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MINIREVIEW

Shiga Toxin-Producing *Escherichia coli* O104:H4: a New Challenge for Microbiology

Maite Muniesa,[a] Jens A. Hammerl,[b] Stefan Hertwig,[b] Bernd Appel,[b] and Harald Brüssow[c]

Department of Microbiology, University of Barcelona, Barcelona, Spain[a]; Bundesinstitut für Risikobewertung, Berlin, Germany[b]; and Nestlé Research Center, Lausanne, Switzerland[c]

In 2011, Germany experienced the largest outbreak with a Shiga toxin-producing *Escherichia coli* (STEC) strain ever recorded. A series of environmental and trace-back and trace-forward investigations linked sprout consumption with the disease, but fecal-oral transmission was also documented. The genome sequences of the pathogen revealed a clonal outbreak with enteroaggregative *E. coli* (EAEC). Some EAEC virulence factors are carried on the virulence plasmid pAA. From an unknown source, the epidemic strains acquired a lambdoid prophage carrying the gene for the Shiga toxin. The resulting strains therefore possess two different mobile elements, a phage and a plasmid, contributing essential virulence genes. Shiga toxin is released by decaying bacteria in the gut, migrates through the intestinal barrier, and is transported via the blood to target organs, like the kidney. In a mouse model, probiotic bifidobacteria interfered with transport of the toxin through the gut mucosa. Researchers explored bacteriophages, bacteriocins, and low-molecular-weight inhibitors against STEC. Randomized controlled clinical trials of enterohemorrhagic *E. coli* (EHEC)-associated hemolytic uremic syndrome (HUS) patients found none of the interventions superior to supportive therapy alone. Antibodies against one subtype of Shiga toxin protected pigs against fatal neurological infection, while treatment with a toxin receptor decoy showed no effect in a clinical trial. Likewise, a monoclonal antibody directed against a complement protein led to mixed results. Plasma exchange and IgG immunoadsoprtion ameliorated the condition in small uncontrolled trials. The epidemic O104:H4 strains were resistant to all penicillins and cephalosporins but susceptible to carbapenems, which were recommended for treatment.

One hundred years ago, infectious diseases were a major cause of mortality in industrialized countries. Several decades later, in the heydays of antibiotics, they were thought diseases of the past. In 1967, U.S. Surgeon General William H. Stewart, speaking before a panel of health officials, declared that we could "close the book on infectious diseases." This hope has not been fulfilled. Today, antibiotic resistance is on the rise, and newly emerging infectious diseases have become so important that the U.S. Centers for Disease Control and Prevention publishes a journal under this name (http://wwwnc.cdc.gov/eid/). When a multiantibiotic-resistant emerging pathogen causes an outbreak, as was recently the case with *Escherichia coli* O104:H4 in Germany, we are nearly returned to the preantibiotic era. Public alert is high, and clinicians and microbiologists come under pressure. When looking back to recently emerged pathogens, several infectious agents could have been linked to a food source. For some infections, the food link has been obvious: avian influenza virus infections spread to humans from live poultry markets (13). In other cases, detective work was needed to establish the food link, such as for the severe acute respiratory syndrome virus, which was traced to bats eaten as a meat delicacy, or for Nipah virus infections transmitted from bats to humans after changes in pig-rearing conditions (14). Also, the enterohemorrhagic *E. coli* (EHEC) O157:H7 serotype showed this food link. First described in 1983 as "hamburger disease" for its association with beef consumption in fast food chains, it was later associated with epidemics linked to such diverse food items as apple juice and spinach leaves (68). Now, the Shiga toxin-producing *E. coli* (STEC) serotype O104:H4 holds public attention, while microbiological detective work traced the German outbreak back to sprout consumption.

Here, we summarize some pertinent features of the German O104:H4 epidemic and the possibilities for treatment and prevention. We also review data from O157:H7 infections for which data on O104:H4 are still lacking.

THE GERMAN OUTBREAK: EPIDEMIOLOGICAL ANALYSIS

In 2011, Germany experienced the largest outbreak of STEC cases ever recorded: a total of 3,842 cases were reported, including 2,987 cases of laboratory-confirmed *E. coli* gastroenteritis with 18 deaths and 855 cases of hemolytic uremic syndrome (HUS) that led to 35 fatal outcomes. The outbreak started on May 8, peaked on May 22, and was declared finished by July 4. One could argue that public health measures stopped the epidemic by alerting people to avoid the consumption of contaminated food, but it is also possible that the epidemic stopped because contaminated foods were no longer present in the markets. The process has been publicly criticized for being too slow and for initial false press announcements linking cucumbers and not sprouts to the outbreak. Retrospectively, this criticism must be viewed with some restraint. In the early days of the outbreak, the median reporting times for HUS cases were 8 days to diagnosis, about 10 days to inform the local health department, and about 12 days for reporting to the Robert Koch Institute (RKI) (3). In a U.S. study on *E. coli* O157 infections, an average reporting time of 7 days was achieved (29). There are two reasons for the slower reporting process in Germany. Germany has a less-centralized public health system, and these cases presented with an

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Address correspondence to Harald Brüssow, harald.bruessow@rdls.nestle.com.

