Exercise : *Enterobacteriaceae- Enterobacteria*

1. Are Oxidase negative.

The family is composed of a large group include: *Escerichia, Enterobacter, Klebsiella, Proteus, Salmonella, Shigella* and *Serratia. Salmonella* and Enterobathogenic The *Enterobacteriaceae* are Gram-negative bacilli which

2. Ferment glucose.

E.coli are primarily pathogenic for man and their precise identification is important. The other *Enterobacteria* are essentially commensals or saprophytes, but can act as opportunistic pathogenic. The ability to ferment Lactose differentiates between the commensals and true intestinal pathogens. There is full range of biochemical tests that can be performed in order to identify all of the Enterobacteria (see textbooks). Tentative differentiation of the most important groups is presented in the table. Definitive identification of species and strain of *Salmonella, Shigella* and others should be done by using agglutination tests with antisera. The most used media for isolation of enteropathogen bacteria from fecal specimens are SS agar, Desoxycholatecitrate agar, MacConkey agar and Selenite Broth.

Non-fermenting Gram-negative Bacilli

In recent years there have been increasing numbers of isolations from clinical specimens, of Gram-negative non-fermenting bacilli that are primarily either free-living saprophytes or opportunistic pathogens in human diseases. For instance, *Pseudomonas* spp., *Alcaligens* spp. and *Acinetobacter* are found frequently in clinical specimens. They are Oxidase-positive and non-Lactose fermenter.

(1)

Materials:

a) Demonstration of growth of *E.coli, Klebsiella, Proteus, Enterobacter, Citrobacter, Pseudomonas, Salmonella* and *Shigella* on MacConkey agar, blood agar, Desoxycholate agar and Kligler's Iron agar.
b) Demonstration of certain biochemical tests used for differentiation of enteric-bacteria.
c) Mixed culture plates.

Procedure:

(First period)

a) Isolate non-lactose fermenter organism as single colony from provided mixed culture plates.
b) Prepare Gram-stain from provided *Klebsiella* and *Pseudomonas* culture.

(Second period)

Identify relatively your culture on Kligler's Iron agar (use the table)

Tentative differentiation of commonly isolation clinical aerobic enteric bacilli by means of Kligler's iron agar and other biochemical tests during 24-hours incubation at 37°C

Organisms	Slant	Butt	Gas	H_2S	Urease	Citrate	Indole	Motility	Oxidase
E.coli	Y*	Y	±	–	–	–	+	±	–
Citrobacter spp.	Y*	Y	+	±	W	+	–	±	–
Enterobacter-serratia	Y*	Y	±	–	–	+	±	±	–
Klebsialla spp.	Y*	Y	±	–	±	+	–	–	–
Proteus spp.	R	Y	+	+	+	±	±	+	–
Morganella spp.	R	Y	–	–	+	–	+	+	–
Providencia spp.	R	Y	±	–	+	+	+	+	–
Salmonella spp.	R	Y	+	+	–	+	–	±	–
Shigella spp.	R	Y	–	–	–	–	±	–	–
Pseudomonas spp.	R	R	–	–	–	–	–	+	+
Vibrio cholera	R	Y	–	–	–	±	+	+	+
Acinetobacter	R	R	–	–	–	+	–	–	–

Y= YELLOW , Y* =Few strains may be fermented after 24 hours , R= RED , W= WEAK.

