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Rapid Microarray-Based Genotyping of Enterohemorrhagic *Escherichia coli* Serotype O156:H25/H−/Hnt Isolates from Cattle and Clonal Relationship Analysis▽†

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Since enterohemorrhagic *Escherichia coli* (EHEC) isolates of serogroup O156 have been obtained from human diarrhea patients and asymptomatic carriers, we studied cattle as a potential reservoir for these bacteria. *E. coli* isolates serotyped by agglutination as O156:H25/H−/Hnt strains ($n = 32$) were isolated from three cattle farms during a period of 21 months and characterized by rapid microarray-based genotyping. The serotyping by agglutination of the O156 isolates was not confirmed in some cases by the results of DNA-based serotyping as only 25 of the 32 isolates were conclusively identified as O156:H25. In the multilocus sequence typing (MLST) analysis, all EHEC O156:H25 isolates were characterized as sequence type 300 (ST300) and ST688, which differ by a single-nucleotide exchange in the *purA* gene. Oligonucleotide microarrays allow simultaneous detection of a wider range of EHEC-associated and other *E. coli* virulence markers than other methods. All O156:H25 isolates showed a wide spectrum of virulence factors typical for EHEC. The stx_1 genes combined with the EHEC *hlyA* ($hlyA_{EHEC}$) gene, the *eae* gene of the ζ subtype, as well as numerous other virulence markers were present in all EHEC O156:H25 strains. The behavior of eight different cluster groups, including four that were EHEC O156:H25, was monitored in space and time. Variations in the O156 cluster groups were detected. The results of the cluster analysis suggest that some O156:H25 strains had the genetic potential for a long persistence in the host and on the farm, while other strains did not. As judged by their pattern of virulence markers, *E. coli* O156:H25 isolates of bovine origin may represent a considerable risk for human infection. Our results showed that the miniaturized *E. coli* oligonucleotide arrays are an excellent tool for the rapid detection of a large number of virulence markers.

Shiga toxin-producing *Escherichia coli* (STEC) strains comprise a group of zoonotic enteric pathogens (45). In humans, infections with some STEC serotypes may result in hemorrhagic or nonhemorrhagic diarrhea, which can be complicated by the hemolytic uremic syndrome (HUS) (32). These STEC strains are also designated enterohemorrhagic *Escherichia coli* (EHEC). Consequently, EHEC strains represent a subgroup of STEC with high pathogenic potential for humans. Although *E. coli* O157:H7 is the most frequent EHEC serotype implicated in HUS, other serotypes can also cause this complication. Non-O157:H7 EHEC strains including serotypes O26:H11/H−, O103:H2/H−, O111:H8/H10/H−, and O145:H28/H25/H− and sorbitol-fermenting *E. coli* O157:H− isolates are present in about 50% of stool cultures from German HUS patients (10, 42). However, STEC strains that cause human infection belong to a large number of *E. coli* serotypes, although a small number of STEC isolates of serogroup O156 were associated with human disease (7). Strains of the serotypes O156:H1/H8/H21/H25 were found in human cases of diarrhea or asymptomatic infections (9, 22, 25, 26). The detection of STEC of serogroup O156 from healthy and diseased ruminants such as cattle, sheep, and goats was reported by several authors (1, 11–13, 21, 39, 46, 50, 52). Additional EHEC-associated virulence genes such as *stx*, *eae*, $hlyA_{EHEC}$, or *nlaA* were found preferentially in the serotypes O156:H25 and O156:H− (11–13, 21, 22, 50, 52).

Numerous methods exist for the detection of pathogenic *E. coli*, including genotypic and phenotypic marker assays for the detection of virulence genes and their products (19, 47, 55, 57). All of these methods have the common drawback of screening a relatively small number of determinants simultaneously. A diagnostic DNA microarray based on the ArrayTube format of CLONDIAG GmbH was developed as a viable alternative due to its ability to screen multiple virulence markers simultaneously (2). Further microarray layouts working with the same principle but different gene targets were developed for the rapid identification of antimicrobial resistance genes in Gram-negative bacteria (5) and for the rapid DNA-based serotyping of *E. coli* (4). In addition, a protein microarray for *E. coli* O serotyping based on the ArrayTube format was described by Anjum et al. (3).

The aim of our study was the molecular genotyping of bovine *E. coli* field isolates of serogroup O156 based on miniaturized *E. coli* oligonucleotide arrays in the ArrayStrip format and to combine the screening of *E. coli* virulence markers, antimicrobial resistance genes, and DNA serotyping targets, some of which were partially described previously for separate arrays (2, 4, 5). The epidemiological situation in the beef herds from which the isolates were obtained and the spatial and

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temporal behavior of the clonal distribution of *E. coli* serogroup O156 were analyzed during the observation period. The potential risk of the isolates inducing disease in humans was assessed.

MATERIALS AND METHODS

Bacterial strains and phenotyping methods. A total of 32 *E. coli* isolates of serogroup O156 were isolated from three cattle farms (farms B, C, and D) during 21 months of monitoring in four German cattle farms. No isolates of this serogroup were found on farm A. Details on the monitoring program, the participating farms, their management practices, and the sampling procedure were reported elsewhere (30). On farm D, two groups of cattle (groups 1 and 2) were investigated. The cattle of group 2 were only born after the animals of group 1 had been slaughtered. *E. coli* O156 isolates were obtained from both groups. All 32 isolates were identified primarily as STEC. The serotypes were determined by National Reference Centre for Salmonella and Other Enterics (Robert Koch-Institut, Wernigerode, Germany). Fermentation of sorbitol was detected on sorbitol McConkey agar (SIFIN GmbH, Berlin, Germany) and the production of EHEC hemolysin was detected on enterohemolysin agar (Haipha GmbH, Eppelheim, Germany). The expression of Shiga toxins was monitored by an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies specific for Stx1 or by the Vero cell neutralization assay described previously (54).