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unusual profile, confronting physicians with a new clinical entity. An early epidemiological investigation consisted of a case-control study involving 26 adults hospitalized with HUS. Univariate analysis linked only the consumption of sprouts with disease. However, no sprout warning was issued at the beginning of the outbreak, since only one-quarter of the patients remembered having consumed sprouts (11). Next was a cohort study of 177 subjects who had eaten at a single restaurant, leading to 33 cases of confirmed STEC diarrhea. According to the restaurant recipe, all 31 cases that could be interviewed had consumed uncooked sprouts (11). A series of environmental and trace-back and trace-forward investigations by the German task group identified a group of Swedish visitors who had consumed a sprout mixture. This finding pointed to a sprout producer in lower Saxony, Germany, where in May one-third of the employees fell ill, with several of them infected with the epidemic strain O104:H4 (11). The next pieces in this puzzle were the distributors served by this sprout producer, linking further clusters to sprouts. Notably, the German sprout producer had a seed supplier that could be linked to 15 cases of O104:H4 infections in Bordeaux, France. These cases were apparently also associated with sprout consumption (24). The pulsed-field gel electrophoresis pattern of the French isolates was identical to that from the German outbreak but different from those of preoutbreak reference O104 strains (48), suggesting a single-source clonal outbreak, consistent with the epidemiological evidence. On 10 June, sprouts of fenugreek seeds imported from Egypt were announced by the German authorities as the culprit source of contamination in this outbreak. However, none of the sprout mixtures (seeds) tested positive for O104:H4.

The power of epidemiology compared with a microbiological approach was highlighted by the inability to grow the epidemic strain from any of the investigated sprouts or from the sprout seeds which were taken from the production chain. Cultivation of the strain was only possible in a few cases where posterior contamination was very likely, such as an opened package of sprouts from a household with disease. Due to the almost universally used culture-based detection methods for epidemics, this failure represents a surveillance problem for health and food safety authorities in general. The problem could be caused by the low infectious dose of the pathogen, its decay in food at the moment of investigation, or a peculiar physiological state of bacteria defined as viable but nonculturable (VBNC). Many different bacterial species, including *E. coli*, enter this VBNC state as a response to stressful environmental conditions. Bacteria in the VBNC state do not grow on microbiological media but regain cultivability when resuscitated after stress relief. Indeed, O104:H4 entered this VBNC state when exposed to nutrient-poor conditions, toxic concentrations of copper ions, or tap water (5). Relieving the stress by copper ion chelating facilitated the resuscitation of O104:H4. However, these experiments should be interpreted with care, since there is so far no direct evidence that *E. coli* O104:H4 is found in the VBNC state in nature.

The epidemiological analysis of an initially basically foodborne infection becomes even more difficult when the initial pathogen transmission via the food chain is replaced by human-to-human transmission. Human-to-human transmission is known to occur in about 20% of households with an O157:H7 primary patient (94). Secondary household transmission from adult patients was also suggested for O104:H4 infections in France (2) and The Netherlands (45), mainly based on the observation of delayed onset compared to the incubation time of 7 to 9 days for O104:H4 infections. Secondary transmissions were also reported in Hessen, Germany, which is situated outside of the main epidemic focus in northern Germany (28). The study documented transmission in families, the hospital, and the microbiological laboratory.

O104:H4 MOLECULAR ANALYSIS

The sequencing of the German epidemic strain was achieved in record time by several groups. The first available draft sequence was reported by the Beijing Genomics Institute (BGI), which received the strain from researchers at the University of Hamburg and finished the sequencing of the genome within 3 days by using their third-generation sequencing platform from Ion Torrent Life Technologies. With this new technology of nonoptical DNA sequencing using integrated circuits, sequence data are obtained by directly sensing the ions produced by template-directed DNA polymerase synthesis by using natural nucleotides on a chip containing ion-sensitive transistor-based sensors. The first analyzed genome sequence published in a scientific journal was from the University of Göttingen, for which 454 DNA libraries were sequenced with the Genome Sequencer Flex system using Titanium chemistry (10), followed by data from the Ion Torrent sequencing system (51) and a data obtained using PacBio RS DNA sequencers (71). With respect to quality (read length, sequencing errors, remaining gaps, and misassemblies), the Ion Torrent technology was not the best.

