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Shiga toxin-producing *Escherichia coli* (STEC) from Humans in the Netherlands

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CHAPTER 8

General Discussion, Summary and Future Perspectives

General Discussion and Summary

The research performed in this thesis describes rapid molecular diagnosis of Shiga toxin-producing *E. coli* (STEC) together with risk assessment and detailed characterization of the bacterium using modern, high resolution whole genome sequencing (WGS). This research focused on two serotypes O157:H7 and O104:H4, which are clinically important and relevant to public health. It also describes the plasticity in virulence and antibiotic resistance properties and the molecular typing of *E. coli* O157:non-H7 strains lacking the Shiga toxin (Stx) encoding gene *stx* to reveal their relation with STEC O157:H7.

STEC infections are usually diagnosed through laboratory testing of stool specimens. Most laboratories can identify STEC O157:H7 by cultural techniques. To detect non-O157 STEC infections, molecular methods, in particular detection of the *stx* gene, is the most reliable way. Improved diagnosis and classification of STEC is necessary for the diagnostic laboratories to implement proper infection control measures in hospitals and to alert public health services, which on their turn need to prevent (further) dissemination of this pathogen in the community. **In chapter 2**, a rapid screening algorithm including both molecular and conventional methods of STEC detection was applied on direct fecal materials. Risk assessment of STEC infection was performed using a presumptive categorization of STEC based on serotyping and presence of virulence genes together with *stx* as described by the European Food Safety Authority (EFSA) in 2013 with slide modifications. Risk groups for disease severity were defined and ranged from high risk pathotype (PT) group I to low risk PT group III, whereas PT group IV consists of not culture confirmed *stx* qPCR-positive samples. It was observed that PT I, defined to have the *escV* or *agg* and/or *aat* gene and belonging to one of the major O serotypes (O26, O103, O104, O111, O121, O145, O157), was significantly associated with bloody diarrhea. *stx* subtypes 2a and 2c were more associated with PT I confirming the association of these subtypes with severe clinical outcomes, which is in concordance with previous findings (1, 2). Therefore, subtyping of the *stx* gene directly on DNA extracts from enriched stool samples could help to increase the speed to obtain data for risk assessment, compared to subtyping performed on isolates. Moreover, it can also determine the subtype in case the *stx* gene is present in a free Stx converting bacteriophage rather than in *E. coli*. In this chapter, it was also observed that an enrichment step of fecal samples increased the chance to obtain a pure isolate for further characterization. Moreover, our study showed the effective use of selective agar medium (CHROM Agar STEC) for easier identification and isolation of *eae* positive STEC as well as enteroaggregative *E. coli* (EAEC) and enteropathogenic *E. coli* (EPEC) pathotypes (3, 4).

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In **chapter 3**, we performed an overall molecular characterization using WGS of the STEC isolates. We confirmed that WGS is a reliable and robust one-step process for characterization of STEC. The most common serotypes found in this study were O91:H14 (14%), O157:H7 (13%), O26:H11 (11%), O103:H2 (8%), O128:H2 (5%) and the most predominant (MLST) sequence types (STs) were ST33 (14%), ST11 (13%), ST21 (13%), ST17 (7%), and ST25 (4%). Previous studies in the Netherlands also observed the predominance of non-O157 over O157 STEC (5-7). Over the period 2007–2012 the most reported non-O157 serotypes were O26 (12%), O63 (10%), O91 (9%), O113 (6%), O103 (5%) and O146 (4%) (7).

The presence of the pathogenicity island LEE (locus of enterocyte effacement) is thought to be an important virulence determinant in STEC. This is also observed in our study as the presence of virulence genes located on LEE were significantly more frequently found in isolates obtained from patients with bloody diarrhea. In addition, several virulence genes were significantly more often present in *eae* negative strains confirming their role in the virulence of these strains. In contrast, no correlation between the severity of the disease and any specific phylogenetic background (e.g. particular ST) of the STEC isolates was observed. Clearly, there is no single factor that could predict the disease outcome. Several factors related to the bacterial isolate, such as its heterogeneity in phage content and competition with intestinal microflora as well as host and environmental factors may play a role in the disease development (8, 9). Therefore, it is difficult to predict if a specific STEC strain can cause an outbreak or not, only based on sequence data. An example of this is *E. coli* O104:H4, supposed not to be associated with outbreaks previously. Indeed, only few sporadic cases with Hemolytic Uremic Syndrome (HUS) in Germany, Korea and Georgia were reported in 2001, 2005 and 2009, respectively, before the outbreak in Germany in 2011 (10). The 2011 outbreak strain showed an unusual combination of virulence factors typically associated with both STEC and EAEC.

