-72 hours, examine your culture plates. Count the number of bacterial colonies on each plates, average this number, and then use this average to calculate the number of bacteria per milliliter (To do this, multiply the average number of bacterial colonies by a factor of 200; 5 µl x 200= 1000µl; 1ml ≈1000µl).
2. If there is any of the bacteria on the plate exceeded 100,000 per milliliter or urine, you may have an organism that caused UTI (urinary tract infection).
3. Do a gram stain of your cultures. Record their morphology and gram reaction. If the bacteria is gram positive cocci, do a catalase test. If the result is catalase-positive, the culture is *Staphylocccus*. If catalase-negative, the culture is other than *Staphylocccus*. For gram-negative rods, do an oxidative test. If oxidase-positive, the culture is *Pseudomonas*. If oxidase-negative it other than *Pseudomonas*.

Reference

Prescott, LM. 2005. Study Guide for Use With Microbiology. McGraw-Hill: Montana State University.

3rd Lab RESULT

Student name : _____
ID Number : _____
Date : _____

Observation of TSA plate (after 48-72 hours)

1. How many colonies per ml ?

2. What is the Gram-stain result? (explain the catalase test result)

3. What is the bacteria in the urine sample?

4th Lab: Assessing Antibiotic Effectiveness: Kirby Bauer method

Antibiotic have become a standard method used by physician to treat bacterial disease. The first antibiotic was founded by Alexander fleming. It was penicillin that produced by his molds over 60 years ago.

Since the discovery of penicillin, many other useful antibiotics have been developed. Each antibiotics has a specific mechanism of action against bacteria. The action may differ among bacteria. There are broad-spectrum antibiotics or effective against a wide variety of bacteria. Others antibiotics the action is narrow spectrum, or effective against only certain bacteria.

When a disease-causing bacterium is isolated from a patient, suitable antibiotics must be determined by the physician for administer treatment. The most widely used method is the Kirby-Bauer method. In this exercise you will learn how to do the Kirby-Bauer method to test antibiotics effectiveness.

Objectives

- To give a view about antibiotics effectiveness
- To learn the student the antibiotics test technique

Materials

Cultures (24 hours in Trytic soy broth)
- *Bacillus-cereus*, Gram-positive rod
- *Escherichia coli*, Gram-negative rod
- *Pseudomonas aeruginosa*, Gram-negative rod
- *Staphylococcus aureus*, Gram-positive coccus

Mueller-Hinton agar lates, 4 mm thick (25 ml/plate)

Antibiotic disks :
- Ampicillin
- Bacitracin
- Chloramphenicol
- Erythromycin
- Gentamicin
- Penicillin G
- Polymixin B
- Streptomycin
- Tetracycline
- Vancomycin

