Review

Outbreak of Shiga Toxin–Producing *Escherichia coli* (STEC) O104:H4 Infection in Germany Causes a Paradigm Shift with Regard to Human Pathogenicity of STEC Strains

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ABSTRACT

An outbreak that comprised 3,842 cases of human infections with enteroaggregative hemorrhagic *Escherichia coli* (EAHEC) O104:H4 occurred in Germany in May 2011. The high proportion of adults affected in this outbreak and the unusually high number of patients that developed hemolytic uremic syndrome makes this outbreak the most dramatic since enterohemorrhagic *E. coli* (EHEC) strains were first identified as agents of human disease. The characteristics of the outbreak strain, the way it spread among humans, and the clinical signs resulting from EAHEC infections have changed the way Shiga toxin–producing *E. coli* strains are regarded as human pathogens in general. EAHEC O104:H4 is an emerging *E. coli* pathotype that is endemic in Central Africa and has spread to Europe and Asia. EAHEC strains have evolved from enteroaggregative *E. coli* by uptake of a Shiga toxin 2a (Stx2a)–encoding bacteriophage. Except for Stx2a, no other EHEC-specific virulence markers including the locus of enteroaggregative fimbrial pili encoded by the enteroaggregative *E. coli* plasmid. The aggregative adherence fimbrial colonization mechanism substitutes for the locus of aggregative adherence function for bacterial adherence and delivery of Stx2a into the human intestine, resulting clinically in hemolytic uremic syndrome. Humans are the only known natural reservoir known for EAHEC. In contrast, Shiga toxin–producing *E. coli* and EHEC are associated with animals as natural hosts. Contaminated sprouted fenugreek seeds were suspected as the primary vehicle of transmission of the EAHEC O104:H4 outbreak strain in Germany. During the outbreak, secondary transmission (human to human and human to food) was important. Epidemiological investigations revealed fenugreek seeds as the source of entry of EAHEC O104:H4 into the food chain; however, microbiological analysis of seeds for this pathogen produced negative results. The survival of EAHEC in seeds and the frequency of human carriers of EAHEC should be investigated for a better understanding of EAHEC transmission routes.

A large outbreak comprising 3,842 cases of human infection with enteroaggregative hemorrhagic *Escherichia coli* (EAHEC) O104:H4 occurred in Germany in May and June 2011 (6, 50, 51, 87). The life-threatening hemolytic uremic syndrome (HUS), which is a sequela of enterohemorrhagic *E. coli* (EHEC) infection, developed in 855 patients (22.3%), and the infection caused the deaths of 53 people (87, 89). The outbreak spread to other countries inside and outside the European Union (33, 42). The characteristics of the outbreak strain, the way it spread among humans, and the clinical picture resulting from EAHEC O104:H4 infections have changed the way Shiga toxin–producing *E. coli* (STEC) strains are regarded as human pathogens in general. Because of the severity of disease expression and the high proportion of patients that developed HUS, this outbreak was much worse than previous outbreaks (87). The high proportion of adults affected in this outbreak and the unusually high number of adult women who developed HUS (68%) makes this outbreak the most dramatic since EHEC strains were first identified as agents of human disease (51, 87).

**EAHEC O104:H4 STRAIN EVOLVED FROM ENTEROAGGREGATIVE *E. COLI***

The EAHEC O104:H4 infection outbreak raised questions about the criteria commonly used to determine what causes an STEC strain to become a human pathogen. STEC strains have been divided into hundreds of *E. coli* serotypes, and more than 400 STEC serotypes have been isolated from human patients (94). The diversity of the STEC group is based on the spread of stx genes to unrelated types of *E. coli* by bacteriophage-mediated horizontal gene transfer (56). In retrospect, enteroaggregative and nonaggregative STEC O104:H4 strains have only rarely been isolated and therefore were not regarded as important STEC serotypes. However, sporadic cases of STEC O104:H4 infections have been recorded worldwide, indicating that this hitherto almost unknown pathogen has spread globally (98). The first cases of human EAHEC O104:H4 infection

