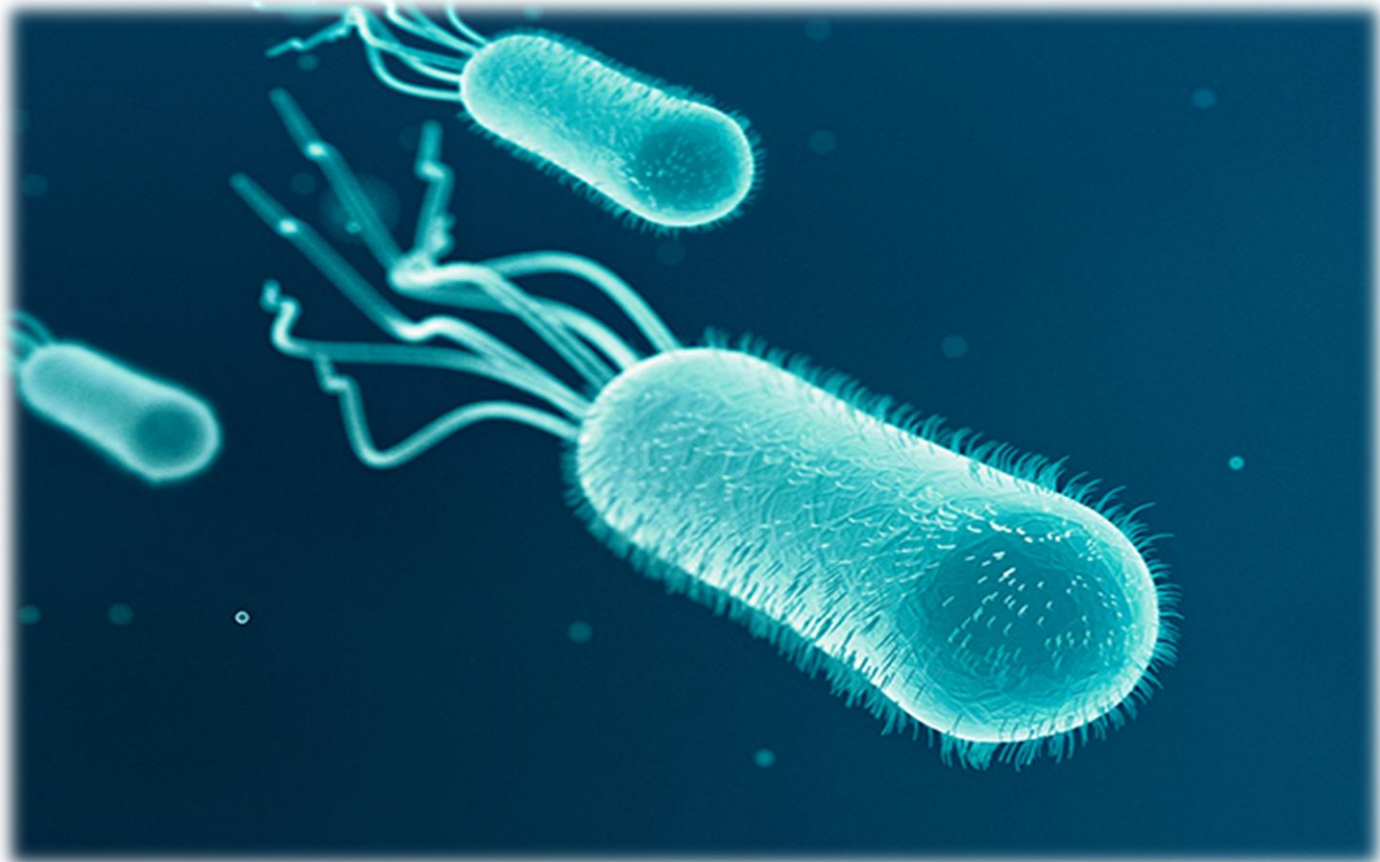
