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Detection and Typing Strategies for Pathogenic Escherichia coli

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Chapter 1
Introduction to Pathogenic *Escherichia coli*

1.1 Pathotypes of *E. coli* Causing Diarrheal Diseases

Escherichia coli is one of the predominant facultative anaerobes in the human gastrointestinal tract. Many strains of *E. coli* are harmless and even provide many health benefits to the host, including preventing colonization of the gut by harmful pathogens. However, there are small groups of *E. coli* that have evolved and developed pathogenic strategies that can cause a broad spectrum of disease, including severe diarrheal disease and serious sequelae, in the human host and are more commonly referred to as pathogenic *E. coli* (Nataro and Kaper 1998). These pathogenic, diarrhea-causing *E. coli* can be broadly classified into six recognized pathotypes for which pathogenicity in outbreaks or volunteer studies has been established: Enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli* (STEC, also referred to as Verotoxigenic or Verocytotoxigenic *E. coli* (VTEC), which includes a subgroup known as Enterohemorrahagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely Adherent *E. coli* (DAEC) (Table 1.1).

There are other extraintestinal pathogenic *E. coli* (ExPEC) that can cause a variety of infections in both humans and animals including urinary tract infections, meningitis, and septicemia (Belanger et al. 2011), but the main focus of this brief will be those *E. coli* that are important for food safety and cause diarrheal disease, with a particular emphasis on the STEC/EHEC group due to their role in major foodborne outbreaks worldwide (Inset 1.1). The pathogenesis of the specific *E. coli* groups will not be described in detail in this review but the reader is referred to the following publications for further information in this area (Nataro and Kaper 1998; Kaper et al. 2004; Steiner et al. 2006; Turner et al. 2006; Gyles 2007).

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Table 1.1 *E. coli* pathotypes associated with human diarrheal diseases. (Adapted from Steiner et al. 2006; Hunt 2010; Mainil 2013)

Pathotype	Predominant serotypes	Key *E. coli*/host interaction	Toxins/virulence	Human disease
Shiga toxigenic/ Enterohemorrhagic *E. coli* (STEC/EHEC)	O157, O26, O103, O111, O113+ approximately 50 others	Attachment to intestinal cells and production of attaching and effacing lesions	At least four; Stx1, Stx2, Stx2c and Stx2d. Multiple variants of Stx2. Locus of enterocyte effacement (LEE), virulence plasmid (pO157), intimin, non-LEE encoding genes (Nles)	Non-bloody to bloody diarrhea, hemorrhagic colitis (HC), sudden onset of severe abdominal pain, vomiting, no fever, hemolytic uremic syndrome (HUS), acute renal failure in children, thrombocytopenia, acute nephropathy, seizures, coma, death
Enteropathogenic *E. coli* (EPEC)	O18ab, O18ac, O26, O44, O55, O86, O114, O119, O125, O126, O127, O128, O142	Attaches to intestinal cells and production of attaching and effacing lesions	No toxins, Type 4 bundle-forming pilus by typical EPEC strains, intimin/Tir	Infant diarrhea, can cause severe dehydration and be fatal. Fever, vomiting and abdominal pain. In adults, severe watery diarrhea with mucus without blood, nausea, vomiting abdominal cramps, headache, fever and chills
Enterotoxigenic *E. coli* (ETEC)	O6, O15, O25, O27, O63, O78, O115, O148, O153, O129	Attachment to intestinal cells and production of toxins eliciting chloride secretion from intestinal cells	Heat-liable enterotoxin (LT), Heat-stable enterotoxin (ST), fimbriae	Traveller's diarrhea: watery diarrhea, low-grade fever, abdominal cramps, malaise, nausea. Severe cases have cholera-like extreme diarrhea with watery stools which can lead to dehydration. Usually self-limiting in adults
Enteroinvasive *E. coli* (EIEC)	O28ac, O29, O112ac, O121, O124, O135, O144, O152, O167, O173	Invades cells in the colon and spread laterally from cell to cell	Invasion and adhesin proteins	Similar to shigellosis with profuse diarrhea or dysentery, chills, fever, headache, muscular pain, abdominal cramps
Entroaggregative *E. coli* (EAEC)	O3, O44, O51, O77, O86, O99, O111, O126	Binds in aggregative clumps to cells of the small intestine and produces toxins	Small fimbrial adhesins, toxins (Pet, EAST1, ShET1) transcriptional activator gene (*agg*R)	Persistent diarrhea in children. Occasionally bloody diarrhea or secretory diarrhea, vomiting
Diffusely adherent *E. coli* (DAEC)	O1, O2, O121, O75	Fimbrial and non-fimbrial adhesins identified	Adhesins of the Afimbrial Adhesin (AFA) family, AIDA adhesin	Childhood diarrhea, septicemia, urinary tract infections

Inset 1.1: Foodborne Disease and *E. coli*

Foodborne diseases, particularly diarrheal diseases, are an important cause of morbidity and mortality and are a public health concern worldwide. Depending on the agent, illness can vary from gastroenteritis to chronic, and in some cases, life-threatening and death. Although a number of countries have conducted studies to determine the burden of foodborne disease, obtaining global estimates has been more challenging (Flint et al. 2005). This is due to the fact that (a) foods can be contaminated by many different agents (i.e., bacteria, viruses, parasites, and chemicals), (b) transmission can occur through a variety of other exposure such as direct contact with animals or with other infected persons, and by exposure to contaminated water, and (c) only a small proportion of illnesses are confirmed and reported (Scallan et al. 2011). Microbes, parasites, and chemicals are the major agents that contribute to the burden of foodborne diseases as a result of the consumption of contaminated food stuffs.

Current estimates from the Centers for Disease Control and Prevention (CDC) state that there are approximately 48 million cases of foodborne illness per year in the USA, with approximately 128,000 hospitalizations and 3000 deaths. An estimation of illness due to 30 pathogens and unspecified agents based on 10 years of data (2000–2010) in Canada found a total of 4.0 million episodes of foodborne illness (Thomas et al. 2006). The World Health Organisation (WHO) reported that 70 % of 2.2 million deaths that occur each year due to acute diarrheal disease are associated with water or foodborne contamination, but because many cases are not reported to the health officials, the true health impact of these foodborne illnesses is unknown (WHO 2008).

The overall burden of pathogenic *E. coli* in foods is unknown, but STEC is one of the main groups of pathogens known to contribute to the vast majority of illnesses, hospitalization, and deaths in the USA and in other countries (CDC 2011). In the USA, *E. coli* O157:H7, the most predominate serotype of STEC was reported to cause 73,000 illnesses annually with 52 % of outbreaks reported to be foodborne (Rangel et al. 2005). Determining the foodborne burden of other non-STEC pathogenic *E. coli* requires information on the incidence of illnesses caused by each pathotype (EPEC, ETEC, EIEC, and EAEC) and attributable fractions to specific foods. This information is not available as few, if any, laboratories examine food or fecal samples for these organisms and infections with these organisms are not categorized as notifiable diseases. It may be possible that other pathotypes of *E. coli* are among the millions of cases of illness where there is insufficient data to estimate the agent-specific disease burden (Thomas et al. 2006; CDC 2011).

Surveillance and outbreak investigations performed in industrialized countries have enabled authorities and industries to study trends over years as well as gain valuable information on the epidemiology of these pathogens by identifying transmission routes, vehicles, and mechanisms of contamination.

Although comparison of surveillance data between different countries is difficult to undertake, given the inherent differences in methodological approaches and data sources, the information obtained from such activities is vital for developing and implementing prevention and control measures in a cost-effective manner (Thomas et al. 2006). In developing countries, foodborne disease is all too common and the lack of efficient surveillance systems demonstrates the significant burden of illness and death due to diarrheal pathogens (Mead et al. 1999). A global estimate of the burden of foodborne disease is therefore unknown.

1.2 *E. coli*

Escherichia coli is a member of the family *Enterobacteriaceae*. The bacterium is a short, nonspore-forming, Gram-negative bacillus that grows readily on simple culture media or synthetic media with as little as glycerol or glucose as its only nutrient. It may also be motile by peritrichous flagella, or nonmotile and is a facultative anaerobe. Other biochemical characteristics include indole production, lack of citrate fermentation, positive methyl red test and negative urease, and Voges–Proskauer reactions (Desmarchelier and Fegan 2003; Steiner et al. 2006).

E. coli is also characterized by a serotyping scheme based on three fundamental antigens, O (lipopolysaccharide), K (capsular), and H (flagellar), all of which can be subdivided into partial antigens. Fimbriated strains can also be classified based on fimbrial antigens. Although there are between 50,000 and 100,000 or more *E. coli* serotypes, the number of pathogenic serotypes in gastrointestinal infections is limited. Each of the major categories of diarrheagenic *E. coli* can be generally grouped based on O:H serotypes, which has proven to be useful in understanding the pathogenesis and epidemiology of enteric *E. coli* infections (Nataro and Kaper 1998; Steiner et al. 2006).

1.3 Pathogenic Types of *E. coli*

Pathogenic *E. coli* strains use a multistep scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defenses, multiplication, and host damage (Kaper et al. 2004; Steiner et al. 2006). Pathogenic *E. coli* are grouped into pathotype lists based on (a) mechanisms of pathogenicity (e.g., patterns of attachment to, and invasion of host cells), (b) virulence properties (e.g., toxin production, presence of virulence plasmids, attachment mechanisms), and (c) clinical syndromes (Kaper et al. 2004). The nomenclature for pathogenic *E. coli* is complex and comprehension is not aided

by the relatively large number of *E. coli* pathotypes described, the similarity in the names given to different pathotypes, inconsistencies in usage in the literature, and the emergence of new pathotypes.

It is important to note that some groups and strains of *E. coli* can share similar virulence traits and there are many overlaps in the mechanisms of pathogenesis for various pathotypes. For example, both EPEC and EHEC produce intimin, a protein that allows the pathogen to attach to intestinal cells. In addition, many of the virulence genes carried by these pathogenic *E. coli* groups are contained within mobile genetic elements and can be transferred between strains to create "emerging" strains. This type of transfer is demonstrated in the 2011 outbreak in Germany that involved an *E. coli* O104:H4 and is described in Insert 1.2.

Inset 1.2: Enterohemorrhagic *E. coli* O104:H4 Outbreak—A Paradigm of Emerging Foodborne Pathogens

Emerging diseases have been described as those whose prevalence has increased in recent decades or is likely to increase in the near future (Altekruse and Swerdlow 1996). New pathogens may emerge due to the uptake of mobile virulence factors found in large regions of DNA known as pathogenicity islands (PAIs). These islands may be shared among various pathogens and contribute to their evolution and adaption to new conditions that the organism may encounter. This transfer of genes is acquired through mobile genetic elements such as plasmids, transposons, conjugative transposons, and bacteriophages (Ahmed et al. 2008; López-Campos et al. 2012). In addition, many pathogens do not cause disease in their animal host but emerged due to ecological changes which brought their exposure and adaptation to various other conditions within the food chain.

Food production and distribution for the industrialized countries occur across complex global networks in increasingly shorter timescales. As a result, the food chain is a dynamic system and foodborne pathogens within this system have unique opportunities to cross-species lines, become resistant to antimicrobial agents, change and adapt to new and existing niches, and thus emerge or reemerge to cause a public health concern. A number of factors have contributed to the current era of emerging infections and include:

1. Rapid population growth and demographic shift toward an ageing population.
2. An increasing global market in fresh produce, meats, ethnic foods, and some of these originating from countries without appropriate food safety procedures.
3. Improved transport logistics and conditions that enable pathogens to survive on food products and increase the risk of exposure to the consumer.
4. An increase in international travel that distributes a transient intestinal flora worldwide.

5. Changes in eating habits, such as an increase in convenience foods and the consumption of raw or lightly cooked foods and the demand for exotic foods.
6. A shift from low- to high-protein foods globally.
7. A greater proportion of immune-compromised individuals either as a result of the increasing elderly population or the increased numbers of highly susceptible individuals with immunosuppressive diseases or treatments.
8. Changing farming practices that include the drive to increase food production at a cheaper cost and the increasing demand for organic or free-range products.
9. The increasing intrusion of man on native wildlife habitats.
10. Climate change that includes the changes in vectors and carriage of diseases to other regions globally (Newell et al. 2010).

Globalization of the food supply has served to expand the range of foodborne pathogens as well as to amplify health and economic impacts of a single contamination event (King 2012). Indeed, surveillance and outbreak investigations have highlighted the changing epidemiology of foodborne diseases; where traditional pathogens are being controlled or reduced in foods while other unknown pathogens appear to emerge and demonstrate the contribution of globalization on the economic and health impacts of foodborne disease outbreaks (Newell et al. 2010; King 2012).

The large foodborne outbreak in 2011 caused by an unusual EHEC O104:H4 is an example of an emerging pathogen. This outbreak was centered in Germany but affected over 4000 people in 17 countries, with 908 cases of hemolytic uremic syndrome (HUS) and 50 deaths (World Health Organisation 2011; CDC 2013c) and has been reported to be one of the deadliest *E. coli*-associated disease outbreaks to date. Early in the outbreak, it became evident that international surveillance would be necessary to determine the extent of the outbreak, characterize the disease, and identify the source. The pathogen was isolated from clinical samples but not from the epidemiologically suspected food vehicle. Epidemiological investigations identified salad sprouts as a possible contamination source; however, the specific EHEC O104:H4 strain was not isolated from sprout products in the market. The source of the contamination remained unclear as the EHEC strain was only identified in leftovers and refuse. Fenugreek sprouts were also identified as a suspected source due to the coincidence between French and German clinical cases. Their origin could be traced back to a common import of seed from Egypt (Karch et al. 2012; King et al. 2012).

Efforts to identify the source implicated in the outbreak quickly resulted in some local authorities warning the public of the possible sources of contaminated produce, which in the end were not genuine. Initial reports had linked the German outbreak to Spanish vegetables, which were later found

to be negative for the O104:H4 serotype (Karch et al. 2012). This confusion over the source of the outbreak caused massive economic losses to industries at a time of global financial hardship. The European Union (EU) approved US$ 287 million in emergency aid for European vegetable farmers affected by the crisis, which was estimated to be only a small fraction of actual losses generated (King 2012). Surveillance conducted in the USA during the outbreak identify six cases associated with the outbreak but none of the patients recalled consumption of sprouts (CDC 2013c).

The EHEC O104:H4 outbreak was unusual in that there were important clinical and microbiological differences between this outbreak and other previous outbreaks of *E. coli* O157:H7. Historically, *E. coli* O104:H4 had been associated with sporadic cases of human disease but not with large-scale outbreaks (Mellmann et al. 2011; Scheutz et al. 2011). Outbreak investigations discovered that the *E. coli* O104:H4 strain possessed a combination of virulence properties of two different diarrhea-causing *E. coli* pathotypes, typical EAEC and STEC. Comparative genomics showed that the outbreak strain carried the chromosomal backbone of a typical EAEC strain but had also acquired the bacteriophage-encoded Shiga toxin and antibiotic-resistant factors from an ancestral precursor of the strain (Frank et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Scheutz et al. 2011). In addition, the strain was identified to be negative for the genes coding for intimin (*eae*), hemolysin A (*hylA*, also known as *ehx*A) and EAST1 toxin (*ast*A), commonly associated with STEC infection but was positive for the *agg*R gene that regulates the expression of aggregative adherence fimbriae associated with EAEC (Gault et al. 2011). Due to the hybrid pathogenicity characteristics, a new pathotype "Entero-Aggregative-Haemorrhagic *E. coli* (EAHEC)" has been proposed (Brzuszkiewicz et al. 2011), but others have suggested that the outbreak strain belonged to the EHEC pathotype as it reflects the major clinical attributes and follows the precedent set by other *eae*-negative EHEC outbreak strains (Mellmann et al. 2011).

Other unusual clinical differences were observed between this outbreak strain compared to previous outbreaks of STEC. First, the number of cases of HUS represented over 20% of cases, which is a much greater percentage compared to other outbreaks. Second, there was a high predominance of adult women among the cases rather than children, and finally a longer median incubation period than expected for cases of STEC (Frank et al. 2011; Gault et al. 2011). Evaluation of the laboratory testing regimes within the EU reported that during the outbreak, many countries lacked the capability at national level to detect and characterize STEC O104:H4. The results highlighted the complexity of the detection and identification of STEC, which made it difficult to diagnose cases. There was also evidence of limited sensitivity of routine diagnostic methods for detecting the outbreak serotype and pathotype, suggesting that further improvements in this area are required in

order to fill surveillance gaps (Rosin et al. 2013). In the USA, the CDC recommended protocols for routine testing of acute community-acquired diarrhea samples that included assays to detect Shiga toxins, and simultaneous culture on selective and differential agar to distinguish STEC O157 and other non-O157 serotypes (CDC 2013c).

This outbreak demonstrates the paradigm of bacterial genome plasticity and the ability of an *E. coli* strain to gain and/or lose chromosomal and plasmid-encoded virulence factors to create a highly pathogenic hybrid of two pathotypes of *E. coli*. Investigations have emphasized the threat posed by newly emergent strains of pathogenic *E. coli* and the need for improved methods to identify such strains within the food chain, as well as the need for public health surveillance of STEC infections and its important role in devising and implementing control measures (Rosin et al. 2013).

1.4 Shiga Toxin-Producing and Enterohemorrhagic *E. coli*

STEC form the majority of the *E. coli* implicated in foodborne disease and can cause illnesses ranging in severity from mild diarrhea to severe kidney complications that can result in death. STEC are a heterogeneous group of *E. coli* linked by a single feature; the ability to produce Shiga toxins (Stx) (Nataro and Kaper 1998). As Shiga toxins are cytotoxic to Vero cells, an alternative nomenclature has been used that includes the terms Vero toxins (VT) and Verotoxigenic *E. coli* (VTEC) (Karmali et al. 2010). Apart from the production of toxins, STEC are quite diverse with respect to other known virulence determinants and are thought to be equally diverse in their capacity to cause disease (Law 2000).

EHEC is a group that has a number of definitions and classifications but it is a term generally used to describe STEC isolated from cases of human infection, such as bloody diarrhea or HUS, and often consists of particular serotypes of *E. coli* such as O157:H7, or O157:H-, O111:H- and O26:H11 or strains that possess certain virulence markers (Desmarchelier and Fegan 2003; Mainil and Daube 2005). Most EHEC can be differentiated from STEC as they possess accessory virulence markers such as a locus of enterocyte effacement (LEE) and a virulence plasmid (pO157), which are common in strains associated with disease (Law 2000). However, not all EHEC contain these accessory virulence markers and it is often difficult to determine which STEC strains have the potential to cause disease. While not all STEC strains are believed to be pathogens, all EHEC strains possessing certain virulence markers are considered to be pathogens. It remains unclear if all STEC present in animal reservoirs present a risk to the human population, though information on virulence continues to expand as more clinical cases arise and other potential virulence factors are identified (Law 2000; Paton et al. 2001).

The STEC seropathotype (SPT) classification is based on a serotype-specific spectrum of disease frequency and severity with discrete intervals ranging from the most pathogenic serotype *E. coli* O157:H7 (SPT-A) to STEC serotypes that are either frequently, occasionally, or infrequently associated with clinical cases (SPTs B-D, respectively) to strains that have never been associated with human disease (SPT E) (Karmali et al. 2003, 2010). The European Food Safety Authority (EFSA) evaluated the seropathotype classification scheme and concluded that the scheme did not define pathogenic STEC or provide an exhaustive list of pathogenic serotypes. It only classifies STEC based on their reported frequency in human disease, their known association with outbreaks, and their severity of the outcome including HUS and hemorrhagic colitis (HC). Therefore, pathogenicity could not be excluded nor confirmed for given STEC serogroups or serotypes based on the seropathotype scheme or analysis of the public health surveillance data (EFSA 2013).

The serogroup O157 is currently the predominant EHEC serogroup to cause infections worldwide and as a result has been the focus of research and regulatory framework (Grant et al. 2011; Vally et al. 2012; Eurosurveillance Editorial Team 2013; Luna-Gierke et al. 2014). However, non-O157 serogroups are increasingly recognized as important foodborne pathogens worldwide, with reports of severe disease and outbreaks linked to produce, meat, and dairy products (Table 1.2). Currently, more than 200 virulent non-O157 serotypes have been isolated from outbreaks and sporadic cases of HUS and severe diarrhea (Kaspar et al. 2010). In the USA, a 6-year study (2005–2010) evaluating an enhanced STEC testing regime in clinical samples found that the most common non-O157 serogroups identified were O103, O26, O111, O45, O121, and O145 (Mingle et al. 2012).

These serogroups have been associated with severe disease in humans and involved in outbreaks, though less frequently than the O157 serogroup (Karmali et al. 2003). These serogroups are also consistently found in other countries, but other serogroups can also be found depending on the country and geographical regions (Mellor et al. 2013). Currently, it is difficult to determine which serotypes of *E. coli* are EHEC and it is equally challenging to predict the emergence of strains that can acquire the genes for Shiga-toxin production or other virulence factors and so cause human illness (Coombes et al. 2008) (Inset 1.2). The true incidence and severity of non-O157 remain unknown due to our current inability to detect all non-O157 cases. Literature suggests the need to identify more predictive virulence factors because serotype does not consistently predict disease severity (Wang et al. 2013b). It is likely that virulence results from a combination of factors (Grant et al. 2011).

1.4.1 Virulence Determinants of Shiga-Toxin Producing E. coli

Human STEC strains can harbor two potent bacteriophage-encoding Shiga toxins (Nataro and Kaper 1998). The two major immunological groups within the Stx family are known as Stx1 and Stx2, with multiple subtypes of Stx2, and strains may express Stx1 only, Stx2 only, or both toxins (Agbodaze 1999; Law 2000). Both

Table 1.2 Selected Shiga-toxin producing *E. coli* outbreaks associated with food

Serotype	Year	Country	No. of cases	No. of hospitalizations	No. of HUS[a]	Deaths	Food Source	Reference
E. coli O121	2013	USA	35	9	2	0	Frozen food product	(CDC 2013a)
E. coli O157	2013	USA	33	7	2	0	Ready-to-eat salads	(CDC 2013b)
E. coli O157	2013	UK	19	7	0	0	Watercress	(Launders et al. 2013)
E. coli O157:H7	2012	USA	33	13	0	0	Organic spinach and spring mix blend	(CDC 2012b)
E. coli O26	2012	USA	29	7	0	0	Raw clover sprouts	(CDC 2012a)
E. coli O104	2011	France, Germany, USA	4075	–	908	50	Fenugreek sprouts	(CDC 2013c)
E. coli O157:H7	2011	USA	58	34	3	0	Romaine lettuce	(Slayton et al. 2013)
E. coli O157:H7	2010	USA	38	15	1	0	Cheese	(CDC 2010)
E. coli O157:H7	2009	USA	17	12	2	0	Beef	(CDC 2009b)
E. coli O157:H7	2009	USA	26	19	5	2	Beef	(CDC 2009a)
E. coli O157:H-	2008–2009	Netherlands	20	7	0	0	Steak tartare	(Greenland et al. 2009)
E. coli O157	2011	UK	250	74	4	1	Vegetables and soil	(Health Protection Agency UK 2011)

Table 1.2 (continued)

Serotype	Year	Country	No. of cases	No. of hospitalizations	No. of HUS[a]	Deaths	Food Source	Reference
E. coli O157:H7	2012	Canada	29	7	0	0	Pork	(Trotz-Williams et al. 2012)
E. coli O157:H7	2009	USA	77	35	10	0	Ready-to-bake cookie dough	(Neil et al. 2012)
E. coli O26:H11	2007	Denmark	20	–	–	–	Beef sausage	(Ethelberg et al. 2007)
E. coli O157:H7	2006	USA	199	102	31	3	Fresh spinach	(CDC 2006)

[a] *HUS* hemolytic uremic syndrome

types of toxins have a similar structure and mode of action, although their effect *in vitro* and *in vivo* varies considerably. There is increasing evidence that Stx2-producing strains are potentially more virulent than strains that produce Stx1 only or that produce both Stx1 and 2 (Werber et al. 2003; Mainil and Daube 2005; Luna-Gierke et al. 2014). Studies have suggested that *E. coli* O157:H7 strains that express Stx2 are more important than Stx1 in the development of HUS and may result in an increase in disease severity (Werber et al. 2003). Shiga toxins act by assisting the bacterium to lyse gastrointestinal epithelial cells that release limiting nutrients such as iron (Torres and Payne 1997).

