Identification of Human-Pathogenic Strains of Shiga Toxin-Producing *Escherichia coli* from Food by a Combination of Serotyping and Molecular Typing of Shiga Toxin Genes[▽]

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We examined 219 Shiga toxin-producing *Escherichia coli* (STEC) strains from meat, milk, and cheese samples collected in Germany between 2005 and 2006. All strains were investigated for their serotypes and for genetic variants of Shiga toxins 1 and 2 (Stx1 and Stx2). stx_1 or variant genes were detected in 88 (40.2%) strains and stx_2 and variants in 177 (80.8%) strains. Typing of stx genes was performed by stx-specific PCRs and by analysis of restriction fragment length polymorphisms (RFLP) of PCR products. Major genotypes of the Stx1 (stx_1, stx_{1c}, and stx_{1d}) and the Stx2 (stx_2, stx_{2d}, stx_{2-O118}, stx_{2e}, and stx_{2g}) families were detected, and multiple types of stx genes coexisted frequently in STEC strains. Only 1.8% of the STEC strains from food belonged to the classical enterohemorrhagic *E. coli* (EHEC) types O26:H11, O103:H2, and O157:H7, and only 5.0% of the STEC strains from food were positive for the *eae* gene, which is a virulence trait of classical EHEC. In contrast, 95 (43.4%) of the food-borne STEC strains carried stx_2 and/or mucus-activatable stx_{2d} genes, an indicator for potential high virulence of STEC for humans. Most of these strains belonged to serotypes associated with severe illness in humans, such as O22:H8, O91:H21, O113:H21, O174:H2, and O174:H21. stx_2 and stx_{2d} STEC strains were found frequently in milk and beef products. Other stx types were associated more frequently with pork (stx_{2e}), lamb, and wildlife meat (stx_{1c}). The combination of serotyping and stx genotyping was found useful for identification and for assignment of food-borne STEC to groups with potential lower and higher levels of virulence for humans.

Shiga toxin-producing *Escherichia coli* (STEC) strains have been known as food-borne disease agents in humans since 1982 (38). STEC strains, also called verotoxin-producing *E. coli* strains, are characterized by production of one or more types of cytotoxins causing tissue damage in humans and animals (34, 46). Humans infected with STEC show symptoms, such as abdominal pain and watery diarrhea, and a number of patients develop a life-threatening disease, such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (46). The natural reservoirs of STEC are domestic and wild ruminant animals, which shed the bacteria with their feces into the environment (10). STEC-infected animals normally do not show signs of disease and can be included in food production. As a consequence, products of animal origin, such as meat and milk, are at risk of contamination with STEC originating from animals (19). Consumption of food containing STEC was identified as a major route of human infections with these pathogens in different countries (10, 19, 29). STEC strains can be divided into more than 200 *E. coli* serotypes. The phenotypes of most STEC strains resemble those of the normal fecal *E. coli* flora, which causes problems with detection (2). Some properties of STEC were shown to facilitate spread into the environment

and to animals and humans. STEC strains are highly resistant to physicochemical stresses, such as acidity and dryness, and can survive for long time periods in soil, manure, and water (27, 28). Relatively few organisms are needed to cause disease, and routes of direct transmission from animals, infected humans, and the environment have been identified as important routes for STEC infections in humans (22, 37, 46).

For STEC, two major types of Shiga toxins, called Stx1 and Stx2, which share 56% homology to each other, have been described previously (34). Genetic variants were detected within members of the Stx1 and Stx2 families, and a growing number of toxin types were defined according to differences in toxicity, toxin receptor, and amino acid composition of StxA and StxB subunits (34, 39). Some Stx types, such as Stx2 and the elastase (mucus)-activatable Stx2d type, are associated with the high virulence of STEC and with HC and HUS (5, 6, 15, 21). Other toxin types, such as Stx1c, Stx2-O118 (formerly Stx2d-Ount), Stx2e, and Stx2f, were found to be associated mainly with uncomplicated diarrhea or asymptomatic excreters (4, 15, 16). STEC strains producing Stx1c and/or Stx2d-O118 were associated with sheep (8, 45), Stx2e with pigs (14), and Stx2f with pigeons (42) as natural reservoirs.

Highly human-virulent STEC strains able to cause HC and HUS are also called enterohemorrhagic *E. coli* (EHEC) strains. Typical EHEC strains possess additional virulence functions encoded by genes of the locus of enterocyte effacement (LEE) pathogenicity island and of a large virulence plasmid. The LEE function enables the bacteria to colonize efficiently the intestine of the host by binding between intimin, the

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TABLE 1. Detection of *stx* genes by PCR