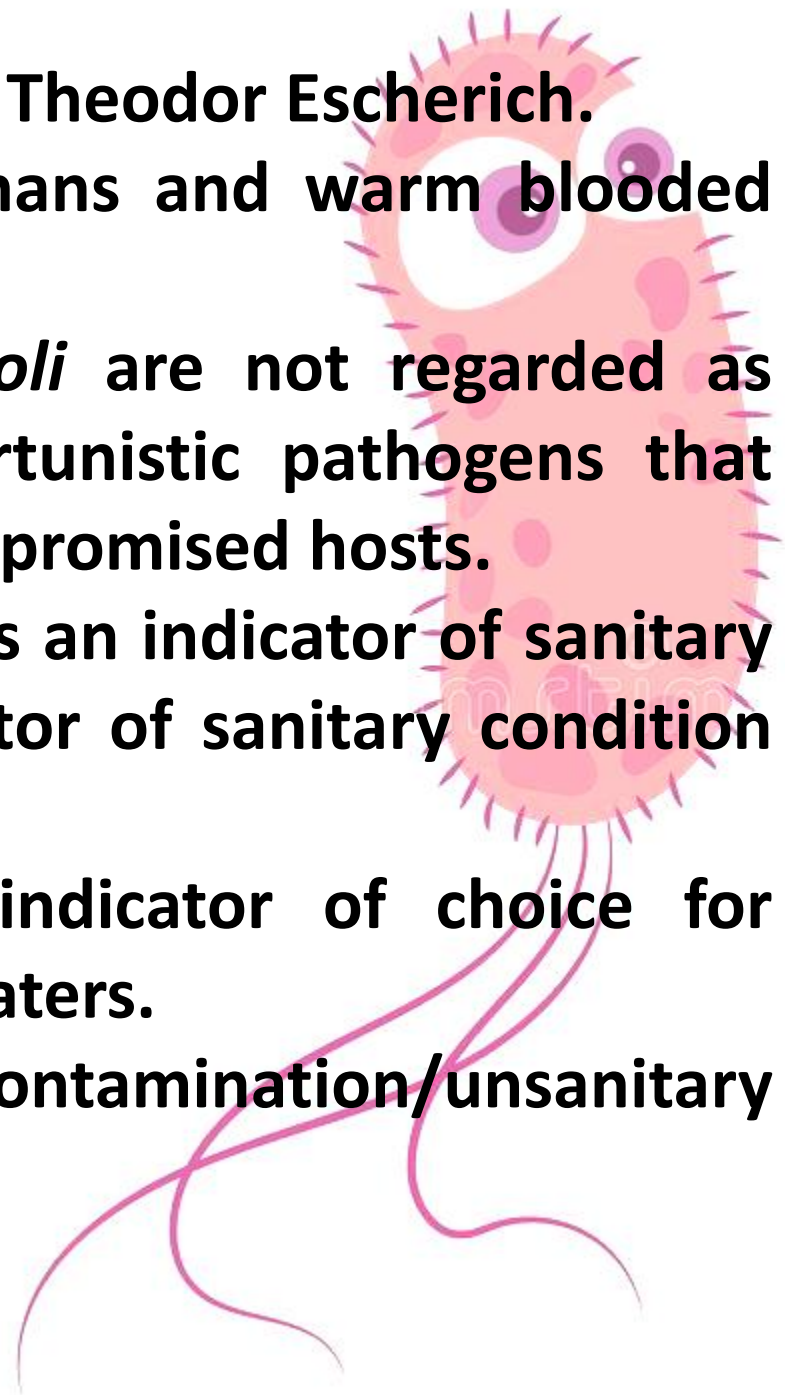


