METHODS OF DETECTION AND CHARACTERIZATION OF PATHOGENIC ESCHERICHIA COLI

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Contents

Summary

Pathogenic *E. coli* that cause diarrheal diseases are most often transmitted via contaminated water and foods. Microbiological testing for the presence of pathogenic bacteria in foods is a complex, multi-step process that entails culture enrichment of the food sample, screening the enriched sample with a target specific assay, and confirming the presence of the pathogen by isolate identification and characterization. To perform this task to detect pathogenic *E. coli* is even more difficult due to the large diversity of strains that belong to the various pathogenic groups. As a result, few assays exist that can be used to test for pathogenic *E. coli* as a whole, but serotype and strain specific assays are available.

This chapter presents background and epidemiological information on the 6 recognized pathogenic *E. coli* groups that causes diarrheal diseases and discusses the logistical details involved at each step to facilitate the detection of these pathogens in foods. In addition, since pathogenic *E. coli* are grouped based on distinct epidemiological and clinical features and/or unique virulence factors, it is often essential to test for virulence factors to identify the strains that belong to the different groups. Lastly, as many of the virulence factors carried by pathogenic *E. coli* reside on mobile genetic elements, such as plasmids and phages, gene transfer among strains occurs, resulting in the emergence of new variants that can cause disease in humans.

1. Introduction

The microbial inhabitants in the digestive tract of humans are comprised mostly of anaerobic bacterial species; however, there are also many other bacteria, of which *Escherichia coli* is one of the dominant enteric bacteria species. Normal intestinal bacterial flora provides many beneficial functions to the human host, such as aiding in digestion and synthesizing vitamins, as well as competing and suppressing the colonization of intestinal linings by pathogenic bacteria that can cause illnesses. *E. coli* was first identified by Theodore Escherich in 1885 from fecal samples of healthy infants. Because it is abundantly found in feces, *E. coli* was proposed to be used as a fecal indicator over a century ago and continues to be used as an indicator of fecal contamination and/or of insanitation, worldwide. The concept of indicators is based on the premise that if *E. coli* is detected in food or water, it is evidence that the product has been contaminated with sewage and indirectly, that pathogenic bacteria may also be present. Over the years, the use of *E. coli* as a fecal indicator has been challenged and scrutinized due to its isolation from non-fecal sources; however, the practice continues to be used worldwide.

As a member of the family *Enterobacteriaceae*, *E. coli* is composed of Gram negative, facultatively anaerobic, non-sporeforming rod shaped bacteria. This species has the ability to ferment many sugars; however, lactose fermentation with the production of acid and gas within 48 h at 37°C is a characteristic trait of *E. coli*, as well as for the other members of the coliform group. Typical biochemical traits of *E. coli* are shown in Table 1. Some of these, such as the IMViC tests (indole, methyl red, Voges-Proskauer and citrate) are key traits that are used to further distinguish *E. coli* into biotypes, where biotype I and II strains have IMViC reactions of ++-- and -+--, respectively, with indole reaction being the only difference. However, as shown in Table 1, since approximately 96% of *E. coli* is indole positive, biotype I strains are much more prevalent. Similarly, atypical *E. coli* strains that do not ferment lactose or exhibit ß-glucuronidase (GUD) activity are rare but do exist.

Media/Assay	**Reaction**
Tryptone broth	Indole positive (96%)*
MR-VP broth	MR positive (100%)
	VP negative (100%)
Simmons citrate	Negative (100%)
ß-glucuronidase	Positive (96%)
Urease	Negative (100%)
Lactose fermentation	Positive (96%)
Sorbitol fermentation	Positive (94%)
Cellobiose fermentation	Negative (98%)
Lysine decarboxylase	Positive (90%)
Ornithine decarboxylase	Positive (60%)
Phenylalanine deaminase	Negative (100%)
Triple sugar iron agar	Yellow butt/yellow slant, no H_2S
Motility test medium	Motile or non-motile (NM)

Table 1. Typical biochemical reactions of *E. coli*.
* denotes the (%) of isolates that exhibit the phenotype.