Target gene(s)	Primer	Nucleotide sequence (5′–3′)	PCR conditions	Size of PCR product (bp)	Source or reference
stx_1, $stx_{1\text{-CB168}}$, $stx_{1\text{-O48}}$, stx_{1c}, stx_{1d}	Stx1com-F Stx1com-R	(C/T)AGTTGAGGGGGGGTAAAATG CG(A/G)AAAATAAC(C/T)TCGCTGAATC	94.0°C, 30 s; 51.3°C, 60 s; 72.0°C, 40 s	282–283	This work
stx_{1c}	Lin-up 1OX3	GAACGAAATAATTTATATGT CTCATTAGGTACAATTCT	94.0°C, 60 s; 48.1°C, 90 s; 72.0°C, 90 s	555	24
stx_{1d}	VT1AvarF VT1AvarR	CTTTTCAGTTAATGCGATTGCT AACCCCATGATATCGACTGC	94.0°C, 60 s; 62.0°C (5 cycles, 60 s each); 58.0°C (5 cycles, 60 s each); 54.0°C (20 cycles, 60 s each); 72°C, 60 s	192	9
stx_2, stx_{2c}, $stx_{2\text{-O118}}$, $stx_{2\text{-Ox3:H21}}$, $stx_{2\text{-OX392}}$, stx_{2e}, stx_{2g}, $stx_{2\text{-NV206}}$, $stx_{2v\text{-ha}}$, $stx_{2v\text{-hb}}$, stx_{2d}	LP43 LP44	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	94.0°C, 30 s; 57.0°C, 60 s; 72.0°C, 60 s	584	3
stx_2, stx_{2c}, $stx_{2\text{-OX392}}$, stx_{2g}, $stx_{2\text{-NV206}}$, $stx_{2v\text{-ha}}$, $stx_{2v\text{-hb}}$, stx_{2d}	GK5 GK6	ATGAAGAAGATGTTTATG TCAGTCATTATTAAACTG	94.0°C, 30 s; 52.0°C, 60 s; 72.0°C, 40 s	269	3
$stx_{2\text{-O118}}$, $stx_{2\text{-Ox3:H21}}$	VTAM1 VTAM2	AGGGCCCACTCTTTAAATACATCC CGTCATTCCTGTTAACTGTGCG	94.0°C, 30 s; 52.0°C, 30 s; 72.0°C, 30 s	246	3
stx_{2e}	SLT-IIBv1 SLT-IIBv2	ATGAAGAAGATGTTTATAGCG GTTAAACTTCACCTGGGCAAAG	94.0°C, 30 s; 62.0°C, 60 s; 72.0°C, 30 s	264	3
stx_{2f}	128-1 128-2	AGATTGGGCGTCATTCACTGGTTG TACTTTAATGGCCGCCCTGTCTCC	94.0°C, 30 s; 57.0°C, 60 s; 72.0°C, 60 s	428	40
stx_{2g}	209F 781R	GTTATATTTCTGTGGATATC GAATAACCGCTACAGTA	94.0°C, 30 s; 46.0°C, 60 s; 72.0°C, 60 s	573	26

product of the *eae* gene, and its translocated receptor (Tir). Binding of LEE-positive bacteria to the colonic cells results in the attaching and effacing lesion (A/E phenotype), with final destruction of colonic enterocytes (12, 34). The EHEC virulence plasmid carries the genes for EHEC hemolysin (*hly*) and other functions which may contribute to the disease process (34). Typical EHEC strains, such as O26, O103, O111, O145, and O157, were shown to express one or more types of Stx, the A/E phenotype, and the plasmid-encoded EHEC hemolysin (10, 31). In humans, typical EHEC strains are isolated frequently from young children, whereas atypical EHEC strains are predominant in older patients (4, 5, 15, 47). Atypical EHEC strains express Stx2 and/or Stx2d but do not carry the LEE and belong to serotypes other than those of typical EHEC strains (31, 33, 36, 43).

Since contaminated foodstuff is one of the major vehicles of STEC transmission to humans, we became interested in investigating STEC strains from food samples for their possible role as human pathogens. In recent years, much data on the epidemiology of human infections with STEC in Germany has become available (4, 5, 15, 16, 47). In this study, we investigated 219 STEC strains isolated from food samples in different parts of Germany for their toxin types, other virulence genes, and serotypes. Typical EHEC strains were found rarely among STEC isolates from food, whereas atypical EHEC strains belonging to known human-pathogenic types were detected frequently.

MATERIALS AND METHODS

Bacteria. A total of 219 STEC strains originating from food samples collected in Germany in 2005 and 2006 were investigated. The strains were isolated in 15 governmental food inspection laboratories situated in different parts of Germany and were sent to the National Reference Laboratory for *Escherichia coli* at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, for examination. Only one isolate per food sample was included in the study in order to avoid investigation of double isolates. The strains originated from beef, lamb, pork, or wildlife meat (*n* = 180), raw milk (*n* = 32), and raw-milk cheese (*n* = 5). Apart from these, one STEC isolate originated from salad and one from a cake sample.

Shiga toxin nomenclature. The nomenclature proposed by Scheutz et al. (39) is used here for the designation of Shiga toxins. The more recently detected toxin variants stx_{1d} (9), $stx_{2\text{-NV206}}$ (1), and stx_{2g} (26) are designated as described previously (3). We use the designation Stx2d for the mucus-activatable Stx2 type, as recently suggested (39, 43). Two types of mucus-activatable genes, called stx_{2d1} and stx_{2d2}, have been described previously (42). stx_{2d1} was associated with the toxin B subunit type $stx_{2v\text{-ha}}$ and stx_{2d2} with the $stx_{2v\text{-hb}}$ B subunit (42). Further investigations have shown that the association of the mucus-activatable stx_{2d} type with B-subunit variants is not absolute and that other variants, except $stx_{2v\text{-ha}}$ and $stx_{2v\text{-hb}}$, can be associated with the mucus-activatable stx_{2d} type (1, 17). Therefore, we use the term stx_{2d} in this work to designate all types of mucus-activatable stx_2 genes. The term Stx2d was used previously for the description of another Stx2 variant (VT2d or VT2d-Ount) (35) that was later renamed Stx2-O118 (39).