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described date back to the year 2001. Two siblings living in Cologne, Germany, were affected and developed hemorrhagic colitis (HC) and subsequently HUS (18). The source of the EAHEC O104:H4 infection in the children could not be identified as was also the case for other EAHEC O104:H4 infections reported between 2001 and 2011. Nucleotide sequencing of the genomes of independent EAHEC isolates revealed similarities of 99.5 to 99.8%, indicating that EAHEC O104:H4 strains isolated in different countries were of the same clonal origin and type (23, 85, 91). Nucleotide sequencing of the genome of an EAHEC O104:H4 patient strain isolated in May 2011 in Germany revealed that it shared 99.8% nucleotide sequence similarity with the Shiga toxin–negative enteric aggregative E. coli (EAEC) O104:H4 strain 55989, which was isolated in 1995 from a diarrheic stool sample from a patient in Central Africa that was infected with human immunodeficiency virus (8, 54, 91). Strains similar to EAEC O104:H4 55989 were isolated in 2009 from diarrheic children in Mali, indicating that EAEC O104:H4 might be common in some Central African countries (85, 93).

The Shiga toxin 2a (Stx2a)–producing EAHEC O104:H4 strains that were isolated in 2001 in Cologne, Germany, and in 2011 as the causative agent of the May and June outbreak in Germany possess the full virulence attributes of EAEC strains (18). However, the 2001 strain differs from the 2011 strain in the type of aggregative adherence fimbrial (AAF) subunits and in the composition of their EAEC virulence plasmids (18, 23). An enhanced resistance to multiple antimicrobial agents resulting in an extended-spectrum β-lactamase phenotype has been found in the 2011 outbreak EAHEC O104:H4 strain when compared with older EAHEC O104:H4 isolates (18, 68).

A number of Stx-producing O104:H4 strains were isolated from humans in Korea, France, Denmark, the Republic of Georgia, and Finland between 2004 and 2010 (7, 18, 33, 92, 93, 97, 98). A table listing the origins, clinical data, and some properties of these strains was published in a joint report from the European Centre for Disease Prevention and Control and the European Food Safety Authority (EFSA) (98). Only some of the EAHEC O104:H4 isolates of other origins were similar to the German May and June 2011 outbreak strain in their virulence attributes and genotypes. The most similar strains were isolated from patients in the Republic of Georgia in 2009 and in Finland in 2010 (93).

The EAHEC O104:H4 German outbreak strain was different in serotype and virulence markers from STEC O104:H2 and STEC O104:H21 strains isolated from outbreaks and sporadic infections of humans in Germany, the United Kingdom, and the United States (98).

**IN CLASSICAL EHEC, THE LOCUS OF ENTEROCYTE EFFACEMENT AND STX2 ARE ASSOCIATED WITH STRAINS CAUSING HC AND HUS IN HUMANS**

All Stx-producing E. coli O104:H4 strains described previously were negative for genetic elements typical of classical EHEC strains, namely the locus of enterocyte effacement (LEE) and the EHEC virulence plasmid (18, 93, 98). The chromosome-encoded LEE pathogenicity island carries the genes (eae and others) coding for the attaching and effacing phenotype. The EHEC plasmid carries genes coding for enterohemolysin (ehxA) and other factors involved in the disease process. The LEE and EHEC plasmid are hallmarks of classical EHEC strains belonging to major serotypes, which cause more than 80% of HC and HUS cases in Europe and the United States (22, 38, 44, 61).

Of these typical EHEC virulence markers, only the stx2a gene is present in the EAHEC O104:H4 strains (23, 85, 91). The stx2a gene was located on a lambdoid bacteriophage, which was integrated into the genome of EAHEC O104:H4 strains. The Stx2a type belongs to a group of Shiga toxin genes in the Stx2acd group (stx2a, stx2c, and stx2d), which are genetically closely related (84). The presence of these Stx2acd types in classical EHEC strains has been reported to be associated with HUS in infected patients (52).

Functional stx2acd genes and toxin production also were detected in 43.4% of 219 STEC strains that were isolated from food samples in Germany (16). The stx2acd–positive strains were distributed over the most frequently occurring serotypes of STEC isolated from food (O113:H21, O8:H9, O22:H8, O174:H21, O8:H19, O178:H19, O179:H8, and O2:H27) (67). Despite their frequent occurrence in food samples, these serotypes of Stx2acd-producing STEC strains play only a minor role in human STEC infections and are rarely implicated in outbreaks and in cases of HC and HUS (13, 61, 107). One explanation for this finding could be that these STEC strains cannot colonize humans efficiently enough to cause disease. The LEE pathogenicity island and non-LEE (nle) effectors were absent in these STEC strains (24, 25, 67). The presence of LEE and non-LEE pathogenicity island–mediated adherence and effector systems in classical EHEC O157 and non-O157 strains was significantly associated with their ability to cause HC and HUS in humans (25, 35, 61). Classical EHEC strains carrying the LEE pathogenicity island were detected only in less than 3% of the foodborne STEC isolates from Germany (16, 67, 69, 107).