Serological typing of *E. coli* is based on the somatic (O), flagellar (H) and capsular (K) antigens. There are at least 181 known O types, 56 H types and 80 K types and an isolate may have any combination of these 3 antigens, and thus, the genus is very complex serologically. However, serotyping remains a very useful classification tool, especially for characterizing and identifying some of the well known pathogenic *E. coli*, such as serotype O157:H7, which has caused food borne illness worldwide.

Although *E. coli* are usually regarded as harmless commensal bacteria, when the intestinal barrier is compromised and they are disseminated elsewhere in the body, even typical *E. coli* may cause infections. For instance, *E. coli* is a common causative agent of urinary tract infections in women and has also caused bacteremia (presence of bacteria in blood); therefore, they are regarded as opportunistic pathogens. There is, however, groups of *E. coli* that have acquired unique virulence traits and causes gastrointestinal illnesses in healthy humans. Commonly known as pathogenic, diarrheagenic, or enterovirulent *E. coli*, there are currently six recognized groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Pathogenic *E. coli* are grouped based on distinct epidemiological and clinical features and/or unique virulence factors, so identification of strains in each of the groups often entails testing for the specific virulence determinants. However, it should be pointed out that since many of the virulence genes are carried on mobile genetic elements, they can be exchanged by horizontal transfer, and therefore, can be present in strains of different pathogenic groups.

This chapter presents the general characteristics, diseases and epidemiology associated with the various pathogenic *E. coli* groups that causes diarrheal diseases. The chapter will also discuss microbiological and molecular methods that can be used for the detection, serotyping and characterization of pathogenic *E. coli* strains.

2. Pathogenic *E. coli* Groups

Almost all infections with pathogenic *E. coli* groups that causes diarrheal diseases begin with the colonization of or attachment to the intestinal mucosa, followed by the elaboration of each groups' distinctive virulence factors that are associated with gastrointestinal symptoms. Pathogenic *E. coli* infections can range from asymptomatic or mild diarrhea to more severe infections such as hemorrhagic colitis (HC) with symptoms of bloody diarrhea that can progress to the potentially fatal complications of hemolytic uremic syndrome (HUS). Of the 6 pathogenic groups, EPEC, ETEC, EHEC, and EIEC are well recognized to be transmitted via contaminated food or water, and EHEC especially, have been implicated in many large, food borne outbreaks worldwide. Some of the other pathogenic *E. coli* groups have only been associated with sporadic cases of gastrointestinal illness in under-developed countries and often with unknown food etiologies, but more recently, even some of these, such as the EAEC strain of O104:H4 serotype that acquired the Shiga toxin 2 gene, has caused a major food-borne outbreak in the European Community (EU). Some of virulence properties and symptoms often associated with these pathogenic *E. coli* groups are summarized in Table 2 and discussed in more detail in each of the respective sections.

Properties/Symptoms	ETEC	EPEC	EHEC	EIEC	EAEC	DAEC
Toxin (s)	LT/ST[a]	-	Shiga/Vero (Stx or VT)	-	EAST1[b]	-
Invasive	-	-	-	+	-	-
Intimin	-	+	+	-	-	-
Enterohemolysin	-	-	+	-	-	-
Stool	Watery	Watery	Watery, very bloody	Mucoid, bloody	Watery, mucoid	Watery
Fever	Low	Low	-	+	Low	-
Fecal leukocytes	-	-	-	+	-	-
Intestinal area affected	Small	Small	Colon	Colon, lower small	unknown	unknown
Major serotypes	O128c, O161, O8 others	O26, O55, O111, O127, others	O157:H7, O26, O111, others	O28c, O29, others	O3, O44, O77, O104 others	O11, O75 others
I_D[c]	High	High	Low	High	unknown	unknown

Table 2. Some properties and symptoms associated with pathogenic *E. coli* groups.