Ethanol 70%

Sterile swabs

Disposable gloves

Facemask

Incubator 35°C

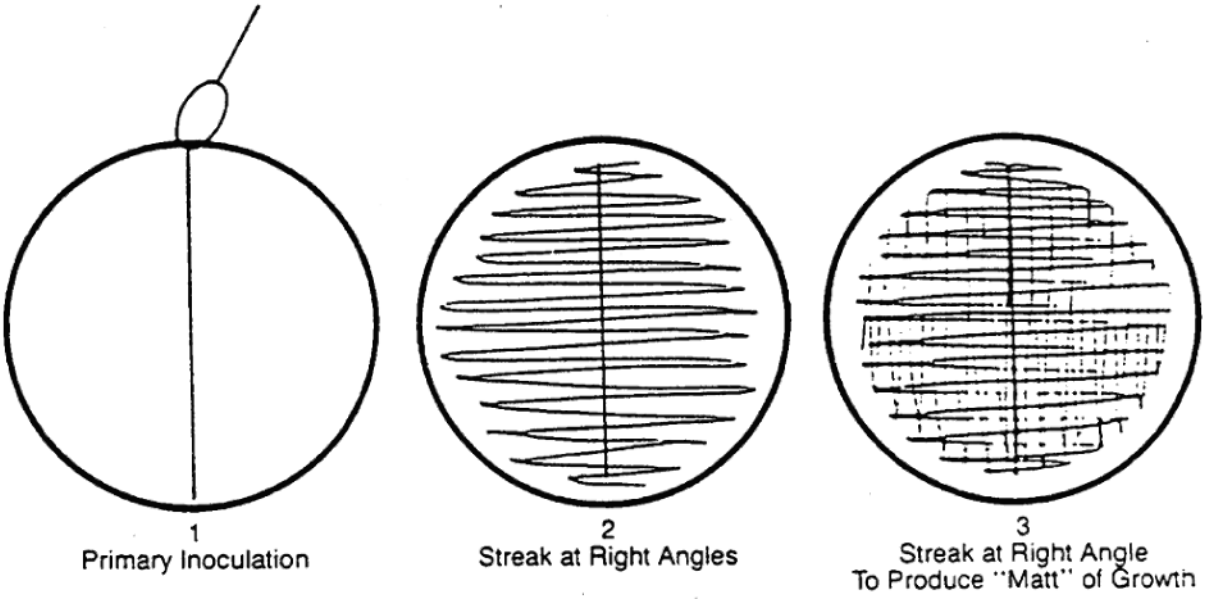
Beaker 250 ml

Ruler millimeter

Procedure

First Session: Preparation of Plates

1. Dip a sterile swab into one of the broth cultures, inoculate a Mueller-Hinton agar plate. Inoculate the plate to entire plate to produce a lawn of bacterial growth see picture.



2. After inoculation, allow all plates to dry for 15 minutes before proceeding to the next step.
3. Pour some 70% ethanol into a 250 ml beaker.
   Caution: Keep this beaker away from your flame.
4. Dip your forceps into the alcohol, and then pass the forceps over the Bunsen burner flame to sterilize them.
5. Pick up an antiobiotic disk from one of the petri dishes, and place it on one of your inoculated plates.
6. Tap it once o make sure it is secure. Place the plate in incubator 35°C.

Second Session: Examination of Plates

1. The plates must be examined after a 16-18 hours of incubation.
2. Examine the plates for zones of inhibition. Measure this with miilmeter ruler accros the disk. Record the diameter zone to the nearest whole millimeter in the laboratory report. If only one side of the zone can be measured multiply the number obtained by 2 to obtain a full zone of inhibition. If there is no zone, record a zero.
3. Compare the zone of inhibition to the interpretive standards for these antibiotics (Table 4). Record whether the each organism is resistant, susceptible, or intermediate to the antibiotic.
4. Compare this exercise by recording for each bacteria the antibiotics the organism is susceptible to. These represent possible drugs of choice to treat infections by these bacteria.

Table 4. Interpretive Standard for Antibiotics Selected for this practicum

Antimicrobial Agents	Abbreviation	Diameter of inhibition (mm)			
		Concentration	Resistant	Intermediate	Susceptible
Ampicillin	AM	10 µg	-	-	-
Gram-negative	-	-	11	12-13	14
Staphyloocci	-	-	20	21-28	29
Bacitracin	B	10 units	8	9-12	13
Chloramphenicol	C	30 µg	12	13-17	18
Erythromycin	E	15 µg	13	14-22	23
Gentamicin	GM	10 µg	12	13-14	15
Penicillin G	P	10 units	-	-	-
Staphylococci	-	-	20	21-28	29
Other organisms	-	-	11	12-21	22
Polymixin B	PB	300 units	8	9-11	12
Streptomycin	S	10 µg	11	12-14	15
Tetracycline	TE	30 µg	14	15-18	19
Vancomycin	VA	30 µg	9	10-11	12

Source: Antimicrobial Susceptibility Test Discs. Technical information published by Becton Dickinson Microbiology Systems, Cockeysville, Maryland.

Reference

Prescott, LM. 2005. Study Guide for Use With Microbiology. McGraw-Hill: Montana State University.