Microbiological investigation. All 219 strains were identified as *E. coli* by biochemical reactions performed as described by Ewing (13). The strains were investigated for production of Stx with a Vero cell cytotoxicity assay and with a commercially obtainable enzyme immunoassay (Ridascreen-EIA; R-Biopharm AG, Darmstadt, Germany) (3). Serotyping of O (lipopolysaccharide) and H (flagellar) antigens was performed with O-specific and H-specific rabbit antisera prepared at the BfR according to standard methods (32). Antigens O1 to O181 and H1 to H56 were investigated, and nonmotile strains were analyzed for their
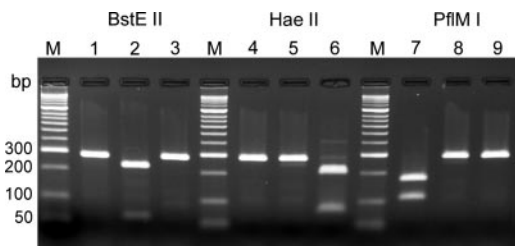
FIG. 1. PCR/RFLP typing of genes of the Stx1 family. PCR amplification of Stx1 family genes with primers Stx1com-F and Stx1com-R results in a 282-bp (stx_1, stx_{1-O48}, $stx_{1-CB168}$, and stx_{1d}) or a 283-bp (stx_{1c}) fragment. The PCR fragments were digested separately with BstEII (lanes 1 to 3), HaeII (lanes 4 to 6), or PflMI (lanes 7 to 9), yielding RFLP patterns characteristic of stx_1, stx_{1c}, and stx_{1d}, respectively (Table 2). Lanes M, molecular size standards; lanes 1, 4, and 7, C600(H19) stx_1 (GenBank accession no. M16625); lanes 2, 5, and 8, DG131/3 stx_{1c} (GenBank accession no. Z36901); lanes 3, 6, and 9, MHI813 stx_{1d} (GenBank accession no. AY17085).

flagellar (*fliC*) genotypes by PCR and restriction fragment length polymorphism (RFLP) of HhaI-digested *fliC* PCR products (4).

PCR detection of STEC virulence genes. All strains were tested by PCR for the presence of the *eae* gene, which encodes intimin, and the EHEC plasmid-encoded hemolysin (*hlyA*) gene (4). Subtyping of *eae* genes was performed as described previously (4). Identification of *stx* genes and subtyping of stx_1 and stx_2 variant genes were performed by PCR as described previously (3) (Table 1). A new PCR/RFLP protocol based on nucleotide differences in the B subunits of the stx_1, stx_{1c}, and stx_{1d} genes was used for distinguishing between genes of the Stx1 family. Common primers (Stx1com-F and Stx1com-R) for amplification of the B subunits of the different stx_1 genes (Table 1) were derived from published DNA sequences (GenBank accession numbers are in parentheses) for stx_1 (M16625), stx_{1-O48} (Z36899), $stx_{1-CB168}$ (Z36900), stx_{1c} (AJ413986 and Z36901), and stx_{1d} (AY170851 and AY986980). These primers were shown to amplify all known types of stx_1 and stx_1 variant genes, yielding a 282-bp (stx_1, stx_{1-O48}, $stx_{1-CB168}$, and stx_{1d}) or a 283-bp (stx_{1c}) PCR fragment. The PCR fragments were digested separately with BstEII, HaeII, and PflMI, yielding RFLP patterns characteristic of stx_1, stx_{1c}, and stx_{1d}, respectively (Fig. 1; Table 2). As a control, the stx_{1c} and stx_{1d} genes were specifically amplified by PCR with their genotype-specific primers as listed in Table 1.

Primers LP43 and LP44 were used for the detection of all known genetic variants of the Stx2 family except stx_{2f}. stx_{2f} genes were searched separately by PCR with specific primers 128-1 and 128-2 (Table 1). All strains found positive by use of primers LP43 and LP44 were examined additionally in PCRs with primers GK5 and GK6 (stx_2 and stx_{2d}), VTAM1 and VTAM2 (stx_{2-O118}), SLT-IIBv1 and SLT-IIBv2 (stx_{2e}), and 209F and 781R (stx_{2g}) (Table 1).

