CHAPTER 7

Isolation and Identification of *Escherichia coli*

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Escherichia coli is one of the predominant species of facultative anaerobes in the human gut and usually harmless to the host; however, a group of pathogenic *E. coli* has emerged that causes diarrheal disease in humans. Referred to as Diarrheagenic *E. coli* or commonly as pathogenic *E. coli*, these groups are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* often requires that the isolates be first identified as *E. coli* before testing for virulence markers. The pathogenic groups includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and perhaps others that are not yet well characterized. Of these, only the first 4 groups have been implicated in food or water borne illness.

ETEC is recognized as the causative agent of travelers' diarrhea and illness is characterized by watery diarrhea with little or no fever. ETEC infections occurs commonly in under-developed countries but, in the U.S., it has been implicated in sporadic waterborne outbreaks as well as due to the consumption of soft cheeses, Mexican-style foods and raw vegetables. Pathogenesis of ETEC is due to the production of any of several enterotoxins. ETEC may produce a heat-labile enterotoxin (LT) that is very similar in size (86 kDa), sequence, antigenicity, and function to the cholera toxin (CT). ETEC may also produce a heat stable toxin (ST) that is of low molecular size (4 kDa) and resistant to boiling for 30 min. There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or STh is predominant in human isolates only. The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower levels. Because of its high infectious dose, analysis for ETEC is usually not performed unless high levels of *E. coli* have been found in a food. Also, if ETEC is detected, levels should also be enumerated to assess the potential hazard of the contaminated food. Production of LT can be detected by Y-1 adrenal cell assays (28) or serologically by commercial reverse passive latex agglutination assay and ELISA. The production of ST can also be detected by ELISA or by infant mouse assay. Both LT

and ST genes have also been sequenced and PCR and gene probe assays are available. Analysis of colonies on plating media using gene probe/colony hybridization also allows enumeration of ETEC in foods.

EIEC closely resemble Shigella and causes an invasive, dysenteric form of diarrhea in humans. Like Shigella, there are no known animal reservoirs; hence the primary source for EIEC appears to be infected humans. Although the infective dose of Shigella is low and in the range of 10 to few hundred cells, volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic. Pathogenicity of EIEC is primarily due its ability to invade and destroy colonic tissue. The invasion phenotype, encoded by a high molecular weight plasmid, can be detected by invasion assays using HeLa or Hep-2 tissue culture cells or by PCR and probes specific for invasion genes.

EPEC causes a profuse watery diarrheal disease and it is a leading cause of infantile diarrhea in developing countries. EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Through volunteer feeding studies the infectious dose of EPEC in healthy adults has been estimated to be 10^6 organisms. Pathogenesis of EPEC involves intimin protein (encoded by eae gene) that causes attachment and effacing lesions; but it also involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells. Production of EAF can be demonstrated in Hep-2 cells and the presence of eae gene can be tested by PCR assays.

EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are typified by the production of verotoxin or Shiga toxins (Stx). Although Stx1 and Stx2 are most often implicated in human illness, several variants of Stx1 and Stx2 exist. There are many serotypes of Stx-producing *E. coli* (STEC), but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide. The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef, raw milk, cold sandwiches, water, unpasteurized apple juice and sprouts and vegetables. EHEC O157:H7 are phenotypically distinct from *E. coli* in that they exhibit slow or no fermentation of sorbitol and do not have glucuronidase activity; hence, these traits are often used to isolate this pathogen from foods. The production of

Stx1 and Stx2 can be tested by cytotoxicity assays on vero or HeLa tissue culture cells or by commercially available ELISA or RPLA kits. Gene probes and PCR assays specific for stx1 and stx2 and other trait EHEC markers are also available.

Enrichment for Pathogenic *E. coli*

The approach recommended here permits qualitative determination of the presence of pathogenic *E. coli*. Aseptically weigh 25 g of sample into 225 ml of BHI broth (dilution factor of 1:10). If necessary, sample size may deviate from 25 g depending on availability of the sample, as long as the diluent is adjusted proportionally. Blend or stomach briefly. Incubate the homogenate for 10 min at room temperature with periodic shaking then allow the sample to settle by gravity for 10 min. Decant medium carefully into a sterile container and incubate for 3 h at 35°C to resuscitate injured cells. Transfer contents to 225 mL double strength TP broth in a sterile container and incubate 20 h at 44.0 ± 0.2°C. After incubation, streak to L-EMB and MacConkey agars. Incubate these agars for 20 h at 35°C.

Selection of colonies

Typical lactose-fermenting colonies on L-EMB agar appear dark centered and flat, with or without metallic sheen. Typical colonies on MacConkey agar appear brick red. Lactose non-fermenting biotypes on both agars produce colorless or slightly pink colonies.

NOTE: EIEC do not ferment lactose and there may also be atypical non-lactose fermenting strains in the other pathogenic *E. coli* groups; therefore, as many as 20 colonies (10 typical and 10 atypical) should be picked for further characterization.