Enumeration of *E. coli*/*E. coli* O157:H7



- ✓ *E. coli*, was identified in 1885 by Theodor Escherich.
- ✓ Distributed in intestine of humans and warm blooded animals.
- ✓ Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immune-compromised hosts.
- ✓ Detection of **coliforms** is used as an indicator of sanitary quality of water/general indicator of sanitary condition in food-processing environment.
- ✓ **Faecal coliforms** is standard indicator of choice for shellfish and shellfish harvest waters.
- ✓ *E. coli* indicates faecal contamination/unsanitary processing.



Enumeration and Isolation of *E. coli*

- **Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases.**
- **In the assay, serial dilutions of sample are inoculated into broth media.**
- **3-tube MPN test is used for testing most foods.**
- **5-tube MPN is used for water, shellfish and shellfish harvest water testing and there is also 10-tube MPN method that is used to test bottled water/samples that are not expected to be highly contaminated.**
- **A solid medium plating method for coliforms that uses violet red bile agar (VRBA) which contains neutral red pH indicator, so that lactose fermentation results in formation of pink colonies.**
- **Membrane filtration tests for coliform and faecal coliform that measure aldehyde formation due to fermentation of lactose.**

Presumptive test for *E. coli*:

- Weigh 50 g food into sterile high-speed blender jar.
- Frozen samples can be softened by storing it for <18 h at 2-5°C, but do not thaw.
- Add 450 ml of Butterfield's phosphate-buffered water and blend for 2 min.
- If <50 g of sample are available, weigh portion that is equivalent to half of sample and add sufficient volume of sterile diluents to make 1:10 dilution.
- Total volume in blender jar should completely cover the blades.
- Prepare decimal dilutions with sterile Butterfield's phosphate diluents.
- Number of dilutions to be prepared depends on anticipated coliform density.
- Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 secs.
- Transfer 1 ml portions to three LST (Lauryl Sulfate Tryptose) tubes for each dilution for at least three consecutive dilutions.
- Hold pipette at angle so that its lower edge rests against the tube.

- **Let pipette drain 2-3 secs.**
- **Incubate LST tubes at 35°C.**
- **Examine tubes and record reactions at 24 ± 2 hours for gas, i.e. displacement of medium in fermentation vial or effervescence when tubes are gently agitated.**
- **Re-incubate gas-negative tubes for an additional 24 h and examine and record at 48 ± 2 hours.**
- **Perform confirmed test on all presumptive positive (gas) tubes.**

Confirmed test:

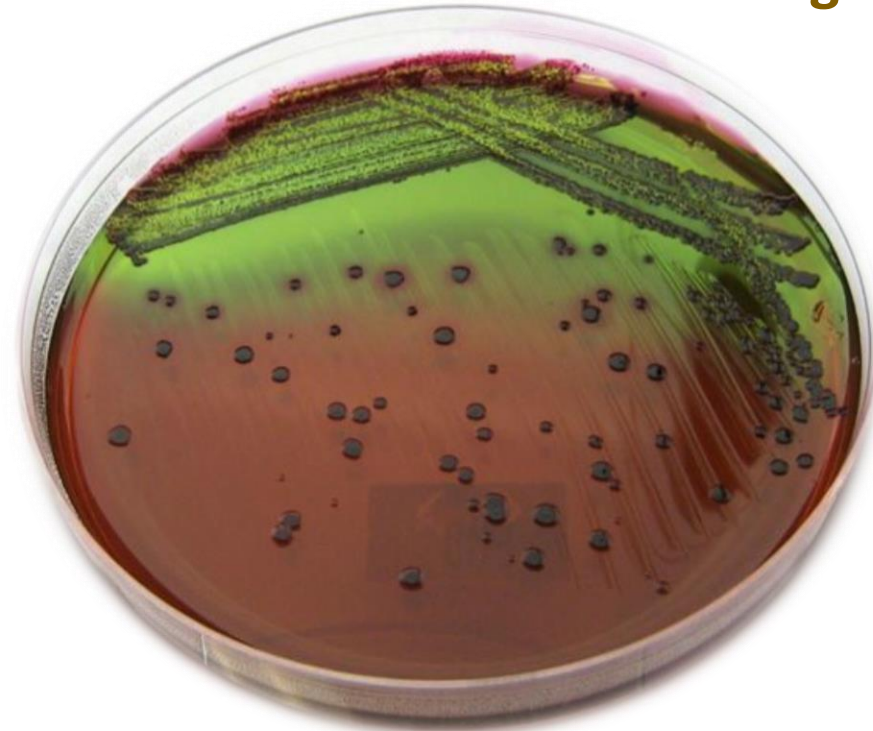
- From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present.
- Incubate BGLB tubes at 35°C and examine for gas production at 48 ± 2 h.
- Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for three consecutive dilutions.

Confirmed test for faecal coliforms and E. coli by Eijkman test:

- From each gassing LST tube from presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers).
- Incubate EC tubes 24 ± 2 h at 45.5°C and examine for gas production.
- If negative, re-incubate and examine again at 48 ± 2 h.
- Use results of this test to calculate faecal coliform MPN.

Completed test for *E. coli*:

- To perform completed test for *E. coli*, gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 h at 35°C.
- Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen.
- Transfer up to five suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35°C and use for further testing.