Genotype characterization. RNA-free genomic DNAs of the *E. coli* O156 isolates were prepared using the Zymo ZR fungal/bacterial DNA kit (Hiss Diagnostic GmbH, Freiburg, Germany) from 2 ml of overnight cultures. The DNA concentration was determined spectrophotometrically at 260 nm and analyzed for fragmentation by agarose gel electrophoresis. Miniaturized *E. coli* oligonucleotide arrays in the ArrayStrip format (CLONDIAG GmbH, Jena, Germany) containing gene targets for the identification of virulence genes (2), antimicrobial resistance genes (5), and DNA-based serotyping (4) were used for the genetic characterization of the O156 *E. coli* isolates. A complete list of primers and probes is available upon request from the corresponding author.

For labeling and biotinylation of the genomic DNA, a site-specific labeling approach was used (43). The primer elongation reaction was performed using the primer mixture and a deoxynucleoside triphosphate (dNTP) solution containing 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and 0.35 mM biotin-16-dUTP (Roche Penzberg, Germany). The mixture for the elongation reaction contained 0.3 μl Therminator polymerase (New England Biolabs, Frankfurt am Main, Germany), 3 μl Therminator polymerase buffer (New England Biolabs), 3 μl primer solution, 3 μl dNTP stock solution, and 1 to 1.5 μg unfragmented genomic DNA free of any RNA of the *E. coli* isolates. The reaction was started with denaturation (5 min at 96°C), and then 45 cycles of 60 s at 96°C, 20 s at 62°C, and 40 s at 72°C followed. The sample was then cooled down to 4°C and hybridized with the DNA array. For hybridization, the HybKit (CLONDIAG GmbH) was used with an adapted protocol. Initially, each ArrayStrip was washed with 200 μl double-distilled water and 150 μl of buffer C1 using a thermomixing device (5 min, 55°C, 550 rpm [Eppendorf GmbH, Hamburg, Germany]). The sample consists of 10 μl labeled sample and 90 μl buffer C1. It was transferred into the ArrayStrip and incubated (60 min, 55°C, 550 rpm). The sample was then removed from the tube, and the array was washed twice (12 min, 40°C, 550 rpm) with buffer C2. Afterwards, 100 μl conjugate solution was added for 15 min at 30°C and 550 rpm, followed by a washing step with 200 μl buffer C5 for 15 min at 30°C and 550 rpm. The ArrayStrip was then stained with buffer D1 (100 μl, 10 min, no agitation), aspirated, photographed using the ArrayMate instrument (CLONDIAG GmbH, Jena, Germany), and automatically analyzed. Mean signal intensity (mean) and local background (lbg) were measured for each probe position, and values were calculated by the formula value = 1 − mean/lbg. Resulting values below 0.1 were considered negative, and those above 0.3 were considered positive. Values between 0.1 and 0.3 were regarded as ambiguous. Validation was performed using a collection of sequenced control strains (GenBank accession no. AE005174, LCL_10009, FM180568, U00096, and LCL_10006). These control strains were tested by the method described above.

In addition, the *E. coli* O serogroups were characterized by MboII restriction endonuclease digests of amplified O-antigen gene clusters (*rfb* restriction fragment length polymorphism [RFLP]), as described elsewhere (20). The detection of the *eae* subtypes and the *tir* and *espB* subtypes was performed as described previously (17, 48, 53, 60). The nucleotide sequences of intimin genes of three isolates (WH-02/23/021–2, WH-02/25/010–9, and WH-04/25/005–1) were determined by PCR (Primer-fwd, 5′-GTTTCCCGCTCGATGATGCTAC-3′; Primer-rev, 5′-GCGAATAGAAAAGGTGGCTGGAG-3′) followed by DNA sequencing in a LiCOR 4200 system using the DYEnamic direct cycle sequencing kit (GE Healthcare Europe GmbH, Freiburg, Germany). A plasmid profile analysis was also conducted (28, 29).

MLST analysis. Internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) of all O156 isolates were analyzed, and the genetic relationships between different sequence types (STs) (assigned in accordance with the *E. coli* MLST website [http://mlst.ucc.ie/mlst/dbs/Ecoli] were determined as described elsewhere (10). Phylogenetic analyses were conducted and the minimum-spanning tree was generated using the appropriate tools in the BioNumerics software v6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium).

CHEF-PFGE and cluster analysis. Preparation of genomic DNA for contour-clamped homogeneous electric field–pulsed-field gel electrophoresis (CHEF-PFGE) was done by a protocol described previously (28, 29, 38). Slices of the DNA agarose plugs were equilibrated in the respective restriction endonuclease buffer and then digested for 4 h with XbaI, NotI, BlnI (AvrII), or SpeI (New England Biolabs GmbH, Frankfurt am Main, Germany). The resulting fragments were separated in 1.0% agarose gels (Biozyme Gold agarose; Biozyme GmbH, Germany) in 0.5× Tris-borate-EDTA (TBE) at 10°C in a CHEF Mapper XA system. The pulse times for XbaI and NotI digests were increased from 5 to 50 s (gradient of 6 V/cm) during 25 h at a constant angle of 120°. The switch time values for BlnI and SpeI were set using the Auto Algorithm function of the CHEF Mapper XA to separate fragments in the range of 50 to 450 kb (BlnI) or 30 to 350 kb (SpeI), respectively. After electrophoresis, the gels were stained with 500 ml ethidium bromide solution (50 μg/ml), and the banding patterns were recorded under UV illumination. Interpretation of the PFGE patterns was performed by visual inspection and computer analysis (BioNumerics software v6.0; Applied Maths NV, Sint-Martens-Latem, Belgium). All fragments larger than 45 kb (up to 23 fragments per isolate for XbaI, up to 15 for NotI, up to 14 for BlnI, and up to 16 for SpeI) were included in the clonal analysis of the isolates. Distance matrices were calculated by pairwise comparisons of the fragmentation patterns produced by each of the four restriction endonucleases used for the PFGE analysis. To this end, the total character difference was used, which counts the pairwise differences between two given patterns. A cluster analysis was performed with the distance matrices using the neighbor-joining algorithm, an agglomerative cluster method which generates a phylogenetic tree based on distance matrices (51). The cluster analysis was conducted in PAUP* for Windows (version 4.0).