Conventional Biochemical Screening and identification

Use the IMViC procedures for biochemical and morphological identification of *E. coli*. However, because many enteric bacteria can also grow in the TP enrichment broth, plus anaerogenic, non-motile and slow or lactose non-fermenting strains of *E. coli* must also be considered, additional tests may need to be performed. Some of these new or modified reactions are discussed here.

Primary screening. Transfer suspicious colonies to TSI agar, BAB slant, tryptone broth, arabinose broth, and urea broth. Incubate 20 h at 35°C. Reject H_2S-positive, urease-positive, arabinose non-fermenting, and indole-negative strains. To test for the ONPG reaction, suspend growth from TSI in 0.85% saline to give detectable turbidity. Add an ONPG-impregnated disk and incubate 6 h at 35°C. Yellow color indicates positive reaction. Reject ONPG-negative,

aerogenic cultures. Some Alkalescens-Dispar strains (i.e., anaerogenic *Escherichia*) are ONPG-negative.

Secondary screening (48 h incubation at 35°C unless otherwise specified). To identify cultures, test additional reactions shown in Table 1, Chapter 4, to subdivide *Escherichia* spp. Since it is not known whether these additional species are of pathogenic significance to humans, strains giving typical reactions for *E. coli* should be further investigated. To differentiate *E. coli* from *Shigella*, examine anaerogenic, non-motile, slow lactose fermenters for lysine decarboxylase, mucate, and acetate reactions. *Shigella sonnei*, which may grow in the same enrichment conditions, is anaerogenic and non-motile. It also produces a negative indole reaction and shows slow or non-fermentation of lactose. Alternatively, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*.

Tests for Enterotoxigenic *E. coli* (ETEC)

When *E. coli* levels in foods exceed 10^4 cells/g, perform enumeration for ETEC by colony hybridization analysis using DNA probes for LT and ST. If biological activity assays are necessary, LT can be detected by the Y-l tissue culture test and ST can be detected by the infant mouse test. There are also commercially available RPLA and ELISA tests to detect LT and ST toxins as well as PCR assays.

Tests for Enteroinvasive *E. coli* (EIEC)

If an isolate is suspected to be EIEC, the invasive potential of the isolates may be tested by the Sereny test or the Guinea pig keratoconjunctivitis assay. Invasive potential of the isolates can also be determined by the HeLa tissue culture cell assay as described, or with the *in vitro* staining technique using acridine orange to stain intracellular bacteria in HeLa monolayers. Alternatively, since the *invA* gene sequence of EIEC closely resembles that of *Shigella*, DNA probe and PCR assays for *inv* gene of *Shigella* will also work for EIEC.

Tests for Enteropathogenic *E. coli* (EPEC)

EPEC strains are identified based on 3 key traits: attachment and effacing lesion (A/E), localized adherence on cells and the lack of Shiga toxin (*Stx*) production. This last trait is also used to distinguished strains of EPEC from EHEC. Phenotypically, A/E and localized adherence are tested using Hep-2 or HeLa tissue cells. Absence of Stx can be determined using tests outlined for EHEC

(see below). There are also PCR and probes for the EAF plasmid that encodes for localized adherence and the *eae* gene that encodes for the intimin that causes the A/E phenotype.

Caution: There are several variants of *eae* gene and some EPEC strains carry *eae* variants identical to EHEC serotypes; hence, these tests will detect strains from both pathogenic groups.

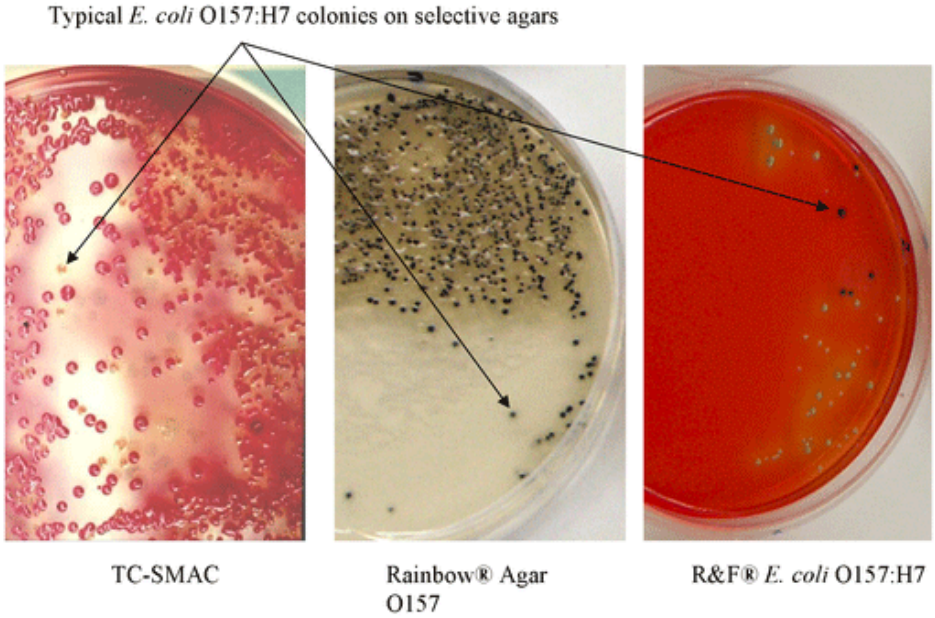
O157:H7 - Cultural Isolation and Presumptive Isolate Screening

For overnight enrichment samples that are found probable positive by the real-time PCR assay, cultural confirmation is required. Similarly, for samples that have not been screened by real-time PCR follow these procedures for culture isolation.