Aside from the production of Shiga toxins, STEC are known to be diverse with respect to other known virulence determinants. Many virulence genes have been found in clusters known as PAIs typically surrounded with unstable or mobile sequence repeats, indicating insertions that are likely to be transferred between bacteria (LeBlanc 2003). A number of STEC virulence factors are responsible for the attachment of the pathogen to the intestinal epithelial cells and destruction or effacement of the bush-border of microvilli. This process is known as the attaching and effacing (A/E) phenotype and is also found to be a mechanism of other bacterial groups, including the EPEC group (LeBlanc 2003). The LEE contains the genes implicated in the A/E phenotypes and can be described as three functional regions: (1) the regions that encode the effector proteins termed the EPEC secreted proteins (Esps), (2) a type III secretion systems (TTSS), required for the secretion of proteins including the products of the Esps, and (3) the region containing *eae* gene that encodes intimin, an outer membrane protein required for intimate attachment, and *Tir* encoding the protein translocated intimin receptor (Tir) that acts as a receptor for intimin (Law 2000; LeBlanc 2003).

Many STEC carry a conserved plasmid such as pO157, pSFO157, and pO113 (Burland et al. 1998, 2006; Newton et al. 2009). The pO157 was the first described and carries a number of putative virulence genes, such as *esp*P, *kat*P and *tox*B (Burland et al. 1998; LeBlanc 2003; Fratamico et al. 2011). This plasmid also encodes a hemolysin (*hly*A or *ehx*A) that has been found to illicit an immune response in clinical disease (Schmidt et al. 1995); however, the role of this hemolysin in disease is not fully understood. Several other potential virulence factors have been implicated in EHEC adherence including the IrgA homologue adhesin (Iha), *E. coli* factor of adherence 1 (Efa1), STEC autoagglutinating adhesin (Saa), production of a serine protease, production of a heat-stable enterotoxin (EAST), and the presence of a special catalase system (Law 2000; Tarr et al. 2000; Paton et al. 2001). Most genes associated with *E. coli* O157:H7 pathogenesis appear to have been acquired from multiple recombination events with foreign DNA originating from other species, through horizontal transfers such as conjugation, bacteriophage transfers, or natural transformations. However their exact roles in the adherence process are not fully understood (LeBlanc 2003). The high virulence of STEC strains such as O157:H7 is also aided by the pathogen's ability to survive environmental stress conditions, such as resistance to low pH levels found in the gastrointestinal tract, which contributes to its very low infectious dose (50–100 colony forming units) (Viazis and Diez-Gonzalez 2011).

Additional studies also indicated that the pathogenesis of STEC/EHEC infection involves other effector molecules that are encoded on PAIs outside the LEE, including many non-encoding effectors (Nles) (Karmali et al. 2003; Gruenheid et al. 2004; Coburn et al. 2007; Coombes et al. 2008; Luzader et al. 2013). The surge of whole genome sequencing technologies has also seen a growing number of *E. coli* O157:H7 and other non-O157 genomes becoming available (Ju et al. 2012a; Eppinger et al. 2013). These technologies are becoming more commonly used during outbreak situations (Frank et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Scheutz et al. 2011), and thus comparative genome analysis will undoubtedly expand the list of putative virulence factors in the near future.

1.4.2 Shiga-Toxin Producing E. coli*-Mediated Disease*

Infections caused by EHEC may be asymptomatic or associated with a variety of gastrointestinal symptoms ranging from mild, non-specific diarrhea to life threatening HC; a bloody diarrhea with inflammation of the large bowel, which is dependent on serotype and a combination of virulence factors (Karch et al. 2005; Gyles 2007). EHEC infection is also the major cause of HUS, a triad comprising intravascular hemolysis, thrombotic thrombocytopenia purpure (TTP, low circulating platelets), and kidney impairment. A systematic review and meta-analysis to determine the proportion that develops chronic sequalae found that the estimated proportion of *E. coli* O157:H7 cases that developed HUS ranged from 17.2 % in extra-small studies (< 50 cases) to 4.2 % in extra-large case studies (> 1000 cases) (Keithlin et al. 2014). HUS has also been reported to have a mortality rate of 2–10 % of cases (Law 2000) and the most severe clinical signs are normally seen in children and the elderly (Karch et al. 2005). The person-to-person spread observed and the small numbers of cells in contaminated foods have shown that the infective dose of *E. coli* O157:H7 is low (Armstrong et al. 1996).

Several serotypes of non-O157 have been associated with sporadic and epidemic human infections worldwide. Some cause clinical disease indistinguishable from that caused by *E. coli* O157:H7, but generally are associated less frequently with bloody diarrhea and HUS (Johnson et al. 2006; Preussel et al. 2013; Wang et al. 2013b), and their animal reservoirs and modes of transmission are not well understood (Karch et al. 2005; Ferens and Hovde 2011). No specific treatment for EHEC infection is available; therapy is symptomatic only, and antibiotic therapy as with many gastrointestinal infections is contraindicated (Nataro and Kaper 1998). Although *E. coli* O157:H7 causes the most EHEC infections, the isolation rates of non-O157 STEC serotypes from foods and animal feces are higher than those of *E. coli* O157:H7 (Johnson et al. 2006; Mathusa et al. 2010; Grant et al. 2011). This suggests that humans are exposed to non-O157 STEC from food and environmental sources more often than *E. coli* O157. However, the incidence of non-O157 STEC infections is lower than *E. coli* O157. The lack of uniformity in the association between non-O157 STEC disease and outbreaks suggests that variability in the

virulence of non-O157 STEC strains is likely and that *E. coli* O157:H7 may be more virulent or transmissible than other STEC (Nataro and Kaper 1998; Johnson et al. 2006; Coombes et al. 2008). It is unclear which factors make *E. coli* O157:H7 more prevalent in disease than non-O157 STEC.

1.4.3 Epidemiology of Shiga Toxin-Producing E. coli

Over 380 different STEC serotypes have now been associated with gastrointestinal disease in humans and both Stx-positive and Stx-negative strains can be found in animals (Karmali et al. 2010). Although isolates belonging to serogroup O157 are regarded as the most clinically significant STEC strains, a number of non-O157 serotypes have been implicated in both sporadic disease and outbreaks (Johnson et al. 2006; Mathusa et al. 2010; Grant et al. 2011; Luna-Gierke et al. 2014). Certainly the increasing detection coincides with a marked rise in reporting of sporadic non-O157 STEC, which is related to improved methods and surveillance (Luna-Gierke et al. 2014). Reporting of non-O157 STEC in the USA has increased every year since it was designated a nationally notifiable infection in 2000 (Wang et al. 2013b). Recent FoodNet data suggest that non-O157 STEC infections have started to gain predominance over O157 (Wang et al. 2013a). Six STEC serogroups, O26, O45, O103, O111, O121, and O145 (often referred to as the "top 6") accounted for 75% of the total non-O157 STEC illnesses in the USA, while other highly pathogenic serogroups, e.g., O91, O104, O113, and O128 are serogroups more prevalent in other countries (Karmali et al. 2003; Johnson et al. 2006; Eurosurveillance Editorial Team 2013). Results from studies have tended to suggest that non-O157 STEC may be associated overall with less severe diseases based on hospitalization rates and serious sequalae (Preussel et al. 2013).

1.4.4 Ecology of Shiga-Toxin Producing E. coli

Ruminants, particularly cattle and sheep, are recognized to be the major reservoir of STEC, where it is mainly harbored in the lower intestine and is intermittently isolated from the rumen and upper gastrointestinal tract (Low et al. 2005; Ferens and Hovde 2011). The rectoanal junction has been found to be a site for the colonization of *E. coli* O157:H7 (Low et al. 2005). *E. coli* O157:H7 has been detected in dairy and beef cattle, both pasture and lot fed and in healthy and diarrheic cattle (Low et al. 2005; Callaway et al. 2009; Wells et al. 2014). It has also been found in other ruminants such as buffalo, goat, sheep, and deer (Mainil and Daube 2005). *E. coli* O157:H7 is uncommon in chicken, but can colonize these animals and wild birds (Best et al. 2005; Ejidokun et al. 2006). STEC strains have also been associated with disease in piglets, and can be shed by swine, although the role that this food animal plays in STEC transmission to humans is unclear (Ho et al. 2013; Tseng et al. 2014). STEC have also been found in various other animals, such as pigeons, flies, horses

and ponies, dogs, and cats, but the great majority of these strains are non-O157 serotypes and are of questionable pathogenicity (Desmarchelier and Fegan 2003).

Cattle appear to be the most important reservoir in terms of human infection (Callaway et al. 2009; Arthur et al. 2010). Stx-positive *E. coli* have been found in bovine herds in many countries (Table 1.3). Isolation rates, which vary between countries, can be as high as 64% and these bacteria are typically associated with healthy animals. The wide range of the reported prevalence rates is very likely a consequence, not only of differences in climate, ecology, and farming practice in different regions, but also of variation in the sampling and testing protocols used in different studies (Gill and Gill 2010). The shedding of STEC from individual animals in feces is transient and sporadic and is affected by diet, age, feeding, and levels of stress (Callaway et al. 2009; Wells et al. 2011, 2014). Fecal shedding of *E. coli* O157:H7 persists longer in calves than in adult cattle and the type of feed consumed by cattle can influence the prevalence and acid resistance of this organism (Callaway et al. 2009). Diet can also influence the microbiotic composition of the feces but little is known about the interaction between the indigenous microbiota and fecal shedding of *E. coli* O157:H7 (Wells et al. 2014). STEC have been reported to survive for a number of months in soil environments and manure, and have been isolated from cattle water troughs and natural water supplies (McGee et al. 2002; Bolton et al. 2011).

1.4.5 Transmission of Shiga-Toxin Producing E. coli

STEC can be transmitted via food, water, person-to-person, and animal-to-person. Many cases involve the consumption of contaminated foods, with undercooked ground beef patties and unpasteurized milk constituting major sources of human infection (Rangel et al. 2005). These bacteria appear to enter the food chain through the contamination of bovine fecal matter during slaughter (Fegan et al. 2005; Arthur et al. 2010; Duffy et al. 2014). Ground beef products present a particular problem as they are prepared from meat obtained from many animals and if one is contaminated, then grinding disperses the bacteria throughout the lot (Duffy et al. 2014). Other types of meats, including those from porcine and avian sources have also been identified, less frequently, as vehicles of infection (Desmarchelier and Fegan 2003; Rangel et al. 2005). *E. coli* O157:H7 can grow and survive in low pH environments in foods such as fermented salami where other pathogens will not survive. Widespread fecal contamination of the environment (soil or water), by farm and wild animals and the use of cattle feces as manure, have also contributed to the contamination of raw foods (Beuchat 2002; CDC 2007; Park et al. 2012). As a result, outbreaks have been reported from contaminated fruit and vegetables, mayonnaise, milk, unpasteurized apple juices, and water (Desmarchelier and Fegan 2003; Berger et al. 2010).

Infections have also been attributed to direct person-to person contact (Seto et al. 2007) and there has been an increased incidence of STEC infection through

Table 1.3 Selected prevalence and concentration data of Shiga-toxin producing *E. coli* in farm animals

Country	Pathogen	Source	Number tested	Positive samples (%)	Enumeration (cfu/g)[a]	Reference
Australia	STEC O26	Cattle, feces	300	0.3		Barlow and Mellor (2010)
	STEC O45		300	1.7		
	STEC O91		300	2.3		
	STEC O103		300	1.7		
	STEC O111		300	0.3		
	STEC O121		300	0.7		
	STEC O145		300	0		
	STEC O157		300	1.3		
Britain	*E. coli* O157	Livestock waste—cattle	428	9.1–13.2	1.2×10^3–2.6×10^2	Hutchison et al. (2005)
		Livestock waste—pig	58	11.9–15.5	3.9×10^3–1.3×10^3	
		Livestock waste—sheep	9	20.8–22.2	7.8×10^2–2.5×10^2	
Canada	*E. coli* O157	Cattle feces—corn fed	4318	1.3	Mean 2.96 $\log_{10}$	Berg et al. (2004)
		Cattle feces—barley fed	4364	2.4	Mean 3.27 $\log_{10}$	
England and Wales	*E. coli* O157	Livestock waste—cattle	810	13.2	2.6×10^8	Hutchison et al. (2004)
		Livestock waste—pig	126	11.9	7.5×10^5	
		Livestock waste—sheep	24	20.8	5.0×10^3	
Ireland	STEC O157	Cattle, feces	301	17.6	1000–1300	Thomas et al. (2012)
	STEC O25		402	0.2	<200	
	STEC O111		402	0		
	STEC O103		402	0.2		
	STEC O145		402	0		
Japan	STEC	Cattle, beef feces	272	23		Kijima-Tanaka et al. (2005)
		Pig, feces	179	14		

Table 1.3 (continued)

Country	Pathogen	Source	Number tested	Positive samples (%)	Enumeration (cfu/g)[a]	Reference
Korea	STEC O157	Cattle, feces	1854	0.04		Jo et al. (2004)
		Pig, feces	345	0		
Mexico	*E. coli* O157	Cattle feces	240	1.25		Callaway et al. (2004)
		Swine feces	240	2.08		
New Zealand	STEC	Cattle—rectoanal mucosa	187	18.7		Cookson et al. (2011)
		Sheep—rectoanal mucosa	132	64.4		
Norway	STEC O157	Cattle feces	156	7.0		Wasteson et al. (2005)
		Sheep feces	117	17		
		Hen feces	22	13.6		
	STEC	Cattle, dairy, rectal feces	680	61		LeJeune et al. (2006)
Scotland	STEC O157	Cattle, beef feces	14856	7.9		Gunn et al. (2007)
	STEC O157	Sheep feces	1082	2.9		Evans et al. (2011)
	STEC O26		1082	5.2		
	STEC O103		1082	0		
	STEC O145		1082	0		
Spain	Non-O157	Cattle, beef, rectal feces	124	46		Oporto et al. (2008)
		Cattle, dairy, rectal feces	82	20.7		
Switzerland	STEC	Cattle, dairy	966	53[b]		Kuhnert et al. (2005)
	E. coli O157		966	4.6		

Table 1.3 (continued)

Country	Pathogen	Source	Number tested	Positive samples (%)	Enumeration (cfu/g)[a]	Reference
USA	*E. coli* O157:H7	Beef cattle, rectal feces	408	4.7		Doane et al. (2007)
		Dairy cattle, rectal feces	408	3.4		
		Poultry, rectal feces	588	2.7		
		Swine, rectal feces	570	8.9		
	E. coli O157:H7	Cattle, feces	10,622	10.2		Sargeant et al. (2003)
	STEC	Cattle, dairy feces	2208	3.2		Cho et al. (2006)
	STEC	Cattle, dairy, rectal feces	750	14.4		LeJeune et al. (2006)
	STEC O26	Cattle, feces	1897	1		Kalchayanand et al. (2013)
	STEC O45		1897	0.1		
	STEC O103		1897	0.3		
	STEC O111		1897	0.05		
	STEC O121		1897	0		
	STEC O145		1897	0.2		

[a] colony forming units per gram
[b] PCR screen only

animal-to-person contact, particularly through petting zoos or agricultural fairs (Ihekweazu et al. 2012; Jaros et al. 2013). There is accumulating evidence that contact with farm animals or farming environments is an important risk factor for sporadic infection with *E. coli* O157:H7 (Locking et al. 2001). The infectious dose of *E. coli* O157:H7 for humans has been found to be as low as 50–100 bacteria (Armstrong et al. 1996). In combination with disease severity, *E. coli* O157:H7 and other STEC are therefore important pathogens in terms of clinical implications.

1.4.6 Shiga-Toxin Producing E. coli *Occurrence in Foods*

Reports of *E. coli* O157:H7 and non-O157 STEC in foods from various countries demonstrate a variation in prevalence estimates (Desmarchelier and Fegan 2003; Erickson and Doyle 2007). Many STEC outbreaks have been linked to the consumption of contaminated meat and the majority of prevalence data available focus on this food commodity (Table 1.4). Meat can become contaminated on the carcass surface, primarily at slaughter and during hide removal. Contamination can also occur during carcass washing, dressing, and processes such as grinding. In addition, cross-contamination can occur through handling and food preparation (Duffy et al. 2014). Prevalence values of *E. coli* O157:H7 and non-O157 in meat samples, including carcass samples, vary significantly between countries, but this may be due to the variation in the sampling and testing protocols used for different studies (Gill and Gill 2010).

In 1994, the United States Department of Agriculture (USDA)'s Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 an adulterant in raw ground beef and began a microbiological testing program later that year. In 2012, the USDA/FSIS expanded the zero-tolerance policy for *E. coli* O157:H7 in raw, non-intact beef products (ground beef, its components, and tenderized steaks) to include six other STEC serotypes ("Big 6"), which include O26, O45, O103, O111, O121, and O145 (Wang et al. 2013a). FSIS undertakes a verification testing for these non-O157 STEC in domestic and imported beef manufacturing trimmings from cattle. Results for 2012 showed that the rate of *E. coli* O157:H7 and non-O157 in trimmings was low (0.65 and 1.09 %, respectively) (USDA-FSIS 2014)

Several recent outbreaks have implicated the consumption of contaminated fruits and vegetables (Beuchat 2002; CDC 2007; Berger et al. 2010). The most notable was the 2011 outbreak caused by EHEC O104:H4 that affected over 4000 people in the EU and USA (refer to Insert 1.2). Another large outbreak involving *E. coli* O157:H7 in the USA involved contaminated spinach, where approximately 200 people were infected and three deaths occurred (CDC 2007). Milk and dairy production from cattle and other milk-producing animals are also potential vehicles of STEC and have been linked to both O157 and non-O157 STEC infections (European Centre for Disease Prevention and Control and European Food Safety Authority 2011).

Table 1.4 Selected studies reporting prevalence of *E. coli* O157:H7 and non-O157 in meat

Country	Products tested	Number tested	% positive		Enumeration (log cfu/g)	Reference
			E. coli O157	non-O157		
Argentina	Beef and chicken burgers	279	6.8	ND	ND	Chinen et al. (2001)
Australia	Sheep carcasses	917	0.7	ND	ND	Phillips et al. (2001a)
	Frozen boneless sheep meat	467	1.3	ND	ND	
	Beef carcasses	1275	0.1	ND	ND	Phillips et al. (2001b)
	Boxed frozen boneless beef	990	0	ND	ND	
	Lamb cutlets, retail	275	0	40	ND	Barlow et al. (2006)
	Ground beef, retail	285	0	16	ND	
Croatia	Chopped beef and baby beef	114	0	ND	ND	Uhitil et al. (2001)
England	Beef products (raw)	3216	1.1	ND	ND	Chapman et al. (2001)
	Lamb products (raw)	1020	2.9	ND	ND	
	Mixed meat products (raw)	857	0.8	ND	ND	
	Beef carcasses	1500	1.4	ND	ND	
	Lamb carcasses	1500	0.7	ND	ND	
	Meat products	4983	0.4	ND	ND	
Ireland	Beef trimmings	1351	2.4	ND	<0.7–1.6	Cagney et al. (2004)
	Beef carcasses	132	3	ND	<0.7–1.4	
	Head meat	100	3	ND	0.7–1.0	
	Minced/ground beef (retail)	1533	2.8	ND	0.5–4.0 for 50% positives, <0.5 for 50% positives	Cagney et al. (2004)
France	Minced beef	3450	0.12	ND	ND	Vernozy-Rozand et al. (2002)

Table 1.4 (continued)

Country	Products tested	Number tested	% positive		Enumeration (log cfu/g)	Reference
			E. coli O157	non-O157		
Holland	Minced pork	260	0	ND	ND	Heuvelink et al. (1999)
	Raw minced beef	571	1.1	ND	ND	
	Raw minced mixed beef and pork	402	0.5	ND	ND	
	Raw minced pork	76	1.3	ND	ND	
	Other pork products	393	0.3	ND	ND	
	Cooked or fermented RTE meats	328	0.3	ND	ND	
	Other raw beef products	223	0	ND	ND	
	Sheep or lamb products	46	0	ND	ND	
Italy	Minced beef	75	0	ND	ND	Stampi et al. (2004)
	Mixed minced beef and chicken	10	0	ND	ND	
	Hamburger	30	3.3	ND	ND	
	Hamburger with vegetables	24	8.3	ND	ND	
	Meatballs	10	0	ND	ND	
	Minced beef	931	0.4	ND	ND	Conedera et al. (2004)
Morocco	Meat and meat products	460	0.9	ND	ND	Badri et al. (2011)
New Zealand	Beef	91	ND	12.1	ND	Brooks et al. (2001)
	Lamb/mutton	37	ND	17.1	ND	
	Pork	35	ND	1	ND	
	Chicken	36	ND	0	ND	

Table 1.4 (continued)

Country	Products tested	Number tested	% positive		Enumeration (log cfu/g)	Reference
			E. coli O157	non-O157		
USA	Ground pork	231	ND	5.2	ND	Ju et al. (2012b)
	Ground beef	249	ND	5.2	ND	
	Ground beef	4133	ND	7.3	ND	Bosilevac and Koohmaraie (2011)
	Ground beef	26,521	0.7	ND	ND	Naugle et al. (2005)
	Ground beef	296	16.8[a]	ND	ND	Samadpour et al. (2002)
	Carcass—pre-eviseration	334	ND	53.9	ND	Arthur et al. (2002)
	Carcass—post-eviseration	326	ND	8.3	ND	
	Beef carcasses	1232	1.2	ND	ND	Barkocy-Gallagher et al. (2003)
	Beef carcasses	1232	16.2[a]	ND	ND	
	Beef carcasses	330	1.8	ND	ND	Elder et al. (2000)

[a] STEC value

Milk can become contaminated from skin, hides, and the dairy environment during milking. As STEC is sensitive to pasteurization, raw milk and raw milk products are the main public health risks (Baylis 2009).

1.5 Enterotoxigenic *E. coli*

ETEC are an important cause of diarrheal disease in infants (6–18 months of age), young children, and the elderly in the developing world (Wenneras and Erling 2004; Qadri et al. 2005). School-age children and adults typically have low incidence of symptomatic ETEC infection. Symptoms include acute watery diarrhea that may be mild and of short duration and which in some cases is similar to cholera. It is also known to be the major cause of "traveler's diarrhea" acquired by tourists visiting developing nations (Qadri et al. 2005). The burden of disease is estimated at 840 million cases per year in the developing world, with an additional 50 million asymptomatic carriers in children less than 5 years of age (Wenneras and Erling 2004). These figures provide a conservative estimate because of high rates of asymptomatic infection (as high as 20% of infections) and the fact that ETEC is a clinically under-recognized pathogen in both developing and industrialized nations. This makes determining the true worldwide incidence difficult.

ETEC is defined as *E. coli* strains that contain at least one member of two defined groups of enterotoxins: heat-labile (LT) and/or heat-stable (ST) toxins (Fratamico et al. 2002). Characteristically, ST-ETEC strains cause the majority of endemic cases (Nataro and Kaper 1998). After ETEC colonizes the surface of the small bowel, these enterotoxins are produced and secreted, eliciting chloride secretion from secretory crypt cells in the intestine, causing the characteristic watery diarrhea associated with ETEC infection (Kaper et al. 2004). Several forms of the enterotoxins have been described and these appear to be associated with particular animal species (Tsen and Jian 1998). Another defining feature of ETEC is their ability to adhere to the intestinal epithelium. This process is mediated by the expression of colonization factor antigens (CFAs), filamentous bacterial surface appendages that are encoded by plasmids and associated with particular animal hosts (Nataro and Kaper 1998). Adherence of the pathogens allows the delivery of the enterotoxins and the subsequent host secretory response that is experienced as diarrhea (Elsinghorst 2002). Indeed, colonization factors currently identified for different animals are clearly different from those of human origin (Qadri et al. 2005). Due to the specificity of these adhesins, animal ETEC strains normally do not infect humans.

