APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2009, p. 6947–6953 Vol. 75, No. 21
0099-2240/09/$12.00 doi:10.1128/AEM.00605-09

Analysis of the Clonal Relationship of Serotype O26:H11 Enterohemorrhagic *Escherichia coli* Isolates from Cattle[▽]

Lutz Geue,[1]* Sabrina Klare,[1] Christina Schnick,[1] Birgit Mintel,[1] Katharina Meyer,[2] and Franz J. Conraths[1]

Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Seestrasse 55, D-16868 Wusterhausen, Germany,[1] and Ernst-Moritz-Arndt University Greifswald, Institute for Mathematics and Informatics, Friedrich-Ludwig-Jahn-Strasse 15a, D-17489 Greifswald, Germany[2]

Received 13 March 2009/Accepted 29 August 2009

Twelve cluster groups of *Escherichia coli* O26 isolates found in three cattle farms were monitored in space and time. Cluster analysis suggests that only some O26:H11 strains had the potential for long-term persistence in hosts and farms. As judged by their virulence markers, bovine enterohemorrhagic O26:H11 isolates may represent a considerable risk for human infection.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains comprise a group of zoonotic enteric pathogens (42). In humans, infections with some STEC serotypes result in hemorrhagic or nonhemorrhagic diarrhea, which can be complicated by hemolytic-uremic syndrome (HUS) (49). These STEC strains are also designated "enterohemorrhagic *E. coli*" (EHEC). Consequently, EHEC strains represent a subgroup of STEC with a high pathogenic potential for humans. Strains of the *E. coli* serogroup O26 were originally classified as enteropathogenic *E. coli* due to their association with outbreaks of infantile diarrhea in the 1940s. In 1977, Konowalchuk et al. (37) recognized that these bacteria produced Stx, and 10 years later, the Stx-producing *E. coli* O26:H11/H− strains were classified as EHEC. EHEC O26 strains constitute the most common non-O157 EHEC group associated with diarrhea and HUS in Europe (12, 21, 23, 24, 26, 27, 55, 60). Reports on an association between EHEC O26 and HUS or diarrhea from North America including the United States (15, 30, 33), South America (51, 57), Australia (22), and Asia (31, 32) provide further evidence for the worldwide spread of these organisms. Studies in Germany and Austria (26, 27) on sporadic HUS cases between 1996 and 2003 found that EHEC O26 accounted for 14% of all EHEC strains and for ~40% of non-O157 EHEC strains obtained from these patients. A proportion of 11% EHEC O26 strains was detected in a case-control study in Germany (59) between 2001 and 2003. In the age group <3 years, the number of EHEC O26 cases was nearly equal to that of EHEC O157 cases, although the incidence of EHEC O26-associated disease is probably underestimated because of diagnostic limitations in comparison to the diagnosis of O157:H7/H− (18, 34). Moreover, EHEC O26 has spread globally (35). Beutin (6) described EHEC O26:H11/H−, among O103:H2, O111:H, O145:H28/H−, and O157:H7/H−, as the well-known pathogenic "gang of five," and Bettelheim (5) warned that we ignore the non-O157 STEC strains at our peril.

EHEC O26 strains produce Stx1, Stx2, or both (15, 63). Moreover, these strains contain the intimin-encoding *eae* gene (11, 63), a characteristic feature of EHEC (44). In addition, EHEC strains possess other markers associated with virulence, such as a large plasmid that carries further potential virulence genes, e.g., genes coding for EHEC hemolysin (*EHEC-hlyA*), a catalase-peroxidase (*katP*), and an extracellular serine protease (*espP*) (17, 52). The *efa1* (*E. coli f*actor for *a*dherence *1*) gene was identified as an intestinal colonization factor in EHEC (43). EHEC O26 represents a highly dynamic group of organisms that rapidly generate new pathogenic clones (7, 8, 63).

Ruminants, especially cattle, are considered the primary reservoir for human infections with EHEC. Therefore, the aim of this study was the molecular characterization of bovine *E. coli* field isolates of serogroup O26 using a panel of typical virulence markers. The epidemiological situation in the beef herds from which the isolates were obtained and the spatial and temporal behavior of the clonal distribution of *E. coli* serogroup O26 were analyzed during the observation period. The potential risk of the isolates inducing disease in humans was assessed.

In our study, 56 bovine *E. coli* O26:H11 isolates and one bovine O26:H32 isolate were analyzed for EHEC virulence-associated factors. The isolates had been obtained from three different beef farms during a long-term study. They were detected in eight different cattle in farm A over a period of 15 months (detected on 10 sampling days), in 3 different animals in farm C over a period of 8 months (detected on 3 sampling days), and in one cow on one sampling day in farm D (Table 1) (28).

The serotyping of the O26 isolates was confirmed by the results of the *fliC* PCR-restriction fragment length polymorphism (RFLP) analysis performed according to Fields et al. (25), with slight modifications described by Zhang et al. (62). All O26:H11 isolates showed the H11 pattern described by Zhang et al. (62). In contrast, the O26:H32 isolate demonstrated a different *fliC* RFLP pattern that was identical to the H32 pattern described by the same authors. It has been demonstrated that EHEC O26:H11 strains belong to at least four

* Corresponding author. Mailing address: Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Seestrasse 55, D-16868 Wusterhausen, Germany. Phone: 49 33979 80189. Fax: 49 33979 80222. E-mail: lutz.geue@fli.bund.de.

▽ Published ahead of print on 4 September 2009.