These findings indicate that an efficient intestinal colonization system such as the LEE is a prerequisite for STEC strains to cause severe illness in the human host. The interaction between the LEE colonization system and Stx2 as virulence factors in classical EHEC and STEC strains has been explored in various studies. All have indicated an increased risk for humans to develop HUS when the stx2 and eae genes both are present in the infecting strain (20, 59, 60). The interaction between the presence of the eae gene (for LEE) and Stx2 as virulence factors in STEC strains was first explored by Boerlin et al. (20). These authors found a significant correlation between severe disease in humans and the concomitant presence of stx2 and eae in a study performed on Canadian STEC strains. Using a multivariate logistic regression model Ethelberg and coworkers (39) identified the presence of eae (odds ratio [OR] = 6.0) and stx2 (OR = 2.5) as risk factors for HC in patients infected with STEC. Risk factors for development of HUS were the
presence of stx₂ (OR = 18.9) and eae (OR undefined) and patient age (≤7 years; OR = 11.4) (39). Similar findings were reported in a study performed on 20 HUS cases in Denmark, indicating that both stx₂ and stx₂c were associated with the development of HUS in a multivariate logistic regression model. The presence of eae was strongly associated with HUS but could not be included as a variable in the model because this gene was identified in isolates from all 20 HUS cases. The patient age of ≤7 years also was a risk factor for the development of HUS (OR = 10.4) in the adjusted logistic regression analysis (86).

A study analyzing the interaction between STEC virulence markers and patient age was performed on a large group of 608 patients infected with STEC in Germany (13). An analysis of the STEC and EHEC virulence markers and the underlying clinical picture and age of 608 human patients infected with STEC in Germany revealed that the presence of Stx2acd toxins alone was not statistically significant as a risk factor for the development HC and HUS (OR = 0.241) (Fig. 1) (66). A multivariate statistical analysis revealed that the concomitant presence of Stx2acd toxins, the LEE gene (eae), and the EHEC hemolysin (e-hlyA) genes, and the age of 108 patients with severe clinical signs (HUS and HC) in comparison to 500 patients with uncomplicated diarrhea, abdominal pain, or no signs of enteric disease.

FIGURE 1. Risk factors for HC and HUS among 608 STEC patients. Odds ratio (OR) with 95% confidence interval (CI) is given. The bold horizontal line indicates an OR of 1 (no effect for HC and HUS). A logistic regression model was obtained by backward elimination to detect significant four-way interaction terms. The model describes the association between STEC virulence factors, i.e., stx, LEE (eae), and EHEC hemolysin (e-hlyA) genes, and the age of 108 patients with severe clinical signs (HUS and HC) in comparison to 500 patients with uncomplicated diarrhea, abdominal pain, or no signs of enteric disease.

IN STX2-PRODUCING EAHEC STRAINS, THE AGGREGATIVE COLONIZATION MECHANISM (AAF-PILI) CAN SUBSTITUTE FOR THE LEE FUNCTIONS AS VIRULENCE ATTRIBUTES FOR CAUSING HC AND HUS IN HUMANS