Optical mapping demonstrated identity with four other outbreak isolates and relatedness to a German isolate predating the outbreak and, notably, an enteroaggregative *E. coli* (EAEC) strain isolated in the late 1990s from an HIV-positive adult living in Central Africa who suffered from persistent diarrhea (54). However, the African strain lacked the prophage carrying the stx_2 gene (10). Mellmann et al. suggested an evolutionary scheme in which an ancestor strain gave rise to an O104:H4 complex by deletion and acquisition of mobile DNA elements (51). According to this scheme, the German outbreak strains had gained a plasmid carrying one aggregative adherence fimbria type (AAF/I) and lost a plasmid carrying AAF/III and the heat-stable enterotoxin AstA. In addition, the outbreak strains had acquired a plasmid carrying the antibiotic resistance genes TEM-1 and CTX-M-15. When the genome sequences from four German outbreak isolates were aligned, only 236 single-nucleotide variants were found in pairwise comparisons, proving that the outbreak was clonal (71). Comparisons between the outbreak strains, however, revealed larger-scale deletions, insertions, and inversions between the isolates, documenting substantial genomic mobility. The researchers observed that these structurally divergent regions contained genes that encode virulence factors. The variable regions included two lambda-like prophages that occupied integration sites within the genes *ynfG* and *wbrA*. The two prophages in the sequenced O104:H4 strain C227-11 shared sequence identity over two-thirds of their lengths, but only the prophage inserted in chromosomal gene *wbrA* contained the stx_{2a} gene. As in the O157 *E. coli* prophage 933W, the Shiga toxin 2 subunit A and B genes in the O104:H4 prophages were inserted between the genes encoding the antitermination protein Q and the S/R lysis proteins of a lambdoid phage. The high degree of nucleotide sequence identity between the O157 and O104 prophages suggests a horizontal gene transfer event.

The researchers then performed whole-genome phylogenetic comparisons of 53 *E. coli* strains (71). Eleven O104:H4 strains derived from African diarrhea patients and the German outbreak formed one cluster. Their nearest neighbors were EAEC isolates. However, EAEC isolates were placed at three separate regions of the *E. coli* phylogenetic tree, suggesting that this pathotype can be realized with strains from widely different genomic backgrounds. EHEC strains were found in two clusters (O157 on one side and O26, O111, and O103 isolates on the other side), and *Shigella* isolates were split evenly into three clusters.

E. coli is known as a remarkably versatile pathogen comprising a number of pathotypes (19). The German epidemic showed that two until-now clearly distinct pathotypes, STEC and EAEC, exchange or share virulence genes. If these virulence genes reside on mobile DNA elements, a highly dynamic situation may occur wherever new pathogens with hitherto-unknown clinical and epidemiological characteristics are expected. The O157:H7 epidemic in Oregon and Michigan in 1982 (73) and the O104:H4 epidemic in 2011 in Germany might thus not be the last in a series of bad surprises involving *E. coli*. The preparedness of the microbiological community is therefore an important issue. The *E. coli* research community has, in that respect, done its homework. The sequencing of strains here was carried out quickly and openly. The annotation of the sequenced genomes from the pathogen was conducted in a community-wide approach using the internet in a creative way, with "crowd sourcing" as rapid outsourcing of analyses to bioinformaticians worldwide (75). This approach elicited curiosity-driven analyses from four continents. After 1 day, the released genome sequence was assembled; after 2 days, it was assigned to an existing sequence type (ST678); after 5 days, strain-specific diagnostic primer sequences were designed. The initial sequencing allowed the development of molecular probes based on the O104, H4, Shiga toxin, and tellurite resistance genes. None of these genes was, however, unique to the epidemic strain. All four had to be present to achieve a positive diagnosis. In one of the community approaches, an O104:H4 draft genome was negatively screened against all *E. coli*, *Salmonella*, and *Shigella* strains from the databases, yielding 13 sequences specific for the query strain. When screened positively against the high-throughput sequencing data from eight O104:H4 genomes, 11 sequences were present in all of them. Three ultimately showed no cross-reactivity with any entries from the database, providing a PCR test for the epidemic strain (32). In addition, the European Center for Disease Prevention and Control (ECDC) did its job with timely publications of new data via the online journal *Eurosurveillance*. Finally, clinical microbiologists had collected many isolates over the previous years, allowing the back-tracing of bacterial lineages in real time. The largest and most relevant is the HUSEC collection, comprising 524 hemolytic uremic syndrome-associated enterohemorrhagic *E. coli* strains, including 169 non-O157 EHEC isolates (50), and this turned out to be a unique reference tool. Comparison of the outbreak strains with this collection brought early insights regarding the nature of this strain.

