Mini-review

Clinical relevance of diarrheagenic *E. coli* detection

A distinction between toxin producing diarrheagenic E. coli *and diarrheagenic* E. coli *types that cause a self-inflicted immune response*

Master Thesis

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Summary

Improving the detection of specific diarrheagenic *E. coli* types has not been the focus of clinical and scientific research. The need for this was not recognized, especially in the western world. Now, the importance of detection and identification has been recognized, mostly due to outbreaks of infections in the western world. The main problem is the fact that there is a large overlap (target genes and symptoms) between different *E. coli* types, which makes identification difficult. Because of these difficulties, the focus in the clinic has not been on linking specific *E. coli* to cases of diarrhea. This gap in clinical detection must be filled. This review covers the detection, pathogenesis and treatment of infections with diarrheagenic *E. coli* (DEC) types in humans, the different DEC types and new molecular tools that can be used for detection and identification, the implications for physicians and the treatment of patients and future challenges to fill this gap.

Background

Infections with diarrheagenic *E. coli* (DEC) types are a threat to public health, especially in the case of an epidemic. Infection can cause morbidity (severe diarrhea, hemolytic uremic syndrome [HUS] and hemorrhagic colitis [HC]) and mortality. In the past, *E. coli* had not been identified as an important cause of diarrhea because there was no screening for *E. coli* in patients with diarrhea. Also, improving the detection of specific diarrheagenic *E. coli* types has not been the focus of clinical and scientific research (Tarr, 1995). The need for this was not recognized, especially in the western world. Now, the importance of detection and identification has been recognized, mostly due to outbreaks of infections in the western world. As an example, the outbreak of enterohemorrhagic *E. coli* (EHEC) O104:H4 in Germany (2011) was a wake-up call. The infections with EHEC (0104:H4) caused more then 30 fatalities and over a 100 people were hospitalized due to the development of severe diarrhea, HUS and HC (Torres *et al.* 2005). Outbreaks proved that *E. coli* is not only the cause of diarrhea in developing countries but also in modern countries. Now, the problem lies in the detection of specific DEC types in cases of diarrhea.

Scientifically, a lot is known about *E. coli* but the used methods are not specific. This is due to the fact that the majority of *E. coli's* are commensal of the intestine. Typing *E. coli* was historically based on serotyping schemes that were both labor-intensive and costly. Methods used in the past were not specific enough and even with new molecular techniques there are difficulties. The main problem is the fact that there is a large overlap (target genes and symptoms) between different *E. coli* types (see figure 1), which makes identification difficult. Because of these difficulties, the focus in the clinic has not been on linking specific *E. coli* to cases of diarrhea. This gap in clinical detection must be filled.

This review will first cover the detection, pathogenesis and treatment of infections with DEC types in humans. Secondly, the different DEC types will be discussed including new molecular tools that can be used for detection and identification. Thirdly, the implications for physicians and the treatment of patients will be discussed. Lastly, the future challenges will be addressed as a conclusion.

BOX 1

Routes of infection with E. coli.

The main route of infection with *E. coli* is via the food. Other routes of infection are human-to-human contact, human-to-animal contact, food and environmental contamination.

Route	***Example situation***	***Reference***
Human-to-human contact	Day-care facility Breast feeding	Belongia *et al.*, 1993 Gindrat, 1972
Human-to-animal contact	Animal farms, pets, faeces contact	Beutin *et al.*, 2007; Licence, 2001
Food	Undercooked meat Vegetables Fermented salami	Chapman *et al.*, 1993; Su and Brandt, 1995 Tilden Jr. *et al.*, 1996
Environment	Contaminated water Contaminate building	Solomon *et al.*, 2002 Varma *et al.*, 2003

Classical diagnostics of diarrheagenic *E. coli* types

Historically, O serotyping was used for the identification of diarrheagenic *E. coli* (DEC) types. Different key sera were used to test for agglutination. Using a mathematical approach, that determines the highest probable scenario, the results were coupled to a serogroup leading to the detection of serotypes (Bettelheim and Thompson, 1987). Serotyping is not used in practice because of high personnel and material costs. Besides O serotyping, serological testing and culturing were also used. However, the identification of DEC types cannot be accomplished by using this technique. The classification of DEC types could also be done by the use of phylogenetic analysis combined with Polymerase Chain Reaction (PCR) (Clermont *et al.* 2000). None of these techniques are routinely applicable in the clinical setting due to high time- and material costs. There is a need for economically efficient techniques that can provide results in a timely fashion. The starting points for developing such a technique is the classification of DEC types, the underlying mechanisms of pathogenicity and methods to detect DEC types.

Classification of diarrheagenic *E. coli*

Diarrheagenic *E. coli* (DEC) types are commonly subdivided into six different categories (Kaper *et al.*, 2004; Nataro and Kaper, 1998): enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Martins *et al.,* 2013). There is genetic overlap between the types and also with *Shigella* spp. (see figure 1), which makes the detection of DEC types more difficult. Different names are used for the same type (STEC and VTEC), which makes the classification confusing. The *Shigella* toxin-producing *E. coli* (STEC) are often also specified as verotoxin producing *E. coli* (VTEC). Putting this confusion aside, detection of some types (EHEC, ETEC, EPEC and EIEC) can be achieved by using a combination of multiplex PCR and/or high-performance liquid chromatography (HPLC) (Xu *et al.*, 2012). Another division can be made between the DEC types, based on their diarrheagenic mechanism of action. The two classes are: toxin producing *E. coli* (depicted as red circles in figure 1) and *E. coli* types that cause a self-inflicted immune response (depicted as blue circles in figure 1). The toxins producing *E. coli* cause damage to the intestinal cells. The *E. coli* attach to the cells via specific mechanisms. Due to changes in the intracellular matrix, specific signaling pathways are initiated, resulting in the production of toxins. These toxins can spread to adjacent cells and cause more damage. These toxins cause diarrhea due to the damage of the cells. The group of *E. coli* types that cause an inflammatory response also attach to the intestinal cells. They either invade the cells of the intestine or cause intracellular changes, which both cause an immune response.