TABLE 1. Typing of *E. coli* O26 isolates

Sampling day, source, and isolate	Serotype	Virulence profile by:															
		fliC PCR-RFLP	stx_1 gene	stx_2 gene	Stx1 (toxin)	Stx2 (toxin)	Subtype(s)					*efa1* gene[b]	*EHEC-hlyA* gene	*katP* gene	*espP* gene	Plasmid size(s) in kb	Cluster
							stx_1/stx_2	*eae*	*tir*	*espA*	*espB*						
Day 15																	
Animal 6 (farm A)																	
WH-01/06/002-1	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	7
WH-01/06/002-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	7
WH-01/06/002-3	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	7
Animal 8 (farm A)																	
WH-01/08/002-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	7
Animal 26 (farm A)																	
WH-01/26/001-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12	7
WH-01/26/001-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	7
WH-01/26/001-6	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	7
WH-01/26/001-7	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/−	+	+	+	110, 12	7
Day 29																	
Animal 2 (farm A)																	
WH-01/02/003-1	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	6
WH-01/02/003-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	6
WH-01/02/003-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	6
WH-01/02/003-6	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	−	+	+	110, 12	6
WH-01/02/003-7	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	6
WH-01/02/003-8	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	−/+	+	+	+	110, 12	6
WH-01/02/003-9	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110	6
WH-01/02/003-10	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110	6
Animal 26 (farm A)																	
WH-01/26/002-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12	5
WH-01/26/002-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12	5
WH-01/26/002-8	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12	5
WH-01/26/002-9	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	−	−	110, 12	5
WH-01/26/002-10	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12	5
Day 64																	
Animal 20 (farm A)																	
WH-01/20/005-3	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	−	−	−	130, 2.5	2
Day 78																	
Animal 29 (farm A)																	
WH-01/29/002-1	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/−	+	−	−	130, 12, 2.5	4
WH-01/29/002-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12, 2.5	4
WH-01/29/002-3	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12, 2.5	4
WH-01/29/002-4	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12, 2.5	4
WH-01/29/002-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	−	−	+	130, 12, 2.5	4
Day 106																	
Animal 27 (farm A)																	
WH-01/27/005-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/−	+	+	+	145, 110, 12	3
WH-01/27/005-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 12, 2.5	5
WH-01/27/005-6	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	−	−	−	130, 12, 2.5	5
Day 113																	
Animal 7 (farm C)																	
WH-04/07/001-2	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	−	+	+	55, 35, 2.5	11
WH-04/07/001-4	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	55	12
WH-04/07/001-6	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	55	12
Day 170																	
Animal 22 (farm C)																	
WH-04/22/001-1	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12, 6.3	12
WH-04/22/001-4	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12, 6.3	12
WH-04/22/001-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12, 6.3	12
Day 176																	
Animal 14 (farm D)																	
WH-03/14/004-8	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	−	+	+	110	10

Continued on following page

TABLE 1—*Continued*

Sampling day, source, and isolate	Serotype	Virulence profile by:															
		fliC PCR-RFLP	stx_1 gene	stx_2 gene	Stx1 (toxin)	Stx2 (toxin)	Subtype(s)					*efa1* gene[b]	*EHEC-hlyA* gene	*katP* gene	*espP* gene	Plasmid size(s) in kb	Cluster
							stx_1/stx_2	*eae*	*tir*	*espA*	*espB*						
Day 218																	
Animal 27 (farm A)																	
WH-01/27/009-1	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110, 12	9
WH-01/27/009-2	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110, 12	9
WH-01/27/009-3	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110, 12	8
WH-01/27/009-8	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	−	−	110, 12	8
WH-01/27/009-9	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110, 12	9
Day 309																	
Animal 29 (farm A)																	
WH-01/29/010-1	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 35, 12	4
WH-01/29/010-2	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	−	−	130, 55, 35	8
WH-01/29/010-3	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	130, 35, 12	8
Day 365																	
Animal 8 (farm C)																	
WH-04/08/008-6	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 55	12
Day 379																	
Animal 9 (farm A)																	
WH-01/09/016-2	O26:H32	H32	+	+	−	−	stx_1/stx_2	−	−	−	−	−/−	−	−	−	145, 130, 1.8	1
Animal 27 (farm A)																	
WH-01/27/014-3	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	9
WH-01/27/014-4	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	9
WH-01/27/014-5	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12	8
Day 407																	
Animal 29 (farm A)																	
WH-01/29/013-4	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12, 2.5	8
WH-01/29/013-7	O26:H11	H11	+	−	+	−	stx_1	β	β	β	β	+/+	+	+	+	110, 12, 2.5	8
Day 478																	
Animal 27 (farm A)																	
WH-01/27/017-1	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110, 12	8
WH-01/27/017-5	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110, 12	8
WH-01/27/017-6	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110	8
WH-01/27/017-7	O26:H11	H11	+	+	+	+	stx_1/stx_2	β	β	β	β	+/+	+	+	+	110	8
WH-01/27/017-10	O26:H11	H11	+	−	+	+	stx_1	β	β	β	β	+/+	+	+	+	130, 12, 2.5	8

[a] stx_1/stx_2, gene stx_1 or stx_2.
[b] *efa1* was detected by two hybridizations (with *lifA1-lifA2* and *lifA3-lifA4* probes). +/+, complete gene; +/− or −/+, incomplete gene; −/−, *efa1* negative.

different sequence types (STs) in the common clone complex 29 (39). In the multilocus sequence typing analysis for *E. coli* (61), the tested five EHEC O26:H11 isolates (WH-01/02/003-1, WH-01/20/005-3, WH-01/27/009-9, WH-03/14/004-8, and WH-04/22/001-1) of different farms and clusters were characterized as two sequence types (ST 21 and ST 396). The isolates from farms A and C belong to ST 21, the most frequent ST of EHEC O26:H11 isolates found in humans and animals (39), but the single isolate from farm D was characterized as ST 396.