TRACING THE SOURCES

For EHEC O157:H7 strains, the primary reservoir is the intestines of ruminants, particularly cattle. No O104:H4 strains were detected in cattle feces collected in the outbreak area, while classical STEC was found in one-quarter of the animals (95). In 2009, Italian physicians isolated an O104:H4 strain from a child who developed bloody diarrhea, HUS, and severe neurological impairments. The strain closely resembled the 2011 epidemic strain but did not produce extended-spectrum beta-lactamases (ESBL) (79). Two O104:H4 isolates from France have been described (in 2009 in Lyon from a child with HUS and in 2004 in Lille from an adult with bloody diarrhea) that were related to the 2011 outbreak strains but showed a slightly different pulsed-field pattern and a distinct plasmid pattern. The epidemic strain lacked the resistance plasmid but still contained the postulated older plasmid harboring *aaf/I* and *astA* (53). In these isolates one might see missing links between the 2011 outbreak strains and the putative ancestor resembling the late 1990 African EAEC isolate 55989. An O104:H4 strain was also isolated from a woman with HUS in Korea (6).

EAEC was first described in 1987, based on the characteristic adherence phenotype with cultured HEp-2 cells. This biological test still remains the gold standard of diagnosis. Subsequently, a number of virulence factors have been described for EAEC strains and include adhesins (e.g., aggregative adherence fimbriae [AAF/I to -III]), heat-stable enterotoxin, transporters, and other secreted proteins (e.g., the serine protease autotransporter Pic, which has mucinase activity) (33). However, EAEC isolates are genetically a heterogeneous group of *E. coli*. Multilocus sequence typing of 150 phenotypic EAEC comprising isolates from children of a Nigerian diarrhea case-control study and of EAEC isolates from various geographic origins revealed multiple lineages despite the shared phenotype (67). None of the described virulence genes was conserved among all EAEC isolates. In surveys, a PCR test based on three genes identified about twice as many EAEC strains as the HEp-2 adherence test (38). The heterogeneity of EAEC isolates (71) has also been noted in volunteer challenge studies, in which not all isolates induced diarrhea in adults (59). However, a large food-borne diarrhea epidemic involving 2,700 Japanese children consuming contaminated school lunches underlined the pathogenic potential of EAEC (37). A review of EAEC reports that compiled data from 17 studies demonstrated the association of EAEC with diarrhea (61). EAEC was found in 20% or more of the diarrhea cases in 12 studies. The pathogenicity index (the percentage of patients with the pathogen divided by the percentage of asymptomatic subjects with the pathogen) was 2 or higher in nine studies. Notably, seven of the reviewed studies emphasized an association of EAEC with persistent diarrhea in children from developing countries. A more recent meta-analysis of the published literature extended the role of EAEC as a cause of diarrhea to adults from and travelers to developing countries, to children from industrialized countries, and to HIV-infected adults (33). EAEC turned out to be the most common bacterial cause of diarrhea in the United States (60) and the United Kingdom. On the basis of the epidemiology of EAEC, one might suspect human carriage also for O104:H4 isolates. However, data are needed to unravel whether this is really the case or whether these strains have a peculiar affinity for sprouts via colonization of plant seedlings.

O104:H4: THE MAKING OF A PATHOGEN

What is the basis of the new and high virulence of the German outbreak strains? Researchers exploring the genome sequence and the virulence gene profiles of the epidemic O104:H4 isolates observed an unusual combination of virulence genes from STEC strains (stx_2; long polar fimbriae [LPF], tellurite resistance, iron uptake system) and EAEC strains (AAF/I, AggR transcription regulator, dispersin Aap, Pic protein, and *Shigella* enterotoxin Set1)

(7). The latter are mostly carried on the virulence plasmid pAA (10). In O104:H4 we have thus the unusual situation that two different mobile elements, a phage and a plasmid, contribute the essential virulence genes to the pathogen. Perhaps the combination of EHEC-specific and EAEC-specific virulence factors created this unusually virulent pathogen, whereby the enhanced adherence and cytological damage of the intestinal epithelia facilitate systemic adsorption of Shiga toxin, which could explain the high prevalence of HUS in the outbreak.

