Shiga toxin-producing *Escherichia coli* O104:H4: An emerging important pathogen in food safety

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In 2011, Shiga toxin-producing *Escherichia coli* O104:H4 resulted in a large outbreak of bloody diarrhea and hemolytic uremic syndrome (HUS) in Germany and 15 other countries in Europe and North America. This event raised a serious public health crisis and caused more than two billion US dollars in economic losses. In this review, we describe the classification of *E. coli*, the Germany outbreak, and the characteristics and epidemical source-tracing of the causative agent. We also discuss the genomics analysis of the outbreak organism and propose an open-source genomics analysis as a new strategy in combating the emerging infectious diseases.

Shiga toxin-producing, *Escherichia coli*, O104:H4, hemolytic uremic syndrome, outbreak, open-source genomic analysis

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The large outbreak of food poisoning caused by the Shiga toxin-producing *Escherichia coli* O104:H4 in Germany from May to July, 2011 [1] threw the public health agencies into an embarrassing situation. Many strains of *E. coli*, with varying virulence, have caused many outbreaks in history [2–10] and this bacterium is one of the most studied pathogens. However, effective measures of identification, prevention, and treatment were insufficient during the whole outbreak process. In the past, Shiga toxin-producing and sorbitol-negative *E. coli* O157:H7 was the main agent responsible for the majority of outbreaks. However, Shiga toxin-producing and sorbitol-fermenting *E. coli* strains other than O157:H7 have recently emerged, with the ability to cause outbreaks [11–23]. The 2011 Germany outbreak of the O104:H4 has brought renewed attention to this specific category of *E. coli*.

1 Classification of *E. coli*

E. coli is a Gram-negative, rod-shaped bacterium, which is

usually serotyped by the somatic (cell wall) “O” antigen and the flagella “H” antigen. Capsular “K” antigen has also been employed to serotype this bacterium. Over 700 serotypes of the bacterium *E. coli* have been identified, with O157:H7 being the most notorious. Most of these serotypes are non-pathogenic facultative bacteria in the human intestine, in plants, and in the environment. A few *E. coli* strains, however, have developed the ability to cause three types of disease in humans: intestinal diseases (gastroenteritis), urinary tract infections (UTI), and neonatal meningitis. The distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, etc., and abilities to withstand host defenses, determine the pathogenicity of a particular strain of *E. coli* (Table 1). Pathogenic *E. coli* can be divided further according to the known pathogenesis, namely, diarrheagenic *E. coli* that caused enteric disease, including ETEC (enterotoxigenic *E. coli*), EIEC (enteroinvasive *E. coli*), EHEC (enterohemorrhagic *E. coli*), EPEC (enteropathogenic *E. coli*), EAEC (enteroaggregative *E. coli*), and DAEC (diffusely adherent *E. coli*) [24]; uropathogenic *E. coli* (UPEC) that caused urinary tract infection [25]; and neonatal meningitis-associated *E. coli* (NMEC)

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Table 1 Selected virulence factors produced by different pathovars of *E. coli*