PCR/RFLP subtyping of stx_2 and stx_{2d} genes according to nucleotide sequence differences in the B subunits was performed with amplified products obtained with PCR primers GK5 and GK6, as described by Gobius et al. (17). The 269-bp PCR products were digested separately with HaeIII, RsaI, FokI, and NciI,

TABLE 3. PCR/RFLP typing of Stx2 family genes with HaeIII, RsaI, FokI, and NciI

Restriction enzyme	Size(s) (bp) of restriction fragment(s)[a]					
	stx_2[b]	$stx_{2v\text{-}ha}$[d]	$stx_{2v\text{-}hb}$[d]	stx_{2g}[e]	$stx_{2\text{-}NV206}$[f]	$stx_{2\text{-}EC1586}$[g]
HaeIII	269[h]	142, 127	142, 127	269[h]	269[h]	269[h]
RsaI	219, 50	139, 80, 50	219, 50	269[h]	219, 50	219, 50
FokI	154, 115	269[h]	269[h]	269[h]	269[h]	269[h]
NciI	269[h]	269[h]	140, 129	269[h]	140, 129	269[h]

[a] Fragments were obtained by enzymatic digestion of PCR products obtained with primers GK5 and GK6.
[b] GenBank accession no. X07865 and AP000422.
[c] GenBank accession no. AJ605767 and AF879828.1.
[d] GenBank accession no. AF479829.2, AF500190, and AF500191.
[e] GenBank accession no. AY286000 and AJ966783.
[f] GenBank accession no. AF329817, AF500187, AF500197, and AY095209.
[g] GenBank accession no. AM498375 and AF500188.
[h] Undigested.

yielding patterns specific for the B subunits of stx_2, $stx_{2v\text{-}ha}$, $stx_{2v\text{-}hb}$, stx_{2g}, and $stx_{2\text{-}NV206}$ genes (Table 3; Fig. 2).

The presence of mucus-activatable stx_{2d} genes in STEC strains from food samples was investigated in all strains typed as stx_2, $stx_{2v\text{-}ha}$, $stx_{2v\text{-}hb}$, or $stx_{2\text{-}NV206}$. It was reported previously that the stx_{2d} variant is found among STEC strains carrying these types of stx_2 genes (3, 17, 21, 30). The activatable stx_{2d} type differs from nonactivatable stx_2 by two amino acid substitutions in the N-terminal region of the toxin A subunit. This results in the absence of a PstI site in the stx_{2d} A-subunit gene, which can be taken as an indicator for the presence of an stx_{2d} genotype (5, 17, 21, 30). For the detection of stx_{2d} toxin types, PCR products of all stx_2, $stx_{2v\text{-}ha}$, $stx_{2v\text{-}hb}$, and $stx_{2\text{-}NV206}$ strains were generated with primers SLT-II-vc and CKS2, yielding an 890-bp product. PCR products generated from nonactivatable stx_2 are cleaved with PstI into 504- and 386-bp fragments, whereas the stx_{2d} gene remains uncleaved (17, 21).
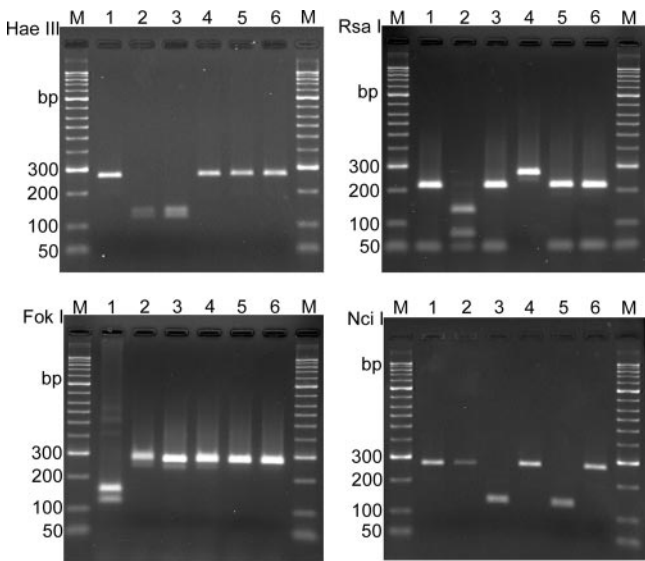

FIG. 2. PCR/RFLP typing of genes of the Stx2 family. PCR amplification of Stx2 family genes was performed with primers GK5 and GK6, resulting in a 269-bp fragment. The PCR fragment was digested separately with HaeIII, RsaI, FokI, and NciI, yielding RFLP patterns characteristic of stx_2, $stx_{2v\text{-}ha}$, $stx_{2v\text{-}hb}$, stx_{2g}, $stx_{2\text{-}NV206}$, and $stx_{2\text{-}EC1586}$ (Table 3). Lanes M, molecular size standards; lanes 1, C600(W34) stx_2 (GenBank accession no. X07865); lanes 2, CB2851 $stx_{2v\text{-}ha}$ (GenBank accession no. AJ605767); lanes 3, CB7753 $stx_{2v\text{-}hb}$ (GenBank accession no. AF479829); lanes 4, 12422 stx_{2g} (GenBank accession no. AY286000); lanes 5, NV206 $stx_{2\text{-}NV206}$ (GenBank accession no. AF329817); lanes 6, RL481/06 $stx_{2\text{-}EC1586}$ (GenBank accession no. AM498375).

TABLE 2. PCR/RFLP typing of Stx1 family genes with BstEII, HaeII, and PflMI

Restriction enzyme	Size(s) (bp) of restriction fragment(s)[a]		
	stx_1[b]	stx_{1c}[c]	stx_{1d}[d]
BstEII	282[e]	224, 59	282[e]
HaeII	282[e]	283[e]	202, 80
PflMI	174, 108	283[e]	282[e]

[a] Fragments were obtained by enzymatic digestion of PCR products obtained with primers Stx1com-F and Stx1com-R.
[b] Results for stx_1 (GenBank accession no. M16625), stx_{1-O48} (GenBank accession no. Z36899), and $stx_{1-CB168}$ (GenBank accession no. Z36900).
[c] Results for stx_{1c} (GenBank accession no. AJ413986 and Z36901).
[d] Results for stx_{1d} (GenBank accession no. AY170851 and AY986980).
[e] Undigested.