The epidemic O104:H4 strains possessed the genetic background of an EAEC strain that has acquired the ability to produce the variant 2a of the Shiga toxin. All the *stx* genes found so far among STEC are carried on inducible or remnant prophages. Stx phages are morphologically and genetically very heterogeneous (30, 56), but all conserve a genetic organization similar to phage lambda and all carry the *stx* operon downstream of the phage late genes (41). *stx* expression is under the control of the late genes (63). The link between induction of Stx phages and toxin production was demonstrated with a wide range of inducers, including environmental conditions like pH, iron depletion, or high hydrostatic pressures (36) and medical interventions, like antibiotics (42, 43). During phage induction, several copies of the phage genome (including *stx*) are generated, and thus *stx* expression increases drastically. Lysis of the infected bacterial cell allows the release of the produced toxin. In addition, the released Stx progeny phage can infect susceptible hosts, thus further enhancing Stx production.

The released Stx phage can convert many different *E. coli* serotypes to be Stx producers (56, 57). Even bacterial genera other than *E. coli* can contain Shiga toxin-encoding phages (56, 57, 87). Stx prophage integration in O104:H4 apparently was a recent evolutionary event (71). For this to occur, it is necessary that the new host strain present a suitable receptor for the Stx phage. Although there is currently limited information about the cell receptors used by Stx phages, YaeT could be such a conserved receptor in *E. coli*, and it is also present on O104:H4 (75). This surface molecule is indeed recognized by about 70% of Stx phages via a conserved tail spike protein in phages with a short tail morphology (85). The source of the Stx phage found in O104:H4 is currently unknown. Some studies indicate that transduction could be more efficient *in vivo* than *in vitro*. Therefore, transduction and not phage conversion could have happened within the intestine of humans (8), particularly because EAEC is frequently found in the human gut. However, infectious Stx phages are also found in several extraintestinal environments (fecally polluted water, soil, commercially purchased vegetables, or minced meat) (34, 35, 75). Many of these phages can transduce *stx* (35).

What are the EAEC virulence factors in O104:H4? One group of gene products, the aggregative adherence fimbriae AAF, lead to bundle-forming fimbriae that are involved in the initial attachment of EAEC to the intestinal mucosa. In fact, EAEC adheres to HEp-2 cells in culture with a unique "stacked brick" pattern that distinguishes EAEC from diffusely adherent and enteropathogenic *E. coli*. O104:H4 displays the same adherence phenotype as EAEC (7). The expression of the fimbriae requires a second plasmid gene, *aggR*. AggR is a member of the AraC class of gene regulators that operate as transcriptional activators (62). In fact, the role of AggR is much larger than in regulating the aggregative adherence phenotype. A gene immediately upstream of *aggR* is also under AggR control. It was initially called *aap*, for antiaggregation protein, but it was later renamed dispersin. This secreted protein remains attached to the lipopolysaccharide (LPS) layer and regulates the structure of the AAF filaments. In *aap* deletion mutants, these filaments collapse on the surface of the bacterium, increasing the interaction with neighboring bacteria. Hyperadherence to the host cell, but with impaired colonization of the mouse intestinal tract (92), is the consequence.

O104:H4 encodes, like EAEC, a serine protease autotransporter of *Enterobacteriaceae* (SPATE) proteins, represented by Pic (protease involved in colonization). Pic is a secreted autotransported protein with mucinase activity. Some EAEC strains cause mucus hypersecretion (reminiscent of the mucoid diarrhea induced by *Shigella*), which is abolished in *pic* mutants. Pic promotes intestinal colonization in streptomycin-treated mice and growth in the presence of mucin (27). On the opposite strand of the *pic* gene, another gene is encoded, *set1* (*Shigella* enterotoxin 1), which like Stx is a bacterial AB_5 toxin; the homologous *Shigella* ShET1 protein causes fluid accumulation in rabbit ileal loops (21). Exceptionally for EAEC, *pic* and *set1* are encoded on the bacterial chromosome and not on the virulence plasmid.

PATHOLOGY

After ingestion of the EHEC pathogen, its adhesion and virulence factors are activated and lead to colonization of the terminal ileum and the epithelium of the Peyer's patches. Thereafter, several virulence factors (e.g., Stx and LPS) are released from the bacterium (69). Until now, the sequence of events leading to the development of HUS was only partially known (88). As bacteremia is uncommon during HUS, it is likely that only the toxin is transported to the site of pathological lesions (the glomerular endothelium, brain, and/or pancreas). Stx released by decaying bacteria in the gut migrates through the intestinal barrier, binds to platelets in the blood, and is transported to the target organs. Shiga toxins interact via subunit B with their cellular receptor (Gb3 [globotriaosylceramide]). Gb3 facilitates the endocytosis and intracellular trafficking of the toxin. Within the host cell, the Stx A-subunit cleaves the rRNA at a specific position (64), and this leads to the inactivation of the protein machinery and results in cell death. *In situ*, Stx acts not only as protein synthesis inhibitor but also as a trigger for cytokine release and tissue factor expression. In HUS, the main affected organs are the kidney and the brain, but other organs can also be involved (23). HUS is mostly associated with EHEC strains producing Stx2 and/or Stx2c (9). Because renal glomerular capillary thrombotic microangiopathy is the hallmark of EHEC-associated HUS, the cytotoxicity of Stx is probably directly linked to the pathogenesis of HUS (40). Several studies have verified the suspected role of the Stx B subunit for the initiation of the apoptotic pathway (58).

