

Virulence Gene Profiles and Clonal Relationships of *Escherichia coli* O26:H11 Isolates from Feedlot Cattle as Determined by Whole-Genome Sequencing

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ABSTRACT

Escherichia coli O26 is the second most important enterohemorrhagic *E. coli* (EHEC) serogroup worldwide. Serogroup O26 strains are categorized mainly into two groups: enteropathogenic (EPEC) O26, carrying a locus of enterocyte effacement (LEE) and mostly causing mild diarrhea, and Shiga-toxigenic (STEC) O26, which carries the Shiga toxin (STX) gene (*stx*), responsible for more severe outcomes. *stx*-negative O26 strains can be further split into two groups. One O26 group differs significantly from O26 EHEC, while the other O26 EHEC-like group shows all the characteristics of EHEC O26 except production of STX. In order to determine the different populations of O26 *E. coli* present in U.S. cattle, we sequenced 42 O26:H11 strains isolated from feedlot cattle and compared them to 37 O26:H11 genomes available in GenBank. Phylogenetic analysis by whole-genome multi-locus sequence typing (wgMLST) showed that O26:H11/H⁻ strains in U.S. cattle were highly diverse. Most strains were sequence type 29 (ST29). By wgMLST, two clear lineages could be distinguished among cattle strains. Lineage 1 consisted of O26:H11 EHEC-like strains (ST29) (4 strains) and O26:H11 EHEC strains (ST21) (2 strains), and lineage 2 (36 strains) consisted of O26:H11 EPEC strains (ST29). Overall, our analysis showed U.S. cattle carried pathogenic (ST21; *stx*₁⁺ *ehxA*⁺ *toxB*⁺) and also potentially pathogenic (ST29; *ehxA*⁺ *toxB*⁺) O26:H11 *E. coli* strains. Furthermore, *in silico* analysis showed that 70% of the cattle strains carried at least one antimicrobial resistance gene. Our results showed that whole-genome sequence analysis is a robust and valid approach to identify and genetically characterize *E. coli* O26:H11, which is of importance for food safety and public health.

IMPORTANCE

Escherichia coli O26 is the second most important type of enterohemorrhagic *E. coli* (EHEC) worldwide. Serogroup O26 strains are categorized into two groups: enteropathogenic (EPEC) carrying LEE, causing mild diarrhea, and Shiga toxin-producing (STEC) carrying the *stx* gene, responsible for more severe outcomes. However, there are currently problems in distinguishing one group from the other. Furthermore, several O26 *stx*-negative strains are consistently misidentified as either EHEC-like or EPEC. The use of whole-genome sequence (WGS) analysis of O26 strains from cattle in the United States (i) allowed identification of O26 strains present in U.S. cattle, (ii) determined O26 strain diversity, (iii) solved the misidentification problem, and (iv) screened for the presence of antimicrobial resistance and virulence genes in the strains. This study provided a framework showing how to easily and rapidly use WGS information to identify and genetically characterize *E. coli* O26:H11, which is important for food safety and public health.

In the United States, enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has caused the majority of food-borne outbreaks in the last 2 decades (1). However, among the non-O157:H7 EHEC strains, Shiga toxin-producing *E. coli* O26:H11/H⁻ (STEC) is the second most common cause of EHEC food-borne infections in the United States (2, 3) and worldwide (4–7). STEC O26 can cause a wide range of infections in humans, from mild diarrhea to hemorrhagic colitis (HC), in some cases leading to hemolytic-uremic syndrome (HUS) (6). Outbreaks of O26 infection have been linked to beef, contact with cattle (4, 8), produce (<http://www.cdc.gov/ecoli/2012/o26-02-12/index.html>), and person-to-person transmission (9). Therefore, in 2011, the U.S. Department of Agriculture Food Safety and Inspection Services (USDA FSIS) declared O26 and five other non-O157 STEC serogroups, O45, O113, O111, O121, and O145, adulterants in ground beef and nonintact beef products and began testing for these pathogens in June 2012 in domestic and imported beef trimmings (10).