Nucleotide sequence accession numbers. The DNA sequences of the complete *eae* genes of the three O156:H25 isolates have been submitted to GenBank under accession no. GU944691, GU944692, and GU944693.

RESULTS

Analyses of EHEC virulence-associated factors. All tested isolates were serotyped by agglutination as O156:H25/H−/Hnt. We detected the *fliC* gene for the H25 antigen in 26 of the isolates by using the oligonucleotide microarrays. In the remaining 6 isolates, the *fliC* genes for H8, H11 (found twice), H43, or H46 (found twice) were detected (Table 1). When the O antigens were typed with the oligonucleotide microarrays, only an O103 antigen was detected in the isolate with H43 antigen; all other hybridization results were negative. The number of O antigens detectable by this method is limited. There are no gene probes for the detection of the O156 antigen, for example. All isolates were therefore also characterized by MboII digests of amplified O-antigen gene clusters (*rfb* RFLP). Twenty-five isolates showed a pattern typical for O156 as described by Coimbra et al. (20). The patterns of some remaining isolates varied, and they also differed from the O156 pattern (Fig. 1). Classification of these isolates using the *rfb* RFLP database described by Coimbra et al. (20) was not possible. All 25 isolates with the O156 pattern were also positive for the H25 antigen. Consequently, these isolates were designated EHEC O156:H25.

Numerous EHEC-associated and other *E. coli* virulence markers were tested using the oligonucleotide microarrays. An stx_1 gene characterized as the stx_1 subtype was found in all 25 EHEC O156:H25 isolates. The detection of the stx_1 genes

TABLE 1. Serotyping and phylogenetic characteristics of tested *E. coli* O156 isolates classified by the time pattern of isolation[a]

Sampling day	Source	Isolate	Serotype by agglutination	Result by:				Plasmid size(s) in kb	Cluster
				MLST		DNA-based serotyping			
				ST	CC	MboII RFLP	*fliC*		
211	Cow 26 (farm B)	WH-02/26/008-10	O156:H25	688	NA	O156	H25	75, 6.9, and 5.1	8
274	Cow 25 (farm B)	WH-02/25/010-9	O156:Hnt	688	NA	O156	H25	75, 6.9, and 5.1	8
		WH-02/25/010-10	O156:H25	688	NA	O156	H25	75, 6.9, and 5.1	8
330	Cow 16 (farm C)	WH-04/16/007-2	O156:H−	58	155	?	H25	155	1
365	Cow 25 (farm C)	WH-04/25/005-1	O156:H25	300	NA	O156	H25	75	8
400	Cow 4 (farm B)	WH-02/04/017-3	O156:Hnt	300	NA	O156	H25	75	7
		WH-02/04/017-9	O156:Hnt	300	NA	O156	H25	75	7
512	Cow 28 (farm B)	WH-02/28//018-1	O156:H25	300	NA	O156	H25	75	6
		WH-02/28//018-3	O156:H25	300	NA	O156	H25	75	6
		WH-02/28//018-4	O156:H25	300	NA	O156	H25	75	6
		WH-02/28//018-6	O156:H−	300	NA	O156	H25	75	6
533	Cow 25 (farm B)	WH-02/25/019-1	O156:H25	300	NA	O156	H25	75	7
		WH-02/25/019-9	O156:H25	300	NA	O156	H25	75	7
547	Cow 12 (farm D, group 1)	WH-03/12/016-1	O156:H−	300	NA	O156	H25	75 and 6.9	7
		WH-03/12/016-2	O156:H−	300	NA	O156	H25	75 and 6.9	7
		WH-03/12/016-8	O156:Hnt	300	NA	O156	H25	75 and 6.9	7
554	Cow 23 (farm B)	WH-02/23/021-2	O156:Hnt	300	NA	O156	H25	75	7
		WH-02/23/021-3	O156:Hnt	300	NA	O156	H25	75	7
		WH-02/23/021-5	O156:Hnt	300	NA	O156	H25	75	7
		WH-02/23/021-9	O156:H25	300	NA	O156	H25	75	7
		WH-02/23/021-10	O156:H25	300	NA	O156	H25	75	7
638	Cow 25 (farm B)	WH-02/25/024-3	O156:H−	300	NA	O156	H25	75	7
		WH-02/25/024-5	O156:H25	300	NA	O156	H25	75	7
708	Cow 2 (farm D, group 2)	WH-05/02/001-2	O156:H25	1308	NA	?	H46	110, 85, and 5.1	2
		WH-05/02/001-3	O156:Hnt	300	NA	O156	H25	75 and 6.9	7
		WH-05/02/001-8	O156:H25	1308	NA	?	H46	110, 85, and 5.1	2
		WH-05/02/001-9	O156:Hnt	300	NA	O156	H25	75 and 6.9	7
764	Cow 23 (farm D, group 2)	WH-05/23/003-1	O156:H25	10	10	?	H11	155, 110, 75, 50, 21, 5.9, and 3.1	3
	Cow 25 (farm D, group 2)	WH-05/25/003-1	O156:H25	48	10	O103	H43	75, 35, 21, 7.5, and 2.9	4
792	Cow 25 (farm D, group 2)	WH-05/25/004-1	O156:H25	300	NA	O156	H25	75 and 6.9	5
		WH-05/25/004-3	O156:H25	10	10	?	H11	155, 110, 75, 50, 21, 5.9, 3.1, and 2.9	3
841	Cow 12 (farm D, group 2)	WH-05/12/006-2	O156:Hnt	327	NA	?	H8	85	4