Several virulence genes promoting intestinal colonization of EHEC are located on pathogenicity islands, but these loci were not found in the epidemic O104:H4 strains (7, 51).

TREATMENT AND PREVENTION

A Cochrane database review analyzing seven randomized controlled clinical trials of pediatric HUS associated with EHEC infections found none of the interventions were superior to supportive therapy alone (52). Since even less is known about the treatment of adult HUS, the clinicians had limited evidence-based treatment options. Here, we provide an overview of experimental

treatment and prevention options that have been discussed in the literature.

Antibodies. Infections with STEC normally do not lead to bacteremia. Systemic complications like HUS are caused by the translocation of Shiga toxin from the intestine into the circulation, where it causes prothrombotic coagulation abnormalities. Even the enteric manifestations of STEC infection, like colitis, can be induced in experimental animals by parenteral administration of Shiga toxin. In fact, bloody diarrhea might be the consequence of mesenteric ischemia induced by circulating Shiga toxin and not a direct injury by the infecting bacterium (88). From that perspective, it was only logic to target Shiga toxin for passive immunity approaches (91). One group selected a specific human monoclonal antibody against the A subunit of Shiga toxin 2 in piglets infected with O157:H7 (83). Practically all placebo-treated piglets developed diarrhea followed by fatal neurological symptoms, while 85% of the antibody-treated piglets survived the infection. Stx2- and Stx1-specific antibodies protected 100 and 0% of piglets, respectively, when they were treated with a Stx1/Stx2-producing strain (39). Clinical trials have not yet been conducted with antibodies against Shiga toxins.

Another human monoclonal antibody was, however, applied in the recent German O104:H4 outbreak. Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5. It was developed for an uncommon form of hemolytic anemia (31). Subsequently, it was discovered that the antibody ameliorated thrombocytopenia in an 18-month-old boy suffering from congenital atypical HUS (26). Eculizumab also showed a strong effect in three children with thrombotic microangiopathy in Shiga toxin-associated HUS. Parenteral injection of the monoclonal antibody led to a recovery of platelet counts. Decreases in leukocyte counts and plasma creatinine and lactate dehydrogenase levels suggested recovery of renal function (46). Encouraged by these results and desperate for a treatment mode in the ongoing German outbreak, Eculizumab was given to about 300 patients (77). Data have not yet been published, but according to a news report in *Nature Medicine*, mixed results were obtained in that study (18).

Plasma exchange and immunoadsorption. For a disease with related pathophysiology (thrombotic thrompocytopenic purpura), plasma exchange is an efficient treatment. For that reason, this treatment mode was also tried during the recent outbreak in five adults with HUS. After plasma exchange, mean platelet counts increased, as did the glomerular filtration rate, and the neurological status improved. Early plasma exchange was correlated with better outcomes (16). It is of course difficult to draw conclusions from so few patients. Another informative study was conducted with 12 patients from the German outbreak, all but one of which were female, who needed intensive care treatment. Most patients showed no response to Eculizumab treatment, suggesting distinct pathophysiological mechanisms in pediatric and adult HUS. Those authors hypothesized a pathological role for an IgG antibody response to the STEC infection in their patients who showed severe neurological symptoms. They therefore performed IgG immunoadsorption followed by IgG replacement. Indeed, the neurological symptoms ameliorated significantly in this prospective noncontrolled trial (25).

Receptor decoys. The receptor for Shiga toxin is the glycolipid Gb3, a trisaccharide linked to ceramide. In a series of clever chemical experiments, subnanomolar inhibitor activity was achieved with five trisaccharide side chains fixed to a central glucose core (44). Modification of the tether structure in the decoy resulted in a compound that protected mice against the lethal effects of both Stx1 and Stx2 injections by inhibiting Stx1 toxin accumulation in kidneys (55). The stool concentration of Stx2 was only minimally affected, while no toxin could be detected in the serum (93). Multiple trisaccharide receptor moieties fixed on silicon dioxide particles were also tested after oral application in a controlled trial with 145 children hospitalized for diarrhea-associated HUS. No significant difference was seen between the oral receptor decoy and the placebo group for primary and secondary outcome variables (90).