Pathogenic O26 strains are divided into two main groups: enteropathogenic *E. coli* (EPEC), characterized by carrying the locus of enterocyte effacement (LEE) and causing mild diarrhea, and

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EHEC, which in addition to the LEE carries the *stx*₁ and/or *stx*₂ gene and is responsible for more severe outcomes, such as HC and HUS (7, 11). EPEC O26 has been further divided into two subgroups. One subgroup carries most virulence markers (including an EHEC virulence plasmid carrying a gene, *ehxA*, encoding an enterohemolysin) for O26 EHEC except the *stx* gene(s). This subgroup has been called EHEC-like O26 (or atypical EPEC O26). They also have the same sequence type (ST), as determined by multilocus sequence typing (MLST), and the same multilocus variable-number tandem-repeat analysis (MLVA) profiles as some O26 EHEC strains (12, 13). It is believed that atypical EPEC O26 evolved into a highly pathogenic clone (EHEC O26) through acquisition of a Shiga toxin (STX)-producing phage (6, 14, 15). The second EPEC group is differentiated from EHEC or EHEC-like O26 based on its fermentative profiles, by carrying a different virulence plasmid (encoding an alpha-hemolysin), and by having the *arcA* allele type 1 gene (13). Serotype O26:H11/H⁻ can be divided, based on MLST, into several STs, with ST21 and ST29 associated with disease in humans (16). Although present worldwide (6), O26:H11/H⁻ has been predominantly described in Europe, with little data on its prevalence on other continents.

Recently, the diversity of O26:H11 strains of cattle origin in the United States based on a combination of molecular markers and clustered regularly interspaced short palindromic repeat (CRISPR) typing has been described (17). It was suggested that some of the strains may have the potential to become pathogenic to humans by acquisition of a *stx*-carrying phage, as they contained a CRISPR PCR target (SP_O26-E) previously identified only in *stx*₂-positive O26:H11 clinical strains (17). Further analysis of the O26 cattle strains from the United States using 48 informative single nucleotide polymorphisms (SNPs) found that EHEC-like O26 strains (*stx* negative) displayed SNP genotypes synonymous with two publicly available clinical strains (ST29; O26:H11 EHEC strains). This result suggested that the EHEC-like O26 strains (*stx* negative) were genetically closer to the clinical O26 EHEC (*stx*⁺) strains (18). However, the actual phylogeny of EHEC, EHEC-like, and EPEC O26 strains has not been determined.

In order to further characterize *E. coli* O26 in U.S. cattle and to confirm previously suggested evolutionary and phylogenetic relationships among EHEC, EHEC-like, and EPEC O26 strains, we conducted a whole-genome sequence (WGS) analysis of selected O26:H11/H⁻ strains isolated from commercial feedlot cattle (19) that had been characterized by various molecular techniques (17, 18) and compared them to other clinical and environmental O26:H11 genomes available at GenBank (37 genomes). The 42 O26:H11/H⁻ strains were analyzed for virulence genes, *in silico* MLST, and antibiotic resistance genes. Finally, their phylogenetic relationships and diversity were determined by whole-genome phylogeny analysis using two methods: (i) allele-based whole-genome MLST (wgMLST) and (ii) targeted SNP analysis.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* O26:H11 strains (*n* = 42) used in this study are listed in Table 1. Each strain was assigned a U.S. FDA Center for Food Safety and Applied Nutrition (CFSAN) number for future tracking. The strains were isolated from cattle feces in a commercial feedlot located in the Midwest (19). The isolation procedure included enrichment of the feces in *E. coli* broth, immunomagnetic separation (IMS) with O26-specific IMS beads, and plating on selective medium, followed by

confirmation of the presumptive isolate by a multiplex PCR targeting the O26 *wzx* gene (*wzx*_{O26}) and the three major virulence genes, *stx*₁, *stx*₂, and *eae* (20). The strains were stored on tryptone soy broth containing glycerol at -70°C until further use. The strains were plated onto blood agar (Remel, Lenexa, KS) and grown overnight at 37°C for further analysis.

DNA preparation. Genomic DNA from each strain was isolated from overnight cultures using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The resultant DNA extract was stored at -20°C until it was used as a template for whole-genome sequencing. The concentration was determined using a Qubit double-stranded DNA HS assay kit and a Qubit 2.0 fluorometer (Thermo Scientific), according to the manufacturer's instructions.

Whole-genome sequencing, contig assembly, and annotation. The genomes of the strains were sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA), with 250-bp paired-end chemistry, according to the manufacturer's instructions, at approximately 80 times the average coverage. The genome libraries were constructed using the Nextera XT DNA sample preparation kit (Illumina). Genomic sequence contigs were *de novo* assembled using default settings within CLC Genomics Workbench v7.6.1 (Qiagen) with a minimum contig size threshold of 500 bp. The draft genomes were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (<http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html>; 21).