As can be seen in figure 1, there is a large amount of overlap between different DEC types. This review will try to disentangle this network of overlap. To provide more clarity, all the DEC types will be discussed separately next.

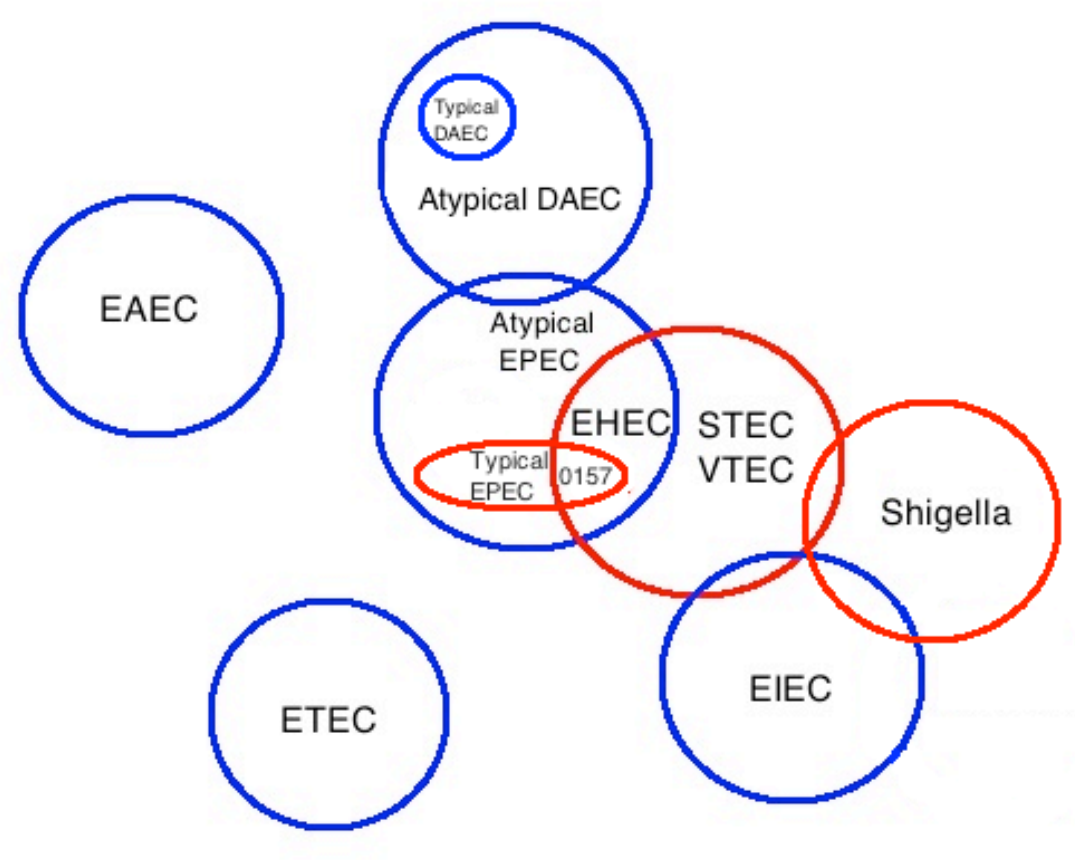
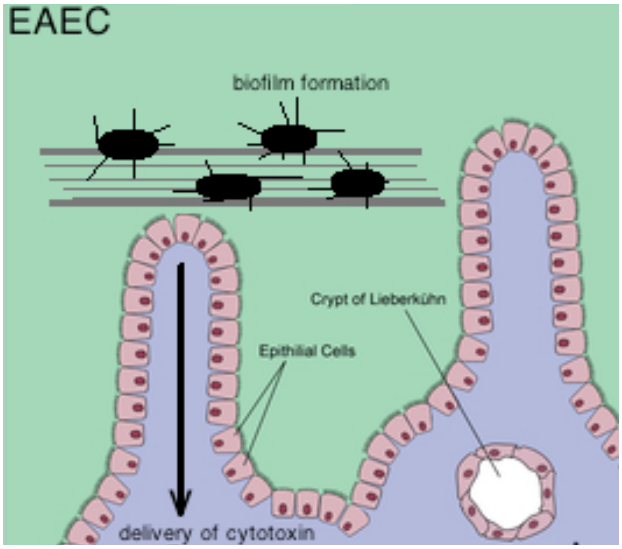


Figure 1: Overview of the genetic overlap of different diarrheagenic *E. coli* types (DEC): enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Two major groups can be distinguished: toxin producing *E. coli* (shown in blue) and *E. coli* that cause a self-inflicted immune response (shown in red).
Based on: http://www.antimicrobialresistance.dk/data/images/protocols/e%20coli%20methods.pdf

E. coli that cause a self-inflicted immune response: enteroaggregative *E. coli* (EAEC)



Main mechanism of pathogenicity	Bioflim formation, cytotoxin producuction leading to a self-inflicted immune response
Specific target genes	*aggR*, *astA*
Clinical symptoms	diarrhea

Figure 2: A schematic overview of the pathogenic mechanism of enteroaggregative *E. coli* (EAEC). The first stage is the formation of a biofilm. The delivery of cytotoxins causes a self-inflicted immune response, leading to diarrhea. Target genes that can be used in detection are *aggR* and *astA*. Adapted from: http://en.wikipedia.org/wiki/File:Coeliac_Disease.png

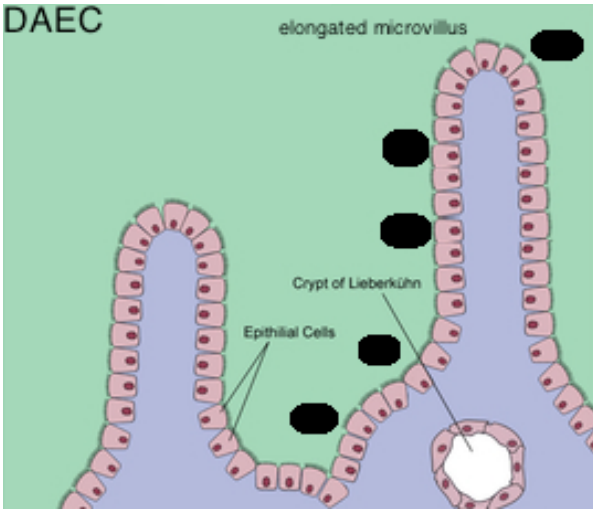
Factors and target genes

Several virulence genes have been discovered in enteroaggregative *E. coli* (EAEC) like *aggR, aggA, aafA, aap,* and *astA.* The *aggR* gene was correlated to diarrhea in more than 73% of the cases (Aslani *et al.*, 2011). These genes can also be used to detect different types of EAEC, as they are typical for these bacteria.