An important lesson to learn from the EAHEC O104:H4 infection outbreak is that LEE and the nle genes present in classical EHEC strains can be substituted by EAEC plasmid-encoded aggregative adherence mechanisms to enable Stx2-producing EAHEC to cause HC and HUS in humans. EAHEC O104:H4 strains possess the typical plasmid and chromosomally inherited virulence markers of EAEC, such as AAF (aggA and agg3a), dispersin (aap), and the Pic (pic) proteins (18, 57). The best studied virulence marker of EAEC is the aggR gene, which is the master regulator of EAEC plasmid and chromosomally inherited virulence genes (34, 57, 75). The aggregative adherence mediated by AAF is efficient for colonization of the human
immunodeficiency virus in Central Africa (54). Stx2 producers were found among the EAEC strains isolated from the African HC and HUS patients, but the serotypes of the African EAHEC strains were not determined (72). Production of Stx2 was associated with EAEC O111:H2 strains that were isolated from children with HUS in France (71). Like EAHEC O104, the EAHEC strains from patients in the Central African Republic and the EAHEC O111 strains from France were negative for LEE (eae) and EHEC plasmid (ehxA) genes (71, 72). The detection of EAHEC strains other than O104:H4 indicates that EAHEC is an emerging group of human pathogens that covers more than one serotype of E. coli. The virulence properties of the EAHEC strains indicate that the combination of an aggregative adherence system and the production of Stx2 is sufficient for to cause HUS in human patients.

**A REVISED CONCEPT IS NEEDED FOR RISK ASSESSMENT OF STEC AND EHEC AS HUMAN PATHOGENS THAT INCLUDES COLONIZATION FACTORS OF THE BACTERIA**

Regarding the importance of intestinal colonization, data suggest that any Stx2acd-producing E. coli strain that colonizes the human intestine might be able to cause HC and HUS. The uptake of the stx2 gene by the bacteria occurs by lysogenization with Stx-encoding bacteriophages, and an EAEC ancestor of the EAHEC O104:H4 strain probably acquired the stx2 phage by horizontal gene transfer (85, 91). The uptake of stx phages is not rare in E. coli and other Enterobacteriaceae (17, 37, 58, 59, 81, 95). Naturally occurring phage uptake has been demonstrated and is favored by the abundance of free stx phages in the environment (58, 59).

The present findings indicate that most of the Stx2acd-producing STEC strains frequently occurring in animals and food are not important human pathogens because they do not colonize the human host efficiently. Most of the LEE-negative STEC strains isolated from animals and food samples were closely associated with certain animal species, and only some of these strains have been found in humans (67). These STEC strains might carry animal-host–specific colonization factors; many of these strains were part of the resident flora of domestic ruminants (12).

Investigation of more than 300 STEC strains isolated from food samples of animal origin (National Reference Laboratory for E. coli, Federal Institute for Risk Assessment, Berlin) for the presence of the aggR gene produced negative results (11). The regulator gene aggR is present in EAEC and EAHEC strains and is considered an indicator for the presence of the aggregative adherence mechanism (34, 57, 74, 75). None of the 608 STEC strains from human patients were positive in a DNA hybridization assay with the EAEC virulence plasmid–specific pCVD432 probe (13). These findings indicate that typical STEC strains isolated from food, animals, and humans do not harbor the EAEC virulence plasmid, which confers aggregative adherence properties. Genetic analysis of known EAHEC strains indicates that they are more likely to be generated from...
EAEC strains by the uptake of stx phages than from STEC strains by the uptake of EAEC plasmids.

The importance of successful intestinal colonization for toxin delivery and subsequent illness in patients was revealed previously for enterotoxigenic E. coli (ETEC) strains. ETEC strains differ in colonization factors that determine animal and human host specificity (49, 73). As a consequence, animal ETEC strains are not pathogens in human patients and vice versa. In contrast, animal and human ETEC strains are very similar regarding the heat-stable and heat-labile enterotoxins they produce, which are virulence markers for watery diarrhea caused by ETEC infection (49, 73).

Because Stx2a is the only EHEC-specific virulence marker found in the EAHEC strains (18, 23, 71, 72, 85, 91), a revision of the current concepts for source attribution and classification of human virulent STEC strains is required (45). The current European and American standards for food safety control are restricted to the detection of classical EHEC serotypes, e.g., strains carrying both stx and eae genes (45, 46, 97, 102). On this basis, all EAHEC strains including EAHEC O104:H4 would have been grouped as having little or no pathogenicity for humans.

A BLAST search revealed that the aggR is conserved in various EAEC and EAHEC strains as a regulator of EAEC plasmid virulence genes, and an aggR-specific real-time PCR was developed for detection (11). Therefore, aggR could be used as a candidate marker in addition to stx and eae for future risk assessment of STEC strains from all kinds of samples to detect EAHEC strains.