***In silico* serotyping.** The serotype of each strain analyzed in this study was confirmed using the genes deposited in the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org>) for *E. coli* as part of their Web-based serotyping tool (SerotypeFinder 1.1 [<https://cge.cbs.dtu.dk/services/SerotypeFinder>]) (22, 23). We used Ridom to perform batch screening of the genomes analyzed. Briefly, all the genes were divided into O-type (*wzx* and *wzy*) and H-type (*fliC*) genes in FASTA format (e.g., all the *wzx* alleles were in a single FASTA file) and used as the task template. For virulence screening, a project was created using all three task templates, and each whole-genome sequence was screened for the presence of each gene type (O-type or H-type gene). The results were similar to those with SerotypeFinder, and as with the virulence genes previously, the data were now in a database and new alleles (if found) could be added to the task templates.

***In silico* MLST phylogenetic analysis.** The initial analysis and identification of the strains were performed using an *in silico* *E. coli* MLST approach, based on the information available at the *E. coli* MLST website (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>) and using Ridom SeqSphere+ software v2.4.0 (Ridom, Münster, Germany). Seven housekeeping genes (*dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC*, and *tnaA*), described previously for *E. coli* (24), were used for MLST analysis. The same *E. coli* MLST database was also used to assign numbers to alleles and STs.

***In silico* determination of virulence genes.** Virulence genes were determined using the genes deposited in the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org>) for *E. coli* as part of their VirulenceFinder 1.5 Web-based tool (<https://cge.cbs.dtu.dk/services/VirulenceFinder>; 23), but we used Ridom to perform batch screening of the genomes analyzed. Briefly, all the genes were divided into classes or groups by homology in FASTA format (e.g., all the *astA* alleles were in a single FASTA file) and used as a task template. Afterward, a project was created using all the task templates, and each WGS was screened for the presence of each gene class (virulence gene). Table S1 in the supplemental material shows the 95 virulence genes analyzed by this method. The *stx* gene variants analyzed are available at <https://cge.cbs.dtu.dk/services/VirulenceFinder>. The result was very similar to the one displayed at VirulenceFinder, except that the data were now in a database and new alleles (if found) could be added to the task templates.

***In silico* antimicrobial resistance gene identification.** Antimicrobial resistance genes present in the sequenced genomes, as well as in those retrieved from GenBank (Table 2), were identified by using the genes deposited in the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org>) as part of their Resfinder 2.1 Web-based tool

TABLE 1 Characteristics of the *E. coli* O26:H11/H⁻ strains of cattle origin analyzed in this study