TABLE 4. Frequencies and combinations of *stx* genotypes in STEC strains from food

stx gene(s)[a]	No. (%) of strains
stx_1	22 (10.0)
stx_{1c}	16 (7.3)
stx_{1d}	3 (1.4)
$stx_1 + stx_2/stx_{2d}$	37 (16.9)
$stx_1 + stx_{2\text{-O118}}$	2 (0.9)
$stx_{1c} + stx_{2\text{-O118}}$	8 (3.7)
stx_2/stx_{2d}	52 (23.7)
$stx_2/stx_{2d} + stx_{2\text{-O118}}$	3 (1.4)
$stx_2/stx_{2d} + stx_{2g}$	3 (1.4)
$stx_{2\text{-O118}}$	25 (11.4)
stx_{2e}	42 (19.2)
stx_{2g}	6 (2.7)

[a] stx_2/stx_{2d} indicates that the strains carry the stx_2 and/or the stx_{2d} gene. Further association of stx_2 and stx_{2d} genes with different *stx* types is presented in Table 5.

Nucleotide sequencing. To obtain the complete coding sequence of the B subunit of the stx_{2d} gene present in strain RL481/06, common primers Stx2B-f (5′-ATGGGAAAGTAATACAGCAGC-3′) and Stx2B-r (5′-CACTTGTTACCCA CATACCAC-3′) were designed from the published gene sequences of stx_2 and stx_{2d} genes. Stx2B-f and Stx2B-r are positioned in highly conserved DNA stretches upstream and downstream of the toxin B subunits of stx_2 and stx_{2d} strains. PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany), used for sequencing by applying dye terminator chemistry (PE Applied Biosystems, Darmstadt, Germany), and separated on an automated DNA sequencer (ABI PRISM 3100 genetic analyzer; Applied Biosystems, Foster City, CA). The sequences were analyzed using Lasergene software (DNASTAR, Madison, WI) and Mac Vector software (Oxford Molecular Group, Campell, CA).

Nucleotide sequence accession number. The nucleotide sequence of the complete coding regions of the stx_2 B subunit of STEC strain RL481/06 was submitted to the EMBL database under the accession number AM498375.

RESULTS

Production of Stx and presence of *stx* genes in STEC strains isolated from food. All 219 *E. coli* strains were shown to carry one or more *stx* genes, as tested by PCR (Table 4). Production of Stx was tested by a Vero cell cytotoxicity assay and an Stx enzyme-linked immunosorbent assay (ELISA). Cytotoxic activity on Vero cells was detected in 216 (98.6%) strains, and a positive Stx ELISA was found with 207 (94.5%) strains. stx_1 or stx_1 variant genes were detected in 88 (40.2%) and stx_2 genes and/or variants in 177 (80.8%) of the 219 STEC strains. Among the 88 strains of the Stx1 family, 61 (69.3%) carried an stx_1 gene, 24 (27.3%) an stx_{1c} gene, and 3 (3.4%) an stx_{1d} gene. Differentiation between stx_1, stx_{1c}, and stx_{1d} genes was performed by PCR/RFLP analysis as described in Materials and Methods (Table 1; Fig. 1). The results from PCR/RFLP typing were in agreement with those obtained with stx_{1c} and stx_{1d} genotype-specific PCRs (Table 1). By PCR/RFLP typing, we could show that only one type of stx_1 gene was present in each of the strains of the Stx1 family (Table 4).

The presence of any stx_2 gene except stx_{2f} was indicated for 177 STEC strains by a positive PCR result with primers LP43 and LP44 (Table 1) (3). Among the members of the Stx2 family, types stx_2 and stx_{2d} are associated with highly virulent STEC strains (5, 21, 30). We were therefore interested to determine whether stx_2 and/or stx_{2d} strains are present in STEC from food. For detection of these strains, we investigated all 177 strains that had reacted with primers LP43 and

TABLE 5. Association of Stx B-subunit types with stx_2 and stx_{2d} genes and serotypes of STEC strains from food

stx_2 and/or stx_{2d} B-subunit genotype (no. of strains)	No. of strains with stx_2 and/or stx_{2d}		
	stx_2	stx_{2d}	stx_2 and stx_{2d}
$stx_{2v\text{-ha}}$ (14)	8[a]	6[b]	0
$stx_2 + stx_{2v\text{-ha}}$ (3)	0	0	3[c]
$stx_{2v\text{-hb}}$ (17)	1[d]	16[e]	0
$stx_2 + stx_{2v\text{-hb}}$ (12)	1[f]	2[g]	9[h]
$stx_2 + stx_{2g}$ (3)	3[i]	0	0
$stx_{2\text{-NV206}}$ (4)	0	4[j]	0
$stx_2 + stx_{2\text{-NV206}}$ (1)	0	0	1[k]
stx_2 (40)	21[l]	16[m]	3[n]
$stx_{2\text{-EC1586}}$ (1)	0	1[o]	0