[a] CC, clonal complex; MLST, multilocus sequence typing; ST, sequence type; NA, CC not assigned; H−, nonmotile; Hnt, not typeable; ?, not detectable.

corresponded with the demonstration of the *eae* gene, the $hlyA_{EHEC}$ gene and the *espP* gene in the same isolates (see Table S1 in the supplemental material). For the subtyping of the intimin genes, a PCR product of 2,430 bp was amplified from all EHEC O156:H25 isolates using SK1/LP6B primers (60). The *eae* genes were therefore considered as members of the *eae*-ζ subgroup. These results were confirmed by DNA sequencing of the complete *eae* genes of the three O156:H25 isolates (GenBank accession no. GU944691, GU944692, and GU944693). The nucleotide sequences were identical or nearly identical relative to the published sequences of ζ-intimin genes of several *E. coli* serotypes (6, 33, 60), but also to that of an O156:H− strain (GenBank accession no. AY520904.1). We detected both locus of enterocyte effacement (LEE)-encoded and non-LEE-encoded genes of the type III secretion system (T3SS) in the EHEC O156:H25 isolates. An *espA* gene (hy-

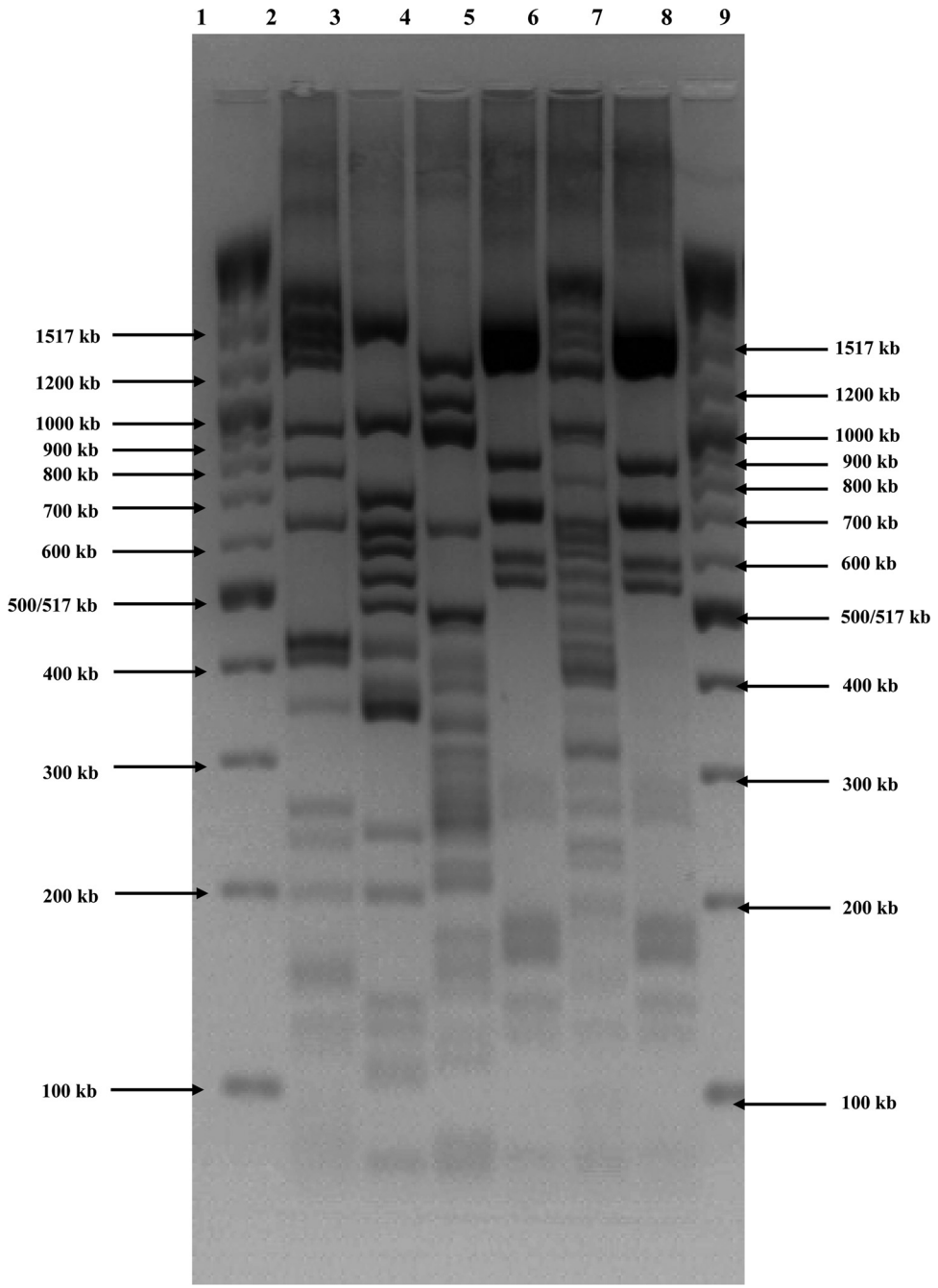

FIG. 1. Electropherogram of MboII restriction of amplified O-antigen gene clusters (*rfb* RFLP). Lane 1, 2-log DNA ladder (New England Biolabs GmbH, Germany); lane 2, WH-02/23/021–2; lane 3, WH-04/16/007–2; lane 4, WH-05/02/001–8; lane 5, WH-05/23/003–1; lane 6, WH-05/25/003–1; lane 7, WH-05/25/004–3; lane 8, 2-log DNA ladder.