Pathovar	Adhesins	Invasive ability and related factors	Toxins or other virulence determinants	Symptoms
ETEC	fimbrial adhesins e.g. CFA I, CFAII, K88, K99	no	heat-labile enterotoxin (LT); heat stable toxin (ST)	watery diarrhea in infants and travelers; no inflammation, no fever
EIEC	nonfimbrial adhesins, possibly outer membrane protein	yes An invasion-related plasmid pInv carrying the mxi and spa loci, which encodes a type III secretion system.	No specific toxin was identified. However, a 63-kD protein encoded by a plasmid-carrying gene <i>sen</i> was proven related to the enterotoxigenic activity.	dysentery-like diarrhea (mucous, blood), severe inflammation, fever
EPEC	nonfimbrial adhesin (intimin) bundle-forming pilus (BFP)	yes, but lower than <i>Shigella</i> or EIEC	some strains to produce Shiga-like toxin; ST-like toxin (EAST1); Four secreted proteins, EspA (25 kD), EspB (38 kD; formerly called EaeB), and EspD (40 kD), are essential for the A/E phenotype.	usually infantile diarrhea; watery diarrhea with blood, some inflammation, no fever; symptoms probably result mainly from invasion rather than toxigenesis
EAEC	aggregative adherence fimbriae (AAF)	not determined	EAST1; Hemolysin; Two possible cytotoxins, 108-kD and 120-kD proteins, were also identified.	persistent diarrhea in young children without inflammation or fever
DAEC	a surface fimbria a 100-kDa OMP	not determined	Not determined	Not epidemiologically defined because some strains could not elicit diarrhea upon human volunteer challenge and no outbreaks of DAEC-associated illness have been documented to date.
EHEC	Intimin; <i>E. coli</i> common pilus (ECP); haemorrhagic coli pilus (HCP)	yes, but lower than <i>Shigella</i> or EIEC	Shiga toxins (also called verotoxins); EAST1; Enterohemolysin	pediatric diarrhea, copious bloody discharge (hemorrhagic colitis), intense inflammatory response, may be complicated by hemolytic uremia
UPEC	P fimbria (or pyelonephritis-associated pili [PAP]); Type 1 fimbriae	yes fimbrial adhesin H (FimH), which is found at the tip of the type 1 fimbriae.	α -hemolysin (HlyA); vacuolating autotransporter toxin (Vat); Siderophores for iron acquisition	urinary tract infections (UTI)
NMEC	outer membrane protein A (OmpA); Type 1 fimbriae	yes Cytotoxic necrotizing factor 1 (CNF1); OmpA; Type 1 fimbriae	Endotoxin; K-1 antigen; Chu haem transport system	Neonatal meningitis

that caused human neonatal meningitis [26]. As molecular genetics technology has advanced in recent years, there has been increasing interest in extending the classification of *E. coli* from phenotype to genotype. *E. coli* was divided into five genetic distinct phylogroups, namely, A, B1, B2, D, and E, through multi-locus enzyme electrophoresis (MLEE) [27]. Furthermore, *Shigella*, a genus of strains that cause dysentery, was proven genetically closely related to *E. coli* and actually can be regarded as another phylogroup of *E. coli* [28,29]. The robustness of phylogroups was validated separately by the multi-locus sequence typing (MLST) analysis [30] and phylogenomics research based on whole-genome sequencing technology [31].

2 Outbreak of Shiga toxin-producing *E. coli* O104:H4

As early as 2001, a hemolytic uremic syndrome-associated

enterohemorrhagic *E. coli* (HUSEC) with serotype O104:H4, HUSEC041 (also designated as 01-09591), was isolated in Germany from patients [32]. This strain could produce Shiga-toxin 2 and belonged to sequence type 678, which was same as the 2011 outbreak strains [33]. Unfortunately, no detailed report exists on the symptoms of the patients from whom this strain was isolated. The first complete report on a case of hemolytic uremic syndrome (HUS) caused by *E. coli* O104:H4 was from Korea in 2006 [34], on a 29-year-old healthy woman. The bacteria were thought to have been transmitted through the consumption of hamburgers; however, the exact origin of the pathogen was not clear. The patient presented with bloody diarrhea, abdominal pain, hemolytic anemia, thrombocytopenia, and acute renal failure. The *E. coli* O104:H4 was isolated from her stool specimen. Fortunately, the patient finally recovered with obvious sequelae through timely proper treatment with plasmapheresis and hemodialysis for three weeks.

From May 1 to July 26, 2011, an unusual outbreak of

diarrhea with HUS hit Germany and spread to other European countries. The outbreak then spread to the United States and Canada. In total, there were 4075 cases reported: 3935 in Germany and 140 from other countries according to a WHO report (Shiga toxin-producing *E. coli* (STEC): Update on outbreak in the EU (27 July 2011, 11:00), <http://www.euro.who.int/en/what-we-do/health-topics/emergencies/international-health-regulations/news/news/2011/07/outbreaks-of-e.-coli-o104h4-infection-update-30>, accessed by Sept 16, 2011). The cases recorded in Germany were 3078 EHEC and 857 HUS cases, with 48 dead; in other countries, there were 89 EHEC and 51 HUS cases. The Robert Koch Institute (RKI), the university medical center Hamburg-Eppendorf, and the health department of the Hamburg northern district reported that the epidemic profile of this outbreak was caused by Shiga toxin-producing *E. coli* O104:H4 [35]. This outbreak is unusual because majority of the cases (89%) were with adults (median age, 43 years) with a preponderance of cases (68%) occurring in women. In addition, HUS developed in a quarter of the symptomatic outbreak cases, contradicting a previous report that HUS usually develops in children under five years old [24]. The infection caused by O104:H4 had an unusually long incubation period (eight days on average) than that of conventional *E. coli* infections (three days on average), which confused clinicians and delayed timely recognition.