Strain ^a	CFSAN no.	Serotype	Accession no. (WGS)	ST ^b	<i>stx</i> ₁ ^b	<i>stx</i> ₂ ^b	<i>ehxA</i> ^b
1895-A	CFSAN025090	O26:H11	LPTU00000000	21	+	-	+
1914-A	CFSAN025091	O26:H11	LPTV00000000	21	+	-	+
1357-A	CFSAN025092	O26:H11	LPTW00000000	29	-	-	-
1270-A	CFSAN025093	O26:H11	LPXY00000000	29	-	-	-
2228-A	CFSAN025094	O26:H11	LPXZ00000000	29	-	-	-
2152-B	CFSAN025095	O26:H11	LPYA00000000	29	-	-	-
1341-A	CFSAN025096	O26:H11	LPYB00000000	29	-	-	+
1676-A	CFSAN025098	O26:H11	LPYC00000000	29	-	-	-
1692-A	CFSAN025099	O26:H11	LPYD00000000	29	-	-	-
1740-A-A	CFSAN025100	O26:H11	LPYE00000000	29	-	-	-
1740-A-B	CFSAN025101	O26:H11	LPYF00000000	29	-	-	-
1668-A-A	CFSAN025102	O26:H11	LPYG00000000	29	-	-	+
1668-A-B	CFSAN025103	O26:H11	LPYH00000000	29	-	-	+
1958-A-B	CFSAN025104	O26:H11	LPYI00000000	29	-	-	-
2194-B	CFSAN025106	O26:H11	LPYJ00000000	29	-	-	-
2105-G	CFSAN025107	O26:H11	LPYK00000000	29	-	-	-
2223-B	CFSAN025108	O26:H11	LPYL00000000	29	-	-	-
2139-A	CFSAN025109	O26:H11	LPYM00000000	29	-	-	-
2176-A	CFSAN025110	O26:H11	LPYN00000000	29	-	-	-
4131	CFSAN025114	O26:H11	LPYO00000000	29	-	-	-
4170	CFSAN025115	O26:H11	LPYP00000000	29	-	-	-
4196	CFSAN025116	O26:H11	LPYQ00000000	29	-	-	-
4277-H-A	CFSAN025117	O26:H11	LPYR00000000	29	-	-	-
4368-A	CFSAN025118	O26:H11	LPYS00000000	29	-	-	-
4435-B	CFSAN025119	O26:H11	LPYT00000000	29	-	-	-
4271-C	CFSAN025121	O26, H2 ^c	LPYU00000000	29	-	-	-
4468-A-B	CFSAN025122	O26:H11	LPYV00000000	29	-	-	-
4848-A-B	CFSAN025123	O26, H2 ^c	LPYW00000000	29	-	-	-
4730-C	CFSAN025124	O26:H11	LPYX00000000	29	-	-	-
4513	CFSAN025126	O26:H11	LPYY00000000	29	-	-	-
4592-A	CFSAN025127	O26:H11	LPYZ00000000	29	-	-	-
5583-H	CFSAN025128	O26:H11	LPZA00000000	29	-	-	-
5687-B	CFSAN025129	O26:H11	LPZB00000000	29	-	-	-
5206-E-B	CFSAN025130	O26:H11	LPZC00000000	29	-	-	-
1802-A	CFSAN025105	O26:H11	LPZD00000000	29	-	-	-
4822-A	CFSAN025125	O26:H2 ^c	LPZE00000000	29	-	-	-
4468-A-A	CFSAN025133	O26:H11	LPZF00000000	29	-	-	-
4860-A	CFSAN025135	O26:H11	LPZG00000000	29	-	-	-
4863-A	CFSAN025136	O26:H11	LPZH00000000	29	-	-	+
5196-B	CFSAN025137	O26:H11	LPZI00000000	29	-	-	-
5019-A	CFSAN025138	O26:H11	LPZJ00000000	29	-	-	-
3674-A	CFSAN025132	O26:H11	LPZK00000000	29	-	-	-

^a All strains were isolated in Nebraska, USA.

^b Determined by *in silico* analysis of the WGS assemblies. +, present; -, absent.

^c Insertion sequence (probably IS2) disrupting *flhC* gene integrity.

(<https://cge.cbs.dtu.dk/services/ResFinder>) (25), but we used Ridom to perform batch screening of the genomes analyzed. Briefly, all the genes were divided into classes or groups by homology in FASTA format (e.g., all the *bla*_{TEM} alleles were located in a single FASTA file) and used as the task template. Later, a project was created using all the task templates, and each WGS was screened for the presence of each gene class (antimicrobial resistance gene). The result was very similar to that of ResFinder, except that the data were now in a database and new alleles (if found) could be added to the task templates.

Phylogenetic relationship of strains by wgMLST analysis. The phylogenetic relationship of the strains was assessed by a wgMLST analysis using Ridom SeqSphere+ v2.4.0 software. The genome of O26:H11 strain 11368 (NC_013361.1) was used as a reference. After eliminating loci that were missing from the genome of any strain used in our analyses, we performed a wgMLST analysis. These remaining loci were considered the core genome shared by the analyzed strains. We used the DNA distance

method of Nei et al. (26) to calculate the matrix of genetic distance, taking only the numbers of same/different alleles in the core genes into consideration. A neighbor-joining (NJ) tree using the appropriate genetic distances was built after the wgMLST analysis. The discriminatory index was calculated with the Ridom software using Simpson's discriminatory index as described previously (27). wgMLST uses the allele number of each locus to determine the genetic distance and to build the phylogenetic tree. The use of allele numbers reduces the influence of recombination in the data set studied and allows fast clustering determination of genomes.