Typing and subtyping of genes (stx_1 and/or stx_2, *eae*, *tir*, *espA*, *espB*, *EHEC-hlyA*, *katP*, and *espP*) associated with EHEC were performed with LightCycler fluorescence PCR (48) and different block-cycler PCRs. To identify the subtypes of the stx_2 genes and of the locus of enterocyte effacement-encoding genes *eae*, *tir*, *espA*, and *espB*, the PCR products were digested by different restriction endonucleases (19, 26, 46). The complete pattern of virulence markers was detected in most bovine isolates examined in our study. An stx_1 gene was present in all O26 isolates. In addition, an stx_2 gene was found in nine O26:H11 isolates in farm A and in three isolates of the same type in farm C, as well as in the O26:H32 isolate. Both Stx1 and Stx2 were closely related to families of Stx1 and Stx2 variants or alleles. EHEC isolates with stx_2 genes are significantly more often associated with HUS and other severe disease manifestations than isolates with an stx_1 gene, which are more frequently associated with uncomplicated diarrhea and healthy individuals (13). In contrast to STEC strains harboring stx_2 gene variants, however, STEC strains of the stx_2 genotype were statistically significantly associated with HUS (26). The stx_2 genotype was found in all O26 isolates with an stx_2 gene, while the GK3/GK4 amplification products after digestion with HaeIII and FokI restriction enzymes showed the typical pattern for this genotype described by Friedrich et al. (26). The nucleotide sequences of the A and B subunits of the stx_2 gene of the selected bovine O26:H11 isolate WH-01/27/017-1 (GenBank accession no. EU700491) were identical to the stx_2

genes of different sorbitol-fermenting EHEC O157:H− strains associated with human HUS cases and other EHEC infections in Germany (10) and 99.3% identical in their DNA sequences to the stx_2 gene of the EHEC type strain EDL933, a typical O157:H7 isolate from an HUS patient. A characteristic stx_1 genotype was present in all O26 isolates. The nucleotide sequences of the A and B subunits of the stx_1 gene of the tested bovine O26:H11 isolate WH-01/27/017-1 (GenBank accession no. EU700490) were nearly identical to those of the stx_1 genes of the EHEC O26:H11 reference type strains H19 and DEC10B, which had been associated with human disease outbreaks in Canada and Australia. Nucleotide exchanges typical for stx_{1c} and stx_{1d} subtypes as described by Kuczius et al. (38) were not found. All bovine O26:H11 strains produced an Stx1 with high cytotoxicity for Vero cells tested by Stx enzyme-linked immunosorbent assay and Vero cell neutralization assay (53). The Stx2 cytotoxicity for Vero cells was also very high in the O26:H11 isolates.

Not only factors influencing the basic and inducible Stx production are important in STEC pathogenesis. It has been suggested that the *eae* and *EHEC-hlyA* genes are likely contributors to STEC pathogenicity (2, 3, 13, 50). Ritchie et al. (50) found both genes in all analyzed HUS-associated STEC isolates. In all O26:H11 isolates we obtained, *stx* genes were present in combination with *eae* genes. Only the O26:H32 isolate lacked an *eae* gene. To date, 10 distinct variants of *eae* have been described (1, 19, 36, 45, 47). Some serotypes were closely associated with a particular intimin variant: the O157 serogroup was linked to γ-*eae*, the O26 serogroup to β-*eae*, and the O103 serogroup to ε-*eae* (4, 19, 20, 58). Our study confirms these associations. All bovine O26:H11 isolates were also typed as members of the β-*eae* subgroup. A translocated intimin receptor gene (*tir* gene) and the type III secreted proteins encoded by the *espA* and *espB* genes were found in all 56 O26:H11 isolates but not in the O26:H32 isolate. These other tested locus of enterocyte effacement-associated genes belonged to the β-subgroups. These results are in accord with the results of China et al. (19), who detected the pathotypes β-*eae*, β-*tir*, β-*espA*, and β-*espB* in all investigated human O26 strains. Like the *eae* gene, the *EHEC-hlyA* gene was found in association with severe clinical disease in humans (52). Aldick et al. (2) showed that EHEC hemolysin is toxic (cytolytic) to human microvascular endothelial cells and may thus contribute to the pathogenesis of HUS. In our study, the *EHEC-hlyA* gene was detected in 50 of the 56 bovine *E. coli* O26:H11 isolates which harbored virulence-associated plasmids of different sizes (Table 1). The presence of virulence-associated plasmids corresponded to the occurrence of additional virulence markers such as the *espP* and *katP* genes (17). The *katP* gene and the *espP* gene were detected in 49 and 50 of the 56 O26:H11 isolates, respectively. The *espP* gene was missing in six of the seven bovine O26:H11 isolates in which the *katP* genes were also absent. Both genes were not found in the O26:H32 isolate (Table 1). Although we found large plasmids of the same size in O26:H11 isolates, they lacked one or more of the plasmid-associated virulence factors (Table 1). Two DNA probes were used to detect the *efa1* genes by colony hybridization. (DNA probes were labeled with digoxigenin [DIG] with *lifA1-lifA2* and *lifA3-lifA4* primers [14] using the PCR DIG probe synthesis kit [Roche Diagnostics, Mannheim, Germany]; DIG Easy Hyb solution [Roche] was used for prehybridization and hybridization.) Positive results with both DNA probes were obtained for 52 of 56 *E. coli* O26:H11 isolates. A positive signal was only found in three isolates with the *lifA1-lifA2* DNA probe and in one isolate with the *lifA3-lifA4* probe. An e*fa1* gene was not detected in the O26:H32 isolate (Table 1).