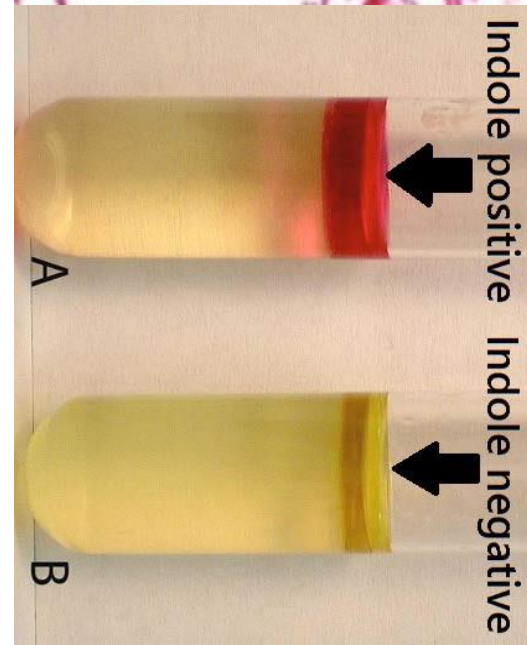
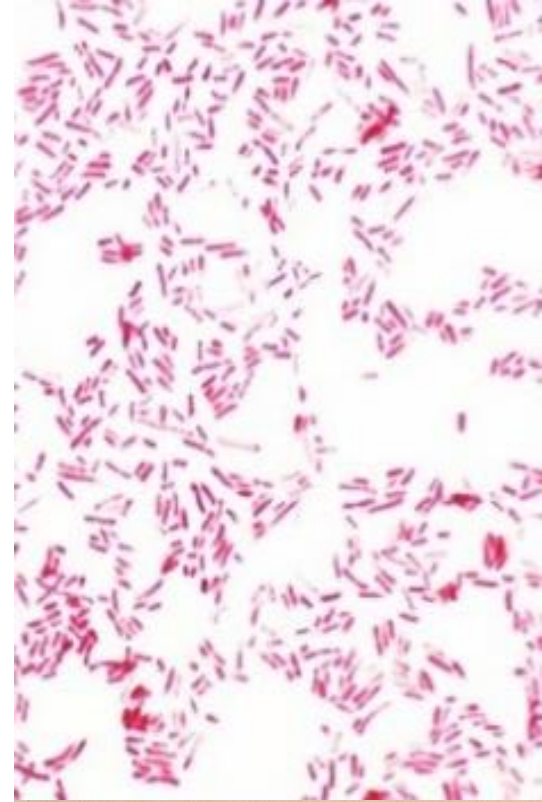


Gram staining

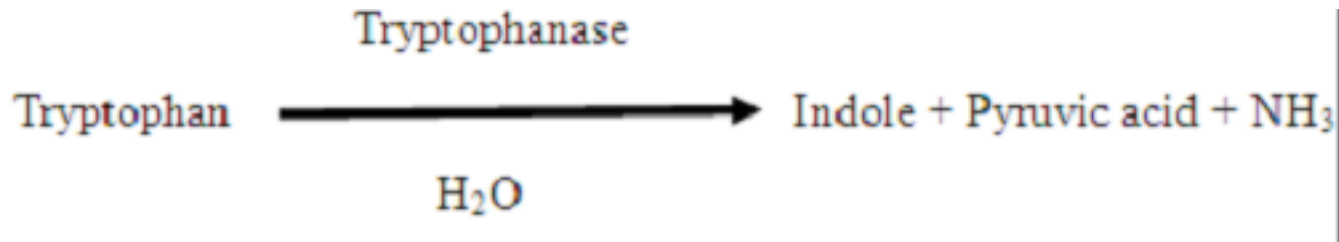
- All cultures appearing as Gram-negative, short rods should be tested for IMViC reactions and also re-inoculated back into LST to confirm gas production.

Indole test

- Some bacteria can produce indole from amino acid tryptophan using tryptophanase.
- Production of indole is detected using Ehrlich's reagent or Kovac's reagent.
- Indole reacts with aldehyde in reagent to give a red colour.
- An alcoholic layer concentrates the red colour as a ring at the top.



- Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°C .
- Test for indole by adding 0.2-0.3 ml of Kovacs' reagent.
- Appearance of distinct red colour in upper layer is positive test.