Identification of Gram Negative Bacteria

- All gram-negative bacteria usually appear as gram-negative small rods with microscope
- 5 biochemical tests are used to identify genus
  1. ~~Carbohydrate Fermentation Test~~ Kligler's Iron
  2. SIM Test (Hydrogen-Sulfide, Indole, Motility)
  3. Citrate Test
  4. ~~Phenolalanine Deaminase Test~~
  5. Urease Test
- Gram negative bacteria are usually cultivated on MacConkey agar, which contains bile salts and crystal violet to inhibit gram-positive bacteria. Agar also contains lactose and a neutral red dye to indicate pH changes. Lactose-positive colonies are red to pink in color, lactose-negative are not.
- Carbohydrate Fermentation Test Kligler's Iron
  - usually in broth with phenol red pH indicator
  - Phenol Red lactose broth or Phenol red glucose broth is used
  - Durham tube inverted in base of test tube traps gas if organism produces gas when fermenting carbohydrate
  - Organism must have beta-galactosidase to break down lactose into glucose and galactose
  - When organism ferments simple sugars, it produces acidic end products, which lower pH and change broth to yellow color.
  - Test result possibilities:
    1. Negative - red broth - no fermentation
    2. A/G Positive - produces acid and gas, forming bubble in Durham tube and changing broth to yellow.
    3. Acid Positive - changes broth to yellow, but no bubble produced.
- SIM Test -
  - SIM agar inoculated by stabbing culture on loop straight into agar.
  - SIM medium is used to determine if organism can metabolize certain amino acids.
  - 3 tests in one:
    1. Hydrogen Sulfide (H_2S)
       - Cystamins and Metathiamine contain sulfur, which allow formation of hydrogen sulfide if organism can metabolize them.
       - Lead nitrate in agar traps hydrogen sulfide, forming a black precipitant when lead sulfide is formed. *Agar turns black in hydrogen sulfide positive test.*
       - Negative result = no change in color of medium.
    2. Indole Test
       - Aromatic amino acid Tryptophan (an ring shaped amino acid with an amine group and indole) is present in medium. If Bacteria can break this molecule amino group and indole, test is positive.
       - After bacteria has had time to grow, Kovac's reagent will react with indole to form a bright red ring at the surface of the tube.
       - If test is negative, no change will occur when Kovac's reagent is added.
    3. Motility test

        - If organism is motility positive, it will spread throughout the medium from the stab
        - If negative, bacteria will only grow where it was stabbed into the medium.
- Citrate Test
    - Surface of citrate agar (green) slant streaked with bacteria
    - Agar contains citric acid, which is a tricarboxylic acid (has 3 carboxyl groups) and Bram Cresol Agar.
    - Bacteria with citrate permease can uptake citric acid, causing alkaline end products that change pH indicator to blue.
    - If test is positive, slant changes to blue
    - If negative, slant remains green.
- Phenylalanine (PA) Deaminase Test
    - Bacteria are smeared on phenylalanine agar slant.
    - Phenylalanine is an aromatic amino acid (has ring structure associated)
    - If bacteria possess phenylalanine deaminase, it can remove amino group, leaving phenylpyruvic acid.
    - To complete test (after bacterial growth), 5% solution of ferric chloride is added to slant.
    - If test is positive, slant will turn olive green on surface
    - If negative, no change
    - Members of the genus *Proteus* are generally positive for this test.
- Urease Test
    - Urea broth (orange), which contains phenol red indicator in low concentration is medium for test.
    - Urea, which is usually toxic, is the end product of amino acid metabolism. Some organisms contain urease, which allows them to break down urea to form CO_2 and ammonia. Ammonia reacts with water to form ammonium hydroxide.
    - *Broth becomes red-purple color if test is positive* due to production of ammonium hydroxide.
    - If negative, broth remains orange.

Bacteria Identification Flow Chart

- Lactose Fermentation
    - positive = coliforms
        - Citrate test
            - positive
                - H_2S test
                    - positive = *Citobacter*
                    - negative
                        - motility test
                            - positive = *Enterobacter*
                            - negative = *Klebsiella*
            - negative (also Indole Positive, motility positive, H_2S negative) = *Escherichia coli.*
    - negative = noncoliforms
        - Urease test
            - positive
                - ~~Phenylalinine deaminase test~~
                    - positive - Proteus
            - negative

- Phenylalinine deaminase test
  - negative
    - Motility test
      - negative = *Shigella* (also produces Acid with glucose)
      - positive = *Salmonella* (also produces Acid and Gas with glucose)

- Glucose negative
  - Oxidase positive = *Pseudomonis*

	Lactose	Glucose	Citrate	H_2S	Indole	Motility	Urease	PA deaminase	Oxidase
Escherichia coli	pos	neg	neg	neg	pos	pos			
Citobacter	pos		pos	pos					
Enterobacter	pos		pos	neg		pos			
Klebsiella	pos	A/G	pos	neg		neg			
Proteus	neg	pos					pos	pos	
Salmonella	neg	A				pos	neg	neg	
Shigella	neg	A/G				neg	neg	neg	
Pseudomonas	neg	neg							pos

Antibiotic Lab

- Kirby Bauer test is used to evaluate antibiotic resistance by placing antibiotic discs on agar where bacteria are growing
- Limitation to Bauer test is that it is only a qualitative test: it cannot indicate how much antibiotic is needed to control microorganism.
- *Candida albicans* is sensitive only to nystatin, which is used to control yeast, not bacteria.
- *Pseudomonas* is resistant to Penicillin, tatracycline, erythromycin: sensitive to cyproflexin and genomycin.
- *E. coli* is resistant to penicillin, but sensitive to ampacillin

Dehydrated Culture Media

KLIGLER IRON AGAR

Code: CM0033

A medium for the identification of Enterobacteriaceae. based on double sugar fermentation and hydrogen sulphide production.