We also analyzed the spatial and temporal behavior of the O26:H11/H32 isolates in the beef herds by cluster analysis (conducted in PAUP* for Windows version 4.0, 2008 [http://paup.csit.fsu.edu/about.html]). This was performed with distance matrices using the neighbor-joining algorithm, an agglomerative cluster method which generates a phylogenetic tree. The distance matrices were calculated by pairwise comparisons of the fragmentation patterns produced by genomic typing through pulsed-field gel electrophoresis analysis with four restriction endonucleases (XbaI, NotI, BlnI, and SpeI) and the presence or absence of potential virulence markers (Fig. 1 and Table 1). To this end, the total character difference was used, which counts the pairwise differences between two given patterns. During a monitoring program of 3 years in four cattle farms (29), different O26:H11 cluster groups and one O26:H32 isolate were detected in three different farms. The genetic distance of the O26:H32 isolate was very high relative to the O26:H11 isolates. Therefore, the O26:H32 isolate was outgrouped. The O26:H11 isolates of each farm represented independent cluster groups. The single isolate from farm D fitted better to the isolates from farm C than to those from farm A. This finding is in accord with the geographical distance between the farms. The fact that the farms were located in neighboring villages may suggest that direct or indirect connections between the farms were possible (e.g., by person contacts or animal trade). However, the isolates from farm C and farm D belonged to different sequence types (ST 21 and ST 396), which may argue against a direct connection. Interestingly, O26:H11 isolates with and without stx_2 genes were detected in the same clusters. This phenomenon was observed in both farm A and farm C. In farm A, the isolates with additional stx_2 genes were found in animal 27 and were grouped in clusters 8 and 9 (day 218). An stx_2 gene was repeatedly found (four isolates) in the same animal (animal 27). The isolates grouped in cluster 8 on a later day of sampling (day 478). All other O26:H11 isolates grouped in the same clusters and obtained from the same animals (27 and 29) on different sampling days lacked an stx_2 gene. Also, the isolates obtained from animal 27 on previous sampling days, which grouped in clusters 3 and 5, exhibited no stx_2 genes. In farm C, the three isolates with additional stx_2 genes obtained from animal 7 grouped in clusters 11 and 12. An stx_2 gene was absent from all other O26:H11 isolates grouped in the same cluster 12 on later sampling days, and no other isolates of cluster 11 were found later on. However, we detected members of many clusters over relatively long periods (clusters 5, 8, and 9 in farm A and cluster 12 in farm C), but members of other clusters were only found on single occasions. This patchy temporal pattern is apparently not a unique property of O26:H11, as we found similar results for cluster groups of other EHEC serotypes of bovine origin (28). The isolates grouped in the dominant cluster 8 were found on 5 of 9 sampling days over a period of 10 months. In contrast, we found the members of clusters 4, 5, 9, and 12 only on two nonconsecutive sampling days. The period during