3 The pathogen of *E. coli* O104:H4

As mentioned above, O104:H4 was found ten years ago in 2001 in Germany and five years ago in 2006 in Korea [32,34], indicating that this bacterium is not a newly-emerged pathogen. Nevertheless, the extensive characterization of this pathogen only began in 2011 due to the widespread outbreak [33,36]. The outbreak strain turned out to be an unusual combination of the pathogenic features of “stacked brick” aggregation of adherence to HEp2-cells in culture, which is typical of EAEC, and of Shiga toxin production, which is typical of EHEC [36]. Compared with HUSEC041 that was isolated in 2001, the outbreak strain acquired an extended-spectrum β -lactamase phenotype as well as resistance to many other types of antibiotics [33]. Detection of other virulence-associated genes characterizing both EAEC and EHEC indicated that the virulence backbone of the outbreak strain should be EAEC. In addition, bacteriophage-encoding Shiga toxin 2 was acquired from EHEC or an unknown environment, along with other antibiotics-resistant genes from unknown sources [33,36–38].

4 Epidemic source-tracing of Shiga toxin-producing *E. coli* O104:H4

After recognizing the unusual outbreak, German scientists

scrambled to find its source. Local authorities in Hamburg announced on May 26 that they had isolated the EHEC bacteria from four cucumbers imported from Spain (<http://news.sciencemag.org/scienceinsider/2011/05/cucumbers-may-be-culprit-in-mass.html>, accessed by Sept 16, 2011). However, it was confirmed five days later that the EHEC isolated from Spanish cucumbers was not same as the outbreak strain; the mistake led to a loss of millions of Euros from Spanish agriculture. German scientists then conducted a series of epidemiological investigations for determining the source of the outbreak strain. Bean sprouts were suspected as the source (http://www.mzh.government.bg/MZH/Libraries/Actual2/E_coli_23_July_ENG_corr.sflb.ashx, accessed by Sept 16, 2011). Although several outbreaks of *Salmonella* and *E. coli* O157:H7 infections had been reported to be associated with raw seed sprouts [39], no O104:H4 strain had been isolated from any sprout. It need further works to confirm if 2011's outbreak was caused by contaminated sprouts or bean seed. After a cluster of patients with bloody diarrhea was reported in Bordeaux, France, the European food safety authority (EFSA) conducted a source-tracing analysis, revealing that sprouts of fenugreek were suspected as a contamination source (www.efsa.europa.eu/en/supporting/doc/176e.pdf, accessed by Sept 16, 2011). When the source-tracing information from both French and German outbreaks was analyzed together, the fenugreek seeds imported from Egypt were the most likely common source. Although extensive epidemiological investigations were implemented, there had previously been no bacterial evidence to confirm any food as the source of *E. coli* outbreak in Germany.

5 Genome sequencing and comparative genomics of O104:H4

Concurrent with the epidemiological investigation, scientists around the world rushed to sequence the whole genome of the outbreak strains to understand their phenotypic and virulent features, as well as acquire the strain-specific sequences using in designation of PCR diagnostic primers [37,38,40–42]. We sequenced one outbreak strain, TY2482, isolated by the University Medical Center Hamburg-Eppendorf during the early phase of the outbreak from a 16-year-old female patient suffering from hemorrhagic enterocolitis. We deciphered the whole genome sequences of the strain within five days after receiving the DNA sample using the Ion Torrent Personal Genome Machine, which is a semiconductor-based third-generation sequencing platform. About a week later, the sequencing data from the Illumina HiSeq 2000 sequencing system was acquired and used to improve the assembly results. Finally, one chromosome and three plasmids were assembled and identified from this strain [37].