[a] O174:H21 (n = 4), O157:[H7] (n = 1), O178:H19 (n = 1), O8:H21 (n = 1), and ONT:HNT (n = 1).
[b] O113:H21 (n = 2), O22:H8 (n = 1), O46:H38 (n = 1), and ONT:H8/48 (n = 2).
[c] O91:H21 (n = 2) and ONT:H21 (n = 1).
[d] O174:H8 (n = 1).
[e] O113:H21 (n = 1), O120:H11 (n = 1), O153:H4 (n = 1), O163:[H27] (n = 1), O174:H2 (n = 2), O22:H8 (n = 3), O22:H16 (n = 1), O91:H21 (n = 2), O91:H42 (n = 1), and ONT:H19/46 (n = 3).
[f] O178:H19 (n = 1).
[g] O104:H21 (n = 1) and ONT:H7 (n = 1).
[h] O91:H21 (n = 4), O113:H21 (n = 1), O153:H1 (n = 1), O8:H19 (n = 1), and O82:H8 (n = 2).
[i] O109:H16 (n = 2) and ONT:NM (n = 1).
[j] O6:H10 (n = 1), O4:H4 (n = 1), O113:H4 (n = 1), and ONT:H29 (n = 1).
[k] ONT:H19 (n = 1).
[l] O113:H21 (n = 1), O172:H25 (n = 1), O174:H2 (n = 1), O178:H19 (n = 2), O179:H8 (n = 4), O2:H27 (n = 1), O2:H42 (n = 1), O46:H38 (n = 1), O8:H19 (n = 1), ONT:H11/18/19/21/28 (n = 7), and O146:H28 (n = 1).
[m] O22:H8 (n = 5), O88:H25 (n = 2), O113:H21 (n = 1), O153:H25 (n = 1), O6:H34 (n = 1), O74:H8 (n = 1), O76:H4 (n = 1), ONT:NM (n = 1), and ONT:H18/19/48 (n = 3).
[n] O113:H21 (n = 3).
[o] ONT:H4 (n = 1).

LP44 by use of a second PCR with primers GK5 and GK6. GK5 and GK6 are specific for the B subunits of the stx_2 and stx_{2d} genes (17). A positive PCR result was obtained with 101 (57.1%) of the 177 Stx2 family strains. It was described previously that multiple copies of the stx_2 and stx_{2d} genes can coexist in the same STEC strain (1, 5, 21, 43). In order to examine this possibility, we digested the 269-bp PCR products obtained with primers GK5 and GK6 with HaeIII, RsaI, FokI, and NciI. The resulting RFLP patterns were attributed to the different subtypes of stx_2 and stx_{2d} genes, such as $stx_{2v\text{-ha}}$, $stx_{2v\text{-hb}}$, and $stx_{2\text{-NV206}}$, as previously described (1, 17) (Table 3; Fig. 2). The PCR/RFLP analysis revealed the presence of single and multiple copies of stx_2, $stx_{2v\text{-ha}}$, $stx_{2v\text{-hb}}$, and $stx_{2\text{-NV206}}$ genes in 94 STEC strains from food (Table 5). In addition, two other Stx2 type genes were identified by the PCR/RFLP analysis (Table 3; Fig. 2). One RFLP pattern characteristic of the recently described stx_{2g} variant was found in nine STEC strains. The other RFLP pattern ($stx_{2\text{-EC1586}}$) was found in only one STEC strain (RL481/06), from raw milk (Table 3; Fig. 2). Because it did not correspond to patterns of previously described stx_2 B variants, we determined the nucleotide sequence of the stx_2 B subunit of RL481/06 (GenBank accession no. AM498375). It was found to be homologous to that of a recently described stx_{2d} variant gene of an STEC O174:H8 strain from livestock sources in Australia (GenBank accession no. AF500188) (17).

Finally, in order to distinguish between stx_2 and stx_{2d} we

TABLE 6. Origins and properties of STEC strains from food belonging to the most important serotypes

Serotype(s) (no. of strains)	Stx2 family gene(s) (no. of strains)	Other virulence gene(s) (no. of strains)	Sources[a] (no. of strains)
O8:H19 (3), O8:H21 (1)	stx_2 (2), $stx_2 + stx_{2d}$ (1), stx_{2e} (1)	$hlyA$ (3)	B (1), B/P (1), P (1), W (1)
O8:H4 (3), O8:H9 (11)	stx_{2e} (14)	None	B (1), B/P (6), M (1), P (6)
O21:H21 (9)	$stx_{2\text{-}O118}$ (9)	$hlyA$ (2)	B (3), M (1), W (5)
O22:[H8] (9)	stx_{2d} (9)	stx_1 (8), $hlyA$ (9)	B (4), B/P (2), M (1), P (1), W (1)
O91:[H14] (6)	$stx_{2\text{-}O118}$ (2)	stx_1 (6), $hlyA$ (2)	B (1), B/P (2), L (1), P (2)
O91:[H21] (8)	$stx_2 + stx_{2d}$ (6), stx_2(2)	stx_1 (6), $hlyA$ (7)	B (1), B/P (1), M (2), P (2), W (2)
O100:[H30] (8)	stx_{2e}	None	B (2), B/P (3), M (1), P (2)
O113:H21 (9)	stx_2 (1), stx_{2d} (4), $stx_2 + stx_{2d}$(4)	stx_1 (1), $hlyA$ (7)	B (3), B/P (1), P (3), W (2)
O146:H21 (4), O146:H28 (5)	$stx_2 + stx_{2\text{-}O118}$ (1), $stx_{2\text{-}O118}$ (8)	stx_{1c} (2), $hlyA$ (3)	B (1), W (8)
O174:H2 (3)	stx_2 (1), stx_{2d} (2)	stx_1 (3), $hlyA$ (3)	B (2), M (1)
O174:H21 (5), O174:[H8] (2)	stx_2 (5), $stx_{2\text{-}O118}$ (2)	stx_{1c} (2), $hlyA$ (3)	B (4), W (3)
O178:H19 (4)	stx_2 (4)	stx_1 (2), $hlyA$ (3)	B (3), L (1)
O179:H8 (4)	stx_2 (4)	$hlyA$ (4)	B (2), W (2)