An exception from current European and American standards for STEC in food is the STEC surveillance system for food samples in Germany. According to German legislation (§64 German Gesetzbuch für Lebensmittel und Futtermittel [Food and Feed Code]), all STEC strains independent of their serotypes and other properties are regarded as potential human pathogens. As a consequence, the presence of any STEC strain in food is regarded as potentially harmful to human health (86). A disadvantage of this approach is that a total surveillance for all STEC types makes the investigation of food samples cumbersome because the methods for detection of all STEC types are laborious and time-consuming (4, 5, 15). One lesson learned from the EAHEC O104 infection outbreak is that the concepts currently used for the risk assessment of STEC strains and for the analysis of food samples urgently need to be revised to detect emerging human pathogenic STEC and EAHEC strains.

NATURAL RESERVOIR FOR EAHEC O104:H4 IS HUMANS IN CONTRAST TO STEC AND EHEC, WHICH HAVE ANIMAL RESERVOIRS

STEC and classical EHEC infections are regarded as zoonotic diseases, and the reservoirs for these strains are mainly domestic animals and wildlife, particularly ruminants (31). Food of all kinds, the environment, and water can be contaminated by STEC and EHEC excreted by animals (9). Humans can get infected by ingestion of STEC- or EHEC-contaminated food and water from a contaminated environmental source or directly from infected humans or animals (43, 47). The minimal infectious dose for EHEC O157 was reported as fewer than 100 cells, in contrast to the high minimal infectious dose (10^{10} CFU) reported for EAEC strains (74, 99). The EAHEC O104:H4 outbreak changes the paradigm of STEC sources because humans constitute the only natural reservoir known for this emerging group of EAHEC strains.

One of the striking features of the EAHEC O104:H4 infection outbreak was that adults were more often affected than in outbreaks of classical O157 and non-O157 EHEC infection (50, 87). Patients between 0 and 9 years of age were less often infected than those of any other age group (87). This finding is in contrast to previous descriptions of outbreaks and sporadic infections with classical EHEC, in which children and the elderly were identified as risk groups for development of HC and HUS (39, 66, 86, 106). Children younger than 11 years and adults older than 60 years of age were significantly more susceptible to the development of HC or HUS after infection with STEC (66). Patients between 12 and 60 years of age were more frequently infected with LEE-negative STEC strains. Most of these infections resulted in uncomplicated watery diarrhea, and severe disease was rarely reported in these patients (13, 66).

The high proportion of young children that become infected with classical EHEC and enteropathogenic E. coli strains indicates that infants are more susceptible than adults to infections with LEE-positive E. coli (100, 106). Protective immunity to intimin, the product of the eae gene, is acquired in early childhood, which might explain why adults are less affected by infections with classical LEE-positive EHEC and enteropathogenic E. coli strains (14, 30, 36, 64, 79, 80). Some LEE-negative STEC strains have other colonization mechanisms that might be more relevant in patients of various age groups (82, 83, 103, 104).

Acquired immunity plays an important role in human infections with EAEC. Healthy and subclinically infected individuals have frequently been identified in geographical regions where EAEC infections are frequent and endemic (77, 78). Experimental infections of humans with EAEC indicate that susceptibility varies among individuals, and EAEC strain-dependent differences play a role in human response. Patients from all age groups were susceptible to EAEC infections, and a high dose (10^{10} CFU) was needed to elicit diarrhea in human volunteers (74). Outbreaks of EAEC infection in humans have been reported from various countries and continents, indicating that EAEC strains are worldwide human pathogens (34, 74). Humans are the only natural reservoir identified for EAEC strains (74, 77). The transmission of EAEC by contaminated food was reported as a frequent cause of EAEC infection in tourists traveling to regions where these pathogens are endemic (57, 78). The spread of EAEC by the fecal-oral route, the ingestion of contaminated food and water, and individual host susceptibility were identified as major risk factors for EAEC infection in humans (57).