bridization with three DNA probes [*espA*-O103:H2, *espA*-O127:H7, and *espA*-O55:H7]) was found in all 25 isolates. Furthermore, the genes *espF* (hybridization of one variant), *tccP* (hybridization of both variants in 23 isolates and of one variant in two isolates), *espJ* (hybridization of one probe), *nleA* (hybridization of all probes), and *nleB* (hybridization of one probe) were presented in all EHEC O156:H25 isolates. The *nleC* gene was found in 16 of the 25 EHEC O156:H25 isolates. Detection of a translocated intimin receptor (*tir*) gene with the gene probes for *tir* genes of the oligonucleotide microarrays failed. We detected, however, a tir_{α} gene and an $espB_{\alpha}$ gene in all EHEC O156:H25 isolates by using the PCRs described previously (17), but the expected sizes of the PstI restriction fragments of the tir_{α} amplicons were not obtained. We found two fragments of ca. 170 kb (data not shown). We also detected the *etpD* gene, which is associated with a type II secretion system, the gene for a heat-stable enterotoxin (*astA* gene), and the gene for fimbria adhesion (*lpfA* gene) in all EHEC O156:

H25 isolates. The *cba* gene (coding for a bacteriocin) was found in five of these isolates; the *katP* gene (15) was found only once (see Table S1 in the supplemental material). The detected virulence markers of the seven non-O156:H25 isolates differed considerably from the EHEC O156:H25 virulence patterns (Table 1; see Table S1 in the supplemental material).

An approximately 75-kb large plasmid was detected in 28 of the tested *E. coli* isolates. This plasmid was found exclusively in 16 of the 25 EHEC 156:H25 isolates; 1 (6.9 kb) or 2 (6.9 kb or 5.1 kb) additional plasmids were detected in the other nine isolates (Table 1). The plasmid profiles of the other three *E. coli* isolates carrying the 75-kb plasmid varied substantially. Several larger plasmids and a number of smaller plasmids were present in these isolates (Table 1). In the remaining isolates, single plasmids of 155 kb or 85 kb, respectively, or two plasmids (110 kb and 5.1 kb) were found in addition to the 85-kb plasmid.

The production of the Shiga toxins was tested by ELISA and Vero cell neutralization assay. All isolates with the stx_1 gene produced Stx1. The cytotoxicity of the Stx1 was very high for Vero cells upon induction with mitomycin C. The EHEC hemolysin was produced by all EHEC O156:H25 isolates harboring $hlyA_{EHEC}$. Sorbitol was fermented by all tested *E. coli* isolates.

MLST analysis. MLST analyses were performed with all 32 *E coli* isolates (Table 1). Twenty-two isolates grouped in the sequence type ST300 and 3 in ST688. The difference between ST300 and ST688 is a single-nucleotide exchange in the *purA* gene (Fig. 2). These 25 isolates were previously identified as EHEC O156:H25 in the DNA-based serotyping. The other seven isolates were grouped into different sequence types (ST). Three isolates were members of the clonal complex (CC) 10 (ST10 and ST48), two were characterized as ST1308, and one isolate each was detected as ST58 (CC155) and ST327. A comparison of all O156 STs with all STEC STs associated with HUS (HUSEC collection described by Mellmann et al. [42]) and with the additional data set entries for O156 strains in the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli) is displayed in Fig. 2.

Analyses of the antimicrobial resistance genes. Antimicrobial resistance genes were rarely detected. An EHEC O156:H25 isolate hybridized with one of three DNA probes (probe blaMOX-CMY-612) for a plasmid encoding AmpC-like β-lactamase gene (bla_{CMY} gene) and with one of three probes detecting resistance genes against sulfonamides (probe sul2-11). Antimicrobial resistance genes were not detected in the remaining O156:H25 strains. Hybridization with a *tetA* gene (encoding tetracycline resistance) and with two probes for sulfonamide resistance genes (probes sul1-11 and sul2-11) were observed in two non-O156:H25 isolates.

Clonal relationships. The tested 32 bovine *E. coli* isolates were found over a period of 21 months in three of four cattle herds monitored at the same time (30). In farm D, we serially investigated two groups of cattle. A total of 8 different clusters were detected. The identified EHEC O156:H25 isolates belonged to four different cluster groups, but the genetic distances to the other four cluster groups comprising the non-O156:H25 isolates were much higher than the distances within the EHEC O156:H25 cluster groups (Fig. 3; see Fig. S1 in the supplemental material).

From farm B, all 18 tested isolates were identified as EHEC O156:H25. These isolates were obtained from seven different animals during a period of 12 months. They belonged to three different clusters (clusters 6, 7, and 8) (Fig. 3; see Fig. S1 in the supplemental material). First, EHEC O156:H25 was found in two different animals (cattle 25 and 26) on sampling days 211 and 274. These isolates were typed as ST688 in the MLST analysis and grouped in cluster 8. Isolates of this sequence type were not detected subsequently during the monitoring. We obtained the other 15 isolates during a period of 9 months. This period started half a year after the isolation of the first EHEC O156:H25 isolate. Certain isolates belonging to cluster 7 were found during this period in the same or different animals (Table 1 and Fig. 3; see Fig. S1 in the supplemental material). Exceptions were four EHEC O156:H25 isolates detected in cow 28 on day 512 which grouped in cluster 6.