Very few studies have investigated the prevalence of ETEC in foods and water, especially in recent years. In 1977, Sack et al. found that 8% of 240 *E. coli* isolates obtained from food of animal origin were ETEC and produced either or both LT and ST (Sack et al. 1977), but none of the food products tested were associated with diarrheal outbreaks. A study from Brazil in 1980 reported that 18 of 1200 (1.5%) *E. coli* strains isolated from hamburger or sausage were found to be ETEC (Reis et al. 1980), while a study in 1996 isolated ETEC from 24 out of 36 (66%) food samples

that included milk, cheese, and ground beef (Alexio and Aver 1996). ETEC has been isolated from drinking water in Bangladesh, where 11 out of 233 (4.7%) *E. coli* isolates obtained were ETEC (Talukdar et al. 2013).

Epidemiologic investigations have implicated contaminated food and water as the most common vehicles for ETEC infection (Nataro and Kaper 1998). In many cases, contamination has been derived from human feces, either directly via an infected food handler or indirectly via contaminated water (Desmarchelier and Fegan 2003). In developing countries, where ETEC infections are endemic, contaminated weaning food is the primary mode of transmission to children (Nataro and Levine 1994). In industrialized countries, large outbreaks of ETEC have been reported in communal dining situations such as catered events, cruise ships, cafeteria meals at schools, and restaurants (Taylor et al. 1982; CDC 1994; Mitsuda et al. 1998; Daniels et al. 2000; Beatty et al. 2006). ETEC is not a common cause of sporadic, endemic diarrhea in industrialized countries with good hygiene. Humans are a major reservoir for ETEC, but person-to-person transmission of ETEC is thought to be rare due to the relatively high dose required to induce infection (Qadri et al. 2005). ETEC have often been detected in the feces of asymptomatic human carriers in developing countries. In endemic areas, young children experience extensive exposure to ETEC and develop immunity (Nataro and Kaper 1998).

In animals, ETEC are a major cause of diarrheal disease in piglets and newborn calves, lambs, and dogs. These animal infections cause significant morbidity and mortality in these animals with substantial economic impact on the livestock industry. Post-weaning ETEC-associated diarrhea is one of the most economically important diseases for the North American swine industry. ETEC are responsible for the death of 10.8% of all pre-weaning pigs and 1.5–2% of all weaned pigs (Tubbs et al. 1993). In pigs, the incidence of neonatal diarrhea and subsequent death has been reduced substantially by the introduction of vaccines (Qadri et al. 2005).

1.6 Enteropathogenic *E. coli*

Many EPEC isolates correspond to certain well-recognized O:H serotypes, however, advances in molecular and cellular detection of EPEC have identified EPEC lineages that include strains that would not have been considered EPEC based on serotype alone (Reid et al. 2000; Scaletsky et al. 2010; Croxen et al. 2013). The main distinguishing feature of EPEC is the ability to induce a characteristic histopathology called the attaching and effacing (A/E) lesion (Trabulsi et al. 2002). This characteristic distinguishes EPEC from most other pathotypes of diarrheagenic *E. coli* such as EAEC, EIEC, and ETEC (Blank et al. 2002). This ability is encoded by a chromosomal genomic PAI called the LEE (McDaniel et al. 1995). Both EPEC and STEC possess the LEE and can produce A/E lesions, however, EPEC do not produce Shiga-like toxin (Campos et al. 2004). EPEC are often described as "typical" or "atypical" EPEC subtypes. Typical EPEC subtypes adhere to intestinal cells by the bundle-forming pilus (BFP), which is encoded on a virulence plasmid called

the EPEC adherence factor plasmid (pEAF) (Clarke et al. 2003). Atypical EPEC do not possess pEAF and do not form a localized adherence pattern, but can possess the LEE plasmid and produce an adherence pattern similar to EAEC (Trabulsi et al. 2002).

Typical EPEC are not only the major cause of acute or chronic enteritis in children in developing countries, but they also cause sporadic cases and outbreaks in industrialized nations (Nataro and Kaper 1998; Sakkejha et al. 2013). Typical EPEC serotypes that cause disease in humans have not been found in animals, so humans are thought to be the only reservoir (Trabulsi et al. 2002). The microorganism is transmitted from host to host via the fecal–oral route through contaminated surfaces, weaning fluids, and human carriers (Levine and Edelman 1984). Advances in water decontamination and improvements in domestic and hospital hygiene have reduced the incidence of EPEC in much of the industrialized world; however, EPEC currently remain a significant contributor to infant diarrhea in developing nations (Levine and Edelman 1984). Very few outbreaks among adults have occurred through the ingestion of contaminated food and water, but a specific environmental reservoir has not been identified as a source of infection (Kaper et al. 2004; Croxen et al. 2013). In contrast, atypical EPEC are rarely isolated from diarrheal patients in the developing world and are more commonly isolated from patients in industrialized countries (Trabulsi et al. 2002). Atypical EPEC serotypes have been isolated from many farm and domestic animal species and they may serve as potential reservoirs for human infection (Trabulsi et al. 2002; Monaghan et al. 2013).

There is very little evidence that indicates that typical EPEC is a foodborne pathogen for adults and a limited number of outbreaks of EPEC disease have been linked to food- and waterborne transmission in Europe and North America. In Korea, it was recently reported that EPEC was the most common subtype of *E. coli* identified as a causative organism in 26 outbreaks involving food and water (Lee et al. 2012). Cold meat and meat pie have also been implicated in two British outbreaks (Desmarchelier and Fegan 2003). Very few studies have investigated the presence of EPEC in foods but they have been detected in ready-to-eat lettuce (Althaus et al. 2012). A study in Brazil isolated EPEC from 11 out of 36 (30%) food samples including milk, cheese, and ground beef (Alexio and Aver 1996).

In contrast, atypical EPEC have been implicated with raw chicken and beef (O'Sullivan et al. 2007; Alonso et al. 2011; Comery et al. 2013). The study of Wedley et al. (2013) described an unusual atypical EPEC that was isolated from affected individuals and linked to food consumption. The strain was identified as *eae*-positive *E. coli* O111 that was negative for the EAEC EAST-1 toxin present in other strains of EPEC associated with foodborne outbreaks but demonstrated bacterial attachment to HEp-2 cell monolayers through the induction of actin at the site of attachment (Wedley et al. 2013). Detection of EPEC in food is difficult especially if it is present in low numbers. The complex tests necessary to establish potential pathogenicity of EPEC isolates have led to infrequent attempts to examine food for EPEC unless a food is incriminated in an outbreak (Desmarchelier and Fegan 2003).

1.7 Enteroinvasive *E. coli*

EIEC are genetically and phenotypically more closely related to *Shigella* than to other *E. coli* (Kaper et al. 2004). EIEC strains possess some of the biochemical characteristics of *E. coli* and yet can cause dysentery using the same method of invasion as *Shigella*. Sequencing of various housekeeping genes has indicated that EIEC is more related to *Shigella* than to non-invasive *E. coli* and that *Shigella* and EIEC evolved from the same ancestor and form a single pathovar within *E. coli* (van den Beld and Reubsaet 2012).

Humans are the main reservoir for EIEC and this pathotype has not been found to be carried in animals or foods. Unlike other *E. coli*, EIEC are mainly non-motile, lysine decarboxylase negative, and 70 % of strains do not ferment lactose (O'Sullivan et al. 2007). EIEC is also the only *E. coli* pathotype to invade and multiply within host epithelial cells, and can cause invasive inflammatory colitis and dysentery, but most symptomatic infections are characterized by watery diarrhea indistinguishable from that produced by other diarrheagenic *E. coli* pathotypes (Bolton 2011). Outbreaks of EIEC are usually associated with water or food contaminated with human feces or person-to-person transmission, but its incidence in the industrialized world is low. In the USA, a large outbreak occurred following the consumption of imported contaminated Brie and Camembert cheese (Desmarchelier and Fegan 2003). An outbreak in Italy in 2012 reported 109 cases being attributed to cooked vegetables served in a canteen (Escher et al. 2014). Seventeen cases and two asymptomatic kitchen workers were positive for the *Shigella* marker gene *iph*H (a multicopy gene found exclusively in all *Shigella* and EIEC). An EIEC strain O96:H19 possessing the *ipa*H gene was isolated from six cases (Escher et al. 2014)

1.8 Enteroaggregative and Diffusely Adherent *E. coli*

Two other diarrheagenic *E. coli* types characteristically adhere to HEp-2 tissue cells in either an aggregative pattern of microcolonies, known as EAEC, or as a diffuse adherence known as DAEC. Little is known about these groups of *E. coli* and whether they are important for foodborne disease. EAEC are increasingly recognized as an emerging enteric pathogen and cause of persistent diarrhea (greater than 2 weeks duration) in children and adults in both developing and industrialized countries (Huang et al. 2006), but very few studies have also investigated the roles of putative EAEC virulence genes in acute diarrheal disease (Law and Chart 1998; Kaur et al. 2010).

Strains belonging to the EAEC group produce specific adhesins (aggregative adherence fimbraie (AAF)) responsible for the adherence to HEp-2 cells and for colonization (Law and Chart 1998; Pierard et al. 2012). Once the organism attaches to the colonic and ileal mucosa, mucus production aids in the formation of a thick biofilm on the mucosal surface. Typical histopathology of EAEC in animal models

is characterized by a thick layer of autoaggregating bacteria loosely adherent to the mucosal surface (Harrington et al. 2006). EAEC do not produce LT, ST, or Shiga toxins, but they are capable of significant mucosal damage via the secretion of different combination of EAEC-specific enterotoxins (the plasmid-encoded autotransporter toxin Pet, the enteroaggregative heat-stable toxin EAST1, and the *Shigella* enterotoxin ShET1). However, the role of these toxins in pathogenesis is unclear, as they can also be produced by other pathogenic *E. coli* strains (Kaper et al. 2004; Pierard et al. 2012). EAEC are a heterogeneous group of strains in terms of their properties and virulence gene repertoires with not all strains likely to be pathogenic (Pierard et al. 2012). To date, the highest correlation between the aggregative adherence phenotype on cell culture and diarrhea in humans is the presence of the *agg*R gene that codes for a transcriptional regulator of the expression of several virulence associated genes (Harrington et al. 2006; Kaur et al. 2010; Pierard et al. 2012).

EAEC have not been reported in animals and very little is known about the ecology of this pathotype of *E. coli*. There are some reports of foodborne diarrheic outbreaks associated with EAEC strains in both developed and industrialized countries after humanborne contamination. In Italy, two EAEC outbreaks affecting 24 individuals were linked to contaminated unpasteurized cheese (Scavia et al. 2008). A large outbreak affecting 2697 children from 16 schools in Japan was caused by EAEC Ountypable:H10. Food that was centrally prepared and distributed to each school was believed to be the vehicle of infection, although EAEC was not isolated from any of the tested foods (Itoh et al. 1997). Two large prospective surveillance studies in the UK and the USA identified EAEC among the most commonly isolated bacterial species from individuals with diarrhea (Wilson et al. 2001; Nataro et al. 2006; Chaudhuri et al. 2010). In addition, a meta-analysis of previously published case control studies from various geographical regions reported a role for EAEC in mediating diarrheal disease and that this group of *E. coli* is an important emerging pathogen (Huang et al. 2006).

DAEC show a diffuse adherent pattern on HEp-2 cells and many carry fimbrial structures but do not produce toxins or other virulence factors described for other pathotypes. The pathogenicity of DAEC strains is generally poorly understood but they have been associated with diarrhea in young children under 12 months in age and which are typically mild without blood in the feces, but can become persistent in both developing and industrialized countries (Kaper et al. 2004). The relative risk of diarrhea associated with DAEC increases with the age of the children and intestinal carriage of these strains has been reported to be widespread in older children and adults. They may contribute to inflammatory intestinal disease (Bouzari et al. 2005; Servin 2005; Steiner et al. 2006).

Little is known about DAEC, and their significance as enteric pathogens is uncertain. DAEC are generally characterized by the absence of virulence properties associated with other *E. coli* pathotypes, and their characteristic diffuse pattern of adherence in the HEp-2 model epithelial cell culture system (Fratamico et al. 2002). Upon attachment, DAEC induce gut epithelial cells to produce finger-like extensions, which wrap around the adherent bacteria, a phenomenon also characteristic of uropathogenic *E. coli*. Induction of these finger-like extensions is dependent on

bacterial expression of a family of fimbriae belonging to the Dr family of related fimbrial adhesins (Bernet-Camard et al. 1996). Approximately 75 % of DAEC produce the same Dr family fimbrial adhesin called F1845. Some DAEC have been shown to produce α-hemolysis and cytotoxic necrotizing factor 1, suggesting that some DAEC might be closely related to, or the same as, necrotoxic *E. coli* (NTEC) and/or cell-detaching *E. coli* (CDEC). However, the exact mechanism by which DAEC produce diarrhea is still unclear (Clark 2001).

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Chapter 2
Isolation and Detection of Pathogenic *Escherichia coli* in Foods

2.1 Introduction

Microbiological analysis and detection of a target microorganism(s) within a food can involve various steps and may include visual, biochemical, immunological, or genetic methods either before enrichment (quantitative or enumerative methods) or after enrichment (qualitative methods or presence/absence tests) (Lopez-Campos et al. 2012). Conventional or traditional methods for detecting microorganisms in foods can often involve enriching in one or more liquid enrichment media that allow for the resuscitation and multiplication of a particular microorganism. Subsequently, the isolation of the target microorganisms can occur when the enrichment is grown on selective and/or differential plating media for visual and additional confirmation steps (Jasson et al. 2010). Many standardized methods for selected foodborne pathogens are available and are considered the reference analytical methods for official controls and often involve conventional methods (Jasson et al. 2010). Although these methods are rather sensitive for the detection of pathogens, they are laborious and time-consuming, often requiring several days before results are known (Lopez-Campos et al. 2012). Therefore, recent research efforts have concentrated on developing methods that can reduce the assay time through the use of alternatives or combinations of isolation and detection methods and even automated versions wherever possible (Jasson et al. 2010; Lopez-Campos et al. 2012).

Pathogenic *E. coli* represents a phenotypically diverse group of pathogens and there is currently no single method that can be used to enrich, isolate, or select for the various pathotypes that exist. Consequently, available methods have been developed to detect or isolate particular pathotypes of interest. Developments of microbiological analysis of pathogenic *E. coli* in food has predominately focused on the Shiga-toxin producing *E. coli* (STEC) group, particularly the serogroup O157 and recently, other serogroups of concern. As a result, a number of methods have been published but various challenges in isolating these pathogens are currently not resolved. Often fecal samples from ill patients will contain large numbers of the pathogen that aid in detection and isolation, but for foods the predominant challenge is that these pathogens are usually present in very low numbers, in a

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non-homogenous distribution among very high levels of background microflora in complex matrices. Various inhibitors within the food matrices can also interfere with isolation and subsequent detection methods. In addition, these STEC cells may be present in an injured or stressed state as a result of unfavorable conditions during food processing, such as exposure to different pH, temperatures, and the presence of preservatives (Grant et al. 2011; Wang et al. 2013).

The complexity of food matrices is the major obstacle for the development of effective sampling and rapid testing methods. Enrichment is primarily used to resuscitate injured/stressed target cells, increase the target cell numbers, as well as dilute the effects of food inhibitors and background flora within the assay (Wang et al. 2013). However, the time taken to enrich samples can lengthen the isolation process to days as opposed to hours (Ge and Meng 2009; Wang et al. 2013). In addition, these problems result in a necessary trade-off between the need to incorporate selective agents such as antibiotics and inhibitory agents to favor the growth of the STEC while suppressing the unwanted background flora without potentially inhibiting the stressed and injured cells by these agents (O'Sullivan et al. 2007). Effective sampling and sample preparation prior to the actual analyses is, therefore, a critical step in the isolation process (Wang et al. 2013).

2.2 General Method of Isolation for *E. coli*

The isolation and identification of the *E. coli* pathotypes that are not STEC is difficult due to the lack of a medium that can be used to enrich or select for specific strains. An isolation method for all pathogenic *E. coli* is outlined in the Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) (Feng et al. 2011a). The FDA-BAM method is a general procedure for the isolation of *E. coli* (excluding STEC) before subsequent testing for specific virulence traits of different pathotypes. In brief, the method recommends pre-enrichment of a 25 g food sample in 225 ml brain heart infusion (BHI) broth at 35 °C for 3 h to facilitate resuscitation of sublethally injured cells. The pre-enrichment is then transferred to 225 ml of tryptone phosphate (TP) broth and incubated at 44 °C for 20 h. A volume of enriched broth is then plated onto Levine's eosin-methylene blue (L-EMB; colonies produce a green metallic sheen) agar and MacConkey agar plates (colonies are brick red in color). These plates should be incubated at 37 °C for 24 h. Colony morphology and color may vary among pathogenic *E. coli* strains and Enteroinvasive *E. coli* (EIEC) do not ferment lactose; therefore, it is recommended that at least 10 typical and 10 atypical colonies should be picked for further analysis. It is important to note that this method is useful for isolating Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), and Enteroaggregative *E. coli* (EAEC), but not STEC O157:H7, which does not grow well at 44 °C. Specific characteristics and methods to identify and confirm presumptive *E. coli* are described for each pathotype below.

2.3 Shiga Toxin-Producing *E. coli*

2.3.1 Culture and Isolation of Shiga Toxin-Producing E. coli

E. coli O157:H7 is phenotypically distinct from other *E. coli* as it can exhibit a delayed (negative) fermentation of D-sorbitol and does not demonstrate glucuronidase activity, hence these traits are often used to selectively isolate *E. coli* O157:H7 from foods (Thompson et al. 1990; Feng et al. 2011a). Unlike *E. coli* O157:H7, there is currently no standardized method for the isolation of non-O157 STEC from foods. The primary limitation for the detection of non-O157 STEC is the lack of known physiological characteristics that distinguish more than 400 serogroups of non-O157 STEC from non-pathogenic or commensal *E. coli,* which hampers the effective detection and enumeration of these organisms (Coombes et al. 2008; Mathusa et al. 2010; Mingle et al. 2012). In addition, to date, it is still not possible to fully define human pathogenic STEC strains, which adds to the difficulty of identifying clinically significant strains in humans (EFSA 2013).

Existing methodology for STEC is based on developments relating to *E. coli* O157:H7, and generally two approaches have been used to detect the pathogens in foods. Firstly, a primary enrichment to recover *E. coli*, including STEC, is used and subsequently screened for selected virulence factors and the subsequent isolation of an STEC is attempted using available methods (O'Sullivan et al. 2007). This can often result in the isolation of non-disease causing strains. The second approach involves serotype-dependent methods that target specific serogroups frequently associated with human disease and outbreaks such as strains belonging to the serogroups O26, O45, O91, O103, O111, O121, O145, and O157 (Farrokh et al. 2013). The disadvantage of this approach is that serogroups that are less frequently associated with disease or are newly emerging (for example, the *E. coli* O104:H4 serotype associated the large European outbreak; Inset 1.2) are missed in these analyses.

The two most common and successful enrichment media used for *E. coli* O157:H7 and other STEC serotypes are tryptone soy broth (TSB) and *E. coli* broth (EC) with or without modifications to their original formulation (O'Sullivan et al. 2007). These modifications include bile salts or dipotassium phosphate in TSB (mTSB) or less bile salts in modified EC broth, as well as various other selective components which aid in the pathogens' recovery. The International Organization for Standardization (ISO) method 16654 for foods and animal stuffs recommends enrichment in mTSB with novobiocin (mTSBn) at 41.5 °C for an initial period of 18–24 h with subsequent analysis at 6 and 24 h.

The FDA-BAM method (Feng et al. 2011a) recommends the use of buffered peptone water with pyruvate (mBPWp) which contains several antimicrobial reagents that effectively suppress normal flora growth and non-target competitors, yet allows the growth of viable *E. coli* O157:H7 and other STEC and is capable of detecting < 1 cfu/g in foods. This method also describes a screening step of food enrichments using a real-time PCR (RT-PCR) protocol to rapidly rule out negative samples or

establish the presumptive presence of *E. coli* O157:H7 in a sample (see Sect. 2.3.2). The enrichment procedure and RT-PCR screening assay has also been validated for the detection and recovery of other non-O157 STEC but is not serogroup specific.

The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) has released a laboratory guidebook for the detection and isolation of STEC from meat products, carcasses, and environmental sponges (MLG 5B.05) (USDA-FSIS 2013). Target STEC strains are often those that have been noted as adulterants of raw non-intact beef products and product components in the USDA-FSIS register (USDA-FSIS 2013). These include STEC belonging to the O157 serogroup or any one of the following six non-O157 serogroups; O26, O45, O103, O111, O121, and O145. Shiga-toxin producing *E. coli* belonging to these six serogroups are often collectively referred to as the "Big 6 STEC" or "Top 7 STEC" when this group includes *E. coli* O157:H7 (Wang et al. 2013; USDA-FSIS 2014b). While the USDA-FSIS protocol (MLG 5B.05) is designed to guide FSIS laboratories required to perform regulatory testing of meat products, many establishments have chosen to implement STEC screen tests using a method based on, or demonstrated to be equivalent to, the FSIS guidebook.

The USDA-FSIS method (MLG 5B.05) recommends the use of mTSB enrichment without any antibiotics such as sodium novobiocin which is commonly used for *E. coli* O157:H7. Exclusion of sodium novobiocin allows the analysis of samples such as raw beef product, environmental and carcass sponges for *E. coli* O157:H7, and the other STEC noted as adulterants in the USDA-FSIS register (USDA-FSIS 2013). Following a screening step of the food enrichment using RT-PCR (refer to Sect. 2.3.2), the positive enrichments are processed using an immunomagnetic separation (IMS) method which is a technique that is also recommended in other methods (Inset 2.1).

Inset 2.1: Immunomagnetic Separation (IMS)

Many pathogens are usually present in low cell numbers within a food sample and the use of immunomagnetic separation (IMS) has provided an enhanced isolation capacity by aiding in the capture, separation, and concentration of a target pathogen from a sample matrix (Stevens and Jaykus 2004; Grant et al. 2011). The method uses superparamagnetic (i.e., only exhibit magnetic properties in the presence of an external magnetic field) or polystyrene beads that are coated with specific antibodies that capture the intact pathogen present within a complex suspension such as an enrichment broth (Deisingh and Thompson 2004). The application of a magnetic field attracts the beads along with the attached intact and viable pathogen and allows for the cell-bead complex to be extracted and concentrated in a tube. Any non-specific organic or liquid material carried over on the beads is removed through wash steps and the beads are subsequently released and plated onto selective media for further isolation. The method does not yield a pure culture and therefore requires the use of other methods to aid identification of the target bacteria, either

through conventional or molecular assays (Olsvik et al. 1994). Indeed IMS has the advantage of being very versatile and can be used in combination with different rapid and automated assays for the detection of *E. coli* O157:H7 (Fu et al. 2005; Hunter et al. 2011).

Over the years, IMS has seen continual developments for the isolation of *E. coli* O157:H7 from foods and is included as a step in the gold standard cultural method for the pathogen (ISO 16654) and is now also commercially available for other key serotypes (e.g., O26, O103, O111, O121, O145). The utilization of IMS for key non-O157 serotypes is becoming more common and studies have reported the usefulness of serotype-specific enrichment broth, IMS, and selective agar with serological and biochemical confirmation testing for the isolation of these serotypes (Catarame et al. 2003; Bettelheim 2007). Immunomagetic separation in combination with selective enrichment has been found to improve rates of detection and isolation of *E. coli* O157:H7 in complex food matrices (Onoue et al. 1999; Weagant and Bound 2001). However, it has been reported that the detection of some serotypes (O26 and O111) through IMS was affected by enrichment protocol, high numbers of background microflora, and the physiological state of the organism. It was suggested that recovery may be improved by using media with low nutrients, such as buffered peptone water instead of tryptic soy broth and using higher enrichment temperatures (Drysdale et al. 2004).