Phylogenetic relationship of strains by targeted SNP analysis. The SNP analysis was conducted in parallel with the wgMLST analysis in order to validate the results obtained by wgMLST and to conduct phylogenetic analysis. After the identification of core genes among the strains, SNPs present in each locus were extracted using Ridom SeqSphere+ software. This core SNP matrix was used for phylogenetic analysis. The maximum-likelihood (ML) phylogeny was reconstructed with Mega v6 software (28),

TABLE 2 *E. coli* O26:H11 genomes available at GenBank used for phylogenetic and *in silico* virulence analyses

Strain	GenBank accession no.	Serotype	Yr isolated	Country	ST ^b	<i>stx</i> ₁ ^c	<i>stx</i> ₂ ^c	<i>ehxA</i> ^c
11368 ^{a,e}	NC_013369	O26:H11	2001	Japan	21	+	-	+
21765 ^e	CDLB0000000	O26:H11	2005	France	29	-	+	-
05-3646 ^e	JHOE0100000	O26:H11	2005	USA	21	+	-	+
2011C-3506 ^e	JHLS0100000	O26:H11	2011	USA	21	+	-	+
2011C-3387 ^e	JHLV0000000	O26:H11	2011	USA	21	+	-	-
2011C-3655 ^e	JHLN0000000	O26:H11	2011	USA	21	+	-	+
06-3464 ^e	JHNO0000000	O26:H11	2006	USA	21	+	-	+
2009C-3612 ^e	JHGZ0000000	O26:H11	2009	USA	29	-	+	+
2009C-4760 ^e	JHGK0000000	O26:H11	2009	USA	21	+	-	+
2010C-4430 ^e	JHND0000000	O26:H11	2010	USA	21	+	-	+
2010C-4819 ^e	JHMP0000000	O26:H11	2010	USA	21	+	+	+
2011C-3282 ^e	JHLX0000000	O26:H11	2011	USA	21	+	-	+
2011C-3270 ^e	JHLY0000000	O26:H11	2011	USA	21	+	-	+
2010EL-1699 ^e	JHMF0000000	O26:H11	2010	USA	21	+	-	+
2010C-5028 ^e	JHMI0000000	O26:H11	2010	USA	21	+	-	+
2010C-4834 ^e	JHMN0000000	O26:H11	2010	USA	21	+	-	+
2010C-4788 ^e	JHMS0000000	O26:H11	2010	USA	21	+	-	+
2010C-4347 ^e	JHFB0000000	O26:H11	2010	USA	21	+	-	+
2010C-4244 ^e	JHFD0000000	O26:H11	2010	USA	21	+	-	+
2010C-3902 ^e	JHFF0000000	O26:H11	2010	USA	21	+	+	+
2010C-3871 ^e	JHFF0000000	O26:H11	2010	USA	21	+	+	+
2010C-3472 ^e	JHFX0000000	O26:H11	2010	USA	21	+	-	+
2010C-3051 ^e	JHGA0000000	O26:H11	2010	USA	21	+	-	+
2009C-4826 ^e	JHGI0000000	O26:H11	2009	USA	21	+	-	+
2009C-4747 ^e	JHGM0000000	O26:H11	2009	USA	21	+	-	+
2009C-3996 ^e	JHGV0000000	O26:H11	2009	USA	21	+	-	+
2009C-3689 ^e	JHGX0000000	O26:H11	2009	USA	29	-	+	+
03-3500 ^e	JHNT0000000	O26:H11	2001	USA	21	+	-	+
CVM10021 ^d	AKAZ0000000	O26:H11	1995	Unknown	21	+	-	-
CVM9952 ^f	AKBC0000000	O26:H11	1985	Unknown	21	+	-	-
CVM9942 ^d	AJVV0000000	O26:H11	1983	Unknown	21	+	-	-
CVM10026 ^d	AJVX0000000	O26:H11	1995	Unknown	21	+	-	+
CVM10030 ^d	AKBA0000000	O26:H11	1995	Unknown	21	+	-	+
CVM10224 ^e	AKBB0000000	O26:H11	1997	Unknown	21	+	-	-
CFSAN001629 ^e	AMXO0000000	O26:H11	1997	Unknown	21	+	-	-
2011C-3274 ^e	JAST0000000	O26:H11	2011	USA	21	+	-	+
ATCC BAA-2196 ^e	AYOF0000000	O26:H11	2003	USA	21	+	+	+

^a Closed genome. The rest are all draft genomes.

^b *In silico* ST.

^c Determined by *in silico* analysis. +, present; -, absent.

^d Cattle origin.

^e Clinical origin.

^f Pig origin.

using the Kimura 2-parameter model to estimate genetic distances. The statistical support of the nodes in the ML tree was assessed by 1,000 bootstrap resamplings. The use of SNPs allowed the determination of the true phylogeny and the discovery of informative SNPs in the data set.

Nucleotide sequence accession numbers. The draft genome sequences of all 42 *E. coli* O26 strains used in our study are available in GenBank under the accession numbers listed in Table 1.