The three isolates of group 1 from farm D were invariably EHEC O156:H25. In contrast, only three of the nine isolates of group 2 from the same farm were identified as EHEC O156:H25. Interestingly, the isolates of group 1 and two isolates of group 2 belonged to the same cluster, 7, in which most of the isolates from farm B grouped. The EHEC O156:H25 isolate WH-05/25/004-1 represented a separate cluster, 5 (Table 1 and Fig. 3; see Fig. S1 in the supplemental material). The EHEC O156:H25 isolates were detected on 2 days, when non-O156:H25 isolates were also found in the same animals (cow 2, day 708; cow 25, day 792) in group 2. The divergences of the non-O156:H25 isolates were very high relative to all obtained EHEC O156:H25 isolates and particularly among the non-O156:H25 isolates (Table 1 and Fig. 3; see Fig. S1 in the supplemental material).

Two isolates obtained from two different animals on sequential sampling days in farm C were typed as EHEC O156:H25 and non-O156:H25. The EHEC O156:H25 isolate was identified as a member of cluster 8. The non-O156:H25 isolate had the greatest genetic divergence from all other isolates (Table 1 and Fig. 3; see Fig. S1 in the supplemental material).

DISCUSSION

Ruminants, especially cattle, are considered the primary reservoir for human infections with EHEC (34). For EHEC strains of serogroup O156, this fact seems to apply only to a very small number of cases. Several authors have described the detection of isolates of serogroup O156 from healthy and diseased ruminants, particularly cattle and sheep. They are referred to most often in reports from Spain but were also detected in Germany, Canada, and India (1, 8, 12, 13, 21, 30, 36, 39, 46, 50, 52, 59). In contrast, O156 isolates were only sporadically found in cases of human disease. Two isolates associated with bloody diarrhea and asymptomatic disease were reported from Finland (25, 26). Cases of asymptomatic disease and diarrhea associated with O156 were also found in Germany (9, 22, 37). In our study, 32 bovine *E. coli* O156:H25/H−/Hnt isolates were characterized by molecular genotyping based on miniaturized *E. coli* oligonucleotide arrays to assess their potential to cause EHEC diseases in humans.