[a] B, beef; W, wildlife meat; L, lamb; P, pork; B/P, mixed beef/pork meat; M, milk.

amplified the stx_2 genes present in all 95 strains carrying stx_2, $stx_{2v\text{-}ha}$, $stx_{2v\text{-}hb}$, $stx_{2\text{-}NV206}$, and $stx_{2\text{-}EC1586}$ genes with primers SLT-II-vc and CKS2. The 890-bp PCR product was digested with PstI, and stx_{2d} genes were discriminated from nonactivatable stx_2 by lack of a PstI site, as described previously (5, 17, 21). stx_2 was present in 50 (52.6%) and stx_{2d} in 61 (64.2%) of the strains (Table 5). Interestingly, 16 (16.8%) of the 95 STEC strains carried both an stx_2 and an stx_{2d} gene.

Other genetic variants of the Stx2 family that were detected in STEC strains from food were stx_{2e} (42 strains), $stx_{2\text{-}O118}$ (38 strains), and stx_{2g} (nine strains) (Table 4). In contrast, none of the 219 STEC strains carried an stx_{2f} gene.

Association between serotype, stx type, and other virulence markers of STEC. The 219 STEC strains from food belonged to 45 *E. coli* O serogroups and to 31 H types. Fifty-four (24.7%) of the STEC strains showed nontypeable (ONT) O antigens different from O1 to O181, and 35 (16.0%) were nonmotile. However, only 10 serogroups (O8, O21, O22, O91, O100, O113, O146, O174, O178, and O179) accounted for 94 (42.9%) of all STEC isolates (Table 6). The association with the H type was even more pronounced, as only four flagellar types (H8, H9, H19, and H21) accounted for 97 (44.3%) of all STEC strains from food. Other STEC virulence markers were present, such as production of the EHEC hemolysin gene (*hlyA*) in 99 (45.2%) and the *eae* gene in 11 (5.0%) of the STEC strains. Only four (1.8%) STEC strains from food belonged to the group of typical EHEC strains. These were two O26:[H11] (stx_1, *eae*-beta, and *hlyA*), one O103:H2 (stx_1, *eae*-epsilon, and *hlyA*), and one O157:[H7] (stx_1, stx_2, *eae*-gamma, and *hlyA*) strain, all of which originated from meat.

On the other hand, 95 (44.0%) of the STEC strains from food were shown to carry stx_2 and/or stx_{2d} genes (Table 5). stx_2 and/or stx_{2d} genes were found to be closely associated with atypical EHEC serotypes O8:H19, O22:[H8], O91:H21, O113:H21, O174:H2, O174:H21, O178:H19, and O179:H8 (Table 6).

The stx_{2e} gene was present in other *E. coli* serotypes (O2, O8:H4/H9, O36:H19, O59:H21, O100:[H30], O141:H4, and ONT), and none of the 42 (19.2%) STEC isolates from this study carried other virulence markers except stx_{2e} (Table 4). The $stx_{2\text{-}O118}$ variant was detected in 37 (16.9%) of the STEC isolates from food. It was associated with certain serotypes (O21:H21, O22:H16, O91:H14, O128:H2, O146:H21/H28, and ONT) of strains, with toxin types stx_2 or stx_{2d} (three strains) and stx_1 or stx_{1c} (10 strains), and with the *hlyA* gene (14 strains) (Table 4). The stx_{2g} variant was present in six STEC strains belonging to rare serotypes (O179:H31, O74:H28, O15:H16, O36:H14, and O59:H21) and occurred in combination with stx_2 in three strains (Table 5).

