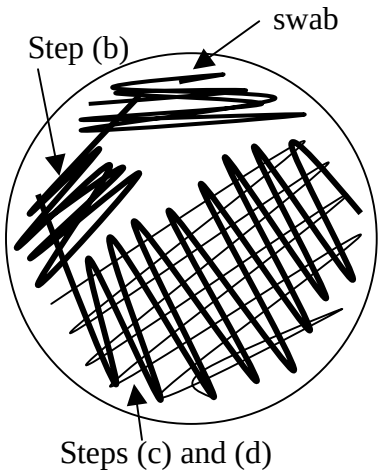
Part 1 - *E. coli* Isolation: Plating fecal specimens on MacConkey agar

Day 1:

1) Obtain sterilized, cotton-tipped swabs and MacConkey Agar plates. Each fecal sample will require 1 swab and 1 plate.
2) Only obtain samples from fecal specimens that have been shed under your direct inspection, to be assured of the origin and freshness of the feces.
   a) If the host is a human, this requirement is not enforced.
   b) Only one sample is to be taken from each host.
3) Carefully remove the sterilized swab from the paper covering or sterile glass jar and swirl the tip into the feces. Start in the middle of the specimen and thoroughly mix the sample as much as possible with the swab.
   a) Only a small amount is required on the tip of the swab - enough to be seen by eye.
4) Swab the sample onto a MacConkey agar such that no more than ¼ of plate is covered (see image below).
   a) Keep the sterile medium covered with the lid as much as possible to prevent the introduction of contaminants.
   b) Avoid adding anything other than the fecal sample itself - remove any introduced sand, dirt, or excess fecal material using the swab.
5) Return the lid and write the following on the agar side of the plate where the inoculum was added:
   a) date (e.g., 7/18/10)
   b) your initials
   c) common name of the host animal
   d) "step 1"
   e) if more than one plate is prepared from the same host, add a number for each fecal sample
6) Repeat steps 3-5 for as many samples as are required for the particular experiment.
7) If not returning immediately to the lab, store the plate at 4˚C for no more than 24 hours.
8) Once you have returned to the lab, streak the samples to obtain isolated colonies using aseptic technique.
   a) Sterilize inoculating loop by incineration, wait 5 seconds for the red color to dissipate and insure that it is cool by touching the loop on a sterile part of the agar.
   b) Drag the loop through the initial streak onto a new part of the agar and make a new patch that is about ¼ of the plate. When streaking onto the new part of the plate do not come into contact with the first streak.
   c) Sterilize and cool the loop as before, but streak from the new patch onto the rest of the agar plate, covering as much area as possible without contacting the other two patches.
   d) Flip the loop over and streak the final patch in the perpendicular direction to insure even distribution of the cells.

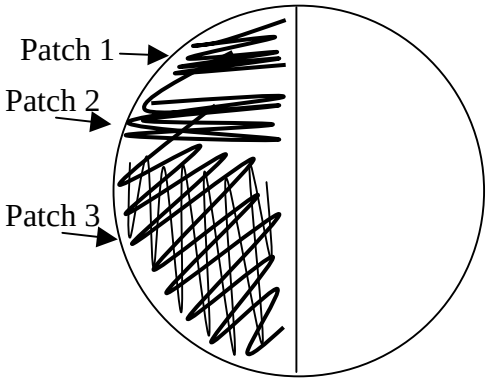


9) Repeat step 8 for all the samples collected.
10) Collect the plates and, if using a convection incubator, either wrap in parafilm or place them in a plate sleeve.
11) Incubate plates inverted such that the lid is on the bottom and the agar side is on top for 24-48 hours at 37˚C.

Part 2 - *E. coli* Isolation: Isolation of strains in MacConkey agar

Day 2:

1) Identify positive samples on streak plates from Part 1 that have 2-4 single, red, well-isolated colonies. (For classes: If no single red colonies are observed, you may borrow from a classmate, but you will need to note that the samples came from the specimen of your partner and keep track of this information throughout the rest of the experiment (only 2 isolates from each specimen are to be kept as stocks)).
2) For each positive sample, obtain 2 new, sterile MacConkey plates and divide each into two halves by drawing a line down the middle of the bottom of the dish.
3) Using aseptic technique, streak each of the 4 isolated colonies from the positive plate to purify the strains by again obtaining isolated colonies. One colony will be streaked for singles on ½ of the plate.
    a) Streak out the colonies by patching, sterilizing the loop, patching again, sterilizing the loop, and streaking the rest onto the remaining area in the halved of the plate to obtain single colonies, as described in step 8 of part 1 above.