RESULTS

***In silico* serotyping, MLST, and virulence gene profiles of cattle O26:H11/H⁻ strains.** The serotypes of cattle O26:H11/H⁻ strains were determined previously by PCR assay for the *wzx*_{O26} and *fliC*_{H11} genes; all the strains were O26, although several strains possessed an undetermined H antigen (20). *In silico* serotyping confirmed the lack of integrity of the H11 marker (*fliC*_{H11}) in the strains with the unknown H antigen (4271-C, 4822-A, and 4848-A-B). This lack of integrity of the *fliC*_{H11} gene explained the ob-

served failure to detect the H11 antigen with the *fliC*_{H11} PCR employed previously (Table 1). Most cattle O26:H11/H⁻ strains analyzed ($n = 40/42$) were ST29, while the remaining two (1895-A and 1914-A) were ST21.

The results of the WGS-based virulence typing are shown in Tables 1 and 3. The genomes were screened for 95 known *E. coli* virulence genes and two *arcA* allele type genes (see Table S1 in the supplemental material). The 42 strains were negative for 74 of the 95 virulence genes tested and described for *E. coli* (23) (see Table S1 in the supplemental material). A majority of the strains were positive for the *eae*- β 1, *tir*, *cif*, *pssA*, *iss*, *lpfA*, *nleA*, *nleB*, *prfB*, *espA*, *espB*, *espJ*, and *espF* genes (Table 3). The exceptions were strain 5019-A, which was negative for *espF*, and strains 5583-H and 4592-A, which were negative for *nleA*. The *stx*₁ gene was present only in the two ST21 strains. Interestingly, the two ST21 strains also carried the microcin H47 system (*mchB*, *mchC*, *mchF*, and *mcmA* genes).

TABLE 3 Virulence genes present in the O26:H11/H⁻ *E. coli* strains sequenced in this study by *in silico* analysis^a

Strain	<i>astA</i>	<i>cba</i>	<i>cma</i>	<i>efa1</i>	<i>espI</i>	<i>espK</i>	<i>espP</i>	<i>f17A</i>	<i>f17G</i>	<i>gad</i>	<i>iha</i>	<i>nleC</i>	<i>tccP</i>	<i>toxB</i>	α - <i>hlyA</i> ^c	<i>arcA</i> allele 2	<i>arcA</i> allele 1
1270-A	-	-	-	+	-	-	-	-	-	+	-	+	-	-	+	-	+
1357-A	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+
1676-A	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+	-	+
1692-A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
1740-A-A	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+	-	+
1740-A-B	-	-	-	+	-	-	-	+	+	+	-	-	-	-	+	-	+
1802-A	-	-	-	+	-	-	-	+	+	-	-	-	+	-	+	-	+
1958-A-B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+
2105-G	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+
2139-A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
2152-B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
2176-A	+	+	+	+	-	-	-	+	+	-	-	-	-	-	+	-	+
2194-B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
2223-B	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
2228-A	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+
3674-A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4131	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+
4170	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+
4196	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+
4271-C	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+
4277-H-A	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
4368-A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4435-B	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
4468-A-A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4468-A-B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4513	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
4592-A	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+
4730-C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4822-A	-	-	+	+	-	-	-	-	-	+	-	-	-	-	+	-	+
4848-A-B	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+
4860-A	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+
5019-A	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
5196-B	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
5206-E-B	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+
5583-H	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+
5687-B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
1341-A	-	-	-	-	+	+	+	-	-	-	+	+	-	+	-	+	-
1668-A-A	-	-	-	-	+	+	+	-	-	-	+	+	-	+	-	+	-
1668-A-B	-	-	-	-	+	+	+	-	-	-	+	+	-	+	-	+	-
1895-A ^b	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	+	-
1914-A ^b	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	+	-
4863-A	-	-	-	-	+	+	+	-	-	+	+	+	-	+	-	+	-

^a +, present; -, absent. The functions of the genes can be found in Table S1 in the supplemental material. All the samples carried the following virulence genes: *cif*, *eae*- β 1, *pssA*, *tir*, *espA*, *espB*, *espF* (except 5019-A), *nleA* (5583-H and 4592-A), *nleB*, *prfB*, *espJ*, *iss*, and *lpfA*. The *exxA* gene content of each strain is shown in Table 1.