4th Lab RESULT

Student name : _____
ID Number : _____
Date : _____

I. Calculate the inhibition zone for each bacteria and its antibiotics, Complete the table

Table 4.1. Inhibition Zone measurement

Bacteria :	
Antibiotics	**Zone of inhibition**
1.	
2.	
3.	
4.	

Table 4.2. Inhibition Zone measurement

Bacteria :	
Antibiotics	**Zone of inhibition**
1.	
2.	
3.	
4.	

II. Question:

1. Which antibiotis is more effective ?

2. Why this antibiotic is more effective?

3. Which antibiotic is less effective ?

4. Why this antibiotic is less effective ?

5th Lab: Koch's Postulates

Microorganisms are the etiologic agent of a wide variety of infectious disease in all form of life. Microbes that cause disease is called pathogens, and the process of disease initiation is called pathogenesis. The interaction between the microbe and host is complex. Whether or not a disease occur is depend on host's vulnerability or susceptibility to the pathogen and on the virulence of the pathogen.

The etiologic agent of many disease is hard to determine. Although many microorganism can be isolated from a diseased tissue, their presence does not prove that any or all of them caused the disease. A microbe may be a secondary invader or part of the normal microbiota or transient microbiota of that area.

Around 100 years ago Robert Koch established four criteria, now called Koch's postulates, to help identify a particular organism as the causative agent for a particular disease. In this exercise we will demonstrate the Koch's postulate with *Erwinia carotrova* isolated from soft rot of carrot.

Objectives

- To know and understand Koch's Postulate
- Practice Koch's postulate to isolate the etiologic agent of disease.

Materials

Culture of *Erwinia carotrova*
Nutrient agar
Carrot
Scalpel or razor blade
Forceps
Alcohol
Disinfectant
Sterile water
Sterile petri with filter paper

Procedure

1. Wash the carrot and peel it to eliminate the outer surface; then dry it, wash it with disinfectant, and rinse it with sterile water. Dip the scalpel with alcohol, and cut the carrot into four cross-sectional slices 5 to 8 mm thick.
2. Put the four slices on filter paper in the bottom of a petri plate.
3. Inoculate the centre of three slices with a loopful of the *Erwinia* culture. Saturate the fiulterpaper with sterile water. Incubate the plate right-side up at room temperature until soft rot appears. More sterile water may be added if the process is slow.
4. Divide the nutrient agar in half. Inoculate one-half of the nutrient agar with the *Erwinia* broth; incubate the plate for 48 hours at room temperature, and then refrigerate it. Make Gram-stain smear of the *Erwinia*, heat fix it and keep it in your drawer.

5. Streak an inoculums from your diseased carrot on the remaining half of the plate. Incubate the plate for 48 hours at room temperature.
6. Make Gram-stain smear of the diseased carrot. Record your observation.
7. Observe the nutrient agar colony, record it.

Reference

Hudson BK. Sherwood LK. 1997. Exploration in Microbiology a discovery based approach. Prentice hall: USA**.**
Case. J. 2004. Laboratory Experiments in Microbiology seventh edition. Pearsons Benjamin Cumings: USA.

5th Lab RESULT

Student name : _____
ID Number : _____
Date : _____

1. Did you isolate one bacterium from the infected carrot ? explain

2. If you isolate more than one bacterium form the infected carrot, which was responsible for causing soft rot ? explain.

3. Did you have any evidence that this bacteria was *Erwinia carotrova* ? explain.

6th Lab Primary Media for Isolation of Microorganisms

As we know, many clinical specimens contain a mixed flora of microorganisms. Thus when the specimen were cultured it will take a great deal of subsequent time to subculture and sort through the isolated bacterial species. Instead, the microbiologist use several types of primary media to culture the specimen initially. In general, the primary media has three bsic purposes, accomplished simultaneously: (1) to culture all bacterial species present and see which if any predominate; (2) to differentiate species by certain characteristic responses to ingredients of the culture medium; and (3) to selectively encourage growth of those species of interest while suppressing the normal flora.