2.1. Enteropathogenic *E. coli* (EPEC).

EPEC was first characterized as a pathogenic group in 1945, and it is most commonly associated with diarrhea in infants, especially those under 2 years of age. Infantile diarrhea caused by EPEC is a prevalent problem in developing countries, and it sometimes surpasses in frequency to diarrheal infections caused by rotavirus. In developed countries or those with good hygienic standards, EPEC has become less significant and only causes sporadic cases of infection. A variety of foods (raw beef and chicken, cold pork, meat pie, coffee substitute drink, etc.) have been implicated in EPEC outbreaks worldwide; however, water is also a common vehicle of infection. The use of contaminated water in the preparation of infant formula is suspected as a common source of EPEC infection in children. The infectious dose of EPEC is estimated to be in the range of 10^6 to 10^8 CFU; but, expected to be lower in infants and immunocompromised individuals. After ingestion of the contaminated source, the average incubation period is 36 h before the onset of illness, but can range from 17 - 72 h. The predominant symptom of EPEC infection is severe diarrhea with watery stools that contain large amount of mucus; but is seldom bloody. Other symptoms include fever, vomiting, nausea, and abdominal cramps. Infected children and adults exhibit similar symptoms, but these may be less severe and shorter lasting in adults. The illness is usually over in a few days; however, in severe cases of infantile diarrhea, it can last up to 14 days. EPEC infections are best treated by preventing dehydration that is

associated with the diarrheal symptoms. Some antibiotics have been shown to be effective against EPEC; however, antibiotic resistant strains are also known to exist. EPEC infection is uncommon in adults either due to lower susceptibility to disease or to the development of immunity to the pathogens, as increases in strain-specific antibodies have been observed with age.

A model proposed for EPEC pathogenesis consists of 3 stages. First, there is localized adherence to epithelial cells, probably via a type of fimbriae known as the bundle-forming pilus (BFP). This then triggers signal transduction activity, encoded by genes on the locus of enterocyte effacement (LEE) pathogenicity island, which includes *tir* and *eae* that encodes for the translocated intimin receptor and intimin, respectively. The Tir protein is secreted and binds to epithelial cells, where it serves as receptor for intimin. These proteins enable intimate adherence of EPEC to cells and the resulting attaching and effacing (A/E) lesion also causes accumulation of polymerized actin at the site of attachment. Typical EPEC are defined as strains that have *eae* and exhibit the A/E phenotype, do not produce Shiga toxins (Stx), and has the EPEC adherence factor (EAF) plasmid, which has the *bfpA* gene that encodes for BFP. Strains that have *eae* but lack the EAF plasmid are known as atypical EPEC (aEPEC), which are suspected to be less virulent, possibly due to lack of the EAF plasmid. Although aEPEC are sometimes more frequently associated with infections in developed and developing countries compared to typical EPEC, additional studies are needed to identify the presence of other virulence factors and to determine the pathogenicity of these aEPEC strains.

2.2 Enterotoxigenic *E. coli* (ETEC).

ETEC is best known as the causative agent of traveler's diarrhea; however, it is also an important diarrheal pathogen in infants. ETEC infections are endemic in many developing countries in the tropics and in areas with poor hygienic standards, and infections are especially prevalent in the warm, wet months. The onset of illness is usually 26 h after ingestion; however, the incubation period can range from 8 to 44 h. ETEC infection is characterized by a sudden onset of cholera-like watery diarrhea, without blood or mucus, and it is rarely accompanied by fever or vomiting. Illness is usually self limiting, mild and brief, lasting only a few days; but in some severe forms, ETEC infections can persist for up to 19 days and can resemble cholera with up to 5 or more daily passages of rice water-like stools, resulting in severe dehydration. Antibiotic treatment is usually not required in ETEC infections; however, it may be effective in reducing the duration and severity of illness. The infectious dose for ETEC is high, and data from volunteer feeding studies estimate it to be 10^8 to 10^{10} cells.