To identify the phylogenetic position of the TY2482, we selected 30 whole genome sequences of *E. coli* strains

(including 12 avirulent strains and 18 that belong to five different pathotypes, which can be used to represent the diversity of the species) and eight *Shigella* strains (Table S1). We used this data to build the maximum likelihood tree based on 177223 whole genome-wide SNPs. Intriguingly, TY2482 is most closely related with a Central Africa-isolated EAEC strain, 55989 although clinical symptoms were reported similar to those caused by EHEC strains [31]. According to the phylogenetic tree, four O157 EHEC strains belong in a different phylogroup (group E) from TY2482, three non-O157 EHEC strains and TY2482 belong to the same group (B1) but still have long genetic distance (Figure 1). Therefore, the strain that caused the outbreak should be defined as EAEC-lineage rather than EHEC in terms of phylogeny.

When we employed the seven conserved housekeeping genes of this new Shiga toxin-producing EAEC strain to compare with the database (<http://mlst.ucc.ie/mlst/dbs/Ecoli>) of more than 3800 *E. coli* strains, we found that the outbreak strain is identical to strain 01-09591, which was isolated 10 years ago in Germany [32]. In addition, TY2482 and 01-09591 have the same profiles in 12 virulence/fitness

genes, whereas the Shiga toxin-producing gene and tellurite-resistance-genes were not detected in 55989. These findings imply that the 01-09591 is closer to the current outbreak strain. Although the genome sequence of the strain 01-09591 indicated that it was different from the 2011 outbreak strain in terms of plasmid content and fimbrial genes [41], the fact that the German-origin O104:H4 isolate is closely related to the Germany outbreak strain is still of scientific interest.

The EAEC strain 55989 was only associated with persistent diarrhea, whereas TY2482 can cause HUS [37]. We carried out whole genome comparative studies between the two strains to explore the possible genetic mechanisms of different virulence phenotypes. Approximately 96% chromosomal regions in strain 55989 can be covered by chromosome sequence of TY2482 with extensive synteny conservation along the chromosome, and only 6% chromosome sequence of TY2482 was strain-specific, indicating highly consistent genomic content between the two strains. Only one among the three TY2482 plasmids reveals similarity with 55989, but carried a rare type of aggregative adherence fimbria gene cluster, AAF/I, rather than the AAF/III in

Figure 1 Phylogenetic tree of 39 *E. coli*/*Shigella* strains based on whole genome wide SNPs. The maximum likelihood phylogeny was built using PHYML, with 1000 repeat bootstrap tests. The numbers near internal nodes indicate the percentage of the supported bootstrap value. The scale bar represents substitutions per SNP site. The colored bar at the right of the tree represents different phylogroups. The tip names were colored according to pathovars as per the legend listed below the tree.

55989. In the case of the other two plasmids absent in 55989, the larger of these encodes a CTX-M-15 extended-spectrum beta-lactamase and is highly homologous with the pEC plasmid identified in other *E. coli* strains [43]. The smaller of these is a selfish plasmid carrying only two genes and one encoding DNA replication protein. On TY2482-specific chromosomal regions, we identified 553 genes, 316 of which are clustered and were annotated as seven regions of difference (RODs). The RODs and the plasmids encoded a variety of virulent determinants, antibiotic resistance, and fitness associated proteins; I-ROD2 (I-ROD, region of difference involving an insertion) in particular is a prophage that contains the *stx2* gene for Shiga toxin, indicating that horizontal gene transfer events play important roles for high virulence and survival of TY2482 [37].