The basic medium used to support the total flora of a clinical specimen contains agar enriched with blood and other nutrient required by pathogenic microorganism. The blood source usually from animal (sheep or rabbits, sometimes horses), but human blood may also be used.

Differential media are formulated with a component, that can be utilized by some microorganisms but not by others. An indicator that will demonstrate any change in this component is added together with basic nutrients.

Selective media contain only one or more components that suppress the growth of some microorganisms without seriously affecting the ability of others to grow.

Objective

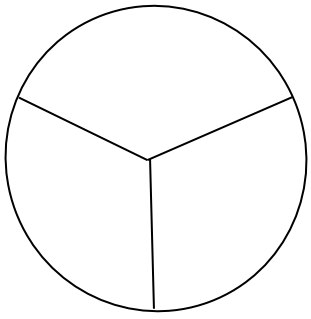
- To see the reponse of a mixed bacterial flora in a clinical specimen using primary media

Materials

Nutrient agar plates
Blood agar plates
Eosin methylene blue agar plates (EMB)
Mannitol salt agar plates (MSA)
Bacterial culture:
Escherichia coli
Pseudomonas aeruginosa
Staphylococcus epidermidis

Procedure

1. Invert each different media plates divide it into three zone as seen in the picture 6. Inoculate different bacteria in each zone. Incubate at 35°C for 24 hours.



Picture 6. Three zone of inoculation

2. Examine the plates. Record it

Reference

Wilson ME, Weisburd MH, Mizer HE. 1974. Laboratory manual and workbook in Microbiology-Applications to patient care. MacMillan Publishing: London.

6th Lab RESULT

Student name : _____
ID Number : _____
Date : _____

1. Observation, fill the table below

Table 6. Colony of different primary media

Medium	Bacterial isolates		
	S. epidermidis	*E. coli*	*P. aeruginosa*
Nutrient agar			
Manitol salt agar			
Eosin-methylene blue agar			
Blood agar			

Question

1. Is blood agar is selective or differential ? explain

2. What are the suitable media for *S. epidermidis*, *E. coli*, and *P. aeurginosa*? explain

7th Lab Precipitin Reaction the ring test

The ring test is a simple serogical technique that illustrates the precipitin reaction in solution. This antigen-antibody reaction can be demonstrated by the formationof a visible precipitate, a flocculent or granular turbidity, in the test fluid. Antiserum is introduced into a small diameter test tube, and the antigen is then carefully added to form a distinct upper layer. After 4 hours incubation a ring of precipitate forms at the point of contact in the presence of antigen-antibody reaction. The rates at which the visible ring forms depends on the concentration of the antigen.

To detect the precipitin reaction, a series of dilutions of the antigen is used, because both insufficient and excessive antigen amounts of antigen will prevent the formation of a visible precipitate. Furthermore, the optimal antibody : antigen ratio by the presence of a pronounced layer of granulation at the interface of the antiserum and antigen solution.

Objective

- To demonstrate a precipitin reaction by means of the ring test

Materials

Saline solution (0.85% NaCl)
Bovine globulin antiserum and Normal bovine serum diluted to 1:25

Procedure

1. Label three serological test tubes according to the antigen dilution to be used (1:25, 1:50; and 1:75) and the fourth test tube as a saline control.
2. Using a different 0,5 ml pipette, transfer 0.3 ml of each of the normal bovine serum dilutions into its appropriately labeled test tube.
3. Using a clean 0.5 ml pipette, transfer 0.3 ml of saline into the test tube labeled as control.
4. Carefully overlay all four test tube with 0.3 ml of bovine globulin antiserum. To prevent mixing of the sera, tilt the test tubeand allow the antiserum to run down the side of the test tube.
5. Incubate all of the test tube for 30 minutes at 37 °C

Reference

Cappucino JG. Sherman N.2002. Microbiolog a laboratory manual sixth edition. Benjamin Cuming: San Fransisco

7th Lab RESULT

Student name : _____
ID Number : _____
Date : _____

1. Examine all test tubes for the development of a ring of precipitation at the interface. Indicate the presence or absence of a ring.