Mechanisms of pathogenicity

It has been reported that EAEC colonizes the gut while forming a biofilm (see figure 2). This biofilm is actually an aggregation of the EAEC bacteria, which ensures the attachment to the intestinal cells. In turn, immune mediators produce cytotoxins, which cause a loss of electrolytes leading to diarrhea. The main mechanism of pathogenicity of EAEC is the self-inflicted immune response. This has been observed in humans, where the Toll-like receptor 5 is triggered causing the attraction of immunological cells (Okhuysen and DuPont, 2010). Also, the Pet and Pic proteins are seen as virulence factors of EAEC (Betancourt-Sanchez and Navarro-Garcia, 2009; Navarro-Garcia *et al.*, 2010) and are linked to diarrhea (Muniesa *et al.*, 2012). EAEC can also cause persistent or chronic diarrhea, which is more often observed in the Western world. Chronic infections with EAEC have similar symptoms as infections with parasites. There is no molecular based diagnosis of this type of diarrhea and the symptoms are often treated as an infection with parasites. In these cases, screening for an infection with EAEC could aid in a better treatment of the symptoms and prevent mistreatment.

E. coli that cause a self-inflicted immune response: diffusely adherent *E. coli* (DAEC)



Main mechanism of pathogenicity	elongation of microvilli via actin filament disassembly
Target genes ***Atypical DAEC subclass I*** ***Atypical DAEC subclass II*** ***Typical DAEC***	 *AfaE-VII, AfaE-VIII, AAF-I, AAF-II, and AAF-III adhesins* *LEE pathogenicity island (Afa/Dr), CEA, DAF* *AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, and NFA-I adhesins*
Clinical symptoms	diarrhea

Figure 3: A schematic overview of the pathogenic mechanism of diffusely adherent *E. coli* (DAEC). DAEC colonize the intestinal cells leading to intracellular changes. This in turn damages the cells causing an inflammatory immune response, leading to diarrhea. Source: http://en.wikipedia.org/wiki/File:Coeliac_Disease.png

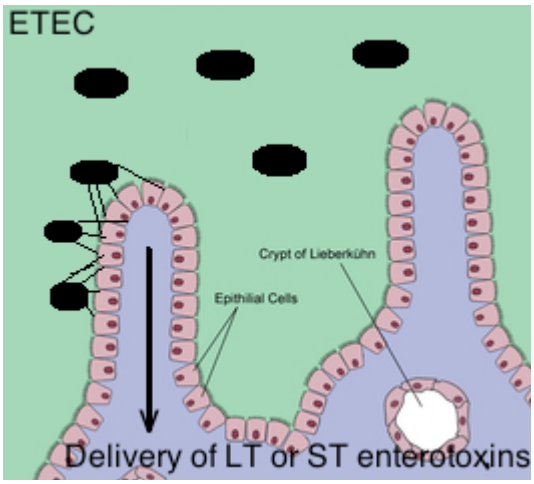
Factors and target genes

Diffusely adherent *E. coli* (DAEC) bacteria rely on the *dae* genes to colonize the intestinal cells of the gut (Rajendran *et al.*, 2010). There are two classes of DAEC, typical and non-typical. The typical class of DAEC includes bacteria harboring AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, and NFA-I adhesins (Afa/Dr DAEC). The atypical class of DAEC includes two subclasses of types; the atypical subclass 1 includes DEC types that express AfaE-VII, AfaE-VIII, AAF-I, AAF-II, and AAF-III adhesins and the atypical subclass 2 includes DEC types that harbor Afa/Dr adhesins or others adhesins promoting diffuse adhesion, together with pathogenicity islands such as the LEE pathogenicity island (DA-EPEC). These specific factors can be used to detect DAEC for they are typical for these bacteria.

Mechanisms of pathogenicity

In general, DAEC uses adhesins to attach to the intestinal cells and are thus essential for colonization (Servin, 2005). Infection with DAEC causes diarrhea, supposedly by binding to parts of the plasma membrane of the intestinal cells. DAEC binds to the intestinal cells using a fimbrial adhesin (F1845) and the decay-accelerating factor (DAF). It is thought that this binding causes the F-actin on the surface of the intestinal cells to disassemble. The signaling pathway of tyrosine kinase Src-like family proteins is activated and causes further actin disassembly inside the cells. Altogether, this results in the elongation of microvilli (see figure 3). Altogether, the damage to the cells causes loss of electrolytes and apoptosis, leading to diarrhea (Fivaz *et al.*, 2000).