Some features of the EAHEC O104:H4 strains causing the large outbreak in Germany are also typical for EAEC infection in humans. Noteworthy are the preponderance of
adult patients, the transmission routes, the high frequency of asymptomatic infected persons, and the long-term carriage and excretion of the pathogen (51, 87). In contrast to EAEC, the median incubation period for EAHEC O104:H4 was 8 days (51). Much shorter incubation periods (8 to 18 h) were reported for human infections with EAEC (57, 74). The minimal infectious dose for EAHEC to cause disease in humans is not known; for EAEC 10⁵ CFU were needed to cause diarrhea in adult volunteers (74). The high proportion of women among patients developing HUS (68%) after infection with EAHEC O104:H4 is surprising and has not yet been explained. Gender-specific eating habits are an unlikely explanation because women and men had similar total numbers of EAHEC O104:H4 infections, but significantly more women were HUS patients (51). In contrast to infections with classical EHEC, the majority of HUS cases (89%) occurred in adults. Only 2% of the patients were younger than 5 years of age compared with 69% of this age group affected by classical EHEC infections (51, 87).

Asymptomatic human carriers also were important in the outbreak of EAHEC O104:H4 infection in May and June 2011 (87). The transmission of the EAHEC O104:H4 strain to food and secondary transmission from infected individuals were identified as major routes of human infection in various countries (3, 55, 63, 87). According to the Robert Koch Institute (89), infection from asymptomatic carriers or recovered patients that no longer show symptoms of disease is possible and may occur from human to human or by the contamination of food by shedders of EAHEC O104:H4 strains. A number of epidemiologically unlinked EAHEC O104:H4 infections have been reported (53, 55, 87). Long-term human excretion of EAHEC O104:H4 occurs, lasting up to 13 weeks (87). Present data indicate that the EAHEC O104:H4 strain has not become endemic in Germany (87). The official end of the EAHEC O104 outbreak was 26 July 2011; no outbreak-related infections were reported after 4 July 2011 (88, 89). However, seven cases of EAHEC O104:H4 infection were reported in the postoutbreak period (17 July to 14 August 2011) (87).

CONTAMINATED FENUGREEK SPROUTS WERE IDENTIFIED AS MAJOR TRANSMISSION VEHICLE OF THE EAHEC O104:H4 OUTBREAK STRAIN

Early in the outbreak, meat and milk were excluded as food sources by epidemiological investigations performed by the Robert Koch Institute, whereas cucumber, tomatoes, and leafy salads were suspected as possible vehicles of infection (50, 87). Consequently, a warning concerning the consumption of cucumber, tomatoes, and leafy salads was issued by official governmental sources, and thousands of samples of cucumber, tomatoes, and leafy salads were analyzed. STEC isolated from suspected cucumber samples from Hamburg turned out to be unrelated to EAHEC O104:H4 and were identified as Stx2a-positive STEC O8:H19 strains (28). STEC O8:H19 strains are frequently found in food samples of animal origin but are unrelated to the current EAHEC O104:H4 outbreak strain (67).

The analysis of 41 outbreak clusters in Germany revealed that EAHEC O104:H4–contaminated sprouted fenugreek seeds produced at a farm (farm A) in Lower Saxony were the initial vehicle of the May and June 2011 outbreak in Germany (41, 87). The sprout-producing facility was investigated thoroughly, but no EAHEC O104:H4 was isolated from hundreds of samples taken from seeds, sprouts, and the environment. However, one sample of mixed sprouts produced at this farm that was retailed at the onset of the outbreak was contaminated with the EAHEC O104:H4 strain. This sprout sample was recovered from the household of patients infected with EAHEC O104:H4 who claimed to have eaten the sprouts before they became ill. The EAHEC O104:H4 strain isolated from the sprout sample was undistinguishable in its virulence attributes and pulsed-field gel electrophoresis patterns from the EAHEC O104:H4 outbreak strain (10).

Early EAHEC O104:H4 infections occurring in May 2011 were associated with the consumption of sprouted seeds produced at farm A (87). Sprouts produced at this farm were withdrawn from the market beginning June 2011. Late infections occurring after mid-June 2011 were attributed to secondary transmission of the pathogen. Contaminated foods of different kinds were identified as vehicles in the secondary transmissions. The transmission of EAHEC O104:H4 to food by infected catering personnel was the cause of nine cases of illness among consumers (55, 87). EAHEC O104:H4 strains that were genetically indistinguishable from the patients’ strains were successfully isolated from smoked and cooked salmon, paprika, and cucumber, which had most probably been contaminated by human excretors (10). Food samples and household items from EAHEC O104:H4–infected patients were frequently contaminated with this strain, highlighting the importance of human carriers for the dissemination of this pathogen. Four cases of laboratory infections with EAHEC O104:H4, which had occurred in Germany, were reported by the Robert Koch Institute (55, 87).