2. Determine and indicate the antigen dilution that produce the greatest degree of precipitation that is indicative of the optimal antibody:antigen ratio

Table 7 The Precipitation

	Antigen dilutions			
Presence of interfacial ring (+) or (-)	1:25	1:50	1:75	Saline control

8th Lab Immunodiffusion: Antigen-Antibody Precipitations Reactions in Gels

Antigen may link together by multiple antibodies and form an insoluble precipitate form. This form is visible to the naked eye. A precipitate also indicates that antibody and antigen molecules are present at optimal proportions for the formation of larger complex, or lattice. This is known as the equivalence zone where each of antibody molecule (two to three molecule) bound to an antigen molecule, leaving no free antigens or antibodies.

In Immunodiffusion tests, antibodies and or soluble antigens are loaded into separate wells of a gel and are allowed to difuse, each reagent moving radially into the gell. An immobile precipitate, visible as a band (precipitin line) in the gel, develops if specific antibody- antigen binding takes place, and if antibody-antigen components are present at optimal proportions. Double immunodiffusion, also known as Ouchterlony, is the most widely used gel precipitation technique in the research laboratory.

In double immunodiffusion, antigen and antibody preparations are loaded into separate wells of an agarose. The antibodies (specific for human serum proteins) are located in the outer wells. Each substance diffuses from its well, and in time, while lines of insoluble precipitate appear at positions where antibodies have bound to their specific antigens at optimal proportions (equivalence zone).

Objecives

- To know about the specific antibody react to its antigen
- To practice the double immunodiffusion test

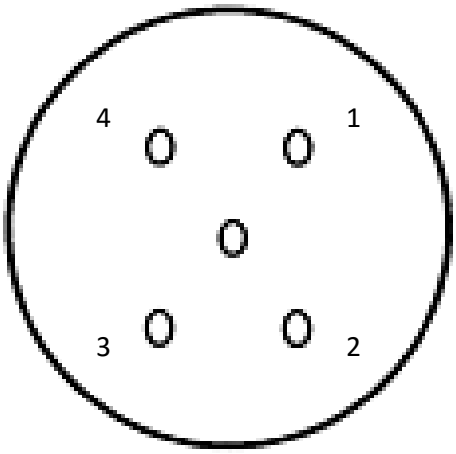
Materials

Agarose: 40 ml 1% (w/v) molten agarose in 0.05 M Tris-Cl, pH 8.6 (per pair)
Antibodies : serum antibodies-set (Carolina Biological Supply: #RG-20-2102), Goat anti-bovine albumin, Goat anti-horse albumin, Goat anti-swine albumin
Antigens : serum antigen set-bovine serum, horse serum, swine serum.
Microwave oven
Water bath at 55°C
60 mm petri dishes
Covered box container for gel storage
Laboratory marker
10 ml pipette
Glass dropper (well cutter)
Micropipettor/tips (1-10µl)

Procedure

1. Prepare 40 ml of 1% agarose: Add0.4 g of agarose to 40 ml of 0.05 M Tris-Cl, pH 8.6, in a 125 ml flask. Microwave the mixture for about 30 seconds, checking to make sure it does not boil over. Using a hot glove, gently swirl the flask, return it to the microwave Heat for 15 seconds, repeating this until no flecks of agarose are visible in the flask. Let the molten agarose cool until the flask is comfortable to handle, but still warm.

2. Obtain three 66 mm diameter petri dishes. Writing with a lab marker on the plate bottom, label the three plates A,B, and C, respectively. Write your initials on all three plates. Pipette 5 ml on slightly cooled molten agarose into each dish. Allow the agarose to solidify, about 20 minutes.
3. Using the large end of a plastic or glass dropper (a diameter of about 0.5 cm),cut wells into each gel as shown in the following template.