Toxin producing *E. coli*: enterotoxigenic *E. coli* (ETEC)



Main mechanism of pathogenicity	colonizing factors aid binding to intestinal cells, plasmid-mediated heat-labile (LT) and heat-stable (ST) enterotoxins cause diarrhea
Specific target genes	*estp, astA*
Clinical symptoms	diarrhea

Figure 4: A schematic overview of the pathogenic mechanism of enterotoxigenic *E. coli* (ETEC). The first stage is adherence to the villus. The delivery of heat-labile (LT) and heat-stable (ST) enterotoxins causes cell damage, leading to diarrhea. Source: http://en.wikipedia.org/wiki/File:Coeliac_Disease.png

Factors and target genes

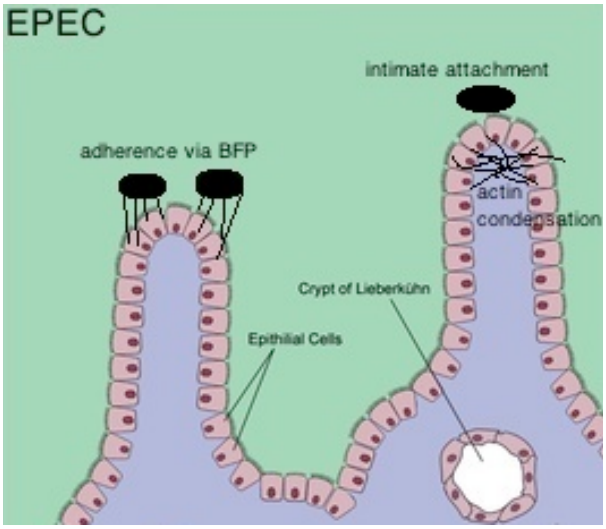
The most important factors for the detection of enterotoxigenic *E. coli* (ETEC) are the genes coding for LT and ST enterotoxins (Rajkhowa *et al.*, 2009). The genes encoding for *invE* (secretion system regulator) and *astA* (arginine succinyltransferase) can also be used as specific target genes to detect the presence of ETEC.

Mechanisms of pathogenicity

Although ETEC produces toxins, diarrhea is caused via adhesion induced cell damage. ETEC is well known for causing travellers' diarrhea especially in developing countries. To colonize the gut, ETEC's produce colonizing factors (CF) that bind to the intestinal cells (Gaastra and Svennerholm, 1996; Torres *et al.*, 2005). These factors govern the attachment and cause intracellular changes in the intestinal cells. Subsequently, plasmid-mediated heat-labile (LT) and heat-stable (ST) enterotoxins are released. These ST toxins must not to be confused with the *stx1/stx2* toxins because they are not identical. As an example, *stx1/stx2* toxins enter the cells and interfere with the inflammation process while LT and ST enterotoxins cause cell damage. Another difference is that *stx1/stx2* toxins are expressed in different *E. coli* types (DAEC, ETEC, EHEC, VTEC/STEC and *Shigella* spp.)

The LT enterotoxins are not stable at temperatures above 60°C. They can be divided into two classes: LT-I and LT-II. LT-I can cause diarrhea in humans and animals, LT-II causes diarrhea in animals (Zhang *et al.*, 2006). It cannot be excluded that LT-II could also cause diarrhea in humans, while no large-scale or specific research has been conducted on human samples. LT enterotoxins are mediated via plasmids and stimulate membrane-bound adenyl cyclase (Seriwatana, 1988). This way, LT damages the intestinal cells and causes diarrhea (Werber *et al.*, 2003). On the other hand, ST enterotoxins stimulate production of cGMP and guanylate cyclase activity (Field *et al.*, 1978). ST enterotoxins also have two subclasses: STa and STb, which can both cause diarrhea in humans and animals. STb is only expressed by ETEC, while STa is expressed in different DEC types (Zhang *et al.*, 2006). Taken together, the LT and ST enterotoxins produced by ETEC cause diarrhea (see figure 4). Infection with ETEC can even be lethal in children via the production of LT and ST enterotoxins (Sánchez and Holmgren, 2005).

E. coli that cause a self-inflicted immune response: enteropathogenic *E. coli* (EPEC)



Main mechanism of pathogenicity	bundle forming protein aids binding to intestinal cells, attachment causes actin condensation leading to an immune response
Specific target genes	*esth, elt, astA*
Clinical symptoms	diarrhea

Figure 5: A schematic overview of the pathogenic mechanism of enteropathogenic *E. coli* (EPEC). The first stage is adherence, via bundle forming protein (BFP), to the villus. Following, intimate attachment occurs. Actin condensation inside the villus finishes the process. These cellular changes cause a self-inflicted immune response leading to diarrhea. Source: http://en.wikipedia.org/wiki/File:Coeliac_Disease.png

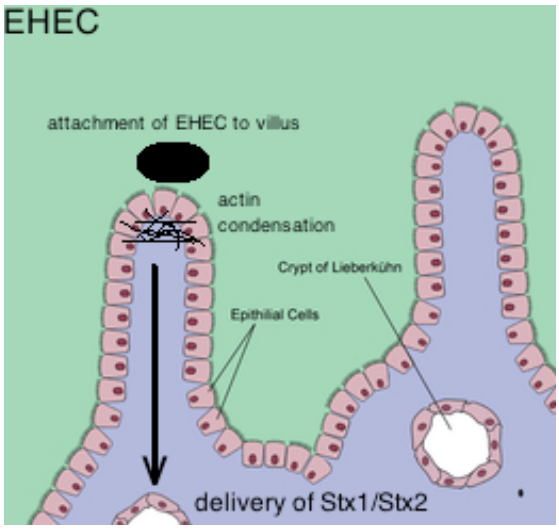
Factors and target genes

For typical enteropathogenic *E. coli* (EPEC), the target gene is *bfp.* For atypical EPEC the *eae* genes can be used. Although the *eae* genes are also found in EHEC, it can still be used to detect EPEC if the analysis includes multiple specific target genes for EPEC.

Mechanisms of pathogenicity

EPEC do not typically produce (*Shigella* like) toxins. Instead their pathogenicity largely depends on eliciting an inflammatory response. The initial step in the colonization of the intestines is the adherence to the cells (see figure 5). The adherence is governed via the bundle forming protein (*bfp*). Also, Higgens *et al.* showed that EPEC uses the Tir receptor and intimin for colonization of the gut. After the adherence, an intimate attachment of EHEC to the intestinal cells is established. This attachment causes actin condensation, which leads to damage to the cells and diarrhea. A self-inflicted immune response is also elicited by EPEC, contributing to the diarrheagenic properties of EPEC. Also, it is known that intimin can bind to T cells (De Grado *et al.*, 1999). Thus, EPEC colonizes the intestinal cells via several factors and causes adhesin induced cell damage and a self-inflicted immune response leading to diarrhea.