The most frequently used magnetic carriers are Dynabeads (produced by Dynal, Oslo, Norway), which are polystyrene-based particles ranging from 2.8–4.5 μM. There are also commercially available automated IMS platforms and include the BeadRetriever™ (Dynal Biotech Ltd, Wirral, UK) and PATHATRIX® (Applied Biosystems, Foster City, CA, USA) The later version has been AOAC Research Institute approved for the detection/isolation of *E. coli* O157:H7 and uses up to 250 ml of recirculating enrichment broth over the trapped beads in order to increase the sensitivities and reduce detection times (Fedio et al. 2011). Other systems such as the Assurance GDS® systems (BioControl, USA) are designed specifically to aid in the confirmation of *E. coli* O157:H7 and the additional "Big 6 STEC" serogroups. The system involves the use of a kit that contains IMS particles and components to be used for a RT-PCR assay that is performed on a specific GDS machine. The kit contains Poly-IMS—Top STEC beads which contain a mixture of IMS particles targeted against the Top 7 STEC. The Assurance GDS PickPen® collects the IMS particles that have captured the Top 7 serogroups from an aliquot of a positive enrichment. The RT-PCR assay can detect *E. coli* O157:H7 or the remaining "Big 6 STEC" as well as the stx_1, stx_2, and *eae* genes to provide an overall confirmation of a positive "Top 7." Following a positive PCR reaction, the IMS procedure is repeated again, either with the Poly-IMS—Top STEC or individual IMS beads (IMS Panel-Top STEC) which allows the capture and isolation of each of the specific 7 target O-groups prior to selective agar plating and confirmation.

The optimization of selective enrichment of non-O157 STEC is ongoing. A review of various enrichment protocols assessed key variables, including type of broth medium, presence of antibiotics and or selective ingredients, and incubation time/temperature conditions, however, no clear conclusions could be drawn from the study. It was highlighted that more extensive work would be required with multiple serotypes and sample matrices (Vimont et al. 2006; Grant et al. 2011) and that all serotypes cannot be detected by one method (Baylis 2008).

The most widely used solid plating medium for the detection of non-sorbitol fermenting *E. coli* O157:H7 is sorbitol MacConkey (SMAC) agar, and selectivity is also improved by the addition of selective supplements cefixime and potassium tellurite (CT-SMAC) (Zadik et al. 1993). However, this media is not appropriate for the detection of non-O157 and sorbitol-fermenting *E. coli* O157:H7 which have also been implicated in human disease (Mathusa et al. 2010). Diagnosis of sorbitol-fermenting non-O157 STEC is complex and requires non-culture screening strategies because selective and differential media are not available for their culture (Gould et al. 2009). This is seldom applied, even in specialized laboratories, resulting in under diagnosis of this pathogen (Werber et al. 2011).

A variety of chromogenic media have become available commercially in recent years for the detection of *E. coli* O157:H7 in humans, food, and animal feed stuffs. These media contain a particular mixture of artificial chromogenic conjugates composed of a substrate for an *E. coli*-specific enzyme coupled to a chromophore. When the *E. coli* enzyme cleaves the colorless conjugate, one or more insoluble chromophores are released, resulting in a distinctive color for the *E. coli* colonies (Gouali et al. 2013). Current chromogenic agars use the characteristic traits such as sorbitol fermentation, glucuronidase or galactosidase activity and are largely effective for the discrimination of *E. coli* O157:H7. Chromogenic agars that are specific for the isolation of *E. coli* O157:H7 from foods include CHROMagar™ O157 (CHROMagar Microbiology, Paris, France), RAPID'*E. coli*™ O157:H7 (Biorad, Hercules, CA, USA), and Rainbow® O157 agar (Biolog, Hayward, CA, USA). Studies have been conducted to determine whether chromogenic culture media developed for the detection of *E. coli* O157:H7 are also applicable to non-O157 serotypes; however the variability of phenotypic characteristics for non-O157 on these media impedes the usefulness of these media for the isolation of these strains. Attempts have, therefore, been made to utilize other phenotypic characteristics for the differentiation of non-O157 on selective media (Gouali et al. 2013).

Posse et al. (2008) developed a set of novel differential media for the isolation and confirmation of *E. coli* O157:H7 and non-O157 strains (O26, O103, O111, and O145) from food and feces. The first differential medium for non-O157 strains is based on a mixture of carbohydrate sources, β-D-galactosidase activity, and selective reagents that result in a color-based differentiation of the four specified non-O157 STEC strains. The growth of the four different non-O157 STEC serotypes on this medium produces different colored colonies. *E. coli* O26 colonies appear as bright red to dark purple, O103 and O111 colonies are blue-purple, and O145 colonies are green. Suspect colonies are subsequently picked from the differential medium and streaked onto one of the more specific confirmation media. These agars

contain phenol red broth base supplemented with dulcitol, L-rhamnose, D-raffinose or D-arabinose (Mathusa et al. 2010). These agars have been reported to result in changes to the original stated colors according to incubation time, how crowded or isolated the colonies on the agars or the medium or food matrix which they are isolated from.

Rainbow® O157 agar has been evaluated as a selective media for the detection of non-O157 serotypes and produce different color reactions for different serotypes present (Mathusa et al. 2010). However, some strains of non-O157 serogroups, mainly from serogroup O103, is inhibited by the concentration of potassium tellurite used in Rainbow® O157 agar (0.8 mg/L) as well as CT-SMAC (2.5 mg/L) (Fukushima et al. 2000; Tillman et al. 2012). A modified version of Rainbow® agar (mRBA) containing 0.05 mg/L cefixime, 0.15 mg/L potassium tellurite, and 5 mg/L novobiocin that supports the growth of various STEC strains is described for the isolation of the *E. coli* and the "top six" non-O157 and recommended in the USDA FSIS Microbiology Laboratory Guidebook (MLG 5B.04) (USDA-FSIS 2013). The procedure also involves an IMS step and a subsequent acid treatment procedure which can reduce the growth of natural microflora while allowing acid-tolerant STEC to grow (Tillman et al. 2012).

CHROMagar™ was developed, and has been found, to allow the growth and presumptive identification (mauve colonies) of approximately 75% of STEC isolates in a vast collection of isolates comprising of 20–40 different serotypes, including the common serotypes of Enterohemorrhagic *E. coli* (EHEC) (Hirvonen et al. 2012; Tzschoppe et al. 2012). This media failed to detect only 5/249 from three published studies (Hirvonen et al. 2012; Tzschoppe et al. 2012; Gouali et al. 2013; Wylie et al. 2013). CHROMagar™ was found to have good performance in a clinical trial of stool specimens and was found to recover different STEC serotypes including non-sorbitol fermenting *E. coli* O157:H7 and O104:H4. The use of this media is also rapid, whereby putative STEC colonies can be clearly visualized the day after sample receipt, and performed well against the "gold standard" method of the laboratory (Gouali et al. 2013). A second medium (CHROMagar™ STEC), derived from the original medium, was developed to characterize *E. coli* O104:H4 following the European outbreak (Inset 1.2), and reported to identify the outbreak strain, which produced an extended-spectrum lactamase (ESBL), but not other sporadic *E. coli* O104:H4 isolates that did not produce ESBL (Gouali et al. 2013).

Enterohemolysin agar was developed based on the observation that many non-O157 and O157 strains produce a narrow zone of hemolysis on blood agar supplemented with the red blood cells of sheep and calcium ions after 18–24 h incubation (Beutin et al. 1989). However, a disadvantage of the media is that not all STEC show hemolysis on this agar and the non-selective properties of the agar allows for the growth of background flora present in the sample. Also enterohemolysin positive colonies must be screened for Stx production as some non-STEC that are alpha-hemolytic can interfere with interpretation of colonies (Grant et al. 2011). Supplementation with vancomycin (30 mg/L), cefixime (20 ug/L), and cefsulodin (3 mg/L) was found to be superior to enterohemolysin agar for the detection of hemolysis by STEC (Lehmacher et al. 1998).

2.3.2 *Molecular Detection of Shiga Toxin-Producing* E. coli

The World Health Organization (WHO) has called the rapid identification of virulent non-O157 STEC a public health priority (WHO 1998). Recent recommendations from the Centers for Disease Control and Prevention (CDC) suggest that future STEC methods should include an assessment of the potential of the organisms to cause severe disease, possibly by detecting virulence factor genes (Mingle et al. 2012). These recommendations also proposed that comparative genomic studies performed on existing and newly sequenced STEC strains may identify gene targets that will likely aid in the identification or predications of pathogenicity in the future (Gould et al. 2009; Mingle et al. 2012). A range of molecular techniques such as conventional polymerase chain reaction (PCR) (Paddock et al. 2012), RT-PCR (Fratamico et al. 2011), PCR coupled to mass spectrometry (Shen et al. 2013), and isothermal nucleic acid amplification (Wang et al. 2012) have been employed in the detection of gene targets associated with STEC strains.

The FDA-BAM method (Feng et al. 2011a) recommends a molecular screening step of food enrichments for *E. coli* O157:H7 and other STEC using an RT-PCR assay. Implementation of an initial screen test can offer time and cost benefits by reducing the number of samples requiring confirmation and the time that it takes to test. The assay detects the stx_1 and stx_2 genes and the +93 single nucleotide polymorphism (SNP) in the *uid*A gene that encodes for the β-D-glucuronidase enzyme (Feng and Lampel 1994; Jinneman et al. 2003). The SNP is highly conserved in *E. coli* O157:H7 strains that produce Stx and is an accurate marker for these pathogens (Feng 1993). This assay also detects other STEC strains where a +93 *uid*A negative but stx_1 and/or stx_2 positive result indicates the sample may contain an STEC, but not necessarily a pathogenic STEC (Feng et al. 2011a). It is therefore essential to isolate and confirm the pathogenic STEC for additional testing.

The USDA-FSIS method (MLG 5B.05) also involves performing a molecular screen of samples targeting specific virulence genes, followed by the isolation and confirmation of STEC from screen test positive broths. The targets utilized in MLG 5B.05 are also extensively used in research and commercial tests and include Shiga toxin encoding genes (*stx*), an attaching and effacing gene (*eae*), and genes specific for each of the Big 6 *E. coli* O serogroups. An RT-PCR test (BAX® System RT-PCR Assay—STEC Suite, Dupont) performed on a commercially available platform (BAX® RT-PCR system) is used to detect each gene and provide the user with an automated interpretation of the result. Any samples that test positive for all three gene targets; *stx*, *eae*, and an O antigen are considered potentially positive for a Big 6 STEC and sent for confirmation.

Numerous RT-PCR methods that use similar approaches to the FSIS protocol have been used for the sensitive detection of STEC in enrichments of ground beef, beef carcass swabs, beef trim, and a range of other food matrices such as apple juice and raw-milk cheeses (Wang et al. 2013). Clinical laboratories have also imple-

mented similar RT-PCR methods to detect the Big 6 STEC serogroups in samples, which has improved timeliness of identification and outbreak investigations (Mingle et al. 2012). In addition, a range of commercially available systems have been developed that utilize a host of different technologies and targets to detect the Big 6 or Top 7 STEC serogroups (Table 2.1).

While most commercial test systems rely on the detection of *stx*, *eae*, and O antigen targets, some utilize additional or alternative targets which may provide a better indication of the presence of a Big 6/Top 7 STEC. For example, the Atlas STEC EG2 Combo Detection Assay designed by Roka Biosciences (San Diego, CA, USA) uses an alternative target that is believed to eliminate false-positive results that may arise from co-contamination of samples with non-STEC organisms that possess *stx* and *eae*. Alternatively, the NeoSEEK™ (Neogen, Lansing, MI, USA) approach to STEC detection and identification employs a high throughput SNP genotyping platform to detect 70 independent targets. The number and range of targets employed is believed to provide sufficient evidence for the non-culture based confirmation of Big 6 STEC. In a modification to the FSIS method, the Assurance GDS system employs an IMS step to separate target O serogroups from enrichment cultures prior to cell lysis and PCR detection of STEC virulence genes by RT-PCR. Many of these commercial screening systems have been issued with a letter of no objection for testing beef products (USDA-FSIS 2014b), and/or have been validated by recognized independent bodies for the detection of STEC in other food types such as poultry, spinach, leafy green vegetables, and sprouts.

Despite their widespread popularity, detection systems that rely solely on *stx*, *eae*, and O antigen markers to detect STEC in complex matrices have a number of limitations associated with these gene targets. Beef cattle feces and beef trim enrichments represent two examples of complex matrices that can contain multiple isolates of *E. coli* harboring different combinations of genetic markers (*stx*, *eae*, and O antigen genes) that are not all associated with a single isolate. Isolates meeting this description, while not considered a Big 6 STEC, are likely to contribute to the potential positive status of a sample. To further confound screening protocols, *stx* and *eae* gene targets may also be harbored by non-Top 7 STEC and bacterial species other than *E. coli* (Schmidt et al. 1993; Paton and Paton 1996; Karch et al. 1999; Gyles 2007; Chandry et al. 2012), further contributing to the potential positive status of a sample. Samples may also contain free *stx*-phages that interfere with the detection of STEC (Martinez-Castillo and Muniesa 2014), and *stx* specific primers may not detect all *stx* variants (Feng et al. 2011b). Therefore, any method that solely rely on *stx*, *eae*, and O antigen targets to screen for Top 7 STEC in complex matrices should confirm the presence of pathogens in screen test positive samples. If no attempt is made to confirm screen positive samples, then those samples are considered positive for a Top 7 STEC serogroup by the FSIS (USDA-FSIS 2014c). Since studies have reported a large number of potential positive samples that cannot be culturally confirmed (Bosilevac and Koohmaraie 2012), failure to confirm samples could result in substantial cost to industry.

Table 2.1 Selected commercially available kits for the detection of Shiga toxigenic *Escherichia coli*. (Adapted from Wang et al. 2013; USDA-FSIS 2014b)

Name of test	Supplier	Target STEC serogroups	Test type/technique	Recommended/validated samples types[a]
ACTERO *Salmonella*/ STEC Enrichment Media	FoodChek System Inc.	Top 7 STEC	Magnetic immunochromatographic technology (lateral flow)	Raw ground beef, liquid whole egg, raw ground chicken, raw frozen scallops, sprouts, environmental surfaces (stainless steel, plastic, rubber, ceramic tiles, sealed concrete)
Atlas STEC EG2 Combo Detection Assay	Roka Bioscience, Inc	Top 7 STEC	Target capture of nucleic acid, Transcription-Mediated Amplification and Hybridization Protection Assay	Fresh raw ground beef (73% lean), fresh raw beef trim, romaine lettuce
Assurance GDS MPX Top 7 STEC	BioControl Systems	Top 7 STEC	Immunomagnetic capture and RT PCR	Raw beef trim, raw ground beef, raw spinach, and raw mixed greens (baby lettuces and other leafy green vegetables)
BAX® System real-time PCR STEC suite	DuPont Qualicon	Big 6 STEC—O26, O45, O103, O111, O121, O145	Real-time polymerase chain reaction (RT PCR)	Raw beef trim, raw ground beef, raw ground beef plus soy
GeneDisc Plate STEC Top 7	Pall GeneDisc Technologies	Top 7 STEC—O26, O45, O103, O111, O121, O145, O157	RT PCR	Fresh raw ground beef (20% fat), fresh raw beef trim (20% fat)
IEH *E. coli* O157 (Stx-producing *E. coli* (STEC) with Intimin and *Salmonella* Test System	IEH Laboratories & Consulting Group	Top 7 STEC	RT PCR	Raw ground beef, raw beef trim, raw poultry, RTE turkey (omitted for STEC) and mixed leafy greens
iQ-Check™ STEC VirX and iQ-Check™ STEC SerO	Bio-Rad Laboratories	Top 7 STEC	RT PCR	Raw beef trim
NeoSeek STEC confirmation	Neogen Corp.	Big 6 STEC	PCR mass spectrometry	Ground beef trim

Table 2.1 (continued)

Name of test	Supplier	Target STEC serogroups	Test type/technique	Recommended/validated samples types[a]
Rapid-B non-O157 STEC test kit	Vivione Biosciences, Inc	Big 6 STEC	IMS and flow cytometry	Raw ground beef and beef trim
RapidFinder STEC	Life Technologies	Top 7 STEC	Immunomagnetic capture and RT PCR	Raw ground beef and beef trim

[a] Systems listed may have been validated for a particular test portion of the matrices listed

2.4 Enumeration of Shiga Toxin-Producing *E. coli*

The importance of quantitative data of food pathogens, such as *E. coli* O157:H7 and other STEC at different points within the food chain is becoming evident as quantitative risk assessment models are increasingly being developed. There is currently no standard protocol for the enumeration of *E. coli* O157:H7 or other STEC serogroups from food or environmental samples. Cultural approaches have involved directly plating the sample onto selective plating media but the sensitivity of this approach is often low (approximately 10^2 colony forming units (CFU) per gram). Bacterial cells that are stressed or injured may not be detected unless initially plated onto a non-selective agar (such as tryptone soya agar) and incubated for 3–4 h to aid recovery of the stressed cells before over-pouring a layer of selective agar and incubated for a longer period.

An alternative method is the most probable number (MPN) method, which calculates the number of viable microorganisms in a sample by preparing decimal dilutions of the samples and transferring subsamples of three dilutions into 9 or 15 tubes containing liquid culture medium, to perform three or five tubes assays, respectively. The tubes are then incubated and those that show growth (turbidity) are counted. Taking into account the dilution factor, the final results are compared to a standard MPN table, which will indicate the MPN of bacteria in the product (Blodgett 2010). This method is often more labor-intensive and expensive than the direct plate method and the confidence limits of the MPN method can be large, even when many replicate samples are included for each dilution. However, the method has been extensively used to enumerate *E. coli* O157:H7 in different sample types (Fegan et al. 2004; Duffy et al. 2010; Nkere et al. 2011).

Enumeration of non-O157 serogroups is more difficult due to the lack of media which can differentiate the morphologically different colonies produced by these serogroups. As a result, serological and other biochemical or molecular methods are required to confirm colonies on media which can be very laborious and expensive to undertake (Caro et al. 2011). Alternatively, various studies have reported the use of molecular methods to enumerate STEC cells within samples but the limit of detection can be very high (10^2–10^7 CFU per gram) (O'Sullivan et al. 2007; Guy et al. 2014; Russo et al. 2014).

2.5 Immunological Detection Methods for Pathogenic *E. coli*

The laborious and time-consuming nature of isolating STEC from foods has seen the increasing development of rapid methods for the detection of the pathogens in various food matrices. Along with targeting specific genes (e.g., *stx* or an antigen) via molecular methods, are biological (cytotoxicity tests) and serological/immunologically based methods (Bettelheim and Beutin 2003). Many of these immunoas-

says are commercially available for STEC and other pathogenic *E. coli* as "ready-to-use kits" (Bettelheim and Beutin 2003; Scheutz et al. 2011; Table 2.2). These kits utilize specific poly- or monocolonal antibodies targeting surface antigens and thus can detect specific STEC serogroups (most kits predominately target *E. coli* O157) while some detect the toxins produced by pathogens.

Many of the commercial kits require prior enrichment of the target cells to reach detectable levels and are generally less sensitive than traditional culture methods. However, the kits offer the advantage of speed, reduced labor costs, and high volume throughput (O'Sullivan et al. 2007). The kits are also easy to use and do not require specific materials or skills, and are therefore widely used in routine laboratory testing for STEC in different countries (Bettelheim and Beutin 2003). The primary disadvantage of the methods, however, is that the infecting organism is not isolated for subsequent serotyping and a specific diagnosis of *E. coli* O157:H7 and the isolation of the pathogen of interest following a positive detection can be challenging (Gould et al. 2009). All immunological techniques should be adequately standardized and controlled to reduce the possibility and impact of false positive and false negative results. This is more common in samples with high levels of background microflora. In all cases, presumptive positive samples should undergo further confirmation tests, as the antibody may cross-react with other microorganisms within the sample. False positive results can occur when the immunological material cross-reacts with a non-STEC organism (i.e., antibody is insufficiently specific). False negative results occur when the immunological material does not detect STEC cells when present in the test sample (i.e., the antibody can find a target binding site on the STEC cell) (O'Sullivan et al. 2007).

Some of the immunoassays can detect and differentiate between Stx1 and/or Stx2 in supernatants of stool or from bacterial cultures in a microtitre plate format or a lateral flow device. Overnight enrichment of the sample is recommended and the subsequent time to undertake the assays can vary from 20 min to 4 h, depending on the test format used (Gould et al. 2009). Essentially, sample supernatant is placed into wells coated with Stx1- and Stx2-specific (monoclonal) antibodies and left to incubate at room temperature for a given time (approximately 1 h) to permit binding of any Shiga toxin present in the sample bound antibodies. Secondary antibodies that are conjugated with an enzyme (such as horse-radish peroxidase or alkaline phosphatase) are then added, and the resulting complex sandwiches the Shiga toxin between two sets of antibodies. Following another short incubation time (approximately 30 min) at room temperature and washing, an enzyme substrate and a chromogen are added, which produce a blue or yellow color, at an intensity is proportional to the amount of toxin present in the original sample (Bettelheim and Beutin 2003).

The FDA introduced the use of enzyme immunoassay (EIA) capable of detecting any STEC for use in clinical laboratories. It was found that the identification and characterization of STEC within clinical laboratories was enhanced by the combined use of the EIA and the confirmatory tests (Schaffzin et al. 2012). This improvement in detection methods is likely to have contributed to the change observed between published estimations of foodborne infections in 1999 and 2011, with a

Table 2.2 Selected commercially available immunological based kits for *E. coli*. (Adapted from (Bell and Kyriakides 2000; USDA-FSIS 2014a)

Name of test	Supplier	Test target	Test type/technique	Recommended/validated samples types
Reveal for *E. coli* O157 systems	Neogen Corporation	*E. coli* O157:H7 and O157:NM	Lateral flow immunoassay	Raw ground and cubed beef, apple cider, lettuce rinse, environmental swabs
Visual Immunopreciptate Assay (VIP®) for EHEC	BioControl Systems	*E. coli* O157:H7	Lateral flow immunoassay	Dairy foods, meats, poultry products, fruits, nutmeats, seafood, pasta, liquid eggs, raw and cooked beef
Assurance® EIA EHEC	Biocontrol	*E. coli* O157:H7	Enzyme immunoassay (microtitre plate)	Dairy foods, meats, poultry products, fruits, nutmeats, seafood, pasta, liquid eggs, raw and cooked beef
SinglePath	Merck KGaA	*E. coli* O157:H7	Lateral flow immunoassay	Raw ground beef and paseurized milk
3M™ Tecra™ *E. coli* O157 Visual Immunoassay	3M Tecra International Pty Ltd	*E. coli* O157	Enzyme immunoassay (microtitre plate)	Raw and cooked ground beef, raw and cooked ground chicken
DuPont Lateral Flow System *E. coli* O157 Test Kit	Dupont Qualicon	*E. coli* O157	Lateral flow immunoassay	Boneless beef, ground beef, apple cider
FoodChek™ *E. coli* O157	FoodChek Systems, Inc.	*E. coli* O157	Lateral flow immunoassay	Raw ground beef, raw beef trim
RapidChek® *E. coli* O157	Romer Labs	*E. coli* O157:H7	Lateral flow immunoassay	Raw ground beef and boneless beef
VIDAS®UP (ECPT) *E. coli* O157	bioMérieux	*E. coli* O157:H7	Enzyme Linked Fluorescent Assay	All human food products and production environmental samples

Table 2.2 (continued)

Name of test	Supplier	Test target	Test type/technique	Recommended/validated samples types
Multipath System™ E for *E. coli* O157	Crystal Dignostics Corporate	*E. coli* O157:H7	Liquid crystal-based immunodiagnostic assay	Raw ground beef, fresh raw beef trim
DuoPath® Verotoxin	Merck KGaA	STEC (Shiga toxins-can differentiate between 1 and 2)	Lateral flow immunoassay	Pure culture
Immunocard STAT! EHEC	Meridian Diagnostics, Inc.	STEC (Shiga toxins-can differentiate between 1 and 2)	Lateral flow immunoassay	Enrichment broth and isolates
Premier EHEC	Meridian Diagnostics, Inc.	STEC (Shiga toxins-cannot differentiate between 1 and 2)	Enzyme immunoassay (microtitre plate)	Overnight enrichment broths and direct testing of stool samples
ProSpecT STEC	Remel	STEC (Shiga toxins-cannot differentiate between 1 and 2)	Enzyme immunoassay (microtitre plate)	Overnight enrichment broths and direct testing of stool samples
VTEC Screen "Seiken"/ Denka Seiken RPLA	Denka Seiken	STEC (Shiga toxins; can differentiate between 1 and 2)	Reversed passive latex agglutination (RPLA)	Isolate
DRG® *E. coli* Verotoxin 1+2 Ag ELISA	DRG International, Inc.	STEC (Shiga toxins-cannont differentiate between 1 and 2)	Enzyme immunoassay (microtitre plate)	Isolate
E. coli heat-labile Enterotoxin Detection Kit (VET-RPLA)	Denka Seiken	*E. coli* heat-labile enterotoxins(LT)	RPLA Microtitre plate	Isolate
E. coli Heat-stable Enterotoxin Detection Kit (*E. coli* ST-EIA "SEIKEN")	Denka Seiken	*E. coli* heat-stabile enterotoxins (ST)	RPLA Microtitre plate	Isolate

notable increase in the number of infections attributed to non-O157 (Scallan et al. 2011). Further enhanced testing regimes for non-O157 which incorporated the use of EIA on stool samples have allowed for the identification of the serogroup and type as part of non-O157 outbreak investigations that otherwise might not have been supported by laboratory data (Mingle et al. 2012; Schaffzin et al. 2012).