By comparing our collection of STEC isolates with a collection of diarrheagenic *E. coli* (DEC) reference isolates in **chapter 3**, it was revealed that many of the STEC isolates shared a common ancestor with other *E. coli* pathogroups. This finding suggests that STEC cannot be considered as a single *E. coli* pathogroup in evolutionary history, rather originated from multiple pathogroups that have acquired the Stx phage (11, 12). The genomic diversity pattern of STEC isolates of this study was similar to that of DEC isolates but was less diverse than the extended spectrum beta-lactamase (ESBL) producing *E. coli* suggesting that Stx phages integrate preferentially in specific *E. coli* types. Such limitation in host range of the Stx phage can be explained by a preference of this phage for specific integration sites not present in all *E. coli* strains. Alternatively, specific bacteriophages

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provide selective advantages for certain *E. coli* strains to let them survive in the environment (8, 13, 14).

As already shortly mentioned, in 2011 one of the largest and most severe outbreaks of STEC infections occurred in Germany (10, 15) which forced scientists and clinical microbiologists to change the STEC detection scheme into a new direction. Because the outbreak strain was not a classical STEC strain, but actually an EAEC strain with an O104:H4 serotype possessing the *Stx* encoding bacteriophage, the name Enterohaemorrhagic *E. coli* (EAHEC) was proposed for the strain (15). In **chapter 4**, we have described three similar EAHEC strains obtained in the Netherlands from two friends who had been travelling to Turkey just before one of them was diagnosed with HUS. One of the EAHEC O104:H4 strains was ESBL positive as the German 2011 outbreak strain. Interestingly, an *stx* negative ESBL positive strain was also isolated from the friend of the HUS patient indicating a possible transfer of resistance genes between bacteria inside the gut. In fact, by comparing the core genome of the isolates it was observed that the *stx* negative strain was genetically at a large distance from the *stx* positive ones. Although we were not able to prove that the STEC strains originated from Turkey, there are several studies describing EAHEC O104:H4 strains associated with traveling to Turkey, Tunisia, Egypt, and North Africa (16). In the same chapter we used WGS to differentiate between the very closely related isolates that was not possible using conventional methods. Thus, WGS is a powerful and helpful tool for hospitals and public health organizations and facilitates taking the appropriate strategies for infection control especially during outbreak situations.

There were several studies performed during the 2011 outbreak describing the role of WGS to identify and fully characterize outbreak strains within short time periods and to reveal the phylogenetic background of them. Several hypotheses have been made on the evolution of the EAHEC O104:H4 including the derivation of this strain from an EAEC that acquired an *stx2*-phage by horizontal gene transfer (15, 17) and the idea that it originated from an ancestor STEC O104:H4 by stepwise gain and loss of chromosomal and plasmid-encoded virulence factors (18). In **chapter 5**, we have proposed an evolutionary model based on the phylogenetic analysis results of 23 O104:H4 genomes including the outbreak and non-outbreak clones. According to our model, the evolution of three successful clades, including the one containing the 2011 outbreak strain and two non-outbreak clades of EAHEC O104:H4, occurred from a recent common ancestor. Furthermore, we could identify some of the driving forces that lead to evolution of successful clones, the most important ones being use of antibiotics and niche competition. Frequent gain and loss of mobile genetic elements (MGEs) could give rise to a new combination of virulence factors in a pathogen, which could trigger a future outbreak. The data obtained in **chapter 4 and 5** revealed that *E. coli* O104:H4 strains, similar to the

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2011 outbreak clone are still circulating in Europe pointing out the necessity for proper molecular surveillance of STEC.

Another and the best studied STEC serotype is O157:H7 associated with large food-borne outbreaks worldwide. Thirteen percent of our collected STEC isolates were O157:H7, whereas we had four *stx* negative O157:H7 isolates. In **chapter 6**, we have described a detailed genomic comparison of *stx* positive and negative *E. coli* O157:H7, the latter not attracting the attention of most clinical laboratories. These *stx* negative isolates can be considered as EPEC if they contain the *eae* gene. Using WGS and subsequent phylogenetic analysis, we proved that *stx* negative variants of *E. coli* O157:H7 carrying all other accessory virulence genes except *stx*, are in fact closely related to STEC O157:H7. Either they may have lost the Stx encoding bacteriophage or they may be a progenitor for STEC O157:H7 being prepared to acquire the Stx phage. Indeed, *stx* negative isolates were also reported in previous studies even from feces of HUS patients and they may have lost this gene during infection (19). As the *stx* gene is encoded on a bacteriophage that could be gained or lost by other *E. coli* pathogroups, it is not always reliable enough to predict the virulence potential of a strain only based on traditional classification of *E. coli*. Therefore, we suggest to screen for the presence of other additional virulence genes to get an idea about the pathogenic potential of an *E. coli* isolate.