Smaller satellite outbreaks most likely caused by asymptomatic and symptomatic human excretors of EAHEC O104:H4 were reported in Germany, France, and The Netherlands (3, 55, 63). Person-to-person transmission also played a role in human infections with Stx2-producing EAHEC O111:H2 strains in France more than 10 years ago (21). Contaminated sprouts produced from seeds at a private home were suspected as vehicles of transmission in a small outbreak of EAHEC O104:H4 infection starting at the end of June 2011 in southwestern France (53). Sporadic cases of EAHEC O104:H4 infection in patients who had neither traveled to Germany nor consumed sprouts were reported in Sweden and Austria (40).

An examination of the employees working on the incriminated sprout-producing farm in Lower Saxony revealed that three of them had clinical signs of HUS infection in the first 2 weeks of May. Asymptomatic carriers of EAHEC O104:H4 working on the farm also were identified (48). The onset of the EAHEC O104:H4 infection outbreak was dated back to the beginning of May 2011 (87). The period of infection was calculated for 90% of outbreak
cases as before 23 May, and most infections occurred between 12 and 14 May (87). The epidemiological data indicated that the EAHEC O104:H4 infection outbreak originated from contaminated sprouts produced at this particular farm in Lower Saxony. This suspected scenario was constructed based on the evaluation of the company’s distribution chain, which was correlated with the geographical distribution of human cases (29).

Sprout production is extremely susceptible to bacterial contamination because the conditions needed for growing sprouts from seeds are also favorable for the growth of contaminating bacteria (2, 76). The retail sale of an EAHEC O104:H4–contaminated batch of sprouts, which was divided into small portions for and distributed in northern Germany, could explain the geographical incidence and sudden onset of this outbreak with such a high number of cases. These conclusions were derived from the findings of traceback investigations conducted by the Robert Koch Institute and other official governmental bodies (48).

CONTAMINATED FENUGREEK SEEDS IMPORTED FROM EGYPT ARE SUSPECTED AS VEHICLE OF ENTRY OF THE EAHEC O104:H4 STRAIN INTO THE FOOD CHAIN

Epidemiological investigations carried out in Germany and other European countries revealed fenugreek seed sprouts as the most likely source of the EAHEC O104:H4 outbreak in Germany and other European countries (48, 90). Fenugreek sprouts came under suspicion after an outbreak of EAHEC O104:H4 infection in southwestern France that was reported on 12 June 2011 (53). Fifteen cases of HC or HUS were reported, and five of these were caused by EAHEC O104:H4. Eleven of the 15 patients had attended a cold buffet served at a children’s community center on 8 June. Nine reported having consumed fenugreek sprouts that had been grown from a batch of seeds supplied by a distributor from the United Kingdom. The properties of the EAHEC O104:H4 were identical to those of the isolates from Germany (65), and the median incubation period of infection was 9 days, similar to that reported for the EAHEC infection cases in Germany (87). Traceforward and traceback investigation led to the farm and to the seeds and indicated that sprouts grown from fenugreek seeds imported from Egypt were the most probable origin of the outbreaks in Germany and France. The exact point of contamination was not established but may have been before the seeds were exported. Contamination of seeds could have occurred by fecal material of human or animal origin (48). A particular batch (48088) of 15,000 kg of fenugreek seeds exported from Egypt in November 2009 was suspected as the source of the EAHEC O104:H4 strain. The contaminated batch was distributed to 70 companies in 12 European countries (2). The sprout-producing farm A in Lower Saxony, which was identified as the source of the EAHEC O104:H4 infection outbreak in Germany, was supplied with 75 kg of the this batch of fenugreek seeds produced in 2009 in Egypt. Homemade sprouts grown from a single package of 50 g of fenugreek seeds of this particular batch were incriminated as responsible for the EHEC O104:H4 infection outbreak in France (48). During 2009 and 2011, three lots of a total 37,000 kg of fenugreek seeds were received from Egypt by the German importer. The possibility that lots other than 48088 were contaminated with the EAHEC O104:H4 outbreak strain could not be excluded (48).