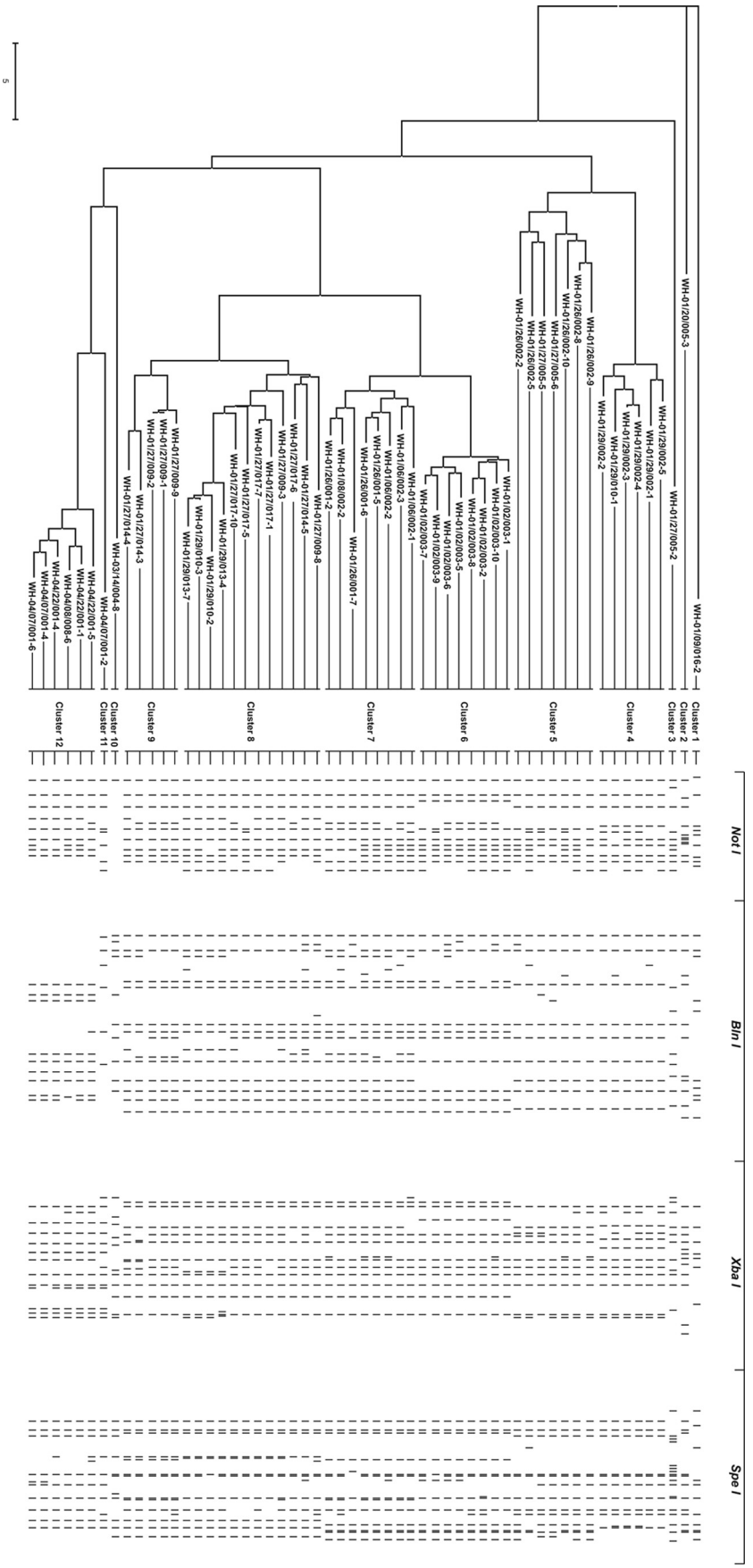

FIG. 1. Neighbor-joining tree of bovine *E. coli* O26:H11/H32 strains based on the restriction pattern obtained after digestion with XbaI, NotI, BlnI, and SpeI.

which isolates of these groups were not detected was particularly long for cluster 4 (231 days). We also observed the coexistence of different clusters over long periods in the same farm and in the same cattle (clusters 8 and 9), while one of the clusters dominated. Transmission of clusters between cattle was also observed. These results suggest that some of the EHEC O26:H11 strains had the potential for a longer persistence in the host population, while others had not. The reasons for this difference are not yet clear. Perhaps the incomplete *efa1* gene found in isolates of clusters which were only detected once might explain why some strains disappeared rapidly. Efa1 has been discussed as a potential *E. coli* colonization factor for the bovine intestine used by non-O157 STEC, including O26 (54, 56). The O165:H25 cluster detected during a longer period in farm B may have disappeared after it had lost its *efa1* gene (28). The precise biological activity of Efa1 in EHEC O26 is not yet known, but it has been demonstrated that the molecule is a non-Stx virulence determinant which can increase the virulence of EHEC O26 in humans (8).

We distinguished 12 different clusters, but complete genetic identity was only found in two isolates. The variations in the O26:H11 clusters may be due to increasing competition between the bacterial populations of the various subtypes in the bovine intestine or to potential interactions between EHEC O26:H11 and the host.

The ephemeral occurrence of additional stx_2 genes in different clusters and farms may be the result of recombination events due to horizontal gene transfer (16). The loss of *stx* genes may occur rapidly in the course of an infection, but the reincorporation by induction of an *stx*-carrying bacteriophage into the O26:H11 strains is possible at any time (9, 40). Nevertheless, an additional stx_2 gene may increase the dangerousness of the respective EHEC O26:H11 strains. While all patients involved in an outbreak caused by an EHEC O26:H11 strain harboring the gene encoding Stx2 developed HUS (41), the persons affected by another outbreak caused by an EHEC O26:H11 strain that produced exclusively Stx1 had only uncomplicated diarrhea (60).

In conclusion, our results showed that bovine O26:H11 isolates can carry virulence factors of EHEC that are strongly associated with EHEC-related disease in humans, particularly with severe clinical manifestations such as hemorrhagic colitis and HUS. Therefore, strains of bovine origin may represent a considerable risk for human infection. Moreover, some clusters of EHEC O26:H11 persisted in cattle and farms over longer periods, which may increase the risk of transmission to other animals and humans even further.

We thank J. Bockemühl, H. Tschäpe, A. Fruth, and T. Kuzius for serotyping the *E. coli* isolates.

This study was funded by the German Federal Ministry of Food, Agriculture and Consumer Protection.