The outbreak strain presented EHEC pathotypes, thus, we investigated the genome content of TY2482 for possible EHEC virulent determinants. There are 136 previously reported EHEC virulence-related genes [44] in TY2482 genome (Table S2), only 19 of which are present in most EHEC strains and less prevalent among non-EHEC strains (*Fisher exact test*, $P < 0.01$, Table 2). Two genes, *stx2* and *lom*, could only be found in EHEC strains and TY2482. The gene *lom* is located on the *stx2*-bearing phage and involved in *E. coli* adhesion [45]. Notably, four other adhesion-related genes present in TY2482 were also identified in almost all EHEC strains, stressing the utmost importance of adhesion for EHEC pathogenicity. However, the most prevalent pathogenicity island in typical EHEC strains, LEE (locus of

enterocyte effacement), which encodes the Type III secretion system responsible for attaching and effacing lesions in the intestine, thought to facilitate the translocation of the Shiga-toxin into the bloodstream of the host [24], was not detected in the TY2482. It was supposed that the enteroaggregative virulence factors in the outbreak strain may compensate for properties of LEE, resulting in similar or even increased virulence. For example, *iha* could promote the adherence of *E. coli* to the bovine-originated epithelial cells [46]. Further, the production of fimbria I gene cluster-encoded fimbriae leads to biofilm formation, which also contributes to the stable adherence of *E. coli* cells to human epithelia [47,48]. Further experimentation is needed to test if and how the unusual combination of EAEC-related adherence mechanisms with *stx2* and additional EHEC-derived virulence determinants contribute to the shift in populations at risk (i.e. the preferential infection of women) and the specific clinical course of the disease.

6 Open-source genomics

We developed a new strategy, open-source genomics [37], for combating emerging infectious diseases, in addition to helping us understand the phenotypic and pathogenic features of the outbreak strain, and developing a rapid nucleic acid-based diagnostic method [42] through rapid whole genome sequencing analysis. In the past, scientists usually release their genome sequencing data after their paper is

Table 2 The EHEC-prevalent virulence genes in TY2482

Gene ID	Product	EHEC(7)*	Non-EHEC(31)*	P value**
Afimbrial adhesins				
GL004286	AidA-I adhesin-like protein	7	5	6.28×10^{-5}
GL005117	Iha adhesion	6	7	3.54×10^{-3}
GL003010	predicted adhesin	7	10	1.54×10^{-3}
pTY2GL0061	adherence factor, Efa1 homolog	7	13	8.66×10^{-3}
Genes the confer resistance to the host defense response				
GL005073	Lom type2	5	0	4.18×10^{-5}
Toxins and their activation and secretion				
GL005048-9	Shiga toxin 2 subunit AB	6	0	2.54×10^{-6}
Regulators				
GL002293	PchD	5	4	4.25×10^{-3}
GL002123	EtrA	7	6	1.36×10^{-4}
T3SS and effector homologues				
GL000529	EspR1	7	10	1.54×10^{-3}
GL003969	EspX1	7	6	1.36×10^{-4}
GL002119-29	ETT2	6	5	1.01×10^{-3}

* The number in parentheses indicates the number of EHEC or Non-EHEC strains used in analysis. ** Two genes with low *P*-value harbored in two multi-gene clusters respectively; as most genes in the clusters are frequently observed in both EHEC and EAEC strains, these two genes were excluded from this table.

accepted for publication. For our strategy, we released the genome data, including raw data, into the public domain under a Creative Commons 0 license, as soon the preliminary sequences were obtained. This elicited a burst of crowd-sourced, curiosity-driven analyses carried out by bioinformaticians on four continents, which provided public society opportunities to propose the possible origins, identification methods, and reasons for the virulence and antibiotic-resistance of the outbreak strain. (<https://github.com/ehc-outbreak-crowdsourced/BGI-data-analysis/wiki>). We then took advantage of intellectual scientists around the world to improve our analysis. This type of internet-connected collaboration based on common interests could play key roles in future combat against infectious diseases.

The Germany O104:H4 outbreak is over, but the war between humans and pathogens never ends. As long as rapid urbanization, environmental change, the expansion of human activity, the rapid development of transportation, and the increasing number poor and hungry people in numerous regions around the world are still problems for us to tackle, pathogens will find more chances to interact with humans, and new infectious diseases will be continually emerging. Therefore, we should take advantage of new advances in technology, new developments in strategy, and our network of intellectuals around the world to develop new mechanisms to achieve victory in the battle against infectious diseases.

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Supporting Information

Table S1 List of strains that used in phylogenetic tree construction

Table S2 EHECs virulent-associated genes harbored in TY2482

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