Despite numerous investigations in Germany, France, and other countries on fenugreek seeds and sprouts grown from seeds, it was not possible to isolate the EAHEC O104:H4 strain responsible. Leftover food and water from the community center outbreak in France also were negative for the incriminated pathogen (2). According to the EFSA “negative test results from the microbiological tests carried out on seeds cannot be interpreted as proof that a batch is not contaminated with STEC O104:H4 since these results depend on and may be limited by both the analytical and diagnostic performance characteristics as well as by nature of the sampling plan” (48).

The public health risks associated with the consumption of STEC-contaminated fresh vegetables was the subject of a report published by the EFSA (47). The report summarized previous findings on the possibility that STEC O104 from manure-contaminated soil and irrigation water could colonize and internalize in plants. Internalization of E. coli in plants has only been demonstrated under experimental laboratory conditions, and the survival of EHEC O157 on and in plants varied drastically between experiments. At present, the survival of STEC on seeds for long time periods remains largely undocumented (2).

LESSONS TO BE LEARNED FROM THE EAHEC O104:H4 INFECTION OUTBREAK

Humans were identified as the primary reservoir of EAHEC O104:H4 strains, suggesting that contamination of food was most likely of human origin. Contamination of food by EAHEC O104:H4–shedding individuals has been demonstrated. Symptomatic and asymptomatic persons excreting EAHEC O104:H4 were identified, and shedding periods of up to 13 weeks were reported. These findings point to the importance of human transmission of EAHEC into the food chain, similar to transmission routes reported for EAEC strains.

Sprouts possibly grown from EAHEC O104:H4–contaminated seeds produced at farm A in Lower Saxony were identified as the source of the outbreak. Warnings concerning the consumption of sprouts and withdrawal from the retail market of food produced at the incriminated farm resulted in a decline of EAHEC O104:H4 infections and the end of the outbreak. Secondary infections were important, but authorities hope that they were not frequent enough to result in an epidemic spread of this pathogen in Germany. Basically the same EAHEC O104:H4 strain caused a family outbreak of HUS in Germany in 2001. This strain reappeared in modified form as the May and June 2011 outbreak agent. Similar findings have been reported in France, where genetically different strains of EAHEC O104:H4 were isolated in 2004 and 2009 (65). We do not
know whether EAHEC O104:H4 strains remain endemic in Germany and other European countries nor do we understand their ecological niche. Epidemiological studies indicate that EAE C O104:H4 and EAHEC O104:H4 strains are endemic in some Central African countries.

If inadequately managed, sprout production is extremely vulnerable to bacterial contamination. When seeds, other inputs, or the production environment are contaminated with enteric pathogens, the conditions of sprouting provide a favorable environment for the growth of these bacteria. The entry of the pathogen into the sprouting system from infected personnel during production cannot be excluded: EAHEC O104:H4–infected humans were found among the personnel working at sprout farm A at the time when the outbreak began. Because the farm workers claimed to have eaten sprouts produced at their own facility regularly, they may have become infected by sprouts grown from the contaminated fenugreek seeds, as suggested by epidemiological investigations. This hypothesis is based on the fact that the same batch of fenugreek seeds used for sprout production at the farm in Germany also was incriminated as the source of a small outbreak of EAHEC O104:H4 infection in France.

Only one sample of sprouts produced at the incriminated farm was identified as positive for EAHEC O104:H4; however, this sample was collected in a household that included EAHEC O104:H4–infected patients. All other samples from seeds and sprouted seeds were negative for EAHEC O104:H4 perhaps due to one or more of the following reasons: (i) a minor fraction of the incriminated batch of fenugreek seeds was contaminated with EAHEC O104:H4 and by chance these contaminated seeds were missed during sampling, (ii) the methods used for investigation were not suitable for detection of EAHEC O104:H4 from the fenugreek seeds, and (iii) the EAHEC contamination was either not present or was below detectable levels or the strain was initially present but died off over time.

What came first, the seeds or the sprouts? Some facts that could help to answer this question remain unknown. First, we do not know much about the natural contamination of plants and seeds with E. coli and about the survival of E. coli on plant seeds. Second, we do not know much about the frequency of asymptptomatically infected humans in the human population and as a consequence their importance as vehicles of transmission of the pathogen to food. Both of these issues should be explored in laboratory and epidemiological research projects.

REFERENCES