REFERENCES

1. **Adu-Bobie, J., G. Frankel, C. Bain, A. G. Goncalves, L. R. Trabulsi, G. Douce, S. Knutton, and G. Dougan.** 1998. Detection of intimins α, β, γ, and δ, four intimin derivatives expressed by attaching and effacing microbial pathogens. J. Clin. Microbiol. **36:**662–668.
2. **Aldick, T., M. Bielaszewska, W. L. Zhang, J. Brockmeyer, H. Schmidt, A. W. Friedrich, K. S. Kim, M. A. Schmidt, and H. Karch.** 2007. Hemolysin from Shiga toxin-negative *Escherichia coli* O26 strains injures microvascular endothelium. Microbes Infect. **9:**282–290.
3. **Barrett, T. J., J. B. Kaper, A. E. Jerse, and I. K. Wachsmuth.** 1992. Virulence factors in Shiga-like toxin-producing *Escherichia coli* isolated from humans and cattle. J. Infect. Dis. **165:**979–980.
4. **Bertin, Y., K. Boukhors, V. Livrelli, and C. Martin.** 2004. Localization of the insertion site and pathotype determination of the locus of enterocyte effacement of Shiga toxin-producing *Escherichia coli* strains. Appl. Environ. Microbiol. **70:**61–68.
5. **Bettelheim, K. A.** 2007. The non-O157 shiga-toxigenic (verocytotoxigenic) *Escherichia coli*: under-rated pathogens. Crit. Rev. Microbiol. **33:**67–87.
6. **Beutin, L.** 2006. Emerging enterohaemorrhagic *Escherichia coli*, causes and effects of the rise of a human pathogen. J. Vet. Med. B **53:**299–305.
7. **Bielaszewska, M., R. Kock, A. W. Friedrich, C. von Eiff, L. B. Zimmerhackl, H. Karch, and A. Mellmann.** 2007. Shiga toxin-mediated hemolytic uremic syndrome: time to change the diagnostic paradigm? PLoS ONE **2:**e1024.
8. **Bielaszewska, M., W. Zhang, A. Mellmann, and H. Karch.** 2007. Enterohaemorrhagic *Escherichia coli* O26:H11/H−: a human pathogen in emergence. Berl. Munch. Tierarztl. Wochenschr. **120:**279–287.
9. **Bielaszewska, M., R. Prager, R. Köck, A. Mellmann, W. Zhang, H. Tschäpe, P. I. Tarr, and H. Karch.** 2007. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. Appl. Environ. Microbiol. **73:**3144–3150.
10. **Bielaszewska, M., R. Prager, W. Zhang, A. W. Friedrich, A. Mellmann, H. Tschäpe, and H. Karch.** 2006. Chromosomal dynamism in progeny of outbreak-related sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:NM. Appl. Environ. Microbiol. **72:**1900–1909.
11. **Bielaszewska, M., W. Zhang, P. I. Tarr, A.-K. Sonntag, and H. Karch.** 2005. Molecular profiling and phenotype analysis of *Escherichia coli* O26:H11 and O26:NM: secular and geographic consistency of enterohemorrhagic and enteropathogenic isolates. J. Clin. Microbiol. **43:**4225–4228.
12. **Blanco, J. E., M. Blanco, M. P. Alonso, A. Mora, G. Dahbi, M. A. Coira, and J. Blanco.** 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J. Clin. Microbiol. **42:**311–319.
13. **Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles.** 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J. Clin. Microbiol. **37:**497–503.
14. **Böhnke, U.** 2002. Untersuchung zur Lokalisation und Regulation des als "lymphocyte inhibitory factor" (*lifA*) oder "EHEC factor for adherence" (*efa1*) bezeichneten Gens des bovinen enterohämorrhagischen *E. coli*-Stammes RW1374 (O103:H2). Ph.D. thesis. Diplomarbeit, Freie Universität Berlin, Berlin, Germany.
15. **Brooks, J. T., E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra, and N. A. Strockbine.** 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. J. Infect. Dis. **192:**1422–1429.
16. **Brunder, W., and H. Karch.** 2000. Genome plasticity in Enterobacteriaceae. Int. J. Med. Microbiol. **290:**153–165.
17. **Brunder, W., H. Schmidt, M. Frosch, and H. Karch.** 1999. The large plasmids of Shiga-toxin-producing *Escherichia coli* (STEC) are highly variable genetic elements. Microbiology **145:**1005–1014.
18. **Carroll, A. M., A. Gibson, and E. B. McNamara.** 2005. Laboratory-based surveillance of human verocytotoxigenic *Escherichia coli* infection in the Republic of Ireland, 2002–2004. J. Med. Microbiol. **54:**1163–1169.
19. **China, B., F. Goffaux, V. Pirson, and J. Mainil.** 1999. Comparison of *eae*, *tir*, *espA* and *espB* genes of bovine and human attaching and effacing *Escherichia coli* by multiplex polymerase chain reaction. FEMS Microbiol. Lett. **178:**177–182.
20. **China, B., E. Jacquemin, A. C. Devrin, V. Pirson, and J. Mainil.** 1999. Heterogeneity of the eae genes in attaching/effacing *Escherichia coli* from cattle: comparison with human strains. Res. Microbiol. **150:**323–332.
21. **Eklund, M., F. Scheutz, and A. Siitonen.** 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. J. Clin. Microbiol. **39:**2829–2834.
22. **Elliott, E. J., R. M. Robins-Browne, E. V. O'Loughlin, V. Bennett-Wood, J. Bourke, P. Henning, G. G. Hogg, J. Knight, H. Powell, and D. Redmond.** 2001. Nationwide study of haemolytic uraemic syndrome: clinical, microbiological, and epidemiological features. Arch. Dis. Child. **85:**125–131.
23. **Espie, E., F. Grimont, P. Mariani-Kurkdjian, P. Bouvet, S. Haeghebaert, I. Filliol, C. Loirat, B. Decludt, N. N. Minh, V. Vaillant, and H. de Valk.** 2008. Surveillance of hemolytic uremic syndrome in children less than 15 years of age, a system to monitor O157 and non-O157 Shiga toxin-producing *Escherichia coli* infections in France, 1996–2006. Pediatr. Infect. Dis. J. **27:**595–601.
24. **Ethelberg, S., K. E. Olsen, F. Scheutz, C. Jensen, P. Schiellerup, J. Enberg, A. M. Petersen, B. Olesen, P. Gerner-Smidt, and K. Molbak.** 2004. Virulence factors for hemolytic uremic syndrome, Denmark. Emerg. Infect. Dis. **10:**842–847.
25. **Fields, P. I., K. Blom, H. J. Hughes, L. O. Helsel, P. Feng, and B. Swaminathan.** 1997. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length poly-