Inhibitors. A pyrrolidin derivative inhibits the enzyme glucosylceramide synthase and thus reduces the Gb3 receptor level in kidneys. This compound decreased the toxin-induced mortality by 50% and prevented the cellular pathology in the kidney and gut of rats (84). In an alternative approach, a cell-permeable peptide was bound to the B subunit of Stx2. When injected together with the toxin into baboons, it prevented the death of the primates. No renal pathology or blood clotting disorders were observed. Even when given 1 day after the toxin, it still saved the majority of the animals. Renal damage and clotting disturbances were observed, but the pathological response was delayed and blunted (86). The smallest, safest, and cheapest Stx inhibitor is zinc. When tested against STEC in ligated rabbit intestinal loops, zinc reduced fluid secretion, mucosal adherence, villus blunting, and Stx toxin production. *In vitro*, zinc decreased the expression of other secreted virulence factors (17).

Antibiotics. Treatment of STEC strains with quinolone, but not with fosfomycin, increased free fecal Stx concentrations and also the death rate of O157:H7-infected mice compared to untreated controls (98). *In vitro* transcription of the type 2 Shiga toxin gene was enhanced by quinolones. Quinolones also mediated induction of the Stx prophage, causing bacterial lysis and thereby release of the Stx toxin from O157 strains (65). Some antibiotics also induced the movement of toxin-encoding prophages to uninfected intestinal *E. coli* commensals, thus increasing Stx production (98). It has been proposed that certain antibiotics induce the bacterial SOS response. This interpretation concurs with the attenuating effect of a mutation in the *recA* gene, which is a critical gene for SOS induction of Stx2 toxin production, as seen in O157:H7-infected piglets treated with ciprofloxacin (97).

The use of antibiotics for the management of human O157:H7 infection is controversial. In one prospective, randomized, controlled trial, antibiotic treatment was neither harmful nor beneficial (70). However, in another prospective cohort study of 71 children hospitalized with O157:H7 diarrhea, antibiotic treatment significantly increased the risk of developing HUS (96). This study strongly influenced the decision of clinicians to avoid antibiotics in these patients, even though a subsequent meta-analysis of nine good-quality studies did not show a higher risk of HUS associated with antibiotic administration (78). Notably, the epidemic O104:H4 strains were resistant to all penicillins and cephalosporins while they were still susceptible to carbapenems (7). During the epidemic, the German Society of Infection (DGI) recommended the use of carbapenem if one suspects invasive disease due to O104:H4. Rifampin was suggested for safe eradication of EHEC from the intestinal tract.

Probiotics. When lactic acid bacteria (LAB) were cocultivated

with O157:H7 in broth culture, the production of organic acids by LAB led to a decrease in both pH and stx_{2A} gene expression (12). In addition, low pH blocks Stx prophage induction to almost zero (36). Mice inoculated with O157:H7 and *Lactobacillus reuteri* showed a decrease in intestinal pathogen count, weight gain, and less kidney damage than seen in infected controls (20). Also, *Bifidobacterium breve* prevented body weight decrease and death (0% versus 90% in the infection control) (4). Treated mice showed 100-fold-lower fecal STEC counts, and the *in vivo* expression levels of stx_1 and stx_2 were totally suppressed. *B. breve* prevented lesions in the ileum, kidney, bone marrow, and lymph nodes. Interestingly, different species of bifidobacteria differed in their protective properties. A Japanese group compared a *Bifidobacterium longum* strain that showed protective activity in an O157:H7 mouse disease model with a *Bifidobacterium adolescentis* strain that lacked this trait (22). *B. longum*-treated mice did not differ from the infection control with respect to fecal O157:H7 cell count or intestinal expression of O157 virulence genes, but they showed significantly lower serum Stx2 concentrations. Those authors then analyzed what factors prevented the translocation of Stx2 into the blood. Comparative genomics demonstrated that the presence or absence of ABC-type carbohydrate transporters correlated with the protective phenotype of bifidobacteria. One specific transporter mutant showed less acetate production in the gut and partial loss of protection. The importance of *in situ* acetate production was demonstrated by feeding animals an acetylated starch, which released acetate into the intestinal tract and thereby protected mice against O157:H7 disease. Feeding animals probiotics might be a practical preharvest control of *E. coli* O157 in cattle (47).

