

MICROBES OF THE BODY: THE ENTERICS

The term **enterics** refers to organisms belonging to the family *Enterobacteriaceae*. This is a family of **Gram-negative, oxidase-negative, facultative rods**. Several groups are included in this family. One group includes genera such as *Escherichia*, *Klebsiella* and *Proteus*, species which are generally found as normal inhabitants of the human body but which may also cause disease under certain circumstances. Other groups include: *Salmonella* and *Shigella* which are usually associated with a disease state in animals; and *Erwinia*, often associated with a disease state in plants. Various members of the genera *Erwinia*, *Enterobacter*, *Klebsiella*, and *Serratia* are common inhabitants of water or soil.

Because of their importance in human disease, the *Enterobacteriaceae* have been studied extensively. Although these bacteria are morphologically alike and in many ways metabolically similar, laboratory procedures for identification of enterics are based on biochemical activities. In this exercise, you will use a limited number of biochemical tests to differentiate between selected members of the *Enterobacteriaceae*.

BIOCHEMICAL REACTIONS USED FOR IDENTIFICATION OF GRAM-NEGATIVE RODS

OXIDASE TEST

Oxidase enzymes play a vital role in the electron transport system during aerobic respiration. Cytochrome oxidase catalyzes the oxidation of a reduced cytochrome by O_2 , resulting in the formation of usually H_2O or sometimes H_2O_2 . Aerobic bacteria, as well as some facultative anaerobes and microaerophiles, exhibit oxidase activity. The oxidase test differentiates between the *Enterobacteriaceae*, which are oxidase-negative, and members of the genus *Pseudomonas*, which are oxidase-positive.

IMViC TESTS

IMViC tests are used primarily for the identification of *Escherichia coli* and *Enterobacter aerogenes*. The IMViC series of tests include **I**ndole production, **M**ethyl red test, **V**ogues-Proskauer test, and **C**itrate utilization.

Indole Production

Certain bacteria such as *E. coli* have the ability to degrade the amino acid tryptophan to indole. Indole can be easily detected with Kovac's reagent. Kovac's reagent is composed of *p*-dimethylaminobenzaldehyde, butanol and HCl. Indole is extracted from the medium (tryptone broth) into the reagent layer by the acidified alcohol component and forms a cherry red complex with the *p*-dimethylamino-benzaldehyde. *E. coli* is indole-positive; *Enterobacter aerogenes* is indole-negative.

Methyl Red Test

Glucose is the major substrate oxidized by enteric bacteria for energy production. The end products of the oxidation process vary depending on the specific enzymatic pathways in the bacteria. The ability to produce a large amount of acid is used to differentiate *E. coli* from *Enterobacter aerogenes*. Both organisms initially produce organic acids. When *E. coli* is grown in glucose broth, mixed acids (lactic, formic and acetic) are formed until a pH of 4-5 is reached. In contrast, *E. aerogenes* converts organic acids to nonacidic endproducts such as 2,3-butanediol and acetoin (acetylmethylcarbinol). The pH of the broth is approximately 6. In this test, the methyl red pH indicator is red between pH 4.4 and 5, and yellow between pH 6 and 7. A red color in the medium indicates a positive MR test; a yellow color indicates a negative MR test.

Vogues-Proskauer Test

The VP test establishes the presence of acetylmethylcarbinol (aka acetoin). This endproduct is oxidized to a diacetyl compound in the presence of O₂, KOH and a guanidine group that is present in the peptone of the MR-VP medium. A pink complex is formed; however, the exact chemistry of the color reaction is not known.

Citrate Utilization

In the absence of fermentable glucose or lactose, some enteric bacteria are capable of using citrate as a carbon source. Citrate agar contains an ammonium salt as the sole nitrogen source, citrate as the sole carbon source, and a bromthymol blue indicator, which is green at pH 6.8 and blue at pH ≥ 7.6 . *E. aerogenes*, but not *E. coli*, utilizes citrate as a sole carbon source and grows on the medium, changing the color of the agar from green to deep “Prussian blue”. The ability to use citrate as a carbon source depends on the presence of a citrate permease that facilitates the transport of citrate into the cell. *E. aerogenes* possesses the enzyme; *E. coli* lacks the transport system or permease that permits citrate to enter the cell.

TRIPLE SUGAR IRON (TSI) AGAR

Triple sugar iron (TSI) is a differential medium used for the identification of Gram-negative enteric pathogens. The medium indicates ability to ferment lactose, sucrose and glucose with the formation of acid and gas, and ability to produce H₂S. These are the characteristics which are used to identify the *Salmonella-Shigella* group of organisms.

TSI agar slants contain lactose and sucrose in 1% concentrations, and glucose in a concentration of 0.1%, which allows detection of glucose fermentation alone. The pH indicator phenol red is also incorporated in the medium. Carbohydrate fermentation is indicated by a change in color of the medium from orange-red to yellow in the presence of acids.

Following incubation, you can determine the fermentative activities of microorganisms as described below.

- 1. Alkaline slant (red) and acid butt (yellow) with or without gas production (breaks in the agar butt):** Only glucose fermentation has occurred. The organisms preferentially degrade glucose first. Since glucose is present in minimal concentration, the small amount of acid produced on the slant surface is oxidized rapidly. The acid produced by glucose fermentation in the butt of the tube will be maintained because of the low oxygen tension and slower growth of the organisms.

2. **Acid slant (yellow) and acid butt (yellow) with or without gas production:** Lactose and/or sucrose fermentation has occurred. As these sugars are present in higher concentrations, they serve as substrates for continued fermentative activities and maintain an acid reaction in both slant and butt.
3. **Alkaline slant (red) and alkaline butt (red) or no change (orange-red):** No carbohydrate fermentation has occurred. Instead, peptones in the medium may have been catabolized; the pH may be alkaline due to production of ammonia.