A 6-year study involving the use of an enhanced method to identify and characterize STEC, which included an algorithm for testing Shiga-toxin EIA positive specimens, found that 41 % of the STEC identified were non-O157 STEC. Without the submission of EIA-positive broths these STEC would have been missed (Mingle et al. 2012). This study assisted in determining the true burden of these serogroups in disease. In contrast, some studies have shown the failure of EIAs to detect *E. coli* O157:H7 that was identified in the same sample through plating and emphasizes the importance of primary isolation (CDC 2001; Klein et al. 2002; Gould et al. 2009).

2.6 Cell Culture Assays Used for Pathogenic *E. coli*

2.6.1 Cell Cytotoxicity Assay for Shiga Toxin-Producing E. coli

Vero (African green monkey kidney) and HeLa cell lines are very sensitive to Shiga toxin because they have high concentrations of globotriaosylceramides Gb3 and Gb4, the receptors for Shiga toxin in eukaryotic cells. Sterile fecal filtrates prepared from stool specimens, broth enrichments, or colonies are inoculated onto cells and observed for typical cytopathic effect. Confirmation that the cytopathic effect is caused by Shiga toxin is performed by neutralization using anti-Stx1 and anti-Stx2 antibodies. Although the method is very sensitive, it is not routinely used in most clinical microbiology laboratories due to the need for specialized skills in tissue culturing and the availability of cell monolayers, and specific antibodies (Bettelheim and Beutin 2003; Gould et al. 2009).

2.6.2 Cell Adherence Assays

One of the most useful phenotypic assays for the diagnosis of EPEC, EAEC, and DAEC (diffusely adherent *E. coli*) is the HEp-2 adherence assay. This assay was originally investigated for EPEC and many modifications to the method such as incubation time and the use of different growth mediums have been reported which can create conflicting results between laboratories (Cravioto et al. 1979; Mathewson and Cravioto 1989; Shariff et al. 1993; Nataro and Kaper 1998; Gomes et al. 2004). However, the assay performed as first described, provides the best ability to differentiate strains amongst the EPEC, EAEC, and DAEC pathotypes (Nataro and

Kaper 1998). This assay involves inoculating the test trains onto a semi-confluent HEp-2 monolayer and incubating for 3 h at 37 °C under 5 % CO_2. Following incubation, the monolayer is washed, fixed, stained, and examined by oil immersion light microscopy for characteristic patterns; namely, localized adherence (LA), aggregative adherence (AA), and diffuse adherence (DA); however, some strains may produce ambiguous results in the assay (Mathewson and Cravioto 1989; Nataro and Kaper 1998).

2.7 Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) are often less reactive biochemically than other *E. coli,* are frequently anaerogenic, and may demonstrate late or no lactose fermentation (Desmarchelier and Fegan 2003). For this reason, the isolation and identification of EIEC strains is difficult due to the lack of a medium that can be used to enrich or specifically select for EIEC strains. Other traits associated with the EIEC group include; failure to decarboxylate lysine, inability to use acetate or ferment mucate, and non-motility. Although the correlation between serogroup and pathogenicity is not perfect, combining serology with biochemical testing can be useful in identifying EIEC isolates (Desmarchelier and Fegan 2003). The EIEC are also related to *Shigella* and are separated from other *E. coli* by molecular methods (PCR of the *ipa*H-gene) and physiological and biochemical typing and serological testing. There are very few biochemical characteristics that differentiate *Shigella* and EIEC from each other and the two most convenient are mucate and acetate tests. EIEC may be positive for either or both, whereas *Shigella* strains are normally negative for both (Lan et al. 2004). Salicin fermentation and esculin hydrolysis have also been used to differentiate the two groups, where *Shigella* is normally negative for both tests (van den Beld and Reubsaet 2012).

Pathogenicity of EIEC is primarily due to its ability to invade and destroy colonic tissue and the invasion phenotype (encoded by a high molecular weight plasmid) involves assessing the ability to cause keratoconjunctvitis in guinea pig eyes and to form plaques in HeLa cell monolayers (Mehlman et al. 1982; Feng et al. 2011a). However, PCR-based detection methods have replaced these phenotypic assays. EIEC and *Shigella* both carry a 140-MDa invasion plasmid. Because this plasmid can be lost during enrichment, genes encoding invasion plasmid antigens (*iap*H), rather than plasmid encoded genes, are commonly used to detect EIEC and *Shigella* and distinguish them from other *E. coli* pathotypes (Venkatesan et al. 1988; Sethabutr et al. 1993; Binet et al. 2014). The *ipa*H target has been used to detect EIEC and *Shigella* in foods including, but not limited to, fresh produce using a TaqMan quantitative PCR (Lin et al. 2010) and ground beef, produce and salad using a multiplex conventional PCR approach (Kim et al. 2010; Binet et al. 2014). Some of these assays have incorporated invasion plasmid targets into multipathotype detection systems. Because invasion plasmids are carried by both EIEC and

Shigella, further analysis is often required in order to distinguish between these two organisms. Additional gene targets have been suggested to discriminate EIEC from *Shigella* using conventional (Yamazaki and Fukasawa 2011) or RT-PCR approaches (Pavlovic et al. 2011).

2.8 Enterotoxigenic *E. coli*

ETEC has a high infective dose for adults (approximately 10^8 cells) and therefore analysis for this pathotype is not usually performed unless high levels of *E. coli* have been found in a food. It is also recommended that if ETEC is detected, levels should be enumerated to assess the potential hazard of the contaminated food (Desmarchelier and Fegan 2003; Feng et al. 2011a). Serotyping is not suitable for the identification of ETEC as isolates can belong to a large number of serotypes and food may contain ETEC strains of animal origin which are not human pathogens (Desmarchelier and Fegan 2003). The ability of ETEC to change serotype profiles over time has been noted (Qadri et al. 2005).

Diagnosis of ETEC is based on the identification of the factors important in pathology and includes the production of LT and/or ST and the possession of colonization factors. ETEC strains that are associated with human disease may have genes for some of these factors within plasmids. Some selective media that are used for the isolation of *E. coli* from foods can cause the loss of these plasmids and the loss of the strains' ability to produce these factors (Desmarchelier and Fegan 2003). The production of LT and ST may be tested using the rabbit ileal loop and infant mouse physiological assays which were initially the gold standards for the identification of these enterotoxins, respectively. These assays are notoriously difficult to perform and time-consuming (Nataro and Kaper 1998). The development of specific and sensitive immunological tests (some are commercially available; Table 2.1) and the Biken test has been used for detecting the LT and ST (Honda et al. 1982; Takeda et al. 1983). Various methods exist to determine the production of colonization factors such as fimbraie and include mannose-resistant agglutination of certain species of erythrocytes, serological tests using monoclonal antibodies, or through molecular methods (Evans et al. 1977; Ahren et al. 1986). Some of these tests are complicated due to the failure of some fimbriae to be readily expressed *in vivo* and the variety of fimbriae that can be produced (Desmarchelier and Fegan 2003).

Molecular detection techniques for ETEC commonly rely on the detection of LT and ST genes. These genes have been used extensively as targets in conventional (Feng and Reddy 2014) and RT-PCR (Grant et al. 2006; Patel et al. 2011), DNA colony hybridization (Ferreira et al. 1986), and microarray assays (Deng et al. 1996; Wang et al. 2010) to detect the presence of ETEC in a range of food and water samples or from pure culture. Although PCR is a popular method for detection of ETEC, its effectiveness has been shown to vary between food matrices. In a large survey of fresh produce collected between 2004 and 2010, isolates were obtained

from less than 40% of samples that tested positive for LT and ST genes using a multiplex PCR approach (Feng and Reddy 2014). The same study noted that isolation rates differed greatly between food sample types and the authors suggested that product type may influence confirmation efficiency. A similar study also reported PCR detection difficulties with more challenging food matrices such as hot sauce (Grant et al. 2006). The latter study suggests that PCR sensitivity may be improved by reducing inhibitors through greater purification of DNA templates. The presence of non-viable ETEC or the death of ETEC in food samples prior to confirmation may also limit the recovery of isolates from screen positive samples (Feng and Reddy 2014). As is the case with most PCR approaches to detect foodborne pathogens in complex matrices, isolation of the target pathogen is recommended to unequivocally determine their presence.

2.9 Enteropathogenic *E. coli*

EPEC were traditionally defined by their serotype based on the O and H antigens which had been traditionally associated with infantile diarrhea, and in general, serotype correlates with specific pathotypes of *E. coli* (O'Sullivan et al. 2007). However, it is now more common to define *E. coli* pathotypes based on their pathogenic characteristics as particular serotypes may belong to different pathotypes. For example, O111:H- may belong to the EPEC and EHEC groups depending on the possession of key virulence genes, such as *stx* and *eae* (O'Sullivan et al. 2007). A study in England indicated that public health laboratories are adopting more molecular assays for the detection of other pathotypes such as EPEC directly from fecal specimens in order to gain further epidemiological information on the pathotype(s) circulating within the population (Sakkejha et al. 2013).

EPEC are genetically defined by the presence of genes encoded on EPEC adherence factor (EAF) plasmids, which are responsible for the localized adherence of EPEC to epithelial cells (Elias et al. 2002; O'Sullivan et al. 2007). EPEC also possess genes commonly associated with EHEC such as *eae* but lack genes encoding Shiga toxins (Elias et al. 2002). *E. coli* that possess *eae* but do not possess the EAF plasmid are classified as atypical EPEC. Despite lacking EAF, this subgroup is still capable of causing human disease. The majority of studies detect EPEC by targeting the bundle-forming pilus gene (*bfp*A) carried by the EAF plasmid. Polymerase chain reaction (PCR) methods have been used to screen a range of food types using the *bfp*A target (Rugeles et al. 2010; Alonso et al. 2011).

EPEC strains show a distinct pattern of localized adherence to HEp-2 cells where cells adhere in clusters in the presence of mannose. The A/E phenotype can also be identified on cultured HEp-2 or HeLa cells by the fluorescent actin staining (FAS) tests (Mathewson and Cravioto 1989). This involves the microscopic observation of the accumulation of actin at an A/E site that binds to a fluorescein. However, this is a labor intensive method which requires specialized skills and equipment.

2.10 Enteroaggregative *E. coli* and Diffusely Adherent *E. coli*

Phenotypically, the HEp-2 cell adherence assay remains the gold standard for identifying enteroaggregative *E. coli* AEC ("stacked-brick" appearance) and diffusely adherent *E. coli* (DAEC) (diffusely adherent appearance). Limitations of the cell assay include time requirement and limited availability in reference laboratories, which have led to the development of other more rapid molecular methods, including PCR and DNA probes (Kaper et al. 2004; Cennimo et al. 2007). Plasmid-encoded genes, *aat*, *agg*R, and *aap*, have been described as suitable genes for the detection of typical EAEC (Jenkins et al. 2006). Due to the important role that the *agg*R gene plays in regulating a range of EAEC virulence factors, isolates that possess this gene are considered typical EAEC (Cennimo et al. 2007). Despite the extensive use of *agg*R in detection methods for typical EAEC, epidemiological data suggests it may not be a suitable marker for disease-causing EAEC as *agg*R containing strains have been isolated from healthy patients. Likewise, strains that lack *agg*R have been isolated from gastrointestinal outbreaks (Cennimo et al. 2007). In addition to this, EAEC are highly heterogeneous pathogens and DNA probes or PCR primers designed to detect single gene targets have been shown to exhibit a wide range of sensitivity (Cennimo et al. 2007). Despite this, *agg*R was recently used as a target in a loop-mediated isothermal amplification (LAMP) method for the detection of EAEC in foods (Yokoyama et al. 2010). This LAMP-based approach to detecting EAEC in various vegetable and meat samples was shown to be significantly more sensitive than a PCR approach (Yokoyama et al. 2010). In addition to *agg*R, a range of alternative plasmid and chromosomally encoded genes have been proposed for EAEC detection (Cerna et al. 2003; Panchalingam et al. 2012). However, the genetic diversity of EAEC continues to present challenges for the rapid, sensitive, and specific molecular detection of pathogenic EAEC in food, clinical, and environmental samples.

Molecular methods that target genes associated with all known Afa/Dr adhesins have been used in the identification of DAEC (Le Bouguenec et al. 2001). Common targets include *daa*C, *daa*E, and *afa*B (Bilge et al. 1989; Le Bouguenec et al. 2001). These markers have been used in the development of DNA probes and PCR based detection methods for screening clinical fecal samples and bacterial colonies (Guion et al. 2008; Rugeles et al. 2010; Souza et al. 2013). However, inherent limitations associated with Afa/Dr genes currently confound efforts to conclusively identify DAEC. For example, not all DAEC hybridize with *daa*C and *daa*E probes (Scaletsky et al. 1999; Lopes et al. 2005) and *daa*C probes have been shown to cross-react with a subset of EAEC (Snelling et al. 2009), which could lead to false positive results. While there are few examples of the use of these or alternative markers for detecting DAEC in foods, *daa*E was used to target DAEC in a large survey of food items (Canizalez-Roman et al. 2013). The reservoir for DAEC is currently unknown, as is its source of transmission to humans (Croxen et al. 2013). Thus, further work is needed to understand the role, if any, food plays as a reservoir or source of transmission to humans.

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Chapter 3
Typing and Subtyping Methods for Pathogenic *Escherichia coli*

3.1 Introduction

It is important to establish relationships between different isolates of bacteria for identification of outbreaks, determining the source of outbreaks, establishing the transmission pathways of pathogens, gaining insights into the global distribution of pathogens, for understanding how such pathogens have evolved, and to aid in the treatment of diseases. Over the past few decades, many different typing methods have been established to aid in this understanding. Some of the earlier methods were based on phenotypic typing, including biochemical-based tests and serotyping, while more modern methods have been based on the genetic components of bacteria, including the presence, absence, or number of specific gene markers, variation within the sequence of specific genes through to variations in the whole genome of individual isolates (Li et al. 2009; Sabat et al. 2013; van Belkum et al. 2001).

Typing methods vary in their discriminatory power, reproducibility, ease of performance, ease of interpretation, and cost (van Belkum et al. 2001; Karama and Gyles 2010). The choice of the most appropriate method to use will depend on the purpose of typing, how reproducible it needs to be, resources required, the desired outcome, and the level of discrimination required. There are many advantages and disadvantages associated with different typing methods (Karama and Gyles 2010; Li et al. 2009; van Belkum et al. 2001) and there is no one single method that currently meets all the needs required of a typing method. The broad range of methods commonly used to type *E. coli* are listed in Table 3.1. The development of new typing methods is constantly occurring particularly in relation to the genetic-based typing methods. Whole genome sequencing (WGS) provides great potential for meeting all the requirements of typing although there is still a way to go in relation to the interpretation of genetic information, and the links between genotypes and phenotypes in relation to pathogenicity and resistance to antibiotics and other stresses (Li et al. 2009). Methods vary in their adoption around the globe, with some used internationally (i.e., pulsed field gel electrophoresis (PFGE)) and others specific to particular laboratories (repetitive element palindromic polymerase chain reaction (Rep-PCR)). The most commonly used methods for typing of pathogenic

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Table 3.1 Methods commonly used for the typing of pathogenic *E. coli*. (Adapted from Hyytia-Trees et al. 2007; Karama and Gyles 2010; Li et al. 2009; van Belkum et al. 2001; Sabat et al. 2013)

Method	Reproducibility	Discriminatory power	Simplicity of the procedure	Data analysis	Availability of method	Application	Covered in this chapter
Phenotypic-based methods							
Biochemical profiling	Relatively good (particularly if using automated or commercial systems)	Poor	Excellent	Easy	Broadly available	Confirmation of isolates as *E. coli* for clinical, epidemiological and research purposes	Yes
Serotyping	Good	Variable	Good	Relatively easy	Variable—some commercial kits for specific serotypes but mostly requires specialized laboratories	Mostly used in disease diagnosis and epidemiological investigations. Applicable to the broad population of *E. coli*	Yes
Bacteriophage typing	Fair	Variable	Poor	Difficult	Variable	Used for subtyping of *E. coli* O157:H7 isolates	Yes
Multilocus enzyme electrophoresis (MLEE)	Good	Good	Good	Excellent	Variable	Phylogenetic and evolutionary analyses of broad populations of *E. coli* isolates	Yes
Antibiotic susceptibility profiling	Good	Poor	Excellent	Easy	Broadly available	Used in management of clinical disease and for epidemiological investigations, applied to clinical isolates of *E. coli*	No, for further information see Woodford et al. (2011)
Genetic-based methods							
Whole genome sequencing (WGS) and Single Nucleotide Polymorphism (SNP) analysis	Excellent	Excellent—ultimate discriminatory power	Good	Difficult but improving	Reasonable	Multiple applications to a broad range of *E. coli*—epidemiological typing, outbreak tracking, phylogenetic analysis, determining presence of virulence factors	Yes

Table 3.1 (continued)

Method	Reproducibility	Discriminatory power	Simplicity of the procedure	Data analysis	Availability of method	Application	Covered in this chapter
Pulsed field gel electrophoresis (PFGE)	Excellent	Excellent	Good	Good	Variable—requires specialized equipment	Globally used for epidemiological studies (identifying outbreaks and determining sources of outbreaks), applicable to the broad population of *E. coli*	Yes
Multiple-locus variable number tandem repeat (MLVA)	Excellent	Very good	Good	Good	Broadly available	Used currently in epidemiological investigations of pathogenic *E. coli*	Yes
Multilocus sequence typing (MLST)	Excellent	Variable	Good	Fair	Reasonable	Phylogenetic and evolutionary analyses of broad populations of *E. coli* isolates	Yes
Repetitive element palindromic polymerase chain reaction (Rep-PCR)	Low	Variable	Good	Variable	Broadly available	Mostly used for establishing relationships between different isolates of *E. coli*, applicable to the broad population of *E. coli*	Yes
Random amplified polymorphic DNA (RAPD)	Low	Variable	Good	Variable	Broadly available	Mostly used for establishing relationships between different isolates of *E. coli*, applicable to the broad population of *E. coli*	Yes
Shiga toxin-encoding bacteriophage insertion (SBI)	Excellent	Fair	Good	Good	Reasonable availability	Understanding phylogenetic relationships among *E. coli* O157:H7 but has applicability to other STEC	Yes
Lineage specific polymorphism analysis (LSPA-6)	Excellent	Fair	Good	Good	Reasonable availability	Understanding phylogenetic relationships and clinical significance of *E. coli* O157:H7	Yes

Table 3.1 (continued)

Method	Reproducibility	Discriminatory power	Simplicity of the procedure	Data analysis	Availability of method	Application	Covered in this chapter
Optical mapping	Excellent	Excellent	Reasonable	Difficult	Low—requires specialized equipment	Phylogenetic analysis of the broad population of *E. coli*	No, for further information see Kotewicz et al. (2007)
Ribotyping	Excellent	Good	Good	Good	Variable—commercial system available	Mostly used for source tracking of broad populations of *E. coli* from different origins and identification of species	No, for further information see Schumann and Pukall (2013); Scott et al. (2003)
Amplified fragment length polymorphism (AFLP)	Good	Good	Good	Fair	Low—requires very specialized equipment	Phylogenetic analysis of the broad population of *E. coli*	No, for further information see Hahm et al. (2003); Leung et al. (2004); Zhao et al. (2000)
Microarrays	Excellent	Excellent	Challenging	Difficult	Low	Phylogenetic analysis and determining presence of virulence factors in a broad range of *E. coli*	No, for further information see Dharmadi and Gonzalez (2004) and Chap. 4

[a] Table adapted from Hyytia-Trees et al. (2007); Karama and Gyles (2010); Li et al. (2009); van Belkum et al. (2001); Sabat et al. (2013)

E. coli and those which have provided the greatest improvement in our knowledge on their evolution have been covered in this chapter.

3.2 Biochemical Profiling

Biochemical profiling involves the reaction of a particular isolate to a range of biochemical tests which can include the fermentation of various carbon sources. The resulting metabolic fingerprint of the isolate can be analyzed by commercially available phenotypic arrays (e.g., Biolog Inc, Hayward, CA, USA) (O'Sullivan et al. 2007). Some studies have attempted to identify differences between Shiga toxin-producing *E. coli* (STEC) serotypes/strains only to report a wide variation in the biochemical properties observed among STEC and no correlation to serogroup or other properties such as enterohaemolysin production and plasmid profile (Souza et al. 2010; Leclercq et al. 2001). However, biochemical profiling is becoming less frequently used as molecular assays can potentially provide more discriminatory information between STEC and other pathogenic *E. coli* pathotypes. The most appropriate application for biochemical profiling in studying pathogenic *E. coli* is to confirm an isolate is *E. coli*, as other enterobacteriaceae often share genetic markers that may be used to different pathotypes (Schmidt et al. 1993; Paton and Paton 1996; Fegan et al. 2006).

3.3 Serotyping

Serotyping is based on the fact that strains of the same species can differ in the antigenic determinants expressed on their cell surface, such as capsular polysaccharides, flagella, and fimbriae. The serotyping of *E. coli* continues to be the predominate approach by which pathogenic strains are differentiated, but classification of pathogenic strains is more commonly occurring through identification of unique virulence factors. The traditional Kauffman scheme of *E. coli* is based on the serotyping of the O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Kaper et al. 2004). Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. Different O antigens each defining a serogroup are currently recognized, whereas a specific combination of O and H antigens defines the "serotype" of an isolate. Specific serogroups of *E. coli* can be associated with certain clinical syndromes but the serological antigens themselves do not confer virulence. Rather, the serotypes and serogroups serve as readily identifiable markers that may correlate with specific virulent clones (Nataro and Kaper 1998). The advantage of serotyping is that most strains are typeable and the method has good reproducibility. However, serotyping can have poor discriminatory power due to the large number of serotypes, cross-reaction of antigens, and untypeable nature of some strains. In addition to its limited sensitivity and specificity, serotyping is

tedious and expensive and is performed reliably only by a small number of reference laboratories. Thus, detection of pathogenic *E. coli* has focused increasingly on the identification of other characteristics, such as virulence markers (Nataro and Kaper 1998).

Kits for O serotyping and H serotyping for *E. coli* are commercially available, but some isolates may be non-motile, requiring supplementary motility tests in specific motility media. There are also latex agglutination kits commercially available for some serotypes, particularly for STEC serotypes (O26, O91, O103, O111, O121, O145 and O157) (Mathusa et al. 2010; Centers for Disease Control and Prevention 2006). These latex agglutination kits are composed of latex particles coated with antisera against an antigen for a particular serotype and are commonly used for screening colonies on selective media after recovery from foods (Catarame et al. 2003; Drysdale et al. 2004). Evaluation of the performance of selected latex kits available for *E. coli* O157:H7 showed a 100% correlation with reference antibodies (Sowers et al. 1996). False positives can arise if latex controls are not routinely used, or due to cross reactivity with some other non-*E. coli* strains. Full serotyping is usually performed by national reference laboratories (O'Sullivan et al. 2007). Molecular serotyping is also becoming more available as a rapid typing method. The continual development and implementation of molecular STEC serogrouping methods will soon eliminate the traditional serotyping techniques and will allow rapid serogrouping capability for STEC (Mingle et al. 2012).