morphism test for identification of *E. coli* O157:H7 and O157:NM. J. Clin. Microbiol. **35:**1066–1070.
26. **Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch.** 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J. Infect. Dis. **185:**74–84.
27. **Gerber, A., H. Karch, F. Allerberger, H. M. Verweyen, and L. B. Zimmerhackl.** 2002. Clinical course and the role of shiga toxin-producing *Escherichia coli* infection in the hemolytic-uremic syndrome in pediatric patients, 1997–2000, in Germany and Austria: a prospective study. J. Infect. Dis. **186:**493–500.
28. **Geue, L., T. Selhorst, C. Schnick, B. Mintel, and F. J. Conraths.** 2006. Analysis of the clonal relationship of Shiga toxin-producing *Escherichia coli* serogroup O165:H25 isolated from cattle. Appl. Environ. Microbiol. **72:**2254–2259.
29. **Geue, L., M. Segura-Alvarez, F. J. Conraths, T. Kuczius, J. Bockemuhl, H. Karch, and P. Gallien.** 2002. A long-term study on the prevalence of shiga toxin-producing *Escherichia coli* (STEC) on four German cattle farms. Epidemiol. Infect. **129:**173–185.
30. **Gilmour, M. W., T. Cote, J. Munro, L. Chui, J. Wylie, J. Isaac-Renton, G. Horsman, D. M. Tracz, A. Andrysiak, and L.-K. Ng.** 2005. Multilocus sequence typing of *Escherichia coli* O26:H11 isolates carrying *stx* in Canada does not identify genetic diversity. J. Clin. Microbiol. **43:**5319–5323.
31. **Hiramatsu, R., M. Matsumoto, Y. Miwa, Y. Suzuki, M. Saito, and Y. Miyazaki.** 2002. Characterization of Shiga toxin-producing *Escherichia coli* O26 strains and establishment of selective isolation media for these strains. J. Clin. Microbiol. **40:**922–925.
32. **Hoshina, K., A. Itagaki, R. Seki, K. Yamamoto, S. Masuda, T. Muku, and N. Okada.** 2001. Enterohemorrhagic *Escherichia coli* O26 outbreak caused by contaminated natural water supplied by facility owned by local community. Jpn. J. Infect. Dis. **54:**247–248.
33. **Jelacic, J. K., T. Damrow, G. S. Chen, S. Jelacic, M. Bielaszewska, M. Ciol, H. M. Carvalho, A. R. Melton-Celsa, A. D. O'Brien, and P. I. Tarr.** 2003. Shiga toxin-producing *Escherichia coli* in Montana: bacterial genotypes and clinical profiles. J. Infect. Dis. **188:**719–729.
34. **Jenkins, C., J. Evans, H. Chart, G. A. Willshaw, and G. Frankel.** 2008. *Escherichia coli* serogroup O26—a new look at an old adversary. J. Appl. Microbiol. **104:**14–25.
35. **Johnson, K. E., C. M. Thorpe, and C. L. Sears.** 2006. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. Clin. Infect. Dis. **43:**1587–1595.
36. **Jores, J., K. Zehmke, J. Eichberg, L. Rumer, and L. H. Wieler.** 2003. Description of a novel intimin variant (type zeta) in the bovine O84:NM verotoxin-producing *Escherichia coli* strain 537/89 and the diagnostic value of intimin typing. Exp. Biol. Med. (Maywood) **228:**370–376.
37. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18:**775–779.
38. **Kuczius, T., M. Bielaszewska, A. W. Friedrich, and W. Zhang.** 2004. A rapid method for the discrimination of genes encoding classical Shiga toxin (Stx) 1 and its variants, Stx1c and Stx1d, in *Escherichia coli*. Mol. Nutr. Food Res. **48:**515–521.
39. **Mellmann, A., M. Bielaszewska, R. Kock, A. W. Friedrich, A. Fruth, B. Middendorf, D. Harmsen, M. A. Schmidt, and H. Karch.** 2008. Analysis of collection of hemolytic uremic syndrome-associated enterohemorrhagic *Escherichia coli*. Emerg. Infect. Dis. **14:**1287–1290.
40. **Mellmann, A., M. Bielaszewska, L. B. Zimmerhackl, R. Prager, D. Harmsen, H. Tschape, and H. Karch.** 2005. Enterohemorrhagic *Escherichia coli* in human infection: in vivo evolution of a bacterial pathogen. Clin. Infect. Dis. **41:**785–792.
41. **Misselwitz, J., H. Karch, M. Bielazewska, U. John, F. Ringelmann, G. Ronnefarth, and L. Patzer.** 2003. Cluster of hemolytic-uremic syndrome caused by Shiga toxin-producing *Escherichia coli* O26:H11. Pediatr. Infect. Dis. **22:**349–354.
42. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
43. **Nicholls, L., T. H. Grant, and R. M. Robins-Browne.** 2000. Identification of a novel genetic locus that is required for in vitro adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. Mol. Microbiol. **35:**275–288.
44. **Orth, D., and R. Würzner.** 2006. What makes an enterohemorrhagic *Escherichia coli*? Clin. Infect. Dis. **43:**1168–1169.
45. **Oswald, E., H. Schmidt, S. Morabito, H. Karch, O. Marchès, and A. Caprioli.** 2000. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infect. Immun. **68:**64–71.