Typical Formula*	gm/litre
'Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.05
Agar	12.0
pH 7.4 ± 0.2 @ 25°C	

* Adjusted as required to meet performance standards

Directions

Suspend 55g in 1 litre of distilled water. Bring to the boil to dissolve completely. Mix well and distribute into containers. Sterilise by autoclaving at 121°C for 15 minutes. Allow to set as slopes with 1 inch butts.

Description

A differential medium for the identification of Enterobacteriaceae on a basis of double sugar fermentation and hydrogen sulphide production.

Oxoid Kligler Iron Agar based on the original medium[1,2,3] combines the principles of Russell[4], double sugar agar, with ferric citrate as an indicator to detect hydrogen sulphide production. The medium is recommended for the identification of colonies picked off from plating media such as MacConkey Agar, Bismuth Sulphite Agar, or Desoxycholate Citrate Agar, etc.

Technique

Smear the surface of a Kligler Iron Agar slope and stab the butt with a colony picked off one of the solid media.

There are three reactions to record when interpreting a KIA tube.

1Carbohydrate utilisation:

(i) slant reaction	(ii) butt reaction
acidity: yellow colour	*acidity*: yellow colour
alkalinity: red colour	*alkalinity*: red colour

2 Gas production

(7)

aerogenic	anaerogenic
bubbles or splitting of agar	no gas production

3 H_2S production:

blackening in whole or part of butt:-

Record the slant reaction/the butt reaction/gas production/H_2S production; in that order.

Red slant/yellow butt - glucose only fermented

Yellow slant/yellow butt - glucose + lactose fermented

Red slant/red butt - neither glucose nor lactose fermented

Reactions after 18 - 24 hours at 35°C

Organism	Slope	Butt	Gas	H_2S
Shigella sonnei	Red	Yellow	-	-
Shigella dysenteriae	Red	Yellow	-	-
Salmonella typhi	Red	Yellow	-	+
Salmonella species	Red	Yellow	+	+
Enterobacter species	Red	Yellow	+	-
Klebsiella species	Yellow	Yellow	+	-
Escherichia coli	Yellow	Yellow	+	-
Proteus mirabilis	Red	Yellow	-	+
Morganella species	Red	Yellow	V	-
Citrobacter freundii	Yellow	Yellow	+	+
Yersinia species	Red	Yellow	V	-

V = variable, + = positive, - = negative.

Storage conditions and Shelf life

Store the dehydrated medium at 10-30°C and use before the expiry date on the label.

Store the prepared medium at 2-8°C.

Appearance

Dehydrated medium: Straw-orange coloured, free-flowing powder

Prepared medium: Red coloured gel

Quality control

Positive controls:

	Expected results			
	Slope	Butt	Gas	H_2S
Citrobacter freundii ATCC® 8090*	Yellow	Yellow	+	+
Shigella sonnei ATCC® 25931*	Red	Yellow	-	-
Alcaligenes feacalis ATCC® 19018	Red	Red	-	-

Negative control:

Uninoculated medium. No change

This organism is available as a Culti-Loop®

Precautions

It is essential that Kligler Iron Agar is examined and reported at 18-24 hours. Early or late readings will give false results.

KIA will grow both oxidative and fermentative organisms. Confusion will result if care is not taken to distinguish between the two groups.

Always use a straight wire to inoculate the butt to avoid splitting the agar with a loop.
Pure cultures are essential to avoid misinterpretation.
Do not use screw-capped tubes or bottles for KIA medium. It is essential that air is freely available for growth on the slant.

References

1. Kligler I. J. (1917) *Am. J. Public Hlth.* 7. 1042-1044.
2. Kligler I. J. (1918) *J. Exper. Med.* 28. 319-322.
3. Bailey Sadie F. and Lacey G. R. (1927) *J. Bact.* 13. 182-189.
4. Russell F. F. (1911) *J. Med. Res.* 25. 217-229.