DISCUSSION

Control of food samples for possible contamination with STEC is performed routinely by governmental laboratories situated in different parts of Germany. Most commonly, these laboratories employ commercially obtainable Stx-specific ELISA kits and colony immunoblotting techniques for detection and subsequent isolation of STEC from food (3, 23, 44). This procedure allows detection of STEC strains independent of their serotypes, which is important to identify virtually all STEC types present in a sample (2). Correspondingly, we obtained a wide spectrum of different serotypes of STEC from food samples for analysis. One interesting finding was that typical EHEC O26, O103, and O157 strains represented only 1.8% of STEC isolates from food. In contrast, EHEC O26:H11 and O157:H7 strains were reported as being isolated most frequently from human patients infected with STEC in Germany and other countries (4, 5, 15). In a similar way, intimin (*eae* gene)-positive strains accounted for only 11 (5.0%) STEC strains from food. Intimin-positive STEC strains were described as most frequently isolated from 1- to 3-year-old pediatric patients suffering from diarrhea and HUS (4, 47). Our data indicate that in Germany food is less important as a source of infection with typical EHEC strains. This finding corroborates a recently published risk analysis for STEC infections in Germany stating that direct transmission from food plays a limited role for infections in children under 3 years of age (47).

On the other hand, about half of the STEC strains from this study were positive for stx_2 and/or stx_{2d}. Production of these toxins is an indicator for severe clinical outcome in the infected patients (5, 6, 15, 21). In our study, stx_2 and stx_{2d} were common in STEC strains of serotypes O22:H8, O91:H21, O113:H21,

O174:H2/H21, and O178:H19 and most of these strains originated from meat (Table 6). STEC strains belonging to these serotypes were associated with cattle as an animal reservoir (http://www.microbionet.com.au/vtectable.htm) and were isolated from patients of all age groups with diarrhea in Germany (4, 5, 47). Some types, such as STEC O91:H21, O113:H21, and O174:H21, were identified as agents of HUS in patients from different countries (7, 20, 33, 36).

In 2005, STEC was detected in 6.7% of 535 investigated meat samples and in 1.9% of 2,681 raw-milk samples in Germany (18). Consumption of meat was identified as a major risk factor of patients older than 10 years for getting infected with STEC (47). More than 80% of STEC strains from our study originated from meat and 87 (48.3%) of 180 STEC-positive meat samples were beef or contained beef together with lamb or pork meat. Seventy-two (82.8%) of the STEC strains isolated from pure beef or beef-containing products carried the stx_2, stx_{2d}, or stx_{2g} gene. Beef and its products may therefore be regarded as most critical in regard to contamination with potentially human-virulent STEC strains.

Twenty-six (14.4%) of the STEC-positive samples from this study contained only pork meat. The stx_{2e} gene was present in 15 (57.7%) of the 26 STEC strains isolated from pork but in only 3 (5.9%) of 51 samples containing only beef. Stx2e, also designated the porcine edema disease toxin (11), is associated mainly with STEC from pigs and is rarely found in other species, including humans (4, 15, 41). The linkage of the stx_{2e} gene with pork meat could indicate that most of the products are contaminated directly by fecal material from pigs after slaughter. The serotypes of stx_{2e} strains from our study were similar to those described to occur in pigs from a study performed in the United States (14). The pathogenicity of Stx2e-producing STEC strains for humans is regarded as low, but some types of Stx2e-producing strains were isolated from more severe cases of diarrhea in patients (4, 15, 41).

In this work, we developed a rapid PCR/RFLP method used for differentiation between members of the Stx1 family in regard to nucleotide sequence differences in the B subunits of stx_1, stx_{1c}, and stx_{1d} (Fig. 1; Table 2). This method was found useful for identification of stx_1 variants and provides the advantage that strains carrying multiple types of stx_1 become detectable. In contrast to findings for genes of the Stx2 family, we did not detect more than one stx_1 gene in any of the 88 investigated strains. This might indicate that the bacteriophages encoding different stx_1 types do not coexist frequently in the same strain. Similar findings were reported for human and animal STEC strains investigated for stx_1 genes (8, 25).

It has been described previously that the stx_{1c} gene is associated with sheep and goats as the animal reservoir (8, 24), and 8 of 24 samples containing stx_{1c} originated from lamb meat products, sheep milk, or goat cheese. The finding that 11 of the samples containing stx_{1c} STEC strains were isolated from deer meat points to wild animals as a possible reservoir for these STEC types.

PCR/RFLP typing of the B subunits of the stx_2 and stx_{2d} genes was found useful for investigation of both the presence of genetic variants and that of multiple types of stx_2 and stx_{2d} in STEC (17). Apart from identification of stx_2 and stx_{2d} B-subunit variants, this method allowed the detection of the stx_{2g} toxin type. Stx2g was recently described as a new, rarely occurring stx_2 variant in bovine STEC that is not activatable by intestinal mucus (26). Like stx_{1d}, stx_{2g} genes carrying STEC have not yet been reported to occur in humans and their role as agents of disease is not clear. In any case, the finding that stx_{1d} and stx_{2g} STEC strains are present in food samples makes it likely that humans can be exposed to these STEC types along the food chain. In contrast, stx_{2f} genes carrying STEC appear to be uncommon in meat and milk samples.

In this work, we have shown that the combination of serotyping and stx genotyping is very suitable for identification and for assignment of food-borne STEC strains to groups of lower and higher levels of potential virulence for humans. The finding that stx_2 and/or stx_{2d} STEC strains of known human-pathogenic serotypes are present in about half of the isolates indicates that raw-meat and -milk products present a risk for consumers of all age groups. Further improvement of molecular detection methods will contribute to the rapid identification of highly virulent STEC strains independent of their serotype. These methods will be very useful in the investigation of food-borne outbreaks and sporadic cases caused by new or unusual types of STEC strains.

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