Toxin producing *E. coli* types: enterohemmorage *E. coli* (EHEC)



Main mechanism of pathogenicity	bundle forming protein aids binding to intestinal cells, attachment causes actin condensation leading production of stx1/stx2
Specific target genes	*stx1, stx2, eae*
Clinical symptoms	diarrhea

Figure 6: A schematic overview of the pathogenic mechanism of enterohemmorage *E. coli* (EHEC). The first stage is the attachment of the EHEC to the villus. Actin condensation inside the villus occurs. *Shigella* toxin 1 or 2 (*stx1/stx2*) is delivered inside the cells, leading to diarrhea.
Source: http://en.wikipedia.org/wiki/File:Coeliac_Disease.png

Factors and target genes

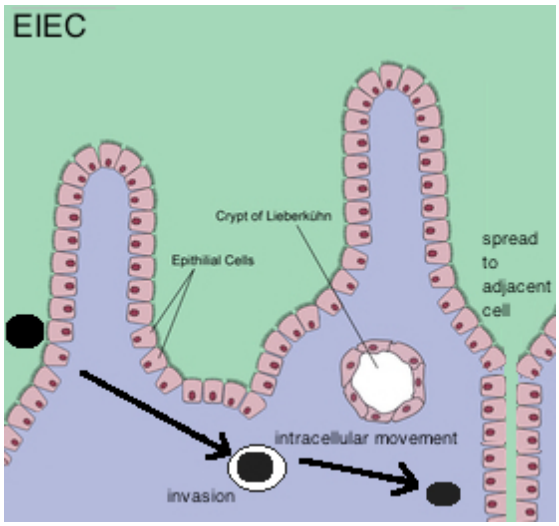
Enterohemmorage *E. coli* (EHEC) belongs to a group of diarrheagenic *E. coli* types that produces toxins. *Stx1* and *stx2* are key factors in the pathogenicity of EHEC. Another factor that can be used are *eae* genes. The *eae* genes encode the intimin protein that is pivotal for the diarrheagenic properties of EHEC. *Stx1/stx2* and *eae* are good candidates to use as target genes in molecular detection methods. *Eae* genes can be subdivided into eleven intimin-subclasses (α1, α2, β, γ, κ, ε, η, ι, λ, θ and ζ). The distribution of these different subclasses in humans differs. The majority consists of the β class (>65%) followed by the other subclasses (percentage values around 5% each). The clinical relevance comes from the observation that specific subclasses of EHEC types posses' specific flagellar antigens. These antigens play a role in the attachment of EHEC to the intestinal cells together with intimin (Ramachandran *et al.*, 2003). A similar division into subclasses is also observed in *stx1/stx2.* The observed subtypes of *stx1* are*: stx1, stx1c* and for *stx2* and *stx1d: stx1d, stx2, stx2c, stx2d, stx2e, and stx2f*. The distribution of subclasses is as following: 50% of the strains expressed *stx1*, 45% expressed both *stx1* and *stx2* and 5% expressed only *stx2.* The strains expressing *stx1* differed in the expression of the subclasses: 38% expressed *stx1c*, 10% expressed *stx1* and 7% expressed *stx1d.* The majority of *stx2* expressing strains expressed only *stx2d* (93%). These distributions can be translated in clinical relevance. Strains expressing *stx1* can cause severe human disease (HUS and HC) and strains expressing *stx1c* can cause mild diarrhea. For *stx2* there is also a difference in virulence between subclasses. The *stx2c* and *stx2* are more virulent in humans then *stx2d* and *stx2e* (Ishii *et al.*, 2007).

Mechanisms of pathogenicity

EHEC delivers toxins to the intestinal cells, which leads to the development of diarrhea (see figure 6). Initial attachment of EHEC is governed by the localized adherence (LA), which involves the formation of bundle-forming pilli (*bfp*) in typical EHEC strains. In a later stage the attachment cause lesions through the condensation of actin filaments and

microvillus effacement. The attaching and effacing (A/E) lesions are responsible for the degeneration of tissue and subsequently results in the loss of electrolytes, which causes diarrhea. The protein involved in this process (intimin, an integrin protein) is encoded on the locus of enterocyte effacement (*lee*) gene (Torres *et al.* 2005). The EHEC uses intimin, which aids the adherence to the cells of the intestine via the Tir receptor. In addition, the pathology of the EHEC is enhanced by Shiga-toxins (*stx1* and *stx2*) that cross the intestinal wall. This can cause hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) through the production of *stx1/stx2* and other factors like verotoxin (Muniesa *et al.*, 2012). Not every type of EHEC produces both factors, while in most cases only one (*stx1/stx2*) is produced. Production of *stx2* is more likely to cause HUS and is therefore more potent in causing disease then *stx1* (Siegler *et al.*, 2003, Fuller *et al.*, 2011). These toxins are key virulence factors and of importance in the symptoms of EHEC infection.

Toxin producing *E. coli*: enteroinvasive *E. coli* (EIEC)



Mechanism of pathogenicity	Invasion of intestinal cells leading to the production of *stx1/stx2*
Target genes	*invE*
Clinical symptoms	diarrhea

Figure 7: A schematic overview of the pathogenic mechanism of enteroinvasive *E. coli* (EIEC). The first stage is the invasion of M-cells followed by intracellular movement. The EIEC's spread to adjacent cells, which causes an inflammatory immune response and ultimately diarrhea. Source: http://en.wikipedia.org/wiki/File:Coeliac_Disease.png

Factors and target genes

The *invE* gene can be used as a target gene for the detection of enteroinvasive *E. coli* (EIEC). This is a secretion system regulator, which is strongly involved in the invasion of EIEC into the cells (Kubori and Galan, 2002). Once the EIEC has invaded the intestinal cells, the production of *stx1/stx2* is stimulated. The invasion and production of toxins leads to diarrhea observed in the clinic.