ETEC strains are often found in the feces of asymptomatic carriers, and hence, humans are the most likely source of this pathogen. ETEC infection does not appear to be transmitted by person-to-person contact but some hospital infections have occurred and are probably caused by insanitary conditions. Consumption of contaminated food or water accounts for most ETEC outbreaks. In 1975, a large outbreak affecting 2000 people was traced to sewage-contaminated water at a national park. Contaminated well water in Japan and the water supply aboard cruise ships have also been implicated in several ETEC outbreaks. From 1996 to 2003, the U.S. Centers for Disease Control and Prevention (CDC) identified 16 ETEC outbreaks, of which three occurred on cruise

ships that docked in the U.S. The vehicles of infection was identified in 11 outbreaks and they included drinking water, ice, and foods such as fresh vegetables, salads, parsley, basil, chicken, tacos, lasagna, and others. Interestingly, ETEC belonging to serotype O161:H41 was identified in 10 of the outbreaks. Food-borne outbreaks of ETEC have also occurred in restaurants and at various food service-catered settings, and implicated foods like Brie cheese, curried turkey, mayonnaise, crabmeat, deli food, salads, and others. In most of these cases, foods became contaminated with ETEC via infected food handlers or through the use of contaminated water during food preparation.

The virulence factors that characterize the ETEC group are the production of enterotoxins and colonization factor antigens (CFA), all of which are encoded by plasmids. The pathogenicity of ETEC resembles that of *Vibrio cholerae*, where the bacteria attach to the intestinal mucosa via fimbrae or CFA, followed by the production of heat-labile (LT) and/or heat-stable (ST) enterotoxins that results in the watery diarrhea symptoms. The LT is a large 86-kD protein that is easily inactivated at 65°C for 30 min. In the LT class, there are 2 serologically distinct types. LT-I is important in causing illness in man and animals and within this type, there are 2 closely related variants, designated LTh-I for human origin or LTp-I for pig origin. The other type, LT-II, is produced mostly by animal isolates of *E. coli*, and it has not been associated with illness. In contrast, ST is a small peptide of about 2 kD, and it is stable to heating at 100°C for 30 min. Like LT, there are also 2 distinct types; STa is produced by ETEC and a few other pathogens and include the human (STh) or pig (STp) variants. STb is produced only by ETEC and is limited mainly to porcine strains**.** ETEC strains may also carry the *ast* gene encoding for the EAST1 toxin; however, the role of this toxin in ETEC pathogenesis is not known. Additional research is needed to elucidate the overall pathogenesis of ETEC, as well as on the identification of regulatory mechanisms that may play a role in colonization and in the secretion and targeting of toxins to host cells. No ETEC vaccine is currently on the market; however, development strategies have focused on adhesin-based antitoxic vaccines and at inducing intestinal immune responses.

2.3. Enterohemorrhagic *E. coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC).

There is a large group called Shiga toxin-producing *E. coli* (STEC), which are characterized by the production of Shiga toxins (Stx), also known as Verocytotoxins. It is estimated that there are 300 STEC serotypes but not all appear to be pathogenic for humans. Various STEC serotypes have been isolated from the feces of healthy humans and they are also found in fresh beef, poultry, seafood, produce and other foods that have not implicated in illness; hence, the health significance of STEC as a whole, is uncertain. There is a subset of STEC that cause serious human illnesses such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). This subset of pathogenic strains has been referred to as EHEC, pathogenic STEC or other terms. In this chapter, we will use the term EHEC.