3.4 Phage Typing

Phage typing is a method that employs individual stocks of whole bacteriophages (bacteriophages are viruses that are specific for bacteria only) applied to bacterial lawns as spots. Certain phages can be very specific and will only infect a few strains of two or more species of a particular genus. The procedure observes the reaction of bacterial strains (susceptibility or resistance) to various known strains of phages. Phage typing has been used as a subtyping method for *E. coli* O157:H7 but not for non-O157. Normally the susceptibility of each *E. coli* O157:H7 to be lysed against a panel of 16 phages is determined and the lytic patterns obtained usually allow typing into one of 82 possible types. Phage typing alone does not usually provide the level of discrimination required for epidemiological and outbreak investigations, as the number of different types identified routinely may not be sufficient to provide confident interpretation of results (O'Sullivan et al. 2007; Khakhria et al. 1990). Phage typing used in combination with other molecular typing techniques such as PFGE, has been found to provide optimal discrimination between *E. coli* O157:H7 (Pearce et al. 2009; Mora et al. 2004; Arthur et al. 2013) and has also been able to differentiate between strains of the same PFGE subtype (Preston et al. 2000). This method is normally performed by reference laboratories as it requires maintenance of biologically active phages.

3.5 Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MLEE) was developed for typing of bacterial isolates, and relies on the differential electrophoretic mobility of specific enzymes which are separated using horizontal starch-gels (Selander et al. 1986; Whittam et al. 1983). Enzymes are extracted from lysed bacterial cells and separated using gel electrophoresis. The gels were generally made of starch to enable easy cutting of the gel for staining of different enzymes, but polyacrylamide has also been used. The gels are generally stained with dyes specific for the enzymes to determine how far they have migrated through the gel. Enzymes produced from different alleles will have slightly different electrophoretic mobility and will migrate through the gel at different rates. These different enzymes are referred to as electromorphs and represent different alleles of the same gene. Each electromorph is assigned a number and in combination with a range of different enzymes, enables distinctive profiles of bacterial isolates to be determined, which are referred to as electrophoretic types (ETs). This method measures multiple loci and provides data used for systematic, epidemiological, and genetic variation studies (Selander et al. 1986).

MLEE was applied to the characterization of a group of 72 *E. coli* isolates, which were from the standard *E. coli* collection of reference (ECOR) (Ochman and Selander 1984). The ECOR collection was analyzed using MLEE of 35 enzymes and grouped into 6 phylogentic groups designated A, B1, B2, C, D, and E (Selander et al. 1983). The phylogentic groups appeared to be associated with the source such that isolates from group B1 were from non-primate mammals (particularly herbivorous) while those in group B2 were primarily from human and primate sources. Further analysis also identified specific phenotypic properties (such as fermentation of sugars) that could be used to differentiate between the phylogentic groups (Selander et al. 1983). Few of the ECOR collection were from patients with diarrhea (most human isolates coming from patients with urinary tract infections) and the method has since been applied to large numbers of *E. coli* isolates from various sources including clinical, animal, and environmental isolates to determine relationships and understand the evolution of these organisms (Whittam et al. 1983). Application of this method to broad populations of *E. coli* from a variety of hosts and countries has determined that the genetic diversity of *E. coli* does not occur randomly, but is associated with the host organism and geographic origin (Souza et al. 1999).

The technique has been used to evaluate pathogenic *E. coli* associated with clinical disease, including providing evidence for the stepwise evolution of *E. coli* O157:H7 from its ancestor *E. coli* O55:H7 (Feng et al. 1998). Strains of different STEC serotypes were analyzed by MLEE and grouped into several different clusters. One group referred to as Enterohaemorrhagic *E. coli* (EHEC) 1, contained serotype O157:H7 and non-motile variants and the ancestor *E. coli* O55:H7, while a divergent group, EHEC 2, comprised serogroups including O26 and O111. Both EHEC 1 and EHEC 2 clusters contained isolates that carry *stx* and *eae* and have been associated with hemolytic uremic syndrome (HUS). Two other clusters were also found, STEC 1 and STEC 2, which comprised strains belonging to multiple

serotypes which carried *stx*, but lack *eae* and their clinical significance was less obvious (Whittam 1998). MLEE analysis of *E. coli* O157:H7 isolates from Brazil and Argentina using 11 enzymes determined that the populations of these bacteria were generally highly related, although the earliest isolates from Brazil obtained in 1997, belonged to distinct ETs. The authors suggested that a lineage of Argentinean *E. coli* O157:H7 may have been introduced into the Brazilian cattle reservoir where it circulated and became established (Regua-Mangia et al. 2012).The application of MLEE to *E. coli* O111 using 20 different enzymes elucidated 16 ETs from 152 isolates representing three different countries (Campos et al. 1994). Certain ETs were found to contain isolates that shared common virulence properties, with the ET12 clone represented by O111 enteropathogenic *E. coli* (EPEC) isolates and the ET 8 clone representing EHEC. This highlights the importance of such methods in being able to resolve the virulence potential and evolution of at least certain serogroups of *E. coli*. Studies of *E. coli* using MLEE have been key to understanding evolutionary relationships within the species and have indicated that pathogenic *E. coli* have arisen many times, with any strain capable of becoming pathogenic through the acquisition of virulence factors (Pupo et al. 1997).

MLEE has mostly been replaced now by gene sequencing methods, particularly multilocus sequence typing (MLST). The major advantage of sequencing methods over those which rely on electrophoresis is that the former provide defined results while the latter rely on migration through a gel which may be difficult to reproduce accurately between different laboratories. Such variation in gel mobility between laboratories has limited the usefulness of MLEE as an internationally comparable typing system. Further investigation of the various phylogenetic groups of *E. coli* determined using MLEE (A, B1, B2, and D), has identified specific genetic markers that are predictive of strains belonging to each of the different groups. The development of PCR for these specific genetic markers has since replaced the use of MLEE for phylogenetic analysis of *E. coli* populations due to the simplicity and greater accessibility of PCR technology (Clermont et al. 2000, 2013).

3.6 Multilocus Sequence Typing

MLST was developed as a portable typing tool allowing comparison between many different laboratories (Maiden et al. 1998). MLST is based on MLEE, but unlike MLEE where the electrophoretic mobility of enzymes is determined, MLST relies on the sequencing of the genes associated with the enzymes. The genes of interest include multiple housekeeping genes that are constitutively expressed genes and are essential for the maintenance of cellular function. Such genes are under stabilizing selection to maintain metabolic function and evolve very slowly, providing a reliable measure of genetic relationships between bacterial isolates (Urwin and Maiden 2003). Each housekeeping gene or locus may consist of multiple alleles which can be differentiated based on gene sequence. Each different allele, whether it varies in sequence (one or multiple nucleotides) or in size, is assigned an arbitrary number.

The sequence type (ST) of each isolate is determined based on the combination of numbers representing each of the alleles present in that particular isolate. STs that contain many of the same alleles can then be further grouped into sequence type complexes (STC) or clonal complexes (CC) or clonal groups (CG) (Hauser et al. 2013). MLST was first described for typing of hypervirulent strains of *Neisseria meningitidis* (Maiden et al. 1998) but has since been applied to a range of different bacteria, including *E. coli*. The seven housekeeping genes most commonly used for MLST typing of *E. coli* include adenylate kinase (*adk*), fumarate hydratase (*fumC*), DNA gyrase (*gyrB*), isocitrate/isopropylmalate dehydrogenase (*icd*), malate dehydrogenase (*mdh*), adenylosuccinate dehydrogenase (*purA*), and ATP/GTP binding motif (*recA*) (Wirth et al. 2006). However, additional genes have also been used to analyze populations of *E. coli* O157:H7 (Foley et al. 2004; Liu et al. 2009).

MLST is reasonably laborious to perform but is highly reproducible and easily standardized for comparison between different laboratories (Karama and Gyles 2010). Sequence-based typing methods such as MLST allow the analysis of bacterial isolates across the globe as the information provided is not ambiguous, is highly reproducible, and can be internationally standardized. MLST has been found to be less discriminating than other methods (such as PFGE) when differentiating within highly clonal populations of *E. coli*. This has been observed with STEC where serotype and ST were highly correlated, with all STEC from the same serotype generally belonging to the same ST. It should be noted that a single ST may be comprised of multiple serotypes (Abu-Ali et al. 2009; Noller et al. 2003; Foley et al. 2004; Hauser et al. 2013). MLST has therefore not been adopted as a typing method and has been used more widely for investigating mostly evolutionary relationships among broader groups of *E. coli* including the enteric pathogens (Karama and Gyles 2010). Interrogation of the relationships between pathogenic *E. coli* using MLST has indicated that acquisition of the same virulence factors over time has led to the parallel evolution of separate lineages of *E. coli* (Reid et al. 2000). MLST has been used to further refine the original model of *E. coli* O157:H7 evolution (developed using MLEE) (Feng et al. 1998) through the inclusion of additional variants of *E. coli* O157:H7 such as the sorbitol fermenting strains from Germany (Feng et al. 2007). Application of MLST to a range of different STEC serotypes determined that lateral gene transfer has played a significant role in the evolution of the different seropathotypes of STEC, which is also related to their differing pathogenic potential (Konczy et al. 2008; Ziebell et al. 2008b). MLST analysis of 117 STEC from a range of serotypes from both human and food sources demonstrated relationships between food and human STEC of the same serotype, reinforcing food as a vehicle for human infection by non-O157 STEC (Hauser et al. 2013). MLST has also been used to investigate the molecular evolution of EPEC strains and the results inferred that horizontal gene transfer of locus of enterocyte effacement (LEE) and the EPEC adherence factor (EAF) plasmid has occurred independently on several occasions (Lacher et al. 2007). An analysis of 1019 ETEC from clinical sources using MLST also established that significant genetic exchange of genes encoding colonization factors and enterotoxins had occurred among the globally distributed lineages of this pathotype (Steinsland et al. 2010).

There are several websites dedicated to *E. coli* that have been developed for researchers to share MLST information (Qi et al. 2004), enabling the creation of large datasets which can be compared across different countries and used to follow the evolution of various *E. coli* populations. These websites include information on how to perform MLST, enable data to be entered online, and provide software for analysis of the data. The EcMLST website is specifically designed for the analysis of STEC and comprises a database including many different serotypes.

3.7 Pulsed-Field Gel Electrophoresis

PFGE is a commonly used technique for generating DNA profiles (DNA "fingerprints") from a range of microorganisms, including *E. coli*. PFGE is a highly effective subtyping tool for discriminating isolates at a subspecies level (e.g., strain) that is widely regarded as the gold standard for subtyping numerous foodborne bacteria (Ribot et al. 2006; Swaminathan et al. 2001). To perform PFGE, bacterial cells are suspended in agarose plugs and treated with cell lysis/proteinase K buffer to disrupt cell walls and inactivate endogenous nucleases such as DNases that can interfere with the process. PFGE plugs are washed multiple times to remove cellular debris after which highly purified intact genomic DNA is cleaved into large variably sized fragments (macrorestriction fragments) using a single, suitable restriction endonuclease (Tenover et al. 1995). Ideally an enzyme will be selected which sparingly cleaves intact genomic DNA to yield a limited number of large fragments (Tenover et al. 1995), typically ranging from 20 kb to >1 Mb in size (Goering 2010). For *E. coli* O157:H7 and non-O157 serotypes, restriction enzymes XbaI and BlnI are commonly used to generate adequate fragment sizes (Centers for Disease Control and Prevention 2013b; Ribot et al. 2006). Fragments are then separated by gel electrophoresis. By alternating the current applied to PFGE gels, much greater separation of large DNA fragments (>20 kb) can be achieved over conventional techniques that employ static currents. Gels are fluorescently stained and fragments are visualized as a series of bands under ultraviolet light that represent different molecular weight fragments of DNA (Fig. 3.1).

DNA fragments form banding patterns, also referred to as restriction/PFGE patterns or profiles that can be used as a fingerprint to isolate comparisons. In some instances, a single endonuclease may fail to provide adequate discrimination between epidemiologically unrelated isolates. To overcome this, washed plugs can be treated with secondary or tertiary restriction endonucleases to provide additional discriminatory PFGE patterns. Using suitable gel imaging software, PFGE bands can be selected (shown as red dots in Fig. 3.2) and normalized against a size standard such as *Salmonella* ser. Braenderup H9812. Restriction digestion of H9812 with XbaI generates fragments ranging from 20.5 to 1135 kb, which cover the fragment ranges observed for the majority of foodborne pathogens tracked by PulseNet (Hunter et al. 2005). For this reason, strain H9812 is universally used as a size standard in PFGE PulseNet protocols for a range of foodborne pathogens (Hunter et al. 2005).

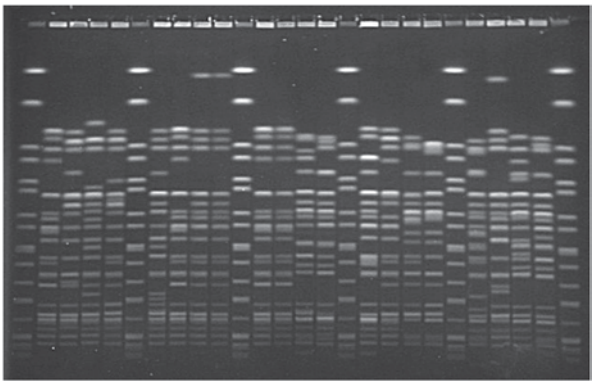

Fig. 3.1 Pulsed-field gel electrophoresis profiles of 20 *Escherichia coli* O157:H7 isolates. The agarose gel was stained with ethidium bromide, destained in water and visualized under ultraviolet light using a transilluminator. *Salmonella* Braenderup was used as a DNA marker in lanes 1, 6, 11, 16, 21, and 26

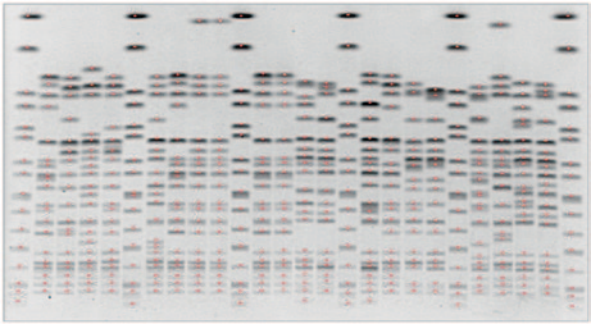

Fig. 3.2 Bionumerics 6.5 (Applied Maths) output of the pulsed-field gel electrophoresis gel shown in Fig. 3.1. *Red dots* represent bands that have been selected using Bionumerics gel analysis software

Once PFGE patterns have been normalized, a cluster analysis with defined similarity coefficients is generally used to produce a tree-like diagram (dendrogram) to illustrate genetic similarities between isolates (Fig. 3.3).

PFGE is most commonly used in molecular epidemiology studies though it has, among other things, been used in phylogenetic investigations (Maatallah et al. 2013; Hall and Barlow 2006) and microbial source tracking through a variety of animal and food production systems (Fegan et al. 2009; Arthur et al. 2008). PFGE has been used to characterize STEC belonging to *E. coli* O157:H7 (Bohm and Karch 1992) and non-O157 (Eklund et al. 2001) serotypes as well other pathotypes such as Enteroaggregative *E. coli* (EAEC), EPEC, Enterotoxigenic *E. coli* (ETEC), and Enteroinvasive *E. coli* (EIEC) (Hien et al. 2008; Shabana et al. 2013). In the mid-1990s, the Centers for Disease Control and Prevention (CDC) and the Association of Public Health Laboratories recognized the value of creating a national laboratory network for the surveillance and investigation of foodborne outbreaks using PFGE (Stephenson 1997; Centers for Disease Control and Prevention 2013a). These organizations jointly developed a national network termed PulseNet USA to provide a depository for US state health departments and other national laboratories to submit PFGE profiles of foodborne pathogens (Stephenson 1997; Swaminathan et al. 2001). This was followed by the inception of an international network (PulseNet

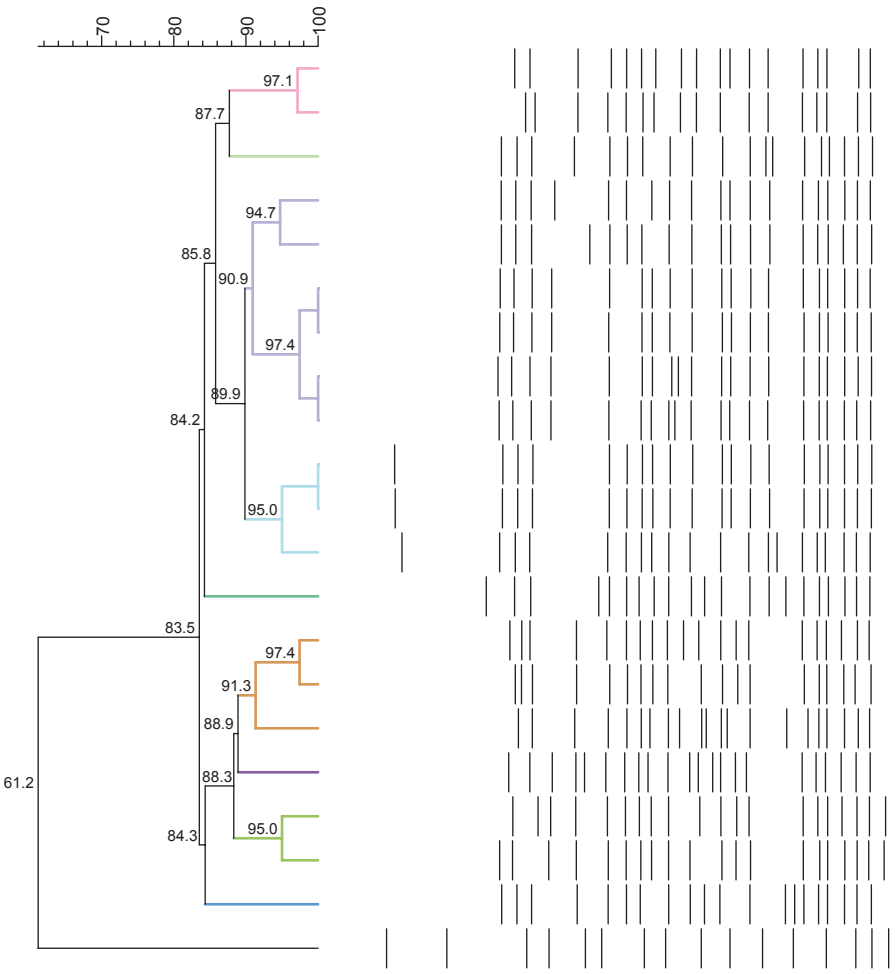

Fig. 3.3 Dendrogram of pulsed-field gel electrophoresis bands shown in Fig. 3.1 using Bionumerics 6.5 software. Pairwise cluster analysis was calculated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with a dice coefficient. Band matching tolerance and optimization were both set at 1.5%. A cut-off value of 90% was used to define PFGE groups and branches were *colored* to represent the different groups

International) for the global surveillance of foodborne pathogens (Swaminathan et al. 2006; Centers for Disease Control and Prevention 2014b) that is presently used by 84 countries spanning seven nations (Centers for Disease Control and Prevention 2012). PFGE is now widely accepted as the best choice for epidemiological surveillance and outbreak investigations of a broad range of pathogens, and as of 2006, the PulseNet database contained over 25,000 PFGE profiles of *E. coli* O157:H7 (Gerner-Smidt et al. 2006).

The extensive collection of PFGE fingerprints available in PulseNet provides a basis for comparing strains from past, present, and future outbreak investigations. The information can be used to identify strains that are frequently associated with beef, cattle, or human disease (Arthur et al. 2013; Lanier et al. 2009), determine the emergence of new clinically important subtypes, or associate an outbreak with a particular vehicle or vector (Wendel et al. 2009). While PFGE has been a critical investigative tool in understanding the molecular epidemiology of foodborne bacterial outbreaks, interpretation of results is confounded by the fact that isolates can undergo genetic alterations throughout the course of an outbreak leading to genetic variants. To overcome this obstacle, a number of criteria are typically used to interpret patterns and assign isolates into categories based on the degree of difference observed between restriction patterns (Tenover et al. 1995). As a result, isolates can be deemed to be epidemiologically related even if they do not share 100 % identity with the original outbreak strain.

Despite its popularity, PFGE is technically demanding, time-consuming, and labor-intensive, and analysis and interpretation of restriction patterns can be subjective. To overcome some of the limitations associated with PFGE, the CDC developed a standardized protocol for subtyping of *E. coli* O157:H7 in 1996 (Centers for Disease Control and Prevention 1996). They later released standardized protocols for a range of other foodborne pathogens including non-O157 STEC serotypes (Centers for Disease Control and Prevention 2013a). These protocols are continually being refined and serve to improve efficiency and facilitate inter-laboratory consistency, reliability, and reproducibility of PFGE methods. Despite this, when compared to common sequence-based methods of typing, PFGE was scored high in discriminatory power, medium in robustness (simplicity/reproducibility of the protocol) but low in portability (repeatability across laboratories), objectivity, and throughput (Hyytia-Trees et al. 2007).

3.8 Multiple-Locus Variable Number Tandem Repeat Analysis

Multiple-locus variable-number tandem repeats analysis (MLVA) is an increasingly popular method of molecularly subtyping bacterial foodborne pathogens. In comparison to other *E. coli* subtyping methods, MLVA is a relatively new technology that is made possible by recent advances in WGS. The technique targets a small number of loci within a bacterial genome that exhibit a broad range of variable number tandem repeats (VNTR). VNTR loci are initially selected by interrogating whole genome sequences for short tandem nucleotide repeats using a specialized software package. Numerous tandem repeats exist within the *E. coli* genome that can be selected for MLVA analysis. A study performed by Keys et al. (2005) detected greater than 20,000 perfect tandem repeats (> 8 bp) in each of the whole genome sequences of *E. coli* strains EDL933, K-12, and Sakai. However, only a small number of satisfactory VNTRs are typically selected for MLVA subtyping. When

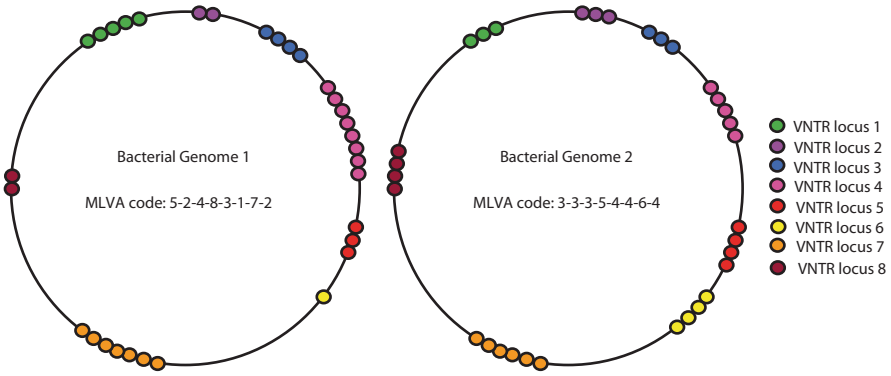

Fig. 3.4 Illustration of eight hypothetical variable number tandem repeat loci carried within the genome of two different bacterial isolates. The MLVA code for each isolate represents a concatenated numeric string of repeat sequences present at each locus and can be used to discriminate isolate A from isolate B

selecting VNTR loci, it is important to consider, among other things, the stability of the locus (Noller et al. 2006), the size of the repeat units (van Belkum et al. 1998; Nadon et al. 2013), the level of conserved sequence in the flanking regions (Nadon et al. 2013), and the location of the VNTR with respect to noncoding or coding sequence (Karama and Gyles 2010). Suitable VNTR loci have been proposed for both *E. coli* O157:H7 (Hyytia-Trees et al. 2006) and other *E. coli* pathotypes including EPEC, non-O157 STEC, EAEC, ETEC, and EIEC (Lobersli et al. 2012; Lindstedt et al. 2007).