4. Label the outer wells 1,2,3,4, and 4 by writing on the plate bottom.
5. Changing micropipette tips between different reagents, pipette 20 µl of antibody to the designated wells according to the loading order in table 8

Table 8. Sampling loading order for double immunodiffusion assay

Patern	Center well*	Outer well*
A	Goat anti-bovine albumin	1. Bovine serum 2. Horse serum 3. Swine serum 4. Swine serum
B	Goat anti-horse albumin	
C	Goat anti-swine albumin and goat anti-bovine albumin	

*The contents of the outer wells are the same for three assays
** The center well antibodies are different for each other

6. Line the bottom of the storage box with a moist paper towel, and place the dishes into the storage container. Make sure the dishes are level. Incubate the gels for 24 to 48 hours at room temperature to allow diffusion and banding. The gels can be store in the refrigerator for several weeks if the box is kept moist.

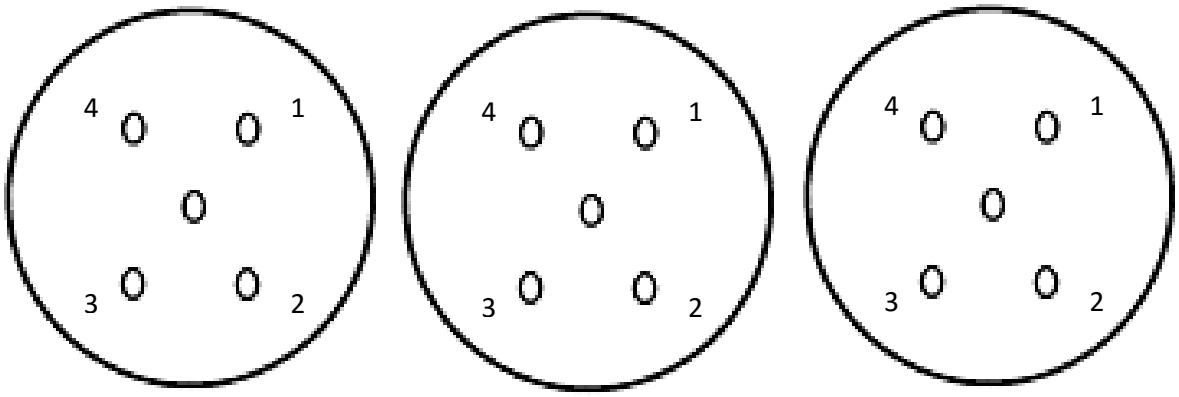
Reference

Case. J. 2004. Laboratory Experiments in Microbiology seventh edition. Pearsons Benjamin Cumings: USA.
Prescott, LM. 2005. Study Guide for Use With Microbiology. McGraw-Hill: Montana State University.

8th Lab RESULT

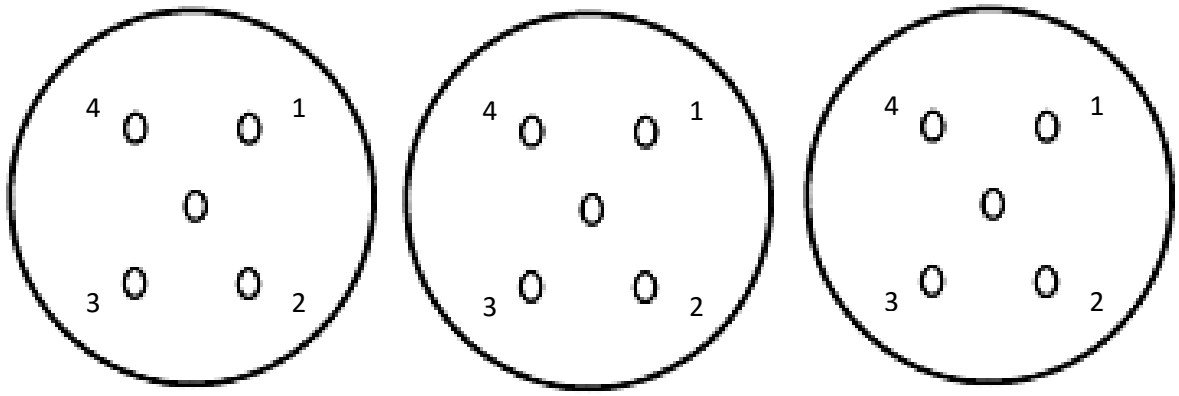
Student name : _____
ID Number : _____
Date : _____