Little is known about *stx* negative *E. coli* O157 that carry a flagellar antigen other than H7. As *stx* negative O157 isolates are usually sorbitol fermenting (SF) and could be non-motile, they could be misidentified as STEC SF O157:HNM if no proper molecular characterization is performed (20). *E. coli* O157 lacking the *stx* gene are very diverse and belong to several STs and H types as described in **chapter 7**. They can be classified as typical or atypical EPEC but also as non-EPEC. However, all of them are distantly related to STEC O157:H7. They appeared to have different virulence properties and some possess genes conferring resistance to multiple antibiotics. We observed the presence of several plasmids, pathogenicity islands, and insertion elements which originated from different *E. coli* types or even from other species. Some of the isolates may not be considered as pathogenic, but the presence of resistance genes in the isolates could play a role in dissemination of these genes to other pathogenic strains in the gut or in the environment.

In this thesis, we have tried to combine advanced diagnostic approaches and comprehensive research in the field of diarrheagenic *E. coli* focusing on STEC and to a lesser extent on EPEC. Instead of using conventional cultural techniques we implemented molecular schemes for rapid diagnosis together with a presumptive risk categorization of STEC that facilitates the health care authorities and scientific communities to get prepared to protect the community from a large epidemic.

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Although some serotypes of STEC are known to cause outbreaks and mortality, none of the other serotypes should be neglected as MGEs carrying virulence markers could be integrated in *E. coli* chromosomes and a combination of them could give rise to new pathogenic clones even of a rare serotype (21). The dissemination of MGEs is rather dynamic and gain and loss of these elements make it hard to define if the organism is a threat for public health or not. Furthermore, we showed that 25% of our STEC isolates carried one or more antibiotic resistance genes, which they may transfer to other clinically significant pathogens. Most of the characterization was performed by WGS, a tool enabling us to study a broad range of characteristics and applicable to a wide range of pathogens (22, 23). Without applying WGS it would not have been possible to compare the detailed virulence, resistance and other molecular features together with the phylogenetic background of so many isolates within a relatively short time span. As WGS is now-a-days becoming less expensive and turnaround times decrease, it is perfectly applicable in routine diagnostics and clinical laboratories. Indeed, primers and probes specific for (outbreak) specific signature sequences to setup a rapid molecular screening test can be developed based on the WGS data. This is of great help for the rapid identification of an outbreak strain (24) enabling taking infection control measures to prevent further spread.

The knowledge obtained from this thesis will be helpful for the rapid identification, risk assessment and understanding of the genomics of STEC leading towards a broad and diverse research field on STEC.

Future Perspective

As most of the large STEC outbreaks and several sporadic cases of STEC infections are linked to food, screening of food, water and food producing animals for the presence of STEC and characterization of the isolates from these sources will contribute to reveal the source of STEC within the food chain and will help to prevent its transmission. Also screening of the healthy human population for the presence of STEC will give insight into possible transmission routes of STEC via asymptomatic carriers.

Stx phage heterogeneity may be responsible for converting the pathogenic profiles of their bacterial hosts (25). The expression of Stx phage genes can be regulated by the presence of other phages in the host genome (26, 27). Therefore, it is worth to analyze the complete phage properties of the isolates of different serotypes that may be of help to define relatively more virulent STEC.

To establish infection, pathogens have mechanisms to interact and compete with the resident microbial community at the site of infection as well as with numerous host factors. Studies have described changes in Stx expression in the presence of other organisms, indicating that the microbial balance has an impact on growth and establishment of STEC infection (28-30). However,

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there is a significant gap in our knowledge regarding the way the intestinal microbiota affects STEC/EPEC persistence, Stx activation and disease outcome. For this, a metagenomics approach based on RNA sequencing can be used. It will help us to understand how the intestinal microbiota affects the likelihood of disease development with regard to a STEC/EPEC infection and will also allow us to observe host mRNA expression. In addition, proteomics and transcriptomics of the selective *E. coli* strains will enable us to observe the gene expression to get an idea about their relevance in disease development and their contribution to survival mechanisms.

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