^b 1895-A and 1914-A were the only ST21 strains among the O26:H11 isolates from cattle. These were positive for Shiga toxin gene type 1 (*stx* type 1, a 100% match to GenBank accession number AF461168) and carried plasmid genes *mchB*, *mchC*, *mchF*, and *mcmA*.

^c Alpha-hemolysin-pEO5 (α -*hlyA*; NG_036728).

According to the classification proposed by Bugarel et al. (6), most O26 cattle strains ($n = 36/42$) analyzed in this study were classified as EPEC (*eae*- β ⁺, alpha-hemolysin gene positive, and *arcA* allele type 1 gene). The remaining six cattle strains were classified as EHEC (1895-A and 1914-A) (*stx*⁺, *eae*- β ⁺, *espK*⁺, and *arcA* allele type 2 gene), and EHEC-like (1341-A, 1668-A-A, 1668-A-B, and 4863-A) (*eae*- β ⁺, *espK*⁺, and *arcA* allele type 2 gene). These six strains also carried the *ehxA* and *toxB* genes (two ST21 and four ST29 strains), and hence, also carried an EHEC virulence plasmid.

***In silico* serotyping, MLST, and virulence of O26 *E. coli* genomes available in GenBank.** Thirty-seven O26:H11 STEC genomes (32 clinical, 4 from cattle, and 1 from pig) were down-

loaded from GenBank, and *in silico* MLST showed that the majority (92%) were ST21 (Table 2). All the strains were confirmed as O26:H11 by *in silico* serotyping. The results of the WGS-based virulence typing are shown in Table 4. Thirty-one of the 37 strains were positive for *stx*₁ variant a (*stx*_{1a}) only and 3 were positive for both *stx*_{1a} and *stx*_{2a}, while the remaining 3 were positive only for *stx*_{2a} (Table 4). The strains positive only for *stx*_{2a} were ST29. Most strains were positive for the presence of *cif*, *eae*- β 1, *pssA* (except CVM9952), *tir* (except 2009C-4826), *espA*, *espB*, *espF* (except 2009C-4826 and 2009C-3689), *nleA* (except CVM10021, CVM9952, CVM9942, 2009C-3612, 2010C-4819, and 2010C-4788), *nleB*, *prfB*, *espJ* (except 2010EL-1699, 2009C-4826, and 2009C-4747), *iss* (except CVM9942), and *lpfA*. All the strains were

TABLE 4 Virulence genes present in the O26:H11/H⁻ *E. coli* strains available at GenBank by *in silico* analysis^a

Strain	<i>astA</i>	<i>cba</i>	<i>celB</i>	<i>cma</i>	<i>efal</i>	<i>espI</i>	<i>espK</i>	<i>espP</i>	<i>gad</i>	<i>iha</i>	<i>nleC</i>	<i>tccP</i>	<i>toxB</i>	<i>katP</i>	<i>arcA</i> allele 2	<i>arcA</i> allele 1
CVM9952	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-
CVM10030	-	-	-	-	+	-	+	-	-	-	+	-	-	-	+	-
CFSAN001629	-	+	-	+	+	-	+	-	-	-	+	-	-	-	+	-
CVM9942	-	-	+	-	+	-	+	-	-	-	+	-	-	-	+	-
2011C-3387	-	-	-	-	+	-	+	-	+	-	-	-	-	+	+	-
05-3646	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+	-
CVM10026	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-
CVM10224	-	-	-	-	+	-	+	-	-	-	+	-	-	-	+	-
CVM10021 ^b	+	-	+	-	+	-	-	-	-	-	-	-	-	+	+	-
21765 ^c	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	+
2010C-3051	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2009C-3689	-	-	-	-	-	+	+	+	-	+	+	-	+	-	+	-
ATCC BAA-2196	-	-	-	-	+	-	-	+	-	-	+	-	+	+	+	-
2010C-4834	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-5028	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-4430	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
11368	-	-	-	-	+	-	+	-	-	-	+	+	+	+	+	-
2011C-3270	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-4788	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2011C-3282	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2011C-3274	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-
2011C-3655	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2011C-3506 ^c	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
03-3500	-	-	+	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-3902	-	-	+	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-4819	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010EL-1699	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-4347	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2009C-4760	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2009C-4747	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-4244	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-3472	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2009C-4826	-	-	+	-	+	-	+	+	-	+	+	-	+	+	+	-
06-3464	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2009C-3996	-	-	-	-	+	-	+	+	-	-	+	-	+	+	+	-
2010C-3871 ^b	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	-
2009C-3612	-	-	-	-	-	+	+	+	-	+	+	-	+	-	+	-