1. A precipitin line represents a specific antibody-antigen reaction occurring between the antibodies diffusing from the center well with antigen diffusing from one of the outer wells. The precipitin line should be perpendicular to an imaginary straight line drawn from an outer well to the center well. Predict the results for pattern A, B, and C.



Pattern A Pattern B Pattern C

2. Diagram your double immunodiffusion result. Do they agree with your prediction ?



Pattern A Pattern B Pattern C

9th Lab Serologic Investigation of Microorganims

Serologic technique may allow rapid and highly specific identification of microorganisms. This involve antibodies and antigen reaction. Antibodies and antigen may react in certain visible ways *in vitro*.

The body of microorganism consist of protein that antigenic. Such antigen are called *somatic* (soma=body) or "O" antigens. Superficial structures of bacterial cells, such as capsules or flagella, also contain specific different antigens. Flagella structure are called "H" antigens (H is from *hauch* Germany word which refers to the motility of the bacteria). Exotoxins and other protein metabolites of bacterial cell also antigenic. The chemical compositions of somatic, capsular, flagellar, and toxin antigen are differ. Therefore each may elicit different antibody production.

Antibodies terminologies are refer to the type of visible reaction produced. For example, agglutinins are antibodies that produce agglutination, a reaction that occurs when bacterial cells or other particles are visibily clumped by antibody combined with antigens on the cell surfaces. Percipitins are antibodies that produce precipitation of soluble antigen (free in solution and unassociated with cells). When antibodies combined with such antigens, the large complexes that result simply precipitate out of solution in visible aggregrates. In this exercise, you will see how a microorganism can be identified by an interaction of its surface antigens with a known agglutinin that produces a visible agglutination of the bacterial cells.

Objective

- To demonstrate identification of microorganism by slide agglutination technique

Materials

Glass slides
70% Alcohol
Saline (85 %)
Pasteur pipettes
Heat-killed suspension of *Escherichia coli* or *Salmonella*
E.coli or *Salmonella* antiserum

Procedure

1. Carefully wash a slide in 70% alcohol and let it air-dry
2. Using a glass marking pencil, draw two circles at opposite ends of the slide.
3. Using a pasteur pipette, place a drop of saline in one circle. Mark this circle "C". For control.
4. With a fresh Pasteur pipette, place a drop of antiserum in the other circle.
5. Use another Pasteur pipette to add a drop of heat-killed bacterial suspension (this is the "antigen") to the material in each circle.
6. Pick up the slides by it edges, with your thumb and forefinger, and rock it gently back and forth for a few seconds.

7. Hold the slide over a good light and observe closely for any change in the appearance of the suspension in the circles.

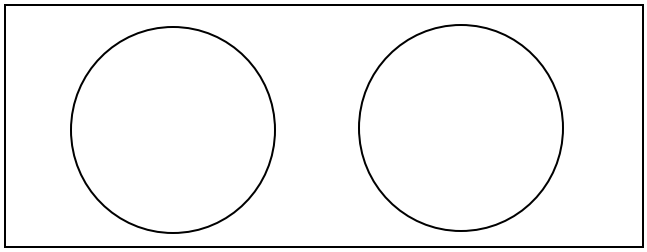
Reference

Wilson ME, Weisburd MH, Mizer HE. 1974. Laboratory manual and workbook in Microbiology-Applications to patient care. MacMillan Publishing: London.