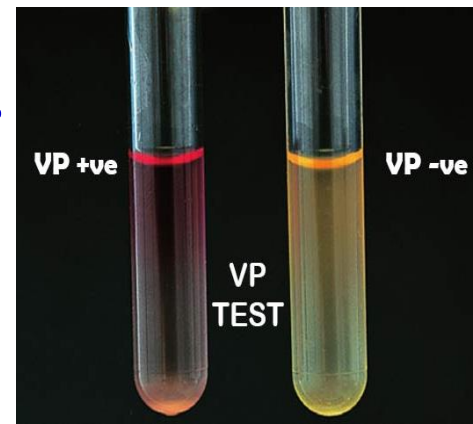


Methyl red test

- Detect ability of an organism to produce and maintain stable acid end products from glucose fermentation.
- Some bacteria produce large amounts of acids from glucose fermentation that they overcome buffering action of system.
- Methyl Red is a pH indicator, which remains red in colour (pH of <4.4).
- Inoculate tube of Glucose peptone water broth and incubate 48 ± 2 h at 35°C .
- Transfer 1 ml to 13 x 100 mm tube and add 5 drops of methyl red solution to each tube.
- Distinct red colour is positive test. Yellow is negative reaction.

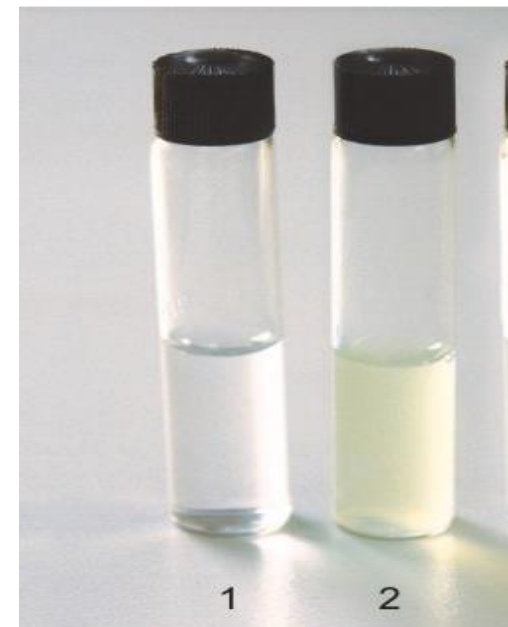
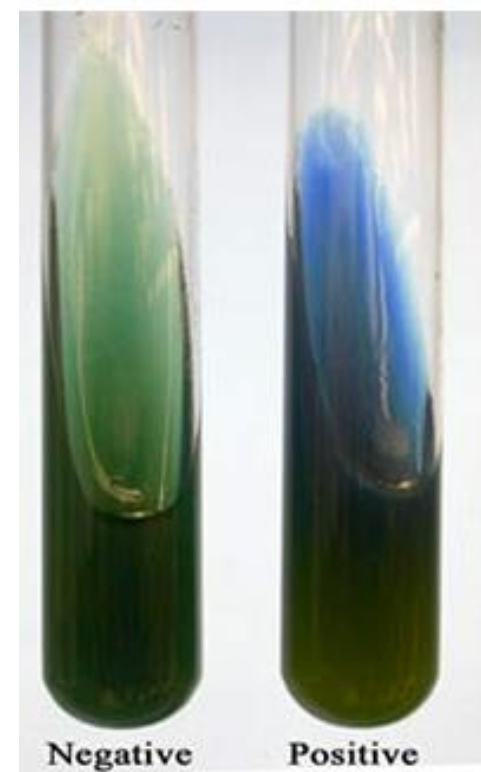
Voges Proskauer (VP) test

- VP test detects butylene glycol producers.
- Acetyl-methyl carbinol (acetoin) is an intermediate in production of butylene glycol.
- Two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen.
- If acetoin is present, it is oxidized in presence of air and KOH to diacetyl.
- Diacetyl then reacts with guanidine components of peptone, in the presence of α -naphthol to produce red colour.
- α -naphthol acts as catalyst and colour intensifier.
- Inoculate tube of glucose peptone water broth and incubate 48 ± 2 h at 35°C .
- Transfer 1 ml to 13 x 100 mm tube and add 0.6 ml α -naphthol and 0.2 ml 40% KOH and shake.
- Add a few crystals of creatine, shake and let stand 2 h.
- Test is positive if eosin pink colour develops.