TSI agar also contains: (a) sodium thiosulfate, a substrate for H₂S production, and (b) ferrous sulfate for detection of the colorless H₂S. Organisms capable of producing H₂S will show an extensive blackening in the butt because of the precipitation of the insoluble ferrous sulfide.

To inoculate a TSI agar slant properly, the inoculating loop must be stabbed into the butt of the slant and then streaked in a zig-zag fashion over the slanted surface. It is **absolutely essential** to observe the cultures and record the reactions within 18 to 24 hours. Doing so will insure that the carbohydrate substrates have not been depleted and that degradation of peptones yielding alkaline end products has not taken place.

BRAIN-HEART INFUSION (BHI) AGAR

Brain-heart infusion (BHI) agar or any similarly nutrient rich media (like blood agar) can be used to test for swarming motility, which yields concentric circles in a series of rings on agar plates. This is a definitive test for the highly motile enteric bacterium, *Proteus mirabilis*.

UREA AGAR

The urease test uses an urea agar slant to test for the ability to hydrolyze urea with the enzyme urease. Urea is the produce of the degradation of certain amino acids. Many enterics can metabolize urea but only *Proteus* (and just a few other enteric genera) are able to rapidly metabolize urea to ammonia with the urease enzyme. Pink is considered a positive, orange or yellow negative.

FIRST PERIOD

Material:

1. One nutrient agar plate
2. Two tubes of tryptone broth
3. Two tubes of MR-VP broth
4. Two citrate slants
5. Three TSI slants
6. One BHI plate
7. Two urea slants
8. Overnight broth cultures of *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella*, *Shigella*, *Proteus* and *Pseudomonas*.

Procedure: (work in pairs)

1. Inoculate individual tubes of tryptone broth with *E. coli* and *Enterobacter aerogenes*.
2. Inoculate individual MR-VP tubes with *E. coli* and *Enterobacter aerogenes*.
3. Streak citrate slants with *E. coli* and *Enterobacter aerogenes*.
4. Stab-and-streak TSI slants with *E. coli*, *Salmonella*, and *Shigella*.
5. Divide the nutrient agar plate into two sections. Inoculate one half of the plate with *E. coli* and the other half with *Pseudomonas*.
6. Incubate tryptone, MR-VP tubes and citrate slants at 37°C for **18-24** hours (only!).
7. Incubate TSI slants at 37°C for **18-24** hours (only!).
8. Incubate the nutrient agar plate at 37°C for **18-24** hours (only!).

9. Inoculate center of a BHI plate with single loop containing *Proteus*, then invert and incubate at 37°C for 24-48 hours.
10. Streak urea slants with *E. coli* and *Proteus*. Incubate urea slants at 37°C for **18-24** hours (only!).

SECOND PERIOD

Material:

1. Oxidase reagent dropper & filter paper
2. Kovac's reagent
3. Methyl red indicator
4. VP reagent

Procedure:

OXIDASE TEST:

Using the nutrient agar plate, place one loop full of the colonies of *E. coli* and of *Pseudomonas*, separately and aseptically onto the filter paper. Apply a few drops of the oxidase reagent (phenylenediamine solution) on the bacterial inoculum spotted onto the filter paper using the oxidase reagent dropper. Oxidase-positive colonies will quickly turn a dark purple or blue. The oxidase reagent is unstable and will eventually oxidize even without cytochrome oxidase present. A false positive can be avoided by reading the test within a few seconds after the reagent is applied.

INDOLE PRODUCTION:

Add 10 drops of Kovac's reagent to each of the tubes of tryptone broth. Allow tubes to stand for 5 minutes. Examine the color of the alcohol "reagent layer" in each culture. A cherry red color indicates the presence of indole.

METHYL RED TEST:

Transfer approximately one-half of each culture into an empty sterile tube and set these tubes aside for the Voges-Proskauer test. Add 5 drops of the methyl red indicator to the remaining aliquot of each culture. A red color in the broth indicates the presence of acid.

VOGES-PROSKAUER TEST:

To each of the other tubes of MR-VP broth, add 10 drops of α -naphthol, **wait 2 minutes**, then add 5 drops of 40% KOH. Mix and reincubate at 37°C for a minimum of 15 minutes. A pink to red color indicates the presence of acetylmethylcarbinol. If no color develops after 15 minutes, cultures can be reincubated at 37°C overnight.

CITRATE TEST:

Check citrate agar slants for growth and change in color.

TRIPLE SUGAR IRON (TSI) AGAR:

See your lab atlas for interpretation of results and aid in scoring of reactions with respect to the three potential outcomes as discussed above.

SWARMING MOTILITY TEST:

Check BHI agar (or blood agar) plate for concentric rings as indicator of swarming motility.

UREA TEST:

Check urea agar slants for growth and change in color.

MULTITEST SYSTEMS FOR IDENTIFICATION OF *ENTERBACTERIACEAE*

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NOTE: Almost all of the rapid multitest ID kits have been developed for the identification of *medically important* microorganisms. If you are trying to identify a saprophytic microorganism of the soil, water, or some other habitat, there is no substitute for the conventional methods.

FIRST PERIOD

Material:

1. Overnight streak plates of *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella*, *Shigella* or *Serratia*.
2. Oxidase ampules
3. One tube with ca. 3 ml sterile saline
4. API RapiD 20E test kit
5. 1.0 ml pipets

Procedure: (work in pairs)

Follow instructions handout for the API RapiD 20E test kit.