46. **Ramachandran, V., K. Brett, M. A. Hornitzky, M. Dowton, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic.** 2003. Distribution of intimin subtypes among *Escherichia coli* isolates from ruminant and human sources. J. Clin. Microbiol. **41:**5022–5032.
47. **Reid, S. D., C. J. Herbelin, A. C. Bumbaugh, R. K. Selander, and T. S. Whittam.** 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature **406:**64–67.
48. **Reischl, U., M. T. Youssef, J. Kilwinski, N. Lehn, W. L. Zhang, H. Karch, and N. A. Strockbine.** 2002. Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **40:**2555–2565.
49. **Remuzzi, G., and P. Ruggenenti.** 1998. The hemolytic uremic syndrome. Kidney Int. Suppl. **66:**S54–S57.
50. **Ritchie, J. M., P. L. Wagner, D. W. K. Acheson, and M. K. Waldor.** 2003. Comparison of Shiga toxin production by hemolytic-uremic syndrome-associated and bovine-associated Shiga toxin-producing *Escherichia coli* isolates. Appl. Environ. Microbiol. **69:**1059–1066.
51. **Rivas, M., E. Miliwebsky, I. Chinen, C. D. Roldan, L. Balbi, B. Garcia, G. Fiorilli, S. Sosa-Estani, J. Kincaid, J. Rangel, and P. M. Griffin.** 2006. The case-control study group. Characterization and epidemiologic subtyping of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina. Foodborne Pathog. Dis. **3:**88–96.
52. **Schmidt, H., L. Beutin, and H. Karch.** 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect. Immun. **63:**1055–1061.
53. **Segura-Alvarez, M., H. Richter, F. J. Conraths, and L. Geue.** 2003. Evaluation of enzyme-linked immunosorbent assays and a PCR test for detection of Shiga toxins for Shiga toxin-producing *Escherichia coli* in cattle herds. J. Clin. Microbiol. **41:**5760–5763.
54. **Stevens, M. P., P. M. van Diemen, G. Frankel, A. D. Phillips, and T. S. Wallis.** 2002. Efa1 influences colonization of the bovine intestine by Shiga toxin-producing *Escherichia coli* serotypes O5 and O111. Infect. Immun. **70:**5158–5166.
55. **Tozzi, A. E., A. Caprioli, F. Minelli, A. Gianviti, L. De Petris, A. Edefonti, G. Montini, A. Ferretti, T. De Palo, M. Gaido, and G. Rizzoni.** 2003. Shiga toxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome, Italy, 1988–2000. Emerg. Infect. Dis. **9:**106–108.
56. **van Diemen, P. M., F. Dziva, M. P. Stevens, and T. S. Wallis.** 2005. Identification of enterohemorrhagic *Escherichia coli* O26:H$^-$ genes required for intestinal colonization in calves. Infect. Immun. **73:**1735–1743.
57. **Vaz, T. M. I., K. Irino, M. A. M. F. Kato, Â. M. G. Dias, T. A. T. Gomes, M. I. C. Medeiros, M. M. M. Rocha, and B. E. C. Guth.** 2004. Virulence properties and characteristics of Shiga toxin-producing *Escherichia coli* in São Paulo, Brazil, from 1976 through 1999. J. Clin. Microbiol. **42:**903–905.
58. **Vernozy-Rozand, C., M. P. Montet, Y. Bertin, F. Trably, J. P. Girardeau, C. Martin, V. Livrelli, and L. Beutin.** 2004. Serotyping, *stx*$_2$ subtyping, and characterization of the locus of enterocyte effacement island of Shiga toxin-producing *Escherichia coli* and *E. coli* O157:H7 strains isolated from the environment in France. Appl. Environ. Microbiol. **70:**2556–2559.
59. **Werber, D., S. C. Behnke, A. Fruth, R. Merle, S. Menzler, S. Glaser, L. Kreienbrock, R. Prager, H. Tschape, P. Roggentin, J. Bockemuhl, and A. Ammon.** 2007. Shiga toxin-producing *Escherichia coli* infection in Germany—different risk factors for different age groups. Am. J. Epidemiol. **165:**425–434.
60. **Werber, D., A. Fruth, A. Liesegang, M. Littmann, U. Buchholz, R. Prager, H. Karch, T. Breuer, H. Tschape, and A. Ammon.** 2002. A multistate outbreak of Shiga toxin-producing *Escherichia coli* O26:H11 infections in Germany, detected by molecular subtyping surveillance. J. Infect. Dis. **186:**419–422.
61. **Wirth, T., D. Falush, R. Lan, F. Colles, P. Mensa, L. H. Wieler, H. Karch, P. R. Reeves, M. C. Maiden, H. Ochman, and M. Achtman.** 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. **60:**1136–1151.
62. **Zhang, W.-L., M. Bielaszewska, J. Bockemühl, H. Schmidt, F. Scheutz, and H. Karch.** 2000. Molecular analysis of H antigens reveals that human diarrheagenic *Escherichia coli* O26 strains that carry the *eae* gene belong to the H11 clonal complex. J. Clin. Microbiol. **38:**2989–2993.
63. **Zhang, W.-L., M. Bielaszewska, A. Liesegang, H. Tschäpe, H. Schmidt, M. Bitzan, and H. Karch.** 2000. Molecular characteristics and epidemiological significance of Shiga toxin-producing *Escherichia coli* O26 strains. J. Clin. Microbiol. **38:**2134–2140.