PulseNet, which was originally set up for the analysis and sharing of PFGE data, has also established protocols for the analysis of VNTR loci. The current PulseNet protocol for *E. coli* O157:H7 is able to achieve a high level of discrimination using eight VNTR loci (Hyytia-Trees et al. 2010). The protocol is performed by amplifying each VNTR using a multiplex PCR with fluorescently labeled primers located in the conserved sequences flanking VNTR loci. The size of each PCR amplicon is determined using high-resolution capillary electrophoresis and the copy number is typically calculated by subtracting the size of both flanking regions from the total amplicon size and dividing by the size of the tandem repeat. The number of VNTRs present at each locus can then be used to generate a concatenated numeric string or MLVA code (Fig. 3.4). MLVA data can be analyzed as categorical values where each different copy number represents a different allele, or as quantitative values that take into account the relatedness of isolates based on the degree of difference between copy numbers (Centers for Disease Control and Prevention 2014d). As with PFGE, a cluster analysis can be used to generate networks (Fig. 3.5) for investigating the relationship between isolates within a bacterial population.

MLVA is appealing to epidemiologists because it offers a high-throughput, high-resolution genotyping method that can be used to rapidly identify the source of an outbreak strain (Kawamori et al. 2008) and thus quickly contain the spread of an

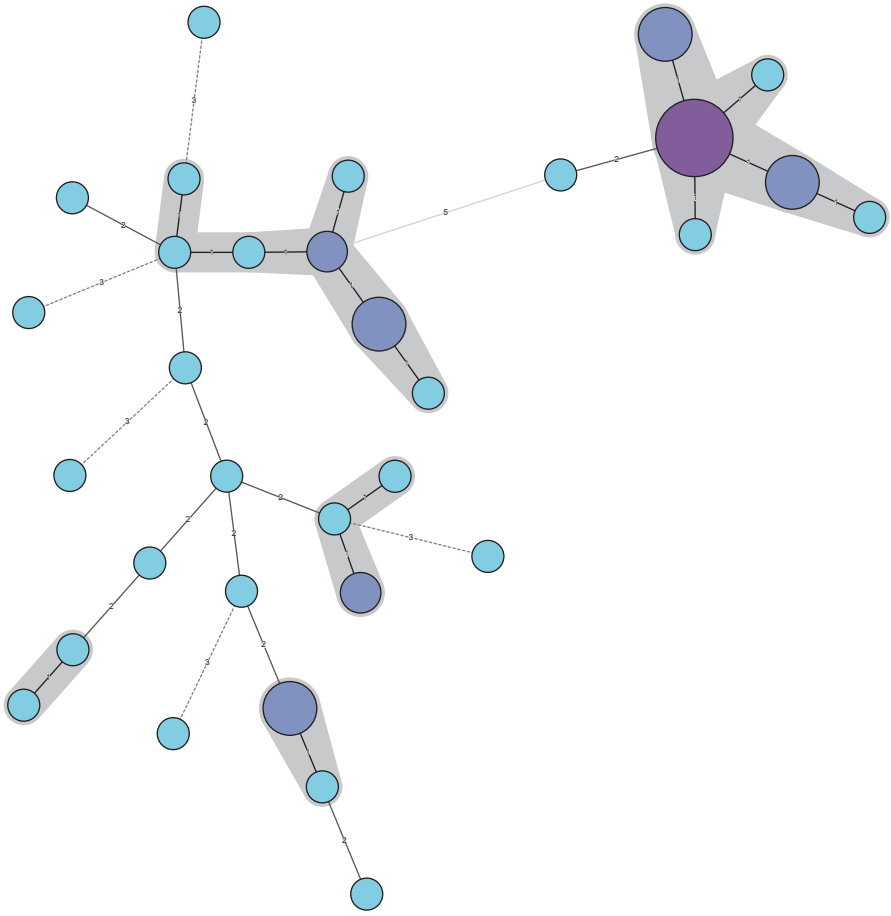

Fig. 3.5 A minimum spanning tree of *E. coli* O157:H7 isolates generated using Bionumerics 6.5 software. Each *node* represents a different MLVA profile. The *branches* represent the number of allele differences between nodes, with *thick solid lines* representing 1 allele difference, *thin solid lines* representing two allele differences, *dashed lines* representing 3 allele differences and *dotted lines* representing four or more allele differences. The number of isolates in each *node* is indicated by size and color with *small blue nodes* representing 1 isolate, *medium light purple nodes* up to 5 isolates and *large maroon nodes* up to 10 isolates. *Nodes* are *shaded* if they differ by no more than 1 loci

outbreak. MLVA scored higher than PFGE in four out of five criteria used to assess the performance of these methods (robustness, portability, objectivity, and throughput) but was regarded as having similar discriminatory power to PFGE (Hyytia-Trees et al. 2007). In addition, MLVA is the only major subtyping technique, other than PFGE, to be used by the PulseNet network for the surveillance of *E. coli* O157. According to PulseNet USA, this technique is currently used in conjunction with PFGE to discriminate bacterial outbreak strains that possess similar PFGE profiles

(Centers for Disease Control and Prevention 2014c). Lobersli et al. (Lobersli et al. 2012) suggest that MLVA may eventually contend with PFGE as the future method of choice for the generic genotyping of *E. coli*. MLVA has also been shown to be an effective method of typing a number of diarrheagenic *E. coli* isolates that were shown to be untypeable by PFGE due to DNA degradation (Lindstedt et al. 2007). However, the lack of standardized protocols for a range of common foodborne pathogens will likely impede the widespread adoption of MLVA as a standalone alternative to PFGE.

At present, *E. coli* O157:H7 is the only foodborne pathogen for which a standardized PulseNet protocol is currently available (Centers for Disease Control and Prevention 2014a) and intrinsic limitations associated with MLVA continue to hinder the development of standardized protocols for other bacterial pathogens. As VNTR cannot be universally applied to all bacterial species, a different set of VNTR loci need to be identified and validated for each new foodborne pathogen targeted. Furthermore, consensus on which VNTRs should be used in a standardized protocol must be reached by the scientific community for widespread adoption of protocols. At present, the number of repeats present in each VNTR is estimated from the size of the amplicon and the nucleotide repeat. Since the size of a VNTR can be affected by genetic mutations unrelated to tandem repeats, the size of each VNTR may not always be proportional to the number of tandem repeats, and the unequivocal demonstration of a repeat copy number can only be achieved by sequencing each amplicon. The comparison of data between laboratories is also confounded by factors such as the use of different PCR reagents, fluorescent labels, thermocyclers, and capillary electrophoresis platforms, each of which can impact the quality and reproducibility of MLVA data. Despite this, a recent multilaboratory validation of a PulseNet STEC O157 MLVA protocol suggests that the challenges currently facing laboratories can be overcome by strict adherence to protocols, training, and implementation of quality control measures (Hyytia-Trees et al. 2010).

3.9 Repetitive Element Palindromic Polymerase Chain Reaction

In the early 1980s, it was reported that the genomes of *E. coli* and *Salmonella* spp. contained short repetitive sequences (usually around 30–35 bp) interspersed throughout the genome. These sequences were referred to as repetitive extragenic palindromic (REP) sequences and were estimated to account for around 1% of the total genome with more than 500 copies present (Stern et al. 1984). Additional repetitive elements were discovered in the genomes of enterobacteria including the enterobacterial repetitive intergenic consensus (ERIC) sequences. The chromosomal locations of ERIC sequences differ between bacterial species although the nucleotide sequence is highly conserved. ERIC sequences are 126 bp long and there are at least 30 copies in the genome of *E. coli* (Hulton et al. 1991). REP and ERIC sequences are used as primer binding sites for PCR amplification of DNA segments

that fall between the repetitive sequences. The amplification products range in size depending on the distance between primer binding sites, which in turn is dependent on the bacterial strain. Electrophoresis of the PCR products produces distinct banding patterns which can then be compared to determine the relationships between different bacterial strains (Versalovic et al. 1991; Versalovic et al. 1998). The process of typing bacteria using Rep-PCR involves the extraction of DNA from the target strain, followed by PCR amplification using primers targeting the repetitive sequences, then separation of the amplified products using gel electrophoresis (Hiett and Seal 2009; Versalovic et al. 1998).

The requirement to amplify targets using PCR and separate bands with gel electrophoresis has meant that Rep-PCR is often difficult to reproduce from day to day, making it difficult to perform comparisons between bacterial strains and also between different laboratories (Johnson and O'Bryan 2000). The automation and commercialization of equipment for conducting and interpreting Rep-PCR has facilitated the typing of bacteria within clinical settings and led to improvements in the reproducibility and interpretation of this technique (Healy et al. 2005).

The application of techniques such as ERIC PCR to differentiate between diarrheagenic *E. coli* was originally reported to align with the findings of MLEE typing (Dalla-Costa et al. 1998), although more recent investigations of a larger number of *E. coli* would suggest that Rep-PCR techniques are not a suitable replacement for MLEE in phylogenetic analysis of pathogenic *E. coli* (Johnson and O'Bryan 2000). Although differentiation of a single clonal type, such as *E. coli* O157:H7 from other serotypes of *E. coli*, has been possible by Rep-PCR methods, the *E. coli* O157:H7 isolates produce very similar patterns which differ from other *E. coli* (Hahm et al. 2003). More discriminatory techniques such as PFGE are better suited to differentiation within a single serotype of *E. coli* (Foley et al. 2004; Hahm et al. 2003).

3.10 Random Amplified Polymorphic DNA

Many PCR-based typing techniques require prior knowledge of the target DNA sequence to enable primer design. The use of arbitrary primers which are not directed to any particular sequence and anneal at random sites throughout the genome overcame this need for sequence information. The amplification of random segments of DNA, referred to as random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990), using arbitrary primers was found to reproducibly amplify segments of DNA in a process referred to as Arbitrarily Primed PCR (AP-PCR; (Welsh and McClelland 1990). This type of PCR is performed using a single primer under low stringency conditions, initially to encourage primer annealing to a variety of sequences with some mismatches occurring. Amplification from the annealing sites will occur when the primer binding sites are within several hundred base pairs of each other and on opposite strands of DNA. The initial low stringency conditions (using annealing temperatures around 40 °C) lead to amplification of sequences between sites where annealing occurs most efficiently, whereas increasing

the stringency of the PCR in later cycles (temperatures around 70 °C) results in more efficient amplification (Welsh and McClelland 1990). AP-PCR or RAPD typing methods result in a series of amplified products of varying sizes based on the number, proximity, and location of primer binding sites within the genome, which can vary between isolates. Agarose gel electrophoresis can be used to separate the bands and generate fingerprinting patterns (Power 1996).

The advantages of RAPD PCR typing over other methods include its speed, ease of use, simplicity, application to a wide variety of microorganisms, and the fact that it is relatively inexpensive to perform (Power 1996; Sabat et al. 2013). There are many factors which can influence the results of RAPD typing methods, including: reaction conditions (such as annealing temperatures); the selection, design and concentration of primer; quality and concentration of template DNA; the type of DNA polymerase used, and the thermal cycler used (Power 1996; Caetanoanolles et al. 1992). All of these variables can lead to difficulties in obtaining reproducible results, particularly between different laboratories. This has been one of the main disadvantages of this method in becoming widely established as an epidemiological typing tool. Standardization of reaction conditions, DNA preparation, and use of the same polymerase can reduce within laboratory reproducibility, but will not reduce this issue between laboratories. The method has therefore been most successfully applied in understanding the epidemiology of local outbreaks under investigation by a single laboratory (Power 1996).

The RAPD fingerprinting technique was used mostly throughout the 1990s and 2000s to further our understanding of pathogenic *E. coli*, although in recent times it has been replaced with more sensitive and reproducible methods such as PFGE, MLST, and MLVA. When applied to a wide variety of diarrheagenic *E. coli*, RAPD PCR was found to be more sensitive at discriminating between isolates than MLEE, with RAPD using 5 primers distinguishing 74 of the 75 isolates and MLEE using 20 enzymes resulting in 15 groups (Wang et al. 1993). An analysis of 73 pathogenic *E. coli*, including typical and atypical EPEC, EHEC, and EAEC using RAPD established two major clusters, one containing only typical EPEC which appeared to be rather homogenous while the other cluster was more heterogeneous, containing representatives of all pathotypes (Bando et al. 2007). Analysis of typical and atypical EPEC isolates from Brazil using RAPD fingerprinting were suggestive of typical and atypical EPEC belonging to distinct bacterial lineages (Dulguer et al. 2003). RAPD PCR typing has also been used to discriminate within pathotypes of *E. coli,* including investigating the diversity of several serotypes of clinical isolates of ETEC and EAEC (Pacheco et al. 1997; Moon et al. 2005) along with providing an understanding of the diversity within a single serotype (O6:H16) of Enterotoxigenic *E. coli* (Pacheco et al. 1998). The genetic diversity of *E. coli* O157:H7 in Canadian feedlots as determined by RAPD was quite variable, with some feedlots having a low genetic diversity of indigenous *E. coli* O157:H7 populations, while others had a broader variety of genetic types present (Vidovic and Korber 2006). The authors suggested that those feedlots with a broad genetic diversity of *E. coli* O157:H7 populations were likely to receive these through introduction by new cattle entering the feedlot.

3.11 Shiga Toxin Subtypes and Bacteriophage Insertion Sites

Numerous serotypes of *E. coli* have been shown to carry genes essential for the production of potent human cytotoxins known as Shiga toxins (Stx) or less commonly Verotoxins (Vtx) (Blanco et al. 2004; Karch et al. 1999). These toxins are generally encoded on genes carried by viruses (bacteriophages) that can infect and replicate within a range of bacterial species (Herold et al. 2004; Schmidt et al. 1993). While this event often leads to cell destruction, in some cases Stx-encoding bacteriophages (Stx bacteriophages) are able to integrate their viral genome with the bacterial chromosome without causing harm to the host cell. The newly integrated Stx-bacteriophage genomes are subsequently termed Stx-prophages while *E. coli* species that have undergone this transformation are commonly referred to as Shiga toxigenic *E. coli* or STEC.

Shiga toxigenic *E. coli* may carry *stx* genes for one or both of the main Shiga toxin branches, Stx1 and Stx2. These branches can be further divided into a number Stx subtypes based on amino acid variations (Scheutz et al. 2012). A variety of molecular methods have been developed for the detection of stx_1, stx_2 (Paton and Paton 1998; Gannon et al. 1992) and their respective subtypes (Scheutz et al. 2012). While the main focus of this work has centered on understanding *stx* types (stx_1, stx_{2a}, and stx_{2c}) commonly associated with the prototype STEC, *E. coli* O157, there is a growing interest in understanding the diversity of *stx* types carried by non-O157 STEC serotypes and other *E. coli* pathotypes. However, the lack of standardized protocols for the detection and nomenclature of all *stx* subtypes has led to confusion in the characterization of isolates and interpretation of published results. In an effort to resolve this, Scheutz et al. (2012) established a standardized protocol for detecting three stx_1 subtypes (stx_{1a}, stx_{1c}, and stx_{1d}) and eight stx_2 variants (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}). This assay has been validated in an external quality assurance program involving numerous laboratories throughout the globe and has since led to the development of a commercially available stx_1 and stx_2 subtyping PCR kit (Statens Serum Institut 2014). Adoption of this typing scheme by the scientific community will help to overcome some of the shortcomings of previous systems by improving accuracy in detection and reporting of future results.

The investigation of Shiga toxin-encoding bacteriophage insertion (SBI) sites, usually in combination with *stx* subtyping, has also been used to characterize *E. coli* isolates (Shringi et al. 2012a; Mellor et al. 2013). While Stx-prophages may be integrated at any one of a number of different loci (Steyert et al. 2012; Ogura et al. 2007), SBI sites are fairly conserved in *E. coli* O157:H7 with phage integration typically limited to one of four loci (*wrbA*, *yehV*, *argW* and *sbcB*) (Shringi et al. 2012b). A multiplex SBI typing system has been used in characterization studies investigating *E. coli* O157:H7 isolated from animals and humans and in geographic studies to investigate the distribution of subtypes in isolates sourced from different countries (Mellor et al. 2013; Shringi et al. 2012a). SBI typing systems have been used to identify a unique *stx* insertion site for stx_1 in Australian isolates that has

been useful in distinguishing Australian isolates from international *E. coli* O157:H7 (Mellor et al. 2012). Specific *stx* subtypes have also been correlated with lineages and virulence clades of *E. coli* O157:H7 (Eppinger et al. 2011b; Zhang et al. 2010; Mellor et al. 2012; Manning et al. 2008) that are differentially associated with disease in humans. To date, *stx* subtyping and SBI analysis has largely focused on *E. coli* O157:H7 and little is known about the distribution of *stx* subtypes and bacteriophage integration sites for non-O157 *E. coli* serotypes. As more focus is placed on understanding non-O157 *E. coli*, similar typing schemes will likely be developed and applied more broadly to cover a number of other clinically relevant *E. coli* serotypes.

Molecular screening for the two main classes of *stx* (stx_1 and stx_2) may be limited by the primers used, as not all *stx* subtypes may be captured by universal primers (Feng et al. 2011). The majority of *stx*-prophage associated with *E. coli* O157:H7 integrate in highly specific locations depending on the *stx* subtype they encode. Due to the difficulties in demonstrating the linkage between *stx* genes and their respective prophage integration, few studies bother to unequivocally link the *stx* gene with prophage, but rather assume that the presence of the *stx* is associated with a particular phage and integration site. This may present a limitation to the development of SBI genotyping systems for non-O157 *E. coli* and other *E. coli* pathotypes, where the integration sites of *stx*-prophage have the potential to be more varied and less specific than *E. coli* O157. However, current and future advancements in protocols for linking *stx* subtypes with prophage integration sites will likely result in substantial improvements to our ability to accurately characterize SBI sites of all *E. coli* pathotypes.

3.12 Lineage Specific Polymorphism Analysis

The lineage specific polymorphism analysis (LSPA-6) is a system that was developed to differentiate between isolates of *E. coli* O157. Like MLST, it relies on the occurrence of variably sized allele combinations in different isolates, but unlike MLST does not focus purely on housekeeping genes and has targeted *E. coli* O157:H7 specific genes. The method evolved from octamer-based genome scanning (OBGS) which was applied in the late 1990s to determine genetic relationships between isolates of *E. coli* O157:H7 from cattle and human sources (Kim et al. 1999). OBGS analysis of *E. coli* O157:H7 populations divided isolates into two groups, one that consisted mostly of human isolates called lineage I, and the other which comprised only isolates from cattle, termed lineage II (Kim et al. 2001). The original OBGS method was applied to a small strain set of 54 *E. coli* O157:H7 isolates as the method was complex to perform and required skilled data analysis. Another method was developed to simplify the differentiation of *E. coli* O157:H7 into the two lineages. This method was called the lineage specific polymorphism assay (LSPA-6) (Yang et al. 2004). The LSPA-6 targeted six different genetic markers that were previously found to differentiate between lineages I and II, hence the

Table 3.2 Alleles sizes (bp) and gene designations used in the LSPA-6 typing scheme. (Adapted from Yang et al. 2004; Zhang et al. 2007)

Allele	folD-sfmA	Z5935	yhcG	rtcB	rbsB	arp-iclR
Lineage I	161	133	394	270	218	315
Designation	1	1	1	1	1	1
Lineage I/II	170	133	394	270	218	315
Designation	2	1	1	1	1	1
Lineage II[a]	170	142	472	279	209 or 214	333 or 324
Designation	2	2	2	2	2 or 3	2 or 3

[a] As new allele sizes have been discovered, additional designations have been determined although there has been no consistency between different publications. Lineage II is quite variable and generally comprises isolates that do not fit the designations of lineage I (111111) and lineage I/II (211111)

name LSPA-6. The presence of different markers was determined using multiplex PCR which allowed testing of all six markers simultaneously. The PCR products generated by the LSPA-6 could not be separated using standard agarose gel electrophoresis due to their similar sizes which originally limited the use of LSPA-6 by many laboratories, as denaturing polyacrylamide gels were required to differentiate between the LSPA-6 products. Capillary electrophoresis is now commonly used to differentiate the products of the LSPA-6 PCR reactions. The LSPA-6 method was able to differentiate *E. coli* O157:H7 into the two lineages with much less effort than the original OBGS method (Yang et al. 2004).

The alleles used for the LSPA-6 typing scheme include *folD-sfmA*, *Z5935*, *yhcG*, *rtcB*, *rbsB*, and *arp-iclR*. Each different allele is given a number depending on its size and each isolate is then represented by a code (similar to other typing methods such as MLST). Isolates with the LSPA-6 designation of 111111 are referred to as lineage I, and those with the LSPA-6 designation of 222222 as lineage II (Yang et al. 2004). Further scrutiny of *E. coli* O157:H7 genomes using comparative genomic hybridization (CGH) built on the work of Yang et al. (2004) and Kim et al. (1999, 2001) by grouping the LSPA-6 subtypes into three different lineages (Zhang et al. 2007) which included the lineage I and II groupings, as well as an additional lineage referred to as lineage I/II. These lineage I/II strains were closely related to the lineage I strains and belonged to one LSPA-6 subtype (211111) and phage type 2 (PT2) (Zhang et al. 2007). The alleles used in LSPA-6 typing are listed in Table 3.2. As more isolates have been typed using LSPA-6, variability in allele sizes have occurred leading to designations other than those that fit strictly within the lineage I, I/II, and II designations (111111, 211111, and 222222 respectively).

The LSPA-6 assay has been used to analyze populations of *E. coli* O157:H7 from a diverse array of countries including Canada, the USA, Australia, Argentina, Japan, and the Netherlands (Franz et al. 2012; Lee et al. 2011; Mellor et al. 2012, 2013; Sharma et al. 2009; Vidovic et al. 2013; Ziebell et al. 2008a). This has led to some interesting observations around geographic diversity of *E. coli* O157:H7 populations and confirmation of the association of clinical isolates with

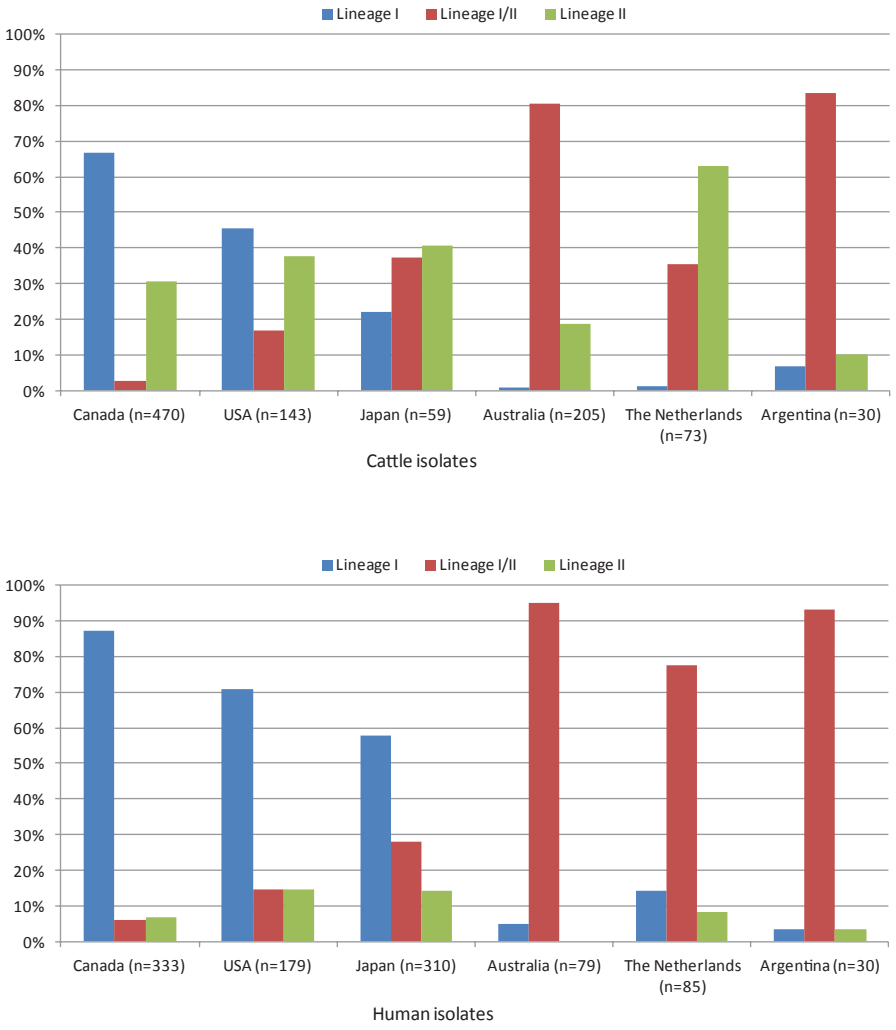

Fig. 3.6 Geographic distribution of LSPA-6 lineages of *E. coli* O157:H7 from cattle (*top*) and human (*bottom*) sources. (Adapted from Franz et al. 2012; Lee et al. 2011; Mellor et al. 2012, 2013; Sharma et al. 2009; Vidovic et al. 2013; Yokoyama et al. 2011; Ziebell et al. 2008a)

LSPA-6 lineages I and I/II and cattle isolates with lineage II, though the distribution of these LSPA-6 lineages between cattle and clinical sources varies between countries (Fig. 3.6). Even within countries, geographical differences in the distribution of *E. coli* O157:H7 LSPA-6 lineages may occur, as isolates of LSPA-6 lineage I were significantly more likely to come from cattle in southern Alberta (92 % of all strains of southern Alberta) than from northern Alberta (28 % of all strains from northern Alberta) (Sharma et al. 2009).