Of those serotypes that are known to be pathogens and regarded as EHEC, serotype O157:H7 is the type strain and has most often been implicated in outbreaks in the U.S. and many other countries. But, other serotypes such as O26:H11, O111:NM, and others

have also been implicated in illness around the world. For example, in 2008, *E. coli* O111:NM caused an outbreak associated with a restaurant in Oklahoma that infected 341 persons with 71 hospitalizations, 26 cases of HUS (8%), and one death. The source of the pathogen was not identified. In 2010, there was an O145 outbreak associated with lettuce that infected over 30 individuals, 40% were hospitalized, and there were 3 cases (ca. 10%) of HUS. Recently, a very large outbreak suspected to have been caused by contaminated sprouts occurred in 2011, in Germany but there were also cases in several other countries. This large outbreak affected over 4,000 individuals with approximately 900 cases of HUS and about 50 deaths. The causative agent was an EAEC strain of O104:H4 serotype that acquired the gene that encodes for Stx.

Serotype O157:H7 was first isolated in the U.S. in 1975; but, was not recognized as a food-borne pathogen until 1982. Cattle are the most important reservoir for O157:H7 and non-O157 STEC serogroups; but, other ruminants, including, sheep, goat, buffalo, guanaco, deer, and, elk also carry STEC. Non-ruminant animals, including cats, dogs, pigs, horses, rabbits, and poultry can also carry and transmit STEC. Infections by O157:H7 are commonly caused by the consumption of undercooked, contaminated ground beef or beef products; however, illness caused by contaminated drinking or recreational water, raw milk, contact with animals carrying the pathogen, and person-to-person contact have also been well documented. As interventions to control the presence of STEC in beef have been implemented, the presence of O157:H7 and the number of outbreaks associated with beef products have slightly declined; however, outbreaks and sporadic cases linked to fresh produce have increased, caused by foods, like vegetables, fruits, alfalfa and radish sprouts, spinach and lettuce. In 1996, a large outbreak in Japan caused by contaminated radish sprouts affected close to 10,000 people. Contamination with animal feces or the use of raw manure on or near fruit and vegetable crops were suspected as sources of the pathogen as O157:H7 and other STEC can be present in animal feces. Outbreaks of O157:H7 have also implicated unpasteurized apple cider, mayonnaise, yogurt, salami and cheeses that are acidic foods with low pH ($\leq$ 4.0), which normally were regarded as safe for consumption. However, O157:H7 have been shown to be able to tolerate and persist in acidic conditions . The infectious dose of O157:H7 in humans is estimated to be very low and in the range of 10 to 100 cells.

The principal illness caused by EHEC is HC, which is characterized by acute abdominal cramps and bloody diarrhea. In some cases, the diarrhea may be very severe, consisting of all blood and occurring every 15 to 30 min. Other symptoms include vomiting, but fever seldom occurs. The incubation period before onset of illness is 3 to 4 days but can range from 1 to 9 days, and the illness can last from 2 to 9 days. Approximately 5 to 10% of the HC infection may progress to more serious complications such as HUS and possibly, thrombotic thrombocytopenic purpura (TTP). HUS can affect all ages, but is more prevalent in children. HUS is characterized by acute renal failure and is a life-threatening sequelae with a mortality rate of 3 to 5% and many survivors suffer permanent disabilities, such as renal insufficiency and neurological deficits. TTP has similar effects as HUS, but it more commonly occurs in adults. TTP also affects the central nervous system; therefore, is often accompanied by fever and other neurological disorders. Treatment for EHEC infection consists mostly of administering fluids and salts to prevent dehydration, but in severe cases or HUS, dialysis and blood transfusions

may be required. Antibiotic therapy has had mixed results in treating EHEC infections, and in some instances, it seems to increase the patient's risk of developing HUS. One speculation for the latter may be that Stx produced by EHEC are mainly accountable for the illness, and antibiotics are lysing the bacteria to release more toxins into the host. EHEC infections by non-O157:H7 serotypes vary from mild, non-bloody diarrhea to HC; however, illness can also be as severe as that caused by O157:H7 and progress to HUS. In some countries, non-O157 EHEC show a higher frequency of infection than O157:H7 and account for the majority of the HUS cases reported.