4) Return the lid and write the following on the agar side of the plate where the inoculum was added:
    a) date (e.g., 7/19/10)
    b) your initials
    c) common name of the host animal
    d) "step 2"
    e) if more than one plate is being prepared from the same host in Part 1 (see step 5e), add the assigned number of the sample followed by the number of each colony taken from the original Part 1 plate. For example, if the colonies came from plate #14 from a given host species, then one half of the plate would be labeled as "14-1" and the other half "14-2".
5) Repeat steps 3-4 for all the samples collected.
6) Collect the plates and, if using a convection incubator, either wrap in parafilm or place them in a plate sleeve.
7) Incubate plates inverted such that the lid is on the bottom and the agar side is on top at 37˚C.
8) Store the original MacConkey plates from Part 1 in a sleeve at 4˚C.
9) After 24-48 hours, observe the MacConkey plates from Part 2 for at least one single colony from each streak.
    a) If less than 2 streaks produce isolated colonies, restreak from the thinnest area of the Part 2 plate onto a new plate as described above.
    b) If less than 2 streaks produce red colonies, return to the original plate from Part 1 and prepare a new streak to get isolated, red colonies.

Part 3 - *E. coli* Isolation: Identification of *E. coli* strains *via* metabolic activity

Day 3:

1) From each streak on the Part 2 plates that produced isolated, red colonies, use aseptic technique to patch ½ of a single colony onto an LB or TSA plate and the other ½ of the same colony onto an EMB plate. Up to four colonies from a single fecal sample (Part 1) may be patched onto the same plate (divide plate accordingly).
   a) The patches will not provide isolated colonies
2) Label each patch on the plates with the media type, date, your initials, and the isolate identifier as provided on the streak plate from Part 2.
3) Prepare a control plate with a known *E. coli* isolate (*e.g.*, C strain or K12) and *Enterobacter aerogenes*.
4) Collect the plates and, if using a convection incubator, either wrap in parafilm or place them in a plate sleeve.
5) Incubate plates inverted such that the lid is on the bottom and the agar side is on top for 24-48 hours at 37˚C.
6) Store the original MacConkey plates form Part 2 in a sleeve at 4˚C

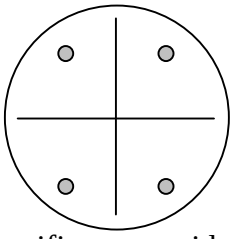
Day 4:

Examine the EMB and LB/TSA plates. Insure that the LB/TSA plates have growth and each patch on the EMB agar produces a green metallic sheen.

a) Verify that the *E. coli* control is green on EMB and the *E. aerogenes* is not.
b) Discard those isolates that do not produce the green sheen on EMB agar.
c) After analysis of isolates on EMB, discard plates in red biohazard bag.
d) Store LB plates in a bag at 4C.

Citrate test

1) Test for the expression of citrate permease by inoculating Simmon's citrate agar with paste from the LB/TSA patch using aseptic technique (only those that produced a green sheen on EMB agar).
   a) Each citrate agar plate may be divided into four sections
   b) Small, pea-sized inoculums are to be used in the middle of each quadrant, as shown to the right.



2) Label each spot on the plates with the date, your initials, and the isolate identifier as provided on the streak plate from Part 2.
3) Prepare a control plate with a known *E. coli* isolate (*e.g.*, C strain or K12) and *Enterobacter aerogenes*.
4) Collect the plates and, if using a convection incubator, either wrap in parafilm or place them in a plate sleeve.
5) Incubate plates inverted such that the lid is on the bottom and the agar side is on top for 24 hours at 37˚C. Plates may be stored after 24 hours at 4˚C until examination.

Day 4, *cont*:

Day 4, *cont*:

Indole test

1) Test for the expression of tryptophanase by inoculating tryptone broth with paste from the LB/TSA patch using aseptic technique (only those that produced a green sheen on EMB agar).
2) Label each tube with the date, your initials, and the isolate identifier as provided on the streak plate from Part 2.
3) Prepare control tubes with a known *E. coli* isolate (*e.g.,* C strain or K12) and *Enterobacter aerogenes*.
4) Incubate tubes for 24-48 hours at 37˚C.