Citrate utilization test:

- Detects ability of an organism to utilize citrate as source of carbon and energy.
- Bacteria are inoculated on medium containing sodium citrate and a pH indicator bromothymol blue.
- Medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen.
- Utilization of citrate involves enzyme citritase, which breaks down citrate to oxaloacetate and acetate.
- Oxaloacetate is further broken down to pyruvate and CO_2 .
- Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's colour from green to blue.
- Lightly inoculate tube of Koser's citrate broth and incubate for 96 h at 35°C .
- Development of distinct turbidity is positive reaction.



Gas from lactose:

- Inoculate a tube of LST and incubate 48 ± 2 h at 35°C .
- Gas production or effervescence after gentle agitation is positive reaction.
- All cultures that ferment lactose with gas production within 48 h at 35°C , appear as Gram-negative non-spore forming rods.

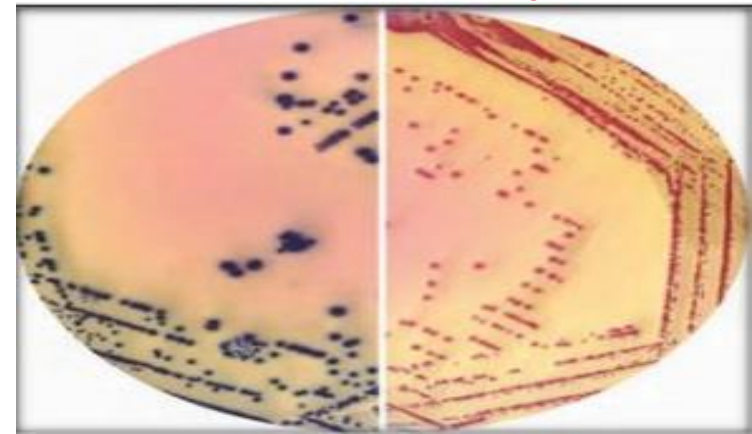


Figure 1. Peptone media with phenol red indicator. From left to right: Uninoculated tube; glucose fermenter with gas production (visible air bubble in the inverted Durham tube);

Various media used for the selective isolation of *E. coli*

- **HiCrome selective ECC base agar**
 - This selective medium recommended for the simultaneous detection of *E. coli* and total coliforms in water and food samples.
 - The chromogenic mixture contains two chromogenic substrates.
 - β -galactosidase produced by coliforms cleaves chromogen resulting in the salmon to red coloration.
 - β -glucuronidase produced by *E. coli*, cleaves Xglucuronide.
 - Colonies of *E. coli* are dark blue to violet colored due to cleavage of both chromogen.
 - Addition of L-tryptophan improves indole reaction, thereby increasing detection reliability.
 - Cefsulodin, when added inhibits *Pseudomonas* and *Aeromonas* sp.

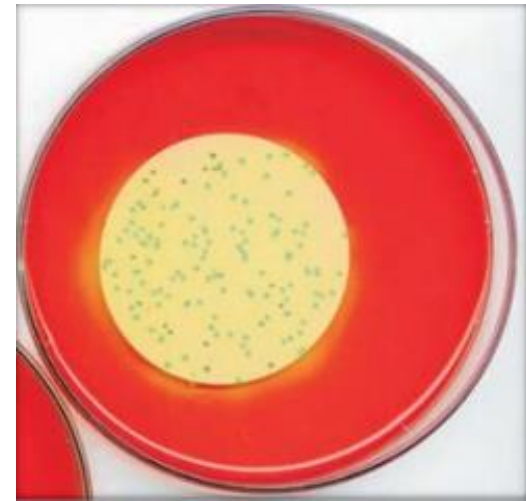
Blue colour colonies of *E. coli* & red colour colonies of *E. aerogenes*



- **HiCrome M-Lauryl sulphate agar**

- A modification of Lauryl tryptose broth, formulated by Mallman and Darby.
- This chromogenic medium is recommended for presumptive identification and differentiation of *E. coli* and other coliforms by single membrane filtration technique.
- Peptic digest of animal tissue and yeast extract provide essential growth nutrients to organisms.
- Lactose acts as a source of fermentable sugar.
- Beta-glucuronidase produced by *E. coli*, cleaves X-glucuronide, imparting a green colour to colonies and along with phenol red indicator aids in detection of lactose fermenter.