9th Lab RESULT

Student name : _____
ID Number : _____
Date : _____

1. In the diagram below indicate any visible difference you observed in the suspensions at each end of the slide



Antiserum Saline Control

2. What is the interpretation of the result? Explain

3. Define the antigen and antibody

4. Name the microbial cells component that are antigenic

10th Lab ELISA

Antibodies can be used to detect disease. These types of tests are called immunoassays. Immunoassays are based on detectable interactions between antigens and antibodies such as precipitation, agglutination, or complement fixation. To increase sensitivity in detecting antigens, antibodies can be labeled with substance such as radioactive chemicals (E.g., Iodine-125), fluorescent compounds, magnetic beds, or enzymes. ELISA (enzyme-linked immunosorbent assay) or ELA (enzyme immunosorbent assay) is the most widely used immunoassay labs today.

The ELISA take advantage of the strong and specific attachment that occurs between an antibody and antigen (thus the term is imumunosorbent) An enzyme covalently attached to the tail portion of the antibody. The enzyme linked to the antibody is one that catalyzes the conversion of a colorless substrate into a colored product.

When the appropriate substrate is added, the enzyme reacts with the substrate to make a colored product. This product can be detected visually or by spectrophotometer. The amount of product produced is directly proportional to the amount of enzyme. Therefore, antibodies also present, this allowing ELISA technique to quantitative as well as qualitative. In this exercise, we will perform an ELISA test to diagnose salmonellosis.

Objectives

- To practice ELISA technique
- To understand how ELISA test can be use clinically to detect antibodies or antigen

Materials

Culture *Salmonella typhimurium* (heated for 30 minutes at 56°C in a water bath)
Coating buffer
Washing buffer
Blocking buffer
Patient's serum
Alkaline phosphatase-labeled anti-antibodies
BCIP/NBT susbtrate
Flat-bottom microtiter plate
Micropipette tips
Latex gloves
Facemask

Procedure

1. Add 100 µl coating buffer to each well of one row (wells 1-12) of the microtiter plate.
2. Add 100 µl *S. typhimurium* to each well.
3. Seal the wells with a strip of plastic tape, and refrigerate the plate at 5°C for 1 to 7 days.
4. Remove your plate from the refrigerator and carefully remove the tape. Shake the inverted plate with a quick shake to remove the liquid into disinfectant.
5. Fill the wells with washing buffer and shake to remove. Wash two more times.

6. Add 100 µl blocking buffer. Leave for 30 to 90 minutes as directed by your instructor.
7. Perform dilution of the patien's serum by placing 100 µl in the first well. Mix up and down three times and, with a new pipette tip, transfer 100 µl to the second well. Mix up and dwon three times, change pipette tips, and transfer 100µl to the third well, and so on. Continue until you have reached the 11^{th} well. Discard 100 µl from that well.
8. Incubate the plate at 35 ℃ for 60 minutes
9. Shake the inverted plate with a quick shake to remove the contents. Wash three times with washing buffer as described in step 5.
10. Add 100 µl of alkaline phosphatase-labeled anti-antibody to each well (1-12). Seal the wells with tape and incubate the plate at 35℃ for 45 minutes. Plates can be sealed and stored at 5℃ until next lab period.
11. Remove the tape carefully shake out the contents, and wash the wells three times with washing buffer.
12. Add 100 µl of the alkaline phosphatase substrate (BCIP/NBT) to each well in the row.
13. Leave at room temperature for 10 to 30 minutes until color develops; well 12 will be colorless.
14. Record the results. The highest dilution with a blue color is the endpoint. The titer is the reciprocal of the dilution of the endpoint.

Reference

Hudson BK. Sherwood LK. 1997. Exploration in Microbiology a discovery based approach. Prentice hall: USA**.**
Prescott, LM. 2005. Study Guide for Use With Microbiology. McGraw-Hill: Montana State University.

10th Lab RESULT

Student name : _____
ID Number : _____
Date : _____

Data

Well	Final dilution	Color
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		

Questions

1. What was the endpoint?

2. What was the antibody titer?

3. Why the control well colorless?