Day 5:

1) Examine the citrate plates.
   a) Verify that the *E. coli* control demonstrates no growth or color change on citrate agar and the *E. aerogenes* has visible signs of growth with a color change in the medium from green to blue.
   b) Discard those isolates that grow on citrate agar and/or show a color change from green to blue.
   c) After analysis of isolates on citrate, discard plates in red biohazard bag.
2) Examine the tryptone broths.
   a) Add 10 drops (or 200-300ul) of Kovac's reagent to each tube without mixing the broth.
   b) Examine tubes for color change at the top of the broth - red color development is positive for indole production and no color change is negative. The color will fade after ~30 minutes.
   c) Verify that the *E. coli* control demonstrates a color change indicating that it is positive for tryptophanase and the *E. aerogenes* has no visible color change.
   d) Discard those isolates that do not turn red after addition of Kovac's reagent.
   e) Dump the tube contents in the 1.5L flask that Dr. Black has set up for this purpose, then wash out the tubes and dry them. Once the volume of the flask reaches 1L, autoclave the contents and separate the organic phase and collect it in a labeled liquid waste bottle. The lower layer containing media and dead bacteria can be discarded in the sink.
3) Return to the LB/TSA plates of the isolated strains and identify all of those that have the following characteristics:
   a) Red colonies on MacConkey agar
   b) Green metallic sheen on EMB agar
   c) No growth or color change in citrate agar
   d) Indole production in tryptone broth, indicated by development of red color following the addition of Kovac's reagent
4) For each fecal specimen obtained in Part 1, keep no more than 2 purified and verified *E. coli* strains.
   a) Clearly identify the positive strains on the LB/TSA plates by circling the strain identifier number and adding "*E. coli* positive" to the appropriate quadrant.

Part 4 - *E. coli* Isolation: Preparation of freezer stocks (*UBL Technician for classes*)

Day 6:

1) (Obtain plates from classes or other students that contain *E. coli* strains that have been purified and their identity verified by metabolic tests).
2) Complete attached table for each strain to be frozen. The below information will be required for each strain:
   a) Strain identifier code (*e.g.*, Pg-004)
   b) Animal source of *E. coli* strain
   c) Host name or number
   d) Sampling date
   e) Sampling location
   f) Date of freezing the strain
   g) Contributor - name of investigator or class section providing the strain (e.g. 224-04-Fall2011)
3) Re-label the quadrants of *E. coli* strains with new strain identifiers using a two-letter abbreviation for the host and a three-digit number (e.g., Pg-004 for the fourth *E. coli* isolate from pigeon).
   a) See attached table to identify the correct two-letter abbreviation code for the host animal
4) To make freezer stocks for each strain, obtain sterile cyrotubes and print out tube label stickers that follow the numbering system of any existing frozen stocks from the host animal (see A. Hamrick).
   a) There will be two sticker formats for each strain, one for the top cap and one for the tube.
5) Place the small round sticker on the cap insert for the cryovials and press the cap to the top of the tube. Place the rectangular sticker with a bar code (accompanied the cap sticker) vertically on the side of the tube.
6) Add 1mL of freezing medium to each labeled cryovial using aseptic technique.
   a) Medium contains 80mL LB broth and 20mL of diluted glycerol mixture (80mL glycerol with 20mL DI water). Sterilize in autoclave prior to use.
   b) For large numbers of tubes, use a10mL serological pipette to add approximately 1mL of the freezing medium to each tube - often best done in an assembly line of 3 students (two working with the tubes and one adding the medium).
7) Organize the LB/TSA plates by new strain identifier.
8) Sterilize and cool a metal loop then take a large balled-up loopful of paste (diameter of ~3mm) from a selected LB/TSA plate.
9) Inoculate the corresponding cyrotube with the *E. coli* paste.
   a) Remove the lid from the cryovial and agitate the loop until the paste has been transferred to the medium (spinning the loop often works best).
   b) Immediately return the lid to the top of the cryovial and screw down tight.
10) Vortex on high for ~30 seconds to fully resuspend the cells in the medium.
11) Repeat steps 5-7 for all additional strains.
12) Add tubes to labeled cryobox and freeze at -70˚C.
13) After frozen, samples may be taken from individual tubes by scraping material from top of frozen medium with sterile pipet tip or wooden stick and streaking cells out for single colonies on LB/TSA plates (as done in Part 1).

Host animal	Abbreviation

Cat	Ct
Cow	Cw
Dog	Dg
Horse	Ho
Human	Hu
Pelican	Pc
Pig	Sw
Pigeon	Pg
Seagull	Sg
Raccoon	Rc
Sheep	Sp
Chicken	Ch
Deer	Dr
Elk	Ek
Rat	Rt
Bat	Bt
Ground Squirrel	GS
Squirrel	Sq