Green colour colonies of *E. coli*



Pathogenic *E. coli*

1. Shiga toxin producing *E. coli* (STEC) are food-borne pathogens that cause serious illness in humans.
2. Bovine animals, which are asymptomatic carriers, are most important reservoir.
3. Infection of humans is mostly caused by consumption of infected foodstuffs derived from bovine animals, such as milk and milk products.
4. Wide range of serogroups is capable of provoking these human infections (important are: O26, O103, O111, O145 and O157).
5. STEC bacteria owes its infective capacity to combination of virulence properties, important of which are: production of type I and II shiga toxins responsible for kidney failure, the proteins encoded on LEE locus, responsible for modification and intimate adherence to gastro-intestinal cells, and enterohemolysin, which plays role in destruction of blood.

- **LST-MUG method for detecting *E. coli*/*E. coli* O157:H7:**
 - Based on enzymatic activity of β -glucuronidase (GUD), which cleaves substrate 4-methylumbelliferyl β -D-glucuronide (MUG), to release 4-methylumbelliferone (MU).
 - When exposed to long wave (365 nm) UV light, MU exhibits a bluish fluorescence that is easily visualized in medium or around colonies.
 - Over 95% of *E. coli* produces GUD, including anaerogenic (non-gas producing) strains.
 - One exception is enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7, which is consistently GUD negative.
 - Lack of GUD phenotype in O157:H7 is often used to differentiate this serotype from other *E. coli*, although GUD positive variants of O157:H7 do exist.

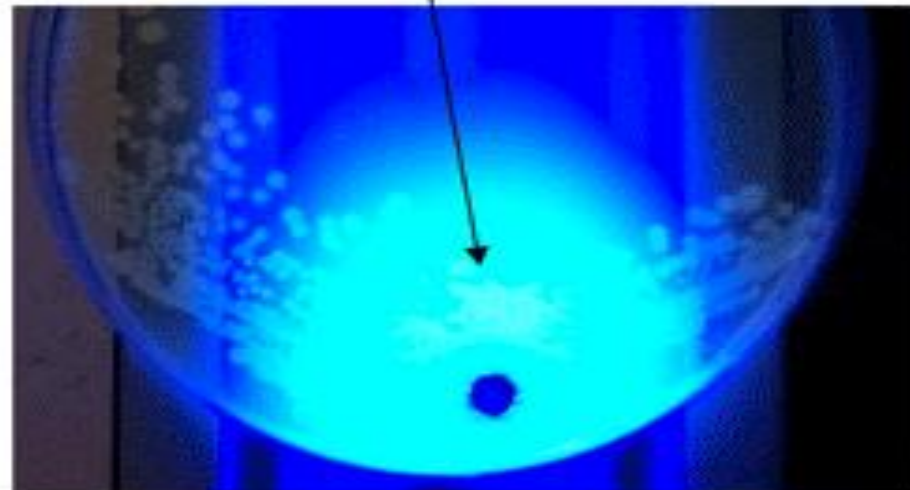
- MUG can be incorporated into almost any medium for use in detecting *E. coli*.
- In EMB which contain fluorescent components, are not suitable, as they will mask fluorescence of MU.
- When MUG is incorporated into LST medium, coliforms can be enumerated on basis of gas production from lactose and *E. coli* are presumptively identified by fluorescence in medium under long wave UV light, so capable of providing presumptive identification of *E. coli* within 24 h.

Appearance of CC disc under UV (365nm) light. *E. coli* O157:H7 is glucuronidase (MUG) negative with no fluorescence, other *E. coli* are MUG positive and fluoresce.

E. coli O157:H7



E. coli



- **Presumptive LST-MUG test for *E. coli*:**

- Prepare food samples and perform MPN Presumptive test, except use LST-MUG tubes instead of LST.
- Inoculate one tube of LST-MUG with a known GUD-positive *E. coli* isolate as positive control (ATCC 25922).
- Inoculate another tube with *Enterobacter aerogenes* (ATCC 13048) as negative control, to facilitate differentiation of sample tubes that show only growth from those showing both growth and fluorescence.
- Incubate tubes for 24 to 48 ± 2 h at 35°C.
- Examine each tube for growth (turbidity, gas), examine tubes in dark under long wave UV lamp (365 nm).
- Bluish fluorescence is a positive presumptive test for *E. coli*.
- After 48 h of incubation, 96-100% of *E. coli*-positive tubes can be identified.
- Perform confirmed test on all presumptive positive tubes by streaking loopful of suspension from each fluorescing tube to L-EMB agar and incubate 24 ± 2 h at 35°C.