The grouping of *E. coli* O157:H7 isolates into LSPA-6 subtypes has led to suggestions that lineage I isolates, which are those most likely to be associated with human illness, are more resistant to stresses such as heat and starvation, than those *E. coli* O157:H7 from lineage II which are less often associated with human illness (Lee et al. 2012). There are also associations of LSPA-6 lineages and other typing methods such as SNP analysis, SBI, and *stx* subtypes, along with the presence or absence of specific gene markers (Wu et al. 2008; Mellor et al. 2012, 2013; Yokoyama et al. 2011; Stanton et al. 2014). Such information provides insights into why there might be a bias for certain types of *E. coli* O157:H7 to be associated with clinical cases and severe disease. LSPA-6 typing has been a useful method for understanding broader relationships between isolates of *E. coli* O157:H7 as discussed above (such as on a global scale and differentiation of isolates between clinical and cattle sources), but as it differentiates isolates at a high level, it does not provide detailed analysis for epidemiological comparisons.

3.13 Whole Genome Sequencing

WGS refers to the ability to accurately compile an ordered sequence of the complete genetic complement of any single organism. The relatively small size of microbial genomes, coupled with dramatic advances in sequencing technologies, now support the widespread application of genome data in microbiology. In particular, WGS is transforming the field of comparative microbial genetic typing through its ability to supersede, but also include, information provided by earlier molecular genetic typing methods such as PFGE, MLST, MLVA, and RAPD.

The WGS revolution is underpinned by the development of next generation sequencing (NGS) technologies, such as pyrosequencing and massively parallel systems (Mardis 2011; Metzker 2010), which replaced the conventional electrophoretic separation of dideoxy terminated DNA fragments used in Sanger sequencing (Sanger et al. 1977). NGS relies on the isolation of DNA templates from an isolate or mixed sample, followed by sequencing and reaction data acquisition, then data analysis (Metzker 2010). There are various types of commercially available sequencing chemistries, including synthesis with reversible or virtual terminators using DNA polymerase dependent methods, sequencing by ligation, semiconductor sequencing, single molecule real time analysis or ionic current sensing (Bertelli and Greub 2013; Metzker 2010). Each of these different chemistries produces reads within a range that is dependent on the type of chemistry used, ranging from very short reads (36–100 bp) to longer reads (250–10,000 bp) (Bertelli and Greub 2013).

NGS technologies generate millions of nucleotide sequence reads at comparatively low cost allowing high throughput in time frames ranging from less than a day to just over a week (Metzker 2010; Sabat et al. 2013). The WGS can then be compiled through assembly of the overlapping short sequences (*de novo* assembly) or alignment with previously assembled "reference" genome/s (resequencing) (Sabat et al. 2013). A limitation of these short read sequencing strategies is their

dependence on overlapping sequences for genome assembly; when *bona fide* repeat regions occur, it is not possible to resolve their position in the genome. Newer sequencing platforms, producing read lengths of 7–10 kb, are now emerging and improving *de novo* assembly (Sabat et al. 2013). Currently, WGS continues to require substantial computer resources and specialist bioinformaticians for the compilation of truly complete (or "closed") genomes. While closed genomes may in time become the new gold standard for bacterial typing (replacing PFGE), unassembled WGS data in various forms has already become extremely useful for several comparative genomic typing methodologies.

Extended MLST (eMLST) has been proposed as a method for the sequence-based comparison of "core" genomes (all genes present in all isolates of a species or pathotype). Alternatively, "pan-genome" comparison uses the full complement of genes present in a species or pathotype, including the "core" genome, as well as accessory genes contributing to the distinct features of different pathotypes (Sabat et al. 2013). Genomic typing of this order is proving to be particularly suited for comparison of the numerous *E. coli* pathotypes (Rasko et al. 2011; Kaas et al. 2012; Gordienko et al. 2013).

Several Single Nucleotide Polymorphism (SNP) typing schemes have been developed for analysis of *E. coli* O157. Through the discovery of SNPs in more variable regions of the genome, it has been possible to discriminate separate lineages of this clonal organism (Eppinger et al. 2011a, b; Manning et al. 2008; Bono et al. 2012). Further, *E. coli* O157:H7 SNP-typing correlates with data showing the nonrandom distribution of lineages, clades, Shiga toxin (Stx)-subtypes, and geographical origin among bovine and clinical isolates (Franz et al. 2012; Mellor et al. 2012, 2013). WGS promises to deliver high-resolution genomic epidemiology as the ultimate method for bacterial typing. However, as with all emergent technology platforms, iterative cycles of innovation and refinement will be necessary to achieve globally acceptable standardized protocols that facilitate efficient and routine *E. coli* pathotype strain comparison.

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Chapter 4
Emerging and Future Trends and Technologies for the Detection and Typing of *Escherichia coli*

4.1 Future Trends in Foodborne Illness and *Escherichia coli*

Foodborne illness is a significant concern worldwide and many of the cases observed can be prevented. Public health efforts have been directed mainly towards the well-known foodborne diseases and pathogens in the food chain. However, it is estimated that 50–60 % of all causative agents of foodborne illnesses are caused by unknown etiological agents (Thomas et al. 2013). Many other pathogens that are known to cause foodborne disease, such as many non-O157 Shiga toxin-producing *E. coli* (STEC) serotypes are certainly underestimated due to the lack of diagnostic tools. The list of known agents that cause foodborne disease is likely to grow as new methods to identify them become available, and already known pathogens may also evolve, thereby adding to the public health risks (Newell et al. 2010). Future trends in lifestyle and globalization of travel and trade, including food, demographic changes, and other factors will influence the emergence and reemergence of various foodborne pathogens. There is also a shift in the traditional association of foodborne pathogens from foods of animal origin to other commodities, such as fresh produce, that is being reported by public health authorities worldwide (Jones et al. 2008).

Assessing the changing levels of foodborne disease through baseline surveillance will become vital and systems to do so will invariably develop to accommodate this need. This will involve a global effort but may be challenging to implement as currently some countries do not routinely collect such data or do not have consistent or comparative data over time in order to identify trends. Consistent reporting by all countries would make an important contribution to international surveillance and would promote global strategies to control infection and determine the public health significance of existing and emerging strains (Karmali et al. 2010). A clear focus and example of improved foodborne disease surveillance efforts is with the STEC group. Although the O157 serotype within the STEC group is widely recognized as an important cause of foodborne disease, improved detection methods have seen an increase in the awareness of other non-O157 serotypes that are also present within different reservoirs such as animals, and their role in foodborne disease. Many of

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these non-O157 serotypes are not commonly isolated, but in light of the *E. coli* O104:H4 outbreak in Europe, (Inset 1.2) it would be useful to elucidate how prevalent these serotypes are in food environments and determine how readily they can exchange genetic information and acquire virulence factors. This would elucidate the potential of these strains to become virulent and cause significant foodborne disease (Kaspar et al. 2010). There is also the potential to correlate disease incidence with the distribution and diversity of specific genomic sequences with a geographical area. Changes in pathogenicity can therefore be determined and aid in new tracking or diagnostic methods to detect emerging pathogens (Franz et al. 2014).

There are also a number of studies that have reported the effects of various intervention strategies on *E. coli* O157:H7, but it is unknown whether these strategies are also effective for non-O157 serotypes. The great diversity within the non-O157 serotypes suggests that further work is needed to elucidate the growth and survival properties within the STEC group and the influence of intervention strategies on their survival through the food chain. This also highlights the need to determine the epidemiology and interactions of non-O157 STEC in animals, environmental, and human sources. This could potentially provide more information into the dissemination, growth, and persistence of these strains within the food chain, which in turn can aid in developing further intervention strategies (Kaspar et al. 2010). It is also important, as with all the diarrheagenic pathotypes that more information is gained on the role and incidence of person-to-person transmission and the role that humans play in the dissemination of these strains. With the exception of the STEC group, the problem with many of the diarrheagenic *E. coli* is that they are not classified as notifiable and therefore their role in foodborne disease is unclear. It is apparent that some of these *E. coli* pathotypes may be emerging pathogens (Huang et al. 2006) and therefore more research is required to investigate the true incidence of these pathogens in foodborne disease. A greater understanding of the potential virulence of an isolate based on the presence of various gene markers will also provide information that is useful to determine the risk of specific *E. coli* isolates to human health. The ability of *E. coli* to exchange genetic information means that new pathogenic strains are likely to occur (as with *E. coli* O104:H4 (Inset 1.2)) and in the future we will be better able to identify these through a greater understanding of the gene complement required to cause disease.

4.2 Future Trends in the Detection and Typing of Pathogens

The advancement of molecular methods is set to replace traditional culturing methods, but there are many challenges to overcome before culturing methods become obsolete. This is because cultural methods are vitally important for the characterization of the isolate, particularly for epidemiological studies but also to determine antibiotic resistance, virulence, growth, and survival properties (Cocolin et al. 2011). The major disadvantage of cultural procedures is the time and labor required to

obtain an isolate for characterization. The development of cultural media to presumptively identify a pathogen based on phenotypic traits is a growing area (Cocolin et al. 2011) and certainly other technologies are emerging with that promise to assist with identifying presumptive isolates within a mixed flora on an agar plate. An example of this technology is *ba*cterial *r*apid *d*etection using *o*ptical scattering *t*echnology (BARDOT) that directs a laser on colonies to produce species or serovar-specific scatter signatures that are compared to those within a library. This technology has been initially evaluated with particular STEC serotypes and can potentially be used as a screening tool during isolation (Tang et al. 2014).

Other technologies, including antibody-based and nucleic acid-based assays, have revolutionized the detection methodology for microbial pathogens in various food matrices (Ge and Meng 2009). These methods have great advantages of ease of use, high throughput, and low cost but their downfall is their detection limit which often requires an enrichment step which requires time for incubation. Design of improved enrichment formulations to facilitate shorter incubation times will decrease the time-to-test results. Alternative concentration technologies other than enrichment will become vital to achieve the desired amount of cells for detection while achieving a short testing time (Ge and Meng 2009). The main goal for such methods is to increase their sensitivities in order to test the sample directly, thus saving time that is otherwise used during the enrichment process. The possibilities of combining different rapid methods, including improved technologies for separation and concentration of specific bacteria will facilitate the direct detection of pathogens in foods. The main focus is to avoid the need for enrichment, providing rapid alternatives to conventional quantitative culture methods (Kaspar et al. 2010).

Detection through the use of real-time PCR provides a near instantaneous amplification and detection at the same time and are likely to be developed to accurately quantitate foodborne pathogens in foods when no enrichment is required. However, one major limitation of the DNA-based molecular detection assay is the inability to differentiate live versus dead cells. Improvements to prevent the detection of dead cell DNAs may be to incorporate chemicals within the sample that selectively intercalate into dead cell DNA and therefore prevent their amplifications in PCR assays (Ge and Meng 2009). Further technological advancements will see new possibilities and solutions for food safety problems for many smaller laboratories previously unable to undertake specialized testing regimes. It is certainly apparent that more commercial systems are focusing on all inclusive kits. These kits contain all the components necessary for the detection of a pathogen, and assays are placed into a “black box” machine that rapidly produces a result that is easily interpreted and does not require specialized skills. These systems also aim to provide multiplexing functions where multiple pathogens will be readily detected in one assay.

4.2.1 Microarrays

Microarray technology plays a significant and growing role in identification and analysis of foodborne microbial pathogens. Microarrays can utilize a variety of

technical formats, but currently incorporate short oligonucleotides (20 to 70 nucleotides) probed onto a glass slide (spotted/chip array) or a single probe attached to a uniquely identified, fluorescently labeled microsphere (bead-based array) (Dunbar 2006; Boxrud 2010). The technology facilitates a significant expansion of the capability of DNA-based methods in terms of the number of DNA sequences that can be analyzed at one time, enabling molecular identification and characterization of multiple pathogens using many genes in a single assay (Rasooly and Herold 2008). Furthermore, the density at which these probes can be arrayed allows for the simultaneous screening of many unique gene sequences from many different species in parallel (Cebula et al. 1995). Microarrays are more frequently being used for the detection of foodborne pathogens and are increasingly being used to genotype strains such as *E. coli* O157:H7 (Garaizar et al. 2006; Boxrud 2010; Zhang et al. 2006). This capability can also expand to incorporate the identification of virulence factors and antibiotics resistance genes. Developments in the manufacture of microarrays will see the production of both spotted and bead arrays become more economical for use in food applications and may offer improvements in foodborne pathogen identification and characterization protocols compared to traditional methods (Rasooly and Herold 2008).

4.2.2 Matrix Assisted Laser-Desorption Ionization-Time-of-Flight Mass Spectrometry

The emergence of matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has enabled rapid and accurate species identification from a single colony and is likely to become an essential identification tool in clinical bacteriology. MALDI-TOF MS machines are costly to purchase and establish within a laboratory but adequate throughput of samples in diagnostic laboratories can see the identification of an organism being significantly cheaper than using traditional biochemical tests or kits. The method is based on the analysis of whole cell mass spectra representing dozens of microbial proteins as peaks with an exactly determinable mass to charge (m/z) ratio (Christner et al. 2014; Pavlovic et al. 2013). Spectral fingerprints vary between microorganisms and some components detected within the spectrum fingerprint are specific to genus, species, and at times subspecies level. These results are also reproducible if bacteria are grown under the same conditions (Carbonnelle et al. 2011). However, the method is poor at differentiating between *E. coli, Shigella* and other related species.

Spectral fingerprints can be compared and matched to known or reference strain profiles within a database that is often created by specialized laboratories. Available databases will grow as the use of the method increases and other relevant organisms in food and clinical microbiology will be included. There is also a potential to use the technology to seek novel “biomarkers” which are distinguishable peaks or proteins that are unique to a group of strains, such as those implicated in outbreaks. This has been demonstrated by Christner et al. (2014) who identified two character-

istic peaks amongst STEC outbreak strains that were found to be of low prevalence among other genome sequenced *E. coli* strains. This technique may therefore be adapted to other typing tasks and assist with epidemiological surveys as a part of routine pathogen identification procedures (Christner et al. 2014). The applicability of the method to detect antibiotic resistance associated to identify bacteria has also been reported (Du et al. 2002). Some studies have evaluated the use of the technology directly to clinical samples (Carbonnelle et al. 2011; Koser et al. 2012; Schubert et al. 2011) and certainly future developments may see similar direct approaches on food products (Pavlovic et al. 2013).

4.2.3 Nanotechnology

The use of nanoscale materials or particles (1–100 nm) and technologies offers a great opportunity to develop fast, accurate, and cost-effective diagnostics for the detection of foodborne pathogens. The properties of nanomaterial used for pathogen detection can be altered by changing the size, shape, composition, and surface modification of the material (Kaittanis et al. 2010). The use of nanoparticles as labels has been combined with novel detection technologies which have led to improvements in sensitivity and multiplexing capabilities (Kaittanis et al. 2010). There are various types of nanosystems and materials that are described in greater detail by Kaittanis et al. 2010.

A growing application of nanoparticles is use as immunosensors (also referred to as "biosensors") which can be described as compact analytical devices that incorporate a biological or biomimetic sensing element, either closely connected to, or integrated within, a transducer system (Velasco-Garcia and Mottram 2003; Tokarskyy and Marshall 2008). There is no strict definition of an immunosensor and various formats and technologies are developing quickly, but many immunosensors use the same principles as an ELISA assay. These assays incorporate the use of nanoparticles which are coupled with specific oligonucleotides that can detect complementary DNA strands through a color change (Chen and Yin 2014; Ma et al. 2014). There are various formats that are described by Tokarskyy and Marshall (2008). Applications of this technology as "proof-of-concept" are appearing more frequently in the literature. For example, Ma et al. (2014) described the use of the paper clip immunoassay that incorporated gold nanoparticles that can quantify bacteria in water samples (Ma et al. 2014). Another example is the use of magnetite–gold nanoparticles bound to *E. coli* O157:H7-specific antibodies which are used in combination with immunomagnetic separation (IMS) methods and surface-enhanced Raman spectroscopy for the detection of *E. coli* O157:H7 in apple juice (Najafi et al. 2014).

The use of quantum dots (QDots) which are a family of nanosized (1–10 nM) particles comprising a few thousand atoms is also being used in different detection systems. QDots exhibit a size-tunable band gap and hence fluorescence spectrum, which allows the exhibition of different colors at one excitation wavelength. To use

QDots in biological applications, crosslinkers (e.g., biotinavidin) have to be used to conjugate them to different biological components (Wang et al. 2012; Fournier-Wirth and Coste 2010). This nanoparticle has been used in combination with IMS to detect 10 cfu/g *E. coli* O157:H7 in ground beef following enrichment (Wang et al. 2012). Another study also described the use of QDots with anti-*E. coli* O157:H7 antibody within glass capillaries to perform a sandwich based assay for the detection of *E. coli* O157:H7 in liquid samples. The QDots were excited by using battery-powered light-emitting-diodes (LED) and the emission from the QDots was then imaged using a cell phone camera (Zhu et al. 2012). The method was able to detect approximately 5–10 cfu/mL in a buffer solution and fat-free milk, without enrichment. The promise is to develop a small, low-cost device that is highly specific and sensitive within a biological system and can offer a rapid alternative to conventional analytical techniques.

A comprehensive review of the use of immunosensors for the detection of *E. coli* O157:H7 with respect to the meat industry, states that the current technology does not offer overwhelming advantages in comparison to immunoassays. If the level of sensitivity becomes better than immunoassays, then immunosensors may become a valuable tool to decrease enrichment procedures and detection times, therefore saving time and money (Tokarskyy and Marshall 2008). Further work in this area is required to elucidate the influence of various food matrices and degree of cross-reactivity with other bacterial species. The major disadvantage of the technology is the cost of expensive equipment, but further development in the technologies may facilitate their use in foods in the future (Tokarskyy and Marshall 2008).

4.2.4 Next-Generation Sequencing of the Whole Bacterial Genome

There have been continuous technological improvements for microbial genomic characterization in the last decades, with various methods such as pulsed-field gel electrophoresis (PFGE), Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) and multilocus sequence typing (MLST) (Chap. 3) commonly applied throughout the world for the investigation of foodborne outbreaks and other epidemiological studies (Struelens and Brisse 2013). Although these methods are often well-established and affordable, the disadvantage is the time required to perform these methods and the lack of discriminatory power when comparing closely related isolates obtained from a single outbreak of a bacterial pathogen. This can hinder the elucidation of precise relationships between these isolates and prevent the identification of source cases or environmental sources (Schurch and Siezen 2010). Recent advances in technology and knowledge in molecular characterization and genetics has seen a rapid development and availability of whole genome sequencing (WGS) methods, such as next-generation sequencing for food safety applications. The decrease in cost of WGS and the development of bench-top sequencing technologies has enabled a fast turnaround of results which is attractive for use in routine diagnostics and typing (Joensen et al. 2014).

WGS has already proven useful in many outbreak investigations and has been reported to be a superior alternative to the current routine typing of pathogens (Chap. 3). The benefit of WGS was evident through the identification, characterization, and elucidation of the source of the highly virulent and unusual STEC O104:H4 outbreak in Germany in 2011 (Joensen et al. 2014; Frank et al. 2011). The application of high-throughput sequencing technologies allowed the genome and origins of the strain to be characterized in relative real time as the outbreak was occurring (Franz et al. 2014; Mellmann et al. 2011; Rasko et al. 2011) (Inset 1.2). This technology can identify changes in a bacterial genome and therefore provides the maximum possible discriminatory power between two isolates. These changes can include single-nucleotide polymorphisms (SNPs) and small insertions and deletions (Schurch and Siezen 2010). This technology shows potential to transform our understanding of the evolution of pathogens and the global spread of strains and their antibiotic resistance. This in turn will significantly influence diagnostic microbiology and surveillance worldwide (Koser et al. 2012; Franz et al. 2014).

Along with the rapid development of WGS in diagnostic microbiology, a number of challenges need to be overcome before it is used as a routine method in laboratories. Firstly, the development of WGS technologies and platforms is rapidly expanding and therefore it can be difficult to keep up to date with the latest information in this area. Implementation is also hindered by the uncertainty of which technologies to use and the cost benefits and requirements of these systems (Wain and Mavrogiorgou 2013). Secondly, WGS can produce huge amounts of information and data, and extraction and identification of useful data can be challenging and involves specialized bioinformatics training and time with current computer programs. Therefore, in order to become a routine method, a system that can automatically convert relevant data quickly in a fully automated and reliable form without human intervention, yet is easily interpreted, is essential (Koser et al. 2012). Such systems or software will require the use of continuously updated databases that would not only be used to compare genomes with others but also detect transmission routes, virulence, and antibiotic properties. Ideally these databases should be available internationally and certainly these avenues and hindrances are currently being investigated by organizations such as the European Centre for Disease Prevention and Control (ECDC) (Koser et al. 2012; Struelens and Brisse 2013; Aarestrup et al. 2012).

As it currently stands, WGS is not suitable for routine primary detection of a pathogen, especially within a clinical sample where the pathogen is present in low numbers and amongst the normal microflora. Current detection methods are also significantly cheaper and rapid in fulfilling this requirement of detection and identification. In order for WGS to become cost-effective within a routine testing regime, other improvements must be considered. Improved throughput of analysis of WGS requires the simplification of sample preparation in order to obtain a product within a few hours that would not require specialized techniques. The resulting products, or read lengths would also have to be sufficiently long (Koser et al. 2012). The technology must also become sensitive enough to sequence DNA from a single colony without the need for subculturing or DNA preamplification steps, which

saves time and labor costs. Therefore, for implementation of WGS as a diagnostic test, technical challenges remain but the estimation of time and cost outcomes also needs to be assessed for each sample type and disease syndrome (Wain and Mavrogiorgou 2013). It is likely that the additional improvements of WGS technologies will replace conventional culture-based and molecular typing methods to provide point-of-care clinical diagnosis and aid in treatment and rapid response of outbreak investigations (Aarestrup et al. 2012).

Future developments in WGS will also see more use of "metagenomics" where the genomic information obtained from the whole sample is analyzed. This type of analysis can identify the microbial diversity and community composition within a sample and assist in establishing an ecological role of pathogens in a particular niche. This is especially relevant for microorganisms that cannot be cultured and identified through traditional methods (Sharma et al. 2008). It is likely that an increase in metagenomic studies of clinical samples will identify many other etiological agents that cause disease (Koser et al. 2012). Most studies in diarrheagenic *E. coli* centers on the STEC pathotype due to its significant role in foodborne disease, but the role in other *E. coli* pathotypes in foodborne disease may become clearer through metagenomic studies. It may be possible that other pathotypes of *E. coli* are among the millions of cases of illness where agents with insufficient data to estimate agent-specific burdens have not been identified (Kaspar et al. 2010). This may also identify other novel virulence genes that in turn create changes in the classification scheme that describes each of these pathotypes and may even identify other pathotypes.

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