The pathogenicity of EHEC begins with intimate attachment of the bacteria to intestinal epithelial cells, mediated by the intimin protein encoded by the chromosomal, LEE-encoded *eae* gene that is the same as that of EPEC. But not all EHEC have *eae*, so attachment probably also involves additional virulence factors. Once attached, Stx are produced, enter the bloodstream, and bind to susceptible epithelial cells via a toxin-binding glycosphingolipid receptors Gb3 or Gb4. Stx inhibit protein synthesis by interfering with the functions of the 28S rRNA, resulting in cell death. EHEC strains also carry a large plasmid (designated pO157 in O157:H7 strains) that encodes for other putative virulence factors, including an enterohemolysin; but the role of this hemolysin in pathogenesis is uncertain.

EHEC produces Stx1 and Stx2 and these are also known as Verocytoxins (Vtx), because of their cytotoxic effects on Vero cells. Stx used to be known as Shiga-like toxins due to the homology of Stx1 to the Shiga toxin of *S. dysenteriae* Type I. Both Stx1 and Stx2 in EHEC are phage encoded but the two toxins only share 55% homology in protein sequence. Stx2 is also thought to be more important as it is most often associated with severe complications such as HUS. There are three known Stx1 subtypes (1a, 1c, and 1d), of which, Stx1c has not been found in O157:H7 strains and it is also the most common subtype in STEC strains from sheep. There are 7 known Stx2 subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g) and thus far, Stx2a, Stx2c and Stx2d look to be associated with more severe illness in humans than the other Stx2 subtypes. Many of the other subtypes seems to be produced by animal or environmental STEC strains, so their health risk is uncertain. In addition to the LEE pathogenicity island, EHEC possess other pathogenicity islands, known as O-islands that encode for potential virulence genes, and these include O-island 122 (OI-122), OI-57, and OI-71; but, the role of these O-islands genes in disease is not clear. Some studies showed that the presence of these three O-islands, particularly OI-122, is closely associated with EHEC that cause severe disease. More specifically, there seemed to be a *nle* (non-LEE effector) gene dose-effect relationship, in which strains associated with severe disease harbored specific O-islands and more *nle* genes. Hence, a molecular risk assessment strategy has been proposed to identify high risk EHEC strains based on the distribution of 16 *nle* genes found on genomic O-islands. EHEC strains may also carry several putative virulence genes that encode for fimbrial adhesins such as the pilin (SfpA) of SFO157 strains and long polar fimbriae (Lpf), and nonfimbrial adhesins, such as the STEC agglutinating adhesin (Saa) and the plasmid-encoded ToxB protein. In addition, they may carry genes encoding for proteases, such as EspP (serine protease) and KatP (catalase peroxidase) and genes encoding for the subtilase cytotoxin (SubAB), cytolethal distending toxin (Cdt), and the enteroaggregative *E. coli* heat-stable

enterotoxin (EAST1), but the role of these proteins in pathogenesis also remains undetermined.

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Nancy Strockbine currently serves as Chief of the *Escherichia* and *Shigella* Reference Unit at the Centers for Disease Control and Prevention in Atlanta, Georgia, which provides bacterial identification, serotyping and virulence profiling services to public health laboratories within the nation and abroad to support surveillance and outbreak investigations. She graduated with a Bachelors of Science from Bucknell University, Lewisburg, Pennsylvania in 1978 and earned a Doctorate of Philosophy in Microbiology and Immunology from Temple University, Philadelphia, Pennsylvania in 1984. After completing her postdoctoral studies in the laboratory of Dr. Alison D. O'Brien, Uniformed Services of the Health Sciences, Bethesda, Maryland, she joined the Centers for Disease Control in 1987 to direct the reference activities for *Escherichia* and *Shigella*. Dr. Strockbine has been an invited speaker at symposia throughout the United States and around the world. She has served as a reviewer for several international, peer-reviewed journals and has authored more than 60 scientific journal articles and book chapters and is a co-inventor on several patents.

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