Mechanisms of pathogenicity

The EIEC bacteria differ from the other *E. coli* types described above. The behaviour resembles *Shigella* species more than other *E. coli* types (van den Beld, and Reubsaet, 2012). The mechanism of colonization of the closely related *Shigella* species is based on the invasion of M-cells. Subsequently, they invade macrophages and induce apoptosis. Further invasion in epithelial cells causes an inflammatory response (Parsot, 2005). This damages the intestinal cells and microvilli leading to diarrhea (see figure 7).

Resemblance to Shigella *spp.*

EIEC strains resemble *Shigella* spp. very closely (see figure 1 and 7) because they have a similar mechanism of action and express certain common genes. Therefore, a description of *Shigella* spp. is given next.

Shigella spp. in relation to diarrheagenic *E. coli* types

Shigella spp. has the same mechanism of pathogenicity as EIEC. Typically, the invasion of intestinal cells is followed by the production of toxins that cause damage to the intestinal cells and lead to diarrhea. There are however some differences between different strains (see table 1). The symptoms of an infection with *Shigella* spp. are also very similar to an infection with EIEC and STEC/VTEC. They include cramps, painful defecation, fever, diarrhea, and dysentery (blood and mucus in the stool). In the last decade, the mechanism of pathogenicity, virulence genes and toxins of *Shigella* spp. has been studied in more detail. A short overview of these results is given in table 1. It must be noted that several diarrheagenic *E. coli* types express the same toxins (*stx1/stx2*) including EIEC, EHEC and STEC/VTEC.

Table 1: Overview of *Shigella* spp. virulence genes, toxins, mechanism of action and symptoms

***Shigella* spp. type**	**Virulence genes**	**Toxin**	**Mechanism of action**	**Symptoms**	**Reference**
Shigella dysenteriae	*stx1 and stx2*	Shiga toxin (*stxA, stxB*)	*Stx* invades cells and causes inflammation and inhibits protein synthesis	Dysentery and diarrhea	(O'Loughlin and Robins-Browne, 2001)
Shigella boydii	*Stx 1/ stx2 and LcsA*	Shiga toxin (*stxA, stxB*) and *IcsA*	*LcsA* promotes actin polymerization	Dysentery	(Parsot, 2005)
Shigella sonnei	*Stx 1/ stx2 and LcsA*	Shiga toxin (*stxA, stxB*) and *IcsA*	Acute inflammation	Diarrhea and dehydration	(Jiang et al., 2005)

Detection of diarrheagenic *E. coli* types

The identification of different *E. coli* types has now been rapidly developing due to the introduction of molecular diagnostic techniques. One of the first techniques used was the High-performance liquid chromatography (HPLC) method. Proteins of *E. coli* types can be detected this way. Their function can be deduced and antigenic proteins can be detected (Marasco *et al.*, 1984). Specific receptors, like the iron receptor, can also be used to detect *E. coli* types (Hantke *et al.*, 2003). However, this approach is not suited for the routine detection of DEC types. On the other hand, DNA hybridization can detect different species. For example, using a DNA probe for specific the genes that encode the virulence (invasion associated) proteins of *Shigella* and EIEIC species, in combination with invasion assays. Boileau *et al.* were able to detect Shigella/EIEC species in the feces of patients this way. This technique cannot determine the serotype because it is based on LPS composition of the bacteria, and many genes are required for the synthesis of a single LPS molecule. Although the sensitivity and specificity of DNA based tests is relatively high, further testing of the bacterial colonies is needed to confirm the serotype (Boileau *et al.*, 1984). Another limitation of DNA hybridization, from colonies on plate, is the large amount of time required to obtain the result.

Another method is based on detection of the antigen against *E.coli* types. Monoclonal antibodies against the antigen for *E. coli* can also be used, in combination with latex agglutination, to determine specific types. LC-MS/MC can be used to analyze the proteomes of *E. coli* types (Nara *et al.*, 2012). The most promising technique is the (multiplex) PCR, which uses specific target genes (see table 2 and 3) for the different DEC types. Detection of diarrheic *E. coli* (DEC) types is based on detection of several genes: *stx1, stx2, eae, bfpA, invE, aggR, esth, estp, elt*, and *astA*. These genes are specific for certain DEC types, see table 1 (Fujioka *et al.*, 2009; Fujioka *et al.*, 2013). The multiplex PCR can simultaneously detect different types of *E.coli* in one sample. However, there is a problem with detection based upon these target genes. As stated before, there is a large overlap between different strains. Another problem comes from practice: fecal samples may consist of more then one DEC type. This mixture of DEC types is thus difficult to analyze. Even when multiple target genes are used some DEC types cannot be discriminated from others. For example, when the target gene *eae* is used to test a fecal sample, detection of these genes can indicate the presence of EHEC, EPEC or VTEC/STEC (see table 2 and 3). This is the major problem in clinical detection of DEC types in fecal samples. To address this problem more closely, the methods that are available to detect DEC types will be discussed next.

Table 2: Overview of target genes used for the detection of diarrheagenic *E. coli* (DEC) types by a multiplex PCR. Adapted from (Fujioka *et al.*, 2009)

Description	DEC Types	Target gene
Atypical DAEC	-	*AfaE-VII, AfaE-VIII, AAF-I, AAF-II, and AAF-III adhesins, LEE pathogenicity island*
Typical DAEC	-	*AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, and NFA-I adhesins (Afa/Dr), CEA, DAF*
EHEC	E1303	*stx1*, *stx2*, *eae*
Typical EPEC	E1231	*bfpA*, *eae*
Atypical EPEC	E-055	*eae*
ETEC	E-O169	*estp*, *astA*
ETEC	E31	*esth*, *elt*, *astA*
EIEC	E33	*invE*
EAggEC	E1407	*aggR*, *astA*

Detection methods for EHEC

The detection of EHEC in a clinical setting can be achieved by the use of (multiplex) PCR, macroarray or by the use of nanoparticles. By using a multiplex PCR method different types can be distinguished. For example, Madic *et al.* developed a multiplex PCR method to detect five different serotypes of EHEC bacteria (O26:H11, O103:H2, O111:H8, O145:H28 and O157:H7). Their method was based on the detection of flagellar antigens and intimin variants (Madic *et al.*, 2010). Another multiplex PCR analysis was developed, which was based on clustered regularly interspaced short palindromic repeats (CRISPR) in the genome of EHEC types. A Meridian assay, based on the detection of *stx1/stx2* can also be used to detect EHEC bacteria (Staples *et al.*, 2012). Other target genes can also be used, like *eae* (Reid *et al.*, 1999) *stx1, stx2, eaeA,* for the detection of EHEC serotypes O111 and O157 (see tables 2 and 3, Paton and Paton, 1998). Multiplex PCR is thus a validated method for the detection of EHEC.