Serotyping results by agglutination of the O156 isolates were

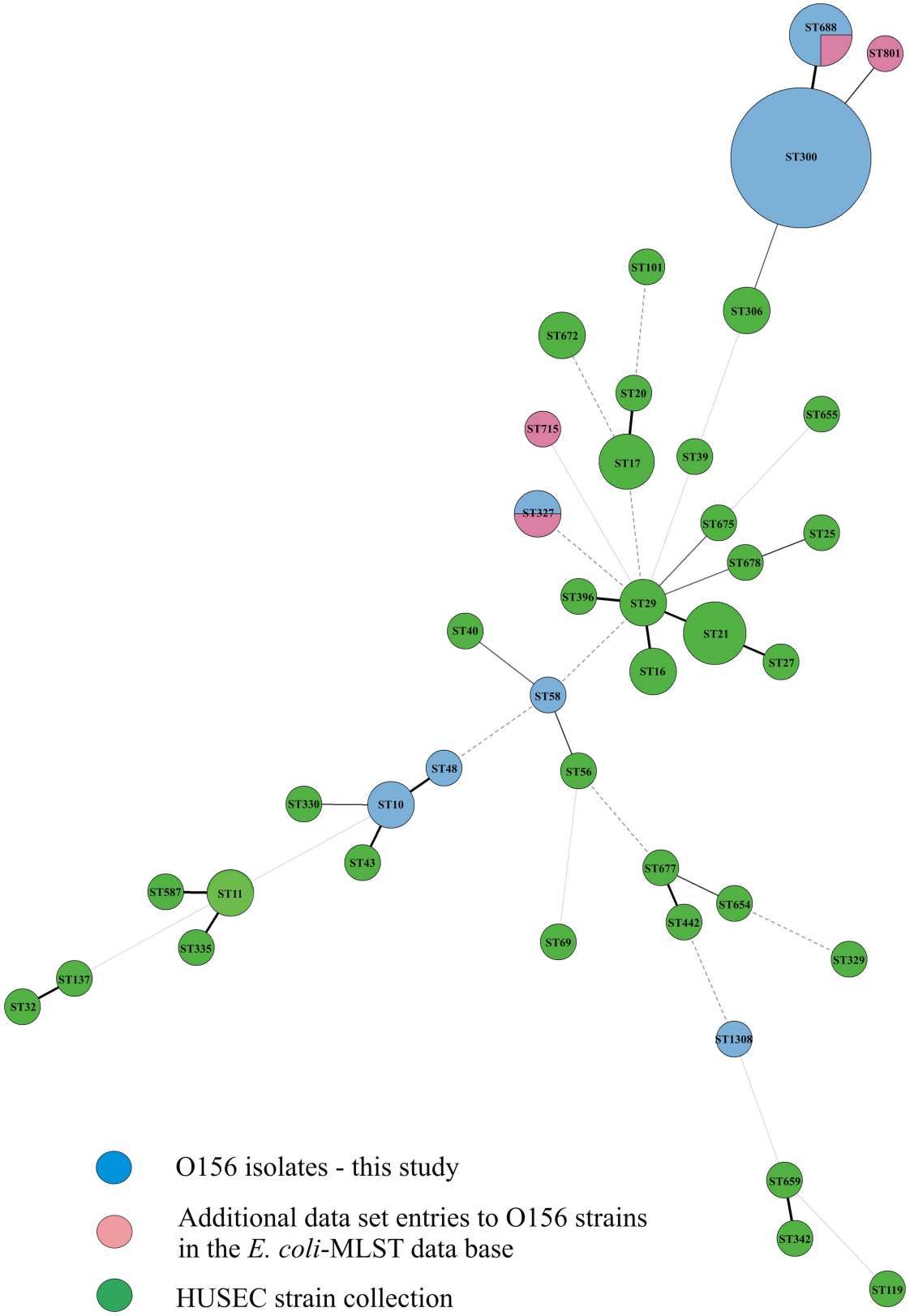

FIG. 2. Minimum-spanning tree based on the multilocus sequence typing allelic profiles that portray the clonal distribution of the tested 32 *E. coli* O156 isolates in relation to the HUSEC collection (described by Mellmann et al. [42]) and to the additional data set entries for O156 in the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). Each circle represents a given sequence type, and the size of each circle is proportional to the number of strains analyzed. Connecting lines show the number of identical alleles between two STs (thick black line, 6 of 7 alleles identical; thinner black lines, 5 alleles identical; thin black lines, 4 alleles identical; thin dashed lines, ≤3 alleles identical).

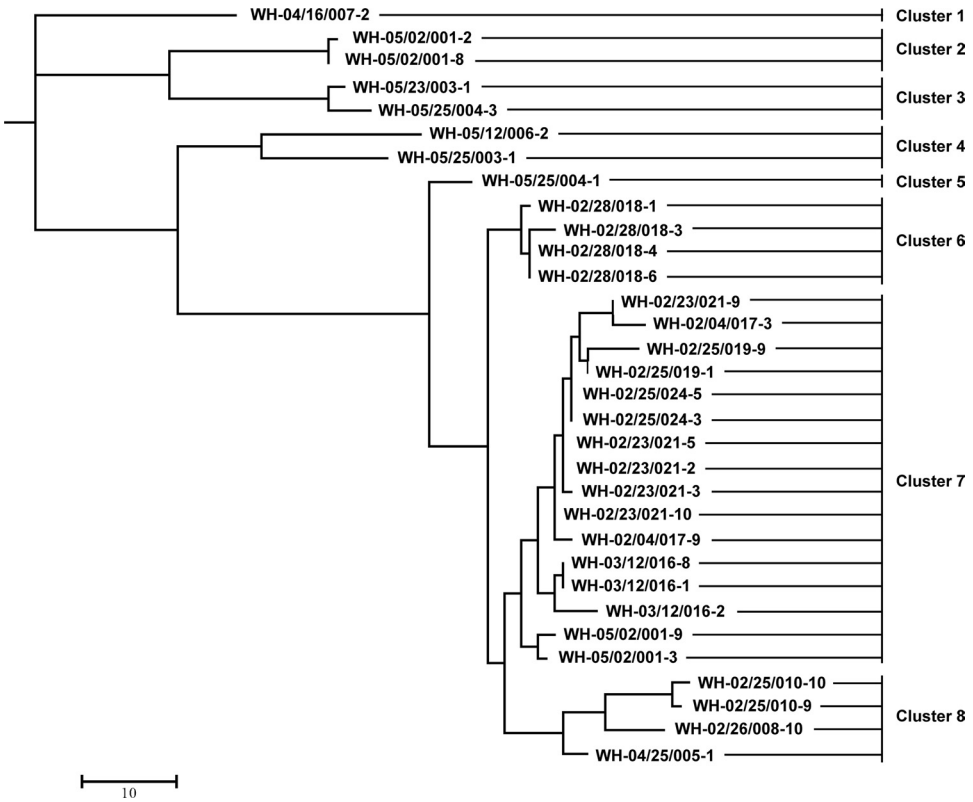

FIG. 3. Neighbor-joining tree of bovine *E. coli* O156 isolates based on the restriction pattern obtained after digestion with XbaI, NotI, BlnI, and SpeI (see Fig. S1 in the supplemental material).

not confirmed in some cases by DNA-based serotyping because only 25 of the 32 isolates were conclusively identified as O156:H25. In contrast, one isolate was typed as O103:H43, and the classification of the O groups by DNA-based serotyping was not possible for the other isolates. There may be several reasons for discrepancies between the serotyping by agglutination and the DNA-based methods. First, it is possible that more than one *rfb* RFLP pattern exists for O156, but not all patterns may be described in the database. Several different patterns were shown for other O groups: for example, for O2, O5, O8, O26, O55, O85, O111, O125, O147, and O149 (20). Second, potential cross-reactions in the agglutination reaction may have led to the false determination of serotypes. Moreover, it is also possible that the originally serotyped isolate has changed and is no longer identical to the isolate genotyped later on.

In the MLST analysis, all EHEC O156:H25 isolates were characterized as ST300 and ST688, which differ by a single nucleotide exchange in the *purA* gene. Both sequence types were previously found in human EHEC O156:H25 isolates from Germany (9). Other data set entries regarding O156 in the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli) are very rare. However, it is interesting that a bovine O156:K+ isolate from the United Kingdom (http://mlst.ucc.ie/mlst/dbs/Ecoli) and four human O156:H8 isolates from Germany (9) were characterized as ST327. We also found the same sequence type in one of our non-O156:H25 isolates.

Oligonucleotide microarrays allow the simultaneous detection of a much wider range of EHEC-associated and other *E. coli* virulence markers than other methods. All of our O156:H25 isolates demonstrated a wide spectrum of virulence factors typical for EHEC. The detection of the combination of genes stx_1, *eae*, and $hlyA_{EHEC}$, as in our isolates, has previously been reported for O156:H21/H25/H− isolates from cattle and sheep (12, 13, 50). To date, 10 distinct variants of *eae* have been described (17, 33, 48, 49). Some serotypes were closely associated with a particular intimin variant (5, 17, 18, 56). Some authors described an *eae*-ζ gene in O156:H25/H− strains (12, 13, 22, 50). Our study confirms these associations. All bovine EHEC O156:H25 isolates were typed as members of the *eae*-ζ subgroup. The DNA sequences of the complete *eae* genes were identical for both the isolates from the two different MLST ST300 and ST688 and for isolates from different farms (farms B and C).