Phages. A cocktail of three *E. coli* phages showed a dose-dependent lytic activity against O157:H7 contamination of various vegetables and meat products (1). Using a high phage dose, lysis rates in excess of 97% were achieved. The surviving cells did not develop phage resistance. Since no phage amplification was seen, the phages probably lysed the cells from the outside without infecting the cells. A follow-up study investigated the effect of this phage cocktail against O157 on fresh-cut fruits and vegetables (82). Phages lysed the pathogen on these food items when maintained at refrigeration, but not at room temperature. Also, meat contaminated with moderately high O157 counts was freed from the food pathogen by phage application (66). Steers that received phage by the rectal route showed a 100-fold fecal titer decrease of O157 compared to controls. Only low fecal titers of phages were seen in steers that received phage orally, and O157 fecal titers were not different from those in untreated control steers (76). Oral T4-like coliphage in sheep reduced the O157 counts in the cecum and rectum (72). However, in a trial with 20 young cattle, no influence of orally applied phage on the fecal O157 titer was observed (74). French scientists isolated three different morphological types of tailed phages that infect O104:H4. The T4-like myovirus showed the best lytic profile against the target cells grown in either broth or as a biofilm. The cocktail of all three phages disrupted *E. coli* cell aggregates on epithelial cells and showed sustained replication in the intestines of mice, but without decreasing the intestinal titer of O104:H4 cells (49).

Bacteriocins. When epidemiologists screened human stools, 17% contained O157-inhibitory *E. coli* isolates (89), leading to the hypothesis that bacteriocin-producing strains contribute to resistance against O157 in human adults. Feeding trials were conducted in calves inoculated with O157 pathogens. A weak although statistically significant reduction was seen when antagonistic *E. coli* was given at a high dose (80). Enterocin from *Enterococcus faecalis* showed weak activity against O157:H7 on soybean sprouts (15). Notably, another group of investigators developed a concept that is halfway between bacteriocins and phages: R-type pyocins (81). Pyocins can be considered prophage remnants that consist only of the tail, base plate, and tail fiber part of a bacteriophage. Pyocins kill target cells by binding to the cell and punching a hole into the cell envelope. These researchers added to the pyocin a tail spike protein from a phage infecting O157:H7. This hybrid pyocin killed O157:H7 *in vitro* and degraded the O157-specific O-antigen (LPS). When high concentrations of the recombinant pyocin were sprayed on artificially contaminated beef, moderate concentrations of O157:H7 were eliminated.

OUTLOOK

The German O104:H4 epidemic is an example of a combination of relatively well-investigated virulence genes derived from two distinct pathogens which, when assembled into one organism, led to new disease manifestations that took the medical community by surprise. The ease with which this combination of two mobile DNA elements can be achieved in *E. coli* suggests that this will not be the last surprise from this versatile pathogen. The research community is well advised to explore this pathogen, even after the epidemic has been declared finished.

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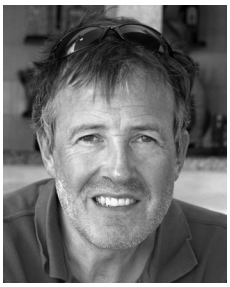
Maite Muniesa received her Ph.D. in Barcelona, followed by postdoctoral fellowships in Würzburg and Giessen, Germany. She is currently an Associate Professor for Microbiology at the University of Barcelona. She is the author of more than 50 research publications. Her research area is the role of mobile genetic elements in bacteria from the environment and of microorganisms as indicators for the fecal pollution of water.

Jens Andre Hammerl obtained his Ph.D. from the Humboldt University of Berlin with work on the genetic switch in linear plasmid prophages. He works now as a postdoctoral fellow at the Federal Institute for Risk Assessment in Berlin (BfR) on the molecular diagnosis of pathogenic bacteria.

Stefan Hertwig studied biology in Göttingen and Kiel in Germany. After receiving his Ph.D. in 1990, he worked as a scientist at the Robert Koch Institute in Berlin, and since 2005 he has worked at the BfR. His research focus is on the biology and genetics of bacteriophages and plasmids from food-borne bacterial pathogens.



Bernd Appel studied at the Free University in Berlin, received a Ph.D. at the Max Planck Institute for Molecular Genetics in Berlin, and held a postdoctoral position at Yale University. He became Professor at the University for Applied Sciences in Berlin, Vice Director of the Department of Genetics at the Robert Koch Institute in Berlin, and since 2005 he has been the head of the department of Biological Safety at the BfR in Berlin.

Harald Brüssow received a Ph.D. in virology at the Max Planck Institute for Biochemistry in Munich and has worked since 1981 at the Nestlé Research Center in Lausanne, Switzerland, on various projects concerning food microbiology, medical microbiology, immunology. and nutrition. He has authored more than 100 scientific articles and 1 book (*The Quest for Food* [Springer, 2007]).