Detection methods for EPEC

The same methods used for the detection of EHEC can be used to detect EPEC types. The multiplex PCR method can also be used to detect different EPEC types. In the experiment of Kong *et al.*, primers were used to detect several genes: *stx1/stx2* and *eae* genes (EHEC and EPEC) (Kong *et al.*, 1999). Multiplex PCR is a promising technique that can detect EPEC. This method should be improved on handling-time and costs so it can be applicable in the clinic.

Detection methods for ETEC

The detection of ETEC in a clinical setting is often done with the use of multiplex PCR (Arenas-Hernández *et al*., 2012; Kong *et al*., 1999; Tilak and Mudaliar, 2012). These methods are often based on detection of genes encoding LS and ST enterotoxins (Caeiro *et al*., 1999). Nowadays, one-step multiplex polymerase chain reaction (mPCR) is also used. The target genes for the detection of ETEC can be found in tables 2 and 3.

Detection methods for EAEC

Multiplex PCR methods are used as a standard for the detection of EAEC (Cerna et al., 2003; Scaletsky *et al.*, 2002). For example, a 16 multiplex PCR method was able to detect diarrheagenic *E. coli* types (EAEC as well as EHEC, EPEC, ETEC, and EIEC respectively) in a single analysis (Antikainen *et al.*, 2009).

Detection methods for EIEC

A target gene for EIEC is *invE*, which is a secretion system regulator (see table 3). The golden standard for the detection of EIEC in stool samples used to be based on culture and DNA hybridization (Echeverria *et al.*, 1989) and DNA probes (Venkatesan *et al.*, 1988). For example, a distinction between *E. coli* and *Shigella* spp. is possible with the use of GenoType® EHEC (Hain Lifeciense, Germany), which is based on DNA hybridization for target genes. This method is not routinely used in the clinic. The current methods of detection are predominantly based on (multiplex) PCR methods (de Boer *et al*., 2010; Nguyen *et al.*, 2005). Also, a combination of PCR and the Luminex bead system can be applied. As seen in the detection of other DEC types, specific types cannot be detected with a standard PCR analysis.

Detection methods for DAEC

DAEC bacteria rely on the *dae* genes to colonize the intestinal cells of the gut (Rajendran *et al.*, 2010) PCR analysis is used to detect DEC types. Not many genes are known for the detection of DAEC in stool samples (Fujioka *et al.*, 2009). The golden standard is still the HEP-2 adherence assay. Two families of target genes have been identified for DAEC: *afa/daa* and AIDA (adhesin-involved in diffuse adhesion)-encoding genes. PCR analysis based on detection of *daaD* can be used to detect DAEC (Barletta *et al.*, 2009; Guion *et al.*, 2008).

Table 3:Target genes for different *E. coli* types. Ordered by prevalence in disease (diarrhea).

***E. Coli* type (% prevalence in disease)**	**Target genes and function**	**Pathogenic mechanism**	**Symptoms**	**References**
DAEC (17.4%, [18])	*daaD* (adhesion protein [1]), *stx 1*(Shiga-like toxin)[2], *eaeA* (intimin [3]), *invE* (secretion system regulator [4]), STP(serine transporter [5]), *astA* (arginine succinyltransferase[6])	Self-inflicted immune response	diarrhea	Fujioka *et al.* 2009
EAEC (5.7%,[18])	*aggR* (putative transcriptional activator [12]), *aggA* (adhesin protein [13]), *aafA* (major fimbrial subunit of aggregative adherence fimbria II [14]), and *astA* (arginine succinyltransferase [6])	Self-inflicted immune response	diarrhea	Okhuysen and DuPont 2010
ETEC (4.2%, [17])	*stx1[2], stx2(*Shiga-like toxin[7]), *bfpA (Bundlin protein [8]), invE*[4], *astA*[6]	Toxic production	diarrhea	Gaastra and Svennerholm; 1996, Torres *et al.* 2005
EHEC (4.2%[19])	*Lee (*intimin protein) [9], *stx2* [7], *eae* [10]	Toxin production	diarrhea, HU and HUS	Muniesa *et al*. 2012
STEC/VTEC (3.4%, [19])	*ehxA* (hemolysin A [10]), *espP* (autotransporter [11]), *eae* [10], *stx1* [2] and *stx2* [7], *invE* [4]	Toxin production	diarrhea	Boerlin *et al.* 1999; Bugarel *et al.* 2010
EPEC (1.8%, [17])	PHO-A (housekeeping gene [15]), LT1/2 (lipid transfer [16])	Self-inflicted immune response	inflammatory response	Higgens *et al.*, De Grado *et al.* 1999

[1] (Snelling *et al.*, 2009)
[2] (Asakura *et al.*, 1998)
[3] (Ramachandran *et al.*, 2003)
[4] (Kubori and Galan, 2002)
[5] (Johnston *et al.*, 1994)
[6] (Ferenci *et al.*, 2009)
[7] (Grad *et al.*, 2012)
[8] (Giron *et al.*, 1991)
[9] (Voss *et al.*, 1998)
[10] (Vander Byl and Kropinski, 2000)
[11] (Kenny *et al.*, 1996)
[12] (Nataro *et al.*, 1994)
[13] (Savarino *et al.*, 1994)
[14] (Chaudhuri *et al.*, 2010)
[15] (Ogura *et al.*, 2009)
[16] (Wasteson and Olsvik, 1991)
[17] (Dutta *et al.*, 2013)
[18] (Snelling *et al.*, 2009)
[19] (Rajendran *et al.*, 2010)