The *espP*/*pssA* gene, which was only recently reported for EHEC strains of serotypes O26:H− (24) and O157:H7 (14), as well as others such as O118:H16/H− (58), was also found in all of our EHEC O156:H25 isolates. The presence of this gene corresponded to the occurrence of a 75-kb virulence-associated plasmid.

In addition to intimin, other LEE-encoded factors such as EspA, EspB, EspC, and Tir were tested by oligonucleotide microarrays. Whereas *espA* genes were detected in all EHEC O156:H25 isolates, the *espB* and *espC* genes were missing. The *espB* genes were previously detected in bovine isolates of serogroup O156 by Orden et al. (46), but the authors tested

enteropathogenic *E. coli* (EPEC) isolates. The lack of a *tir* gene in all EHEC O156:H25 isolates is surprising, because this gene might be expected in combination with the intimin gene too. By using PCRs described by China et al. (17), we also detected a *tir* gene and an *espB* gene of the α subtype in all EHEC O156:H25 isolates. Perhaps the DNA probes used on the array failed to detect yet unsequenced alleles of both the *tir* genes and the *espB* or *espC* genes, because the variability of these genes seems to be high. Therefore, the divergent results obtained by the PstI restriction digests of the tir_{α} amplicons may point in this direction. It has been shown that additional effector proteins encoded by genes outside the LEE in cryptic or intact prophages are translocated by the LEE-encoded T3SS. This group of non-LEE-encoded effectors also includes the cycle-inhibiting factor Cif (41); the Tir cytoskeleton coupling protein TccP/$EspF_U$ (16, 27); the effector proteins NleA/EspI, NleB, and NleC, which are determinants necessary for virulence (31, 35, 40, 44); and EspJ, which may play a role in host survival and pathogen transmission (23). We found some of these factors also in the EHEC O156:H25 isolates. Different *nleA* gene variants and combinations of the variants were previously reported for members of the O156 serogroup, such as O156:H25 (22). Our study confirms these associations. *nleA* genes were detected in all of our EHEC O156:H25 isolates. They hybridized in each case with all four tested DNA probes. In our study, the detection of *nleB*, *nleC*, *tccP*, *espF*, and *espJ* genes in isolates of serogroup O156 is described for the first time. Interestingly, we found an *astA* gene, which encodes a heat-stable enterotoxin in all EHEC O156:H25 isolates. This heat-stable enterotoxin is typically found in enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) isolates.

Two non-O156:H25 isolates, such as O103:H43, were identified as typical enteropathogenic *E. coli* (EPEC) strains. In contrast to the O156:H25 isolates, we detected *eae* genes of the θ subtype and failed to find *stx* and $hlyA_{EHEC}$ genes in these strains. Moreover, the hybridization patterns for the genes of LEE-encoded and non-LEE-encoded T3SS varied. O156 isolates with *eae*-θ and without *stx* genes were previously reported by Creuzburg and Schmidt (22).

We also analyzed the spatial and temporal behavior of the O156 isolates in the beef herds. In contrast to the O26:H11 isolates that we had analyzed in the same monitoring program (28), the identified EHEC O156:H25 isolates did not represent independent cluster groups on each farm. Despite the relatively large geographic distance between the farms, we found members of the same cluster groups in different farms. However, we isolated members of the dominant cluster 7 repeatedly on several sampling days over a relatively long period of 8 months, although other clusters were only detected on single occasions. Interestingly, we found the same cluster 7 isolates first, in group 1 in farm D and later in group 2. The cattle of group 2 were only born after the animals of group 1 had been slaughtered. This patchy temporal pattern is apparently not a unique property of O156:H25 as we found similar results for cluster groups of the EHEC serotypes O26:H11 and O165:H25 of bovine origin during the same monitoring program, as published elsewhere (28, 29). Transmission of clusters between individual animals was also observed. These results suggest that some EHEC O156:H25 strains as well as some EHEC O26:H11 (28) and EHEC O165:H25 (29) strains had the potential for a longer persistence in the host population, while others did not. The reasons for this difference are not yet clear. The tested genes for adherence factors such as *saa*, *efa*, and *ifa* were missing in the EHEC O156:H25 isolates. On the other hand, the gene for a fimbrial adhesion (*lpfA* gene) was found in all these isolates.

We distinguished eight different clusters, including four for O156:H25, but complete genetic identity was only found in few isolates. The variations in the O156:H25 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 O156:H25 and the host.

Antimicrobial resistance genes were rarely found, but we detected such genes in an EHEC O156:H25 isolate which was classified as cluster 7. The isolate was found at the end of the detection period for this cluster. This could be an indication of the incorporation of resistance genes by gene transfer, which may be facilitated by the long persistence period of a strain in a cattle herd.

In conclusion, our results showed that the miniaturized *E. coli* oligonucleotide arrays are an excellent tool for the rapid detection of a large number of virulence markers. In addition, this method provides an overview of the presence of resistance genes. It also allows rapid DNA-based serotyping of important O groups, a technique which can still be improved. Our results further showed that bovine EHEC O156:H25 isolates that can carry virulence factors of EHEC are strongly associated with EHEC-related disease in humans, particularly with severe clinical manifestations such as HC and HUS. Therefore, strains of bovine origin may represent a considerable risk for human infection. Moreover, some clusters of EHEC O156:H25 persisted in cattle and farms over relatively long periods, potentially increasing the risk of transmission to other animals and even to humans.

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