Isolation procedure.

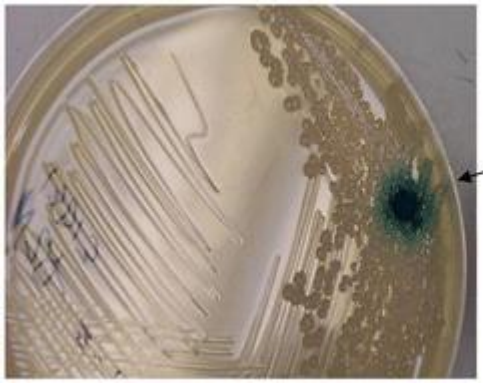
Serially dilute the overnight sample enrichment in phosphate buffer and spread-plate appropriate dilutions (usually 0.05 mL of 10^{-2} and 10^{-4} dilutions should yield approximately 100-300 isolated colonies) in duplicate onto TC-SMAC and one chromogenic agar (Rainbow® Agar O157 or R&F®*E. coli* O157:H7 agar). Optionally, a streak plate may also be included. Incubate plates at 37°C ± 1°C for 18 - 24 h. On TC-SMAC. typical O157:H7 colonies are colorless or neutral/gray with a smoky center and 1-2 mm in diameter. Sorbitol-fermenting bacteria such as most *E. coli* appear as pink to red colonies. On Rainbow® Agar O157 or R&F®*E. coli* O157:H7 agar, *E. coli* O157H7 colonies should appear as black to blue-black colonies.

Figure: Appearance of typical *E. coli* O157:H7 on TC-SMAC, Rainbow® Agar O157 and R&F® *E. coli* O157:H7 agars.

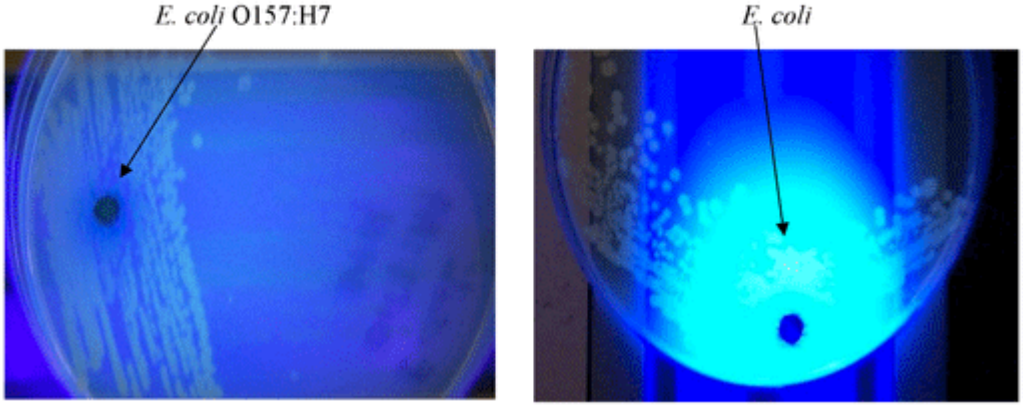


Screen typical colonies by picking a portion of each isolated suspect colony from the isolation agar and testing for O157 antigen by latex agglutination (Remel kit). Pick all typical colonies that screen positive (up to 10, if >10 are present) from isolation agars and streak onto TSAYE plates to check for purity. Place a ColiComplete (CC) disc (BioControl, Bellevue, WA) in the heaviest streak area on the TSAYE plate. Prepare a similar TSAYE plate using a known MUG-positive *E. coli* strain as positive control. Incubate the plates 18-24 h at 37°C ± 1°C. CC has a chromogenic assay for galactopyranosidase (X-gal) and a fluorogenic assay for glucuronidase (MUG) on the same disc. The positive control should show blue color on and around the disc (indicative for coliforms) and blue fluorescence around the disc under long wave UV (365 nm) light (indicative of *E. coli*). Strains of O157:H7 are X-gal (+) but MUG (-).

Figure: Results of ColiComplete (CC) disc for *E. coli* and *E. coli* O157:H7



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



Spot Indole Test: Spot growth from TSAYE plate to a filter wetted with Kovac's reagent. *E. coli* O157:H7 are indole positive.