Treatment of diarrhea symptoms

The treatment of the DEC types (EHEC, EPEC, ETEC, EAEC, EIEC and DAEC respectively) is multi-targeted. The first step is treatment of the symptoms of (bloody) diarrhea. Ensuring that the loss of fluids from the intestine is limited and the amount of electrolytes lost is replaced is essential. This can be done by administration of oral rehydration solutions (ORS) and by administering intravenous fluids. Controlling the symptoms of diarrhea also aids in preventing and controlling hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Goldwater and Bettelheim, 2012). In case of acute renal failure, the physician must act immediately by administering acute renal replacement therapy (ARRT). ARRT includes peritoneal dialysis, hemodialysis, plasma infusion or exchange (Goldwater and Bettelheim, 2012). The next steps are controlling the symptoms of HC and HUS with specific treatments. When a patient has been diagnosed with diarrhea the identification of specific pathogen *E.coli* types can be important for the treatment. Physicians should also focus on identifying the pathogens, not only on treating the symptoms.

Treatment of infections with diarrheagenic *E. coli* types

Infection with DEC types and can be fatal, especially in children and elderly people and can cause hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). The case-fatality rate ranges from 3 to 5% (World Health Organisation, 2011). HC is characterized by diarrhea as main symptom. The treatment is the same as for diarrhea in general. The status quo in treatment of diarrhea caused by bacterial infections with *E. coli* is the administration of (non-specific) antibiotics. However, the emergence of antibiotic resistance is a problem for treatment in the clinic, development of medicines and in scientific research (Korzeniewska *et al.*, 2013; Mahanti *et al.*, 2013; Van der Donk *et al.*, 2013). The use of antibiotics might be applicable for certain *E.* coli types, in which there is no increase in toxin production due to the administration of antibiotics. For EAEC and DAEC antibiotics might be used as treatment. Thus, physicians must be able to detect different DEC types. Instead of using (non-specific) antibiotics, other types of treatment must be used. The use of bacteriophages is a new emerging treatment, which can be used to treat infections with STEC and EHEC (Dini and De Urraza, 2010). The use of bacteriophages as treatment has only been achieved in cattle, but might be applicable in humans. But the use of (non-specific) antibiotics is still the golden standard. The correct selection of antibiotics is crucial for treatment of multidrug resistant *E. coli* types (Qadri *et al.*, 2005). In order to achieve this goal the specific types of *E. coli* must be detected. HUS is a more severe complication caused by DEC infection. HUS causes other complications like hemolytic anemia, thrombocytopenia, and acute renal failure (Rosales *et al.*, 2012). Acute renal failure must be addressed in a rapid manner (Goldwater and Bettelheim, 2012). There are treatments available for thrombocytopenia such as the administration of anti-thrombotic medicines. Hemolytic anemia can be treated by the blood transfusion and the administration of coagulation promoting medicines. Antibiotics are used in the clinic to treat infections with a range of bacteria but the use of antibiotics in infections with diarrheagenic *E. coli* infections is strongly debated. Most often, infections with diarrheagenic *E. coli* are self-limiting and do not require specific treatment. In the case of severe diarrhea, HUS or HC treatment with antibiotics is sometimes applied. The main problem is that the use of antibiotics may increase production of toxins. This occurs via the induction of the bacterial SOS response (Kimmitt *et al.*, 2000), which is a reaction to DNA damage induced by the infection (Zhang *et al.*, 2000). Increasing of the production of Shig-toxins (Stx) can worsen HUS

and increase the change on complications (Bielaszewska *et al.*, 2012) Thus it is important for physicians to identify the DEC types when a patient is diagnosed with diarrhea. This holds true especially in the case of immuno-compromised patients, children and elderly people. These patient groups are more vulnerable to the effects of non-specific antibiotics and complications can occur worsening the progression of the disease. Also, prescribing non-specific antibiotics in otherwise healthy patients can cause more damage due to the increase in toxin production. Patients may experience reassurance from the treatment with antibiotics. Although this might be satisfactory for patients, it is not advised because of the risk of an increase in toxin production. Specific (antibiotic) treatment could be used, targeting only one DEC type. This way, the development of antibiotic resistant types of DEC would decrease. Patients could also be treated more efficiently, saving money and time. With a better detection of DEC types, the treatment of patients would also be improved this way.

Future perspectives

There is little evidence of validated methods for detection of DEC types besides those for the *E. coli* O157:H7. The focus of (clinical) research must be on the development of these techniques. Detection of DEC types has many advantages for treatment of patients with diarrhea.

To improve the detection and identification of DEC types new molecular techniques are available. Requirements for new techniques are: swift analysis, high throughput, multiple strain detection and applicability in the clinic. Fast analysis is important due to the diarrheagenic properties of DEC types. Quick and high-throughput detection of diarrheagenic DEC types is most important to detect hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC) or other complications. Detection of multiples DEC types (multiplex) is pivotal due to the fact that an average fecal sample contains many (commensal) types of *E. coli*. This is not only important for treatment but also offers more insight into the mechanisms behind DEC infections. Applicability in the clinic must be ensured to reach the goal of detection and identification at an early stage as well as improving treatment programs.

The most promising new molecular technique is the multiplex PCR. It has a high throughput and is considered to be one of the fastest analyses available for DNA analysis. There are multiple target genes that can be used to detect different DEC types (see table 2 and 3). These target genes can be included in a multiplex PCR analysis. A multiplex PCR analysis is also applicable in the clinic while the equipment is cost-efficient, space-efficient and the operating is not labor intensive. Also, the analysis can be run in a timely manner ensuring swift detection and identification of DEC types. A future perspective is the modification of a multiplex PCR for DEC types in which requirements for applicability in the clinic must be met.

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