^a +, present; -, absent. A majority of the strains carried the following virulence genes: *cif*, *eae-β1*, *psaA* (except CVM9952), *tir* (except 2009C-4826), *espA*, *espB*, *espF* (except 2009C-4826 and 2009C-3689), *nleA* (except CVM10021, CVM9952, CVM9942, 2009C-3612, 2010C-4819, and 2010C-4788), *nleB*, *prfB*, *espJ* (except 2010EL-1699, 2009C-4826, and 2009C-4747), *iss* (except CVM9942), and *lpfA*. The Shiga toxin and *ehxA* gene contents of each strain are shown in Table 2.

^b Isolate positive for alpha-hemolysin-pEO5 (α -*hlyA*; NG_036728).

^c Isolate positive for *mchB*, *mchC*, *mchF*, and *mcmA*.

considered EHEC (*stx*⁺, *eae-β*⁺, *espK*⁺, and *arcA* allele type 2 gene), except for strain 21765, which was an EPEC carrying a *stx*₂ gene (*eae-β*⁺, alpha-hemolysin gene positive, and *arcA* allele type 1 gene). The virulence genes found in plasmids showed high diversity among the strains, with most carrying *ehxA* ($n = 30$), while only 27 also carried *toxB*, indicating the presence of different virulence plasmids. As observed for cattle O26 strains, three of the GenBank genomes (CVM10021, 2011C-3506, and 2010C-3871) showed the presence of the microcin H47 system (*mchB*, *mchC*, *mchF*, and *mcmA* genes).

Presence of antimicrobial resistance genes. Twenty-nine of the 42 cattle strains (70%) carried at least one antibiotic resistance gene, while only 6 of the 37 genomes available at GenBank carried at least one antibiotic resistance gene (16%) (Table 5). Of the cattle strains positive for antibiotic resistance, 16 carried only a tetracycline resistance gene (*tetC*), while the remaining strains carried multiple antibiotic resistance genes (aminoglycoside

and tetracycline; beta-lactamase and tetracycline; aminoglycoside and tetracycline; or aminoglycoside, phenicol, and sulfonamide) (Table 5). Among the six genomes from GenBank positive for antibiotic resistance, three carried only a single resistance gene (*tetA*, *tetB*, or *bla*_{TEM-1B}), while the remaining three carried multiple antibiotic resistance genes.

Phylogenetic relationship of strains by wgMLST analysis. The phylogenetic relationships among the *E. coli* O26:H11/H⁻ strains sequenced in this study and the genomes available at GenBank as determined by wgMLST analysis are shown in Fig. 1. The genome of strain 11368 (NC_013361.1) was used as a reference. This *E. coli* strain has 4,554 genes, 2,965 of which (core genes) were present in all the analyzed strains, with 1,048 genes identical among all the strains (see Table S2 in the supplemental material). The resultant NJ tree showed that the O26 genomes analyzed were highly diverse and polyphyletic. The genomes were divided into two main lineages (1 and 2), each with multiple clades. This wg-

TABLE 5 Presence of antimicrobial resistance genes identified by *in silico* analysis in the genomes of the strains analyzed in this study^a

Strain ^b	<i>aadA</i> ^c	<i>aph3</i> ^c	<i>aph6</i> ^c	<i>strA</i> ^c	<i>strB</i> ^c	<i>bla</i> _{TEM} ^d	<i>floR</i> ^c	<i>sul2</i> ^f	<i>tetA</i> ^g	<i>tetB</i> ^g	<i>tetC</i> ^g	<i>tetM</i> ^g
1357-A	–	–	–	–	–	–	–	–	–	–	+	–
1676-A	–	–	–	–	–	–	–	–	–	–	+	–
1692-A	–	+	+	–	–	–	–	–	–	+	–	–
1740-A-A	–	–	–	–	–	–	–	–	–	–	+	–
1740-A-B	–	–	–	–	–	–	–	–	–	–	+	–
1802-A	–	–	–	–	–	–	–	–	–	–	+	–
1895-A	–	–	–	+	+	–	–	–	+	–	–	–
1914-A	–	–	–	+	+	–	–	–	+	–	–	–
1958-A-B	–	–	–	–	–	–	–	–	–	–	+	–
2139-A	–	–	–	+	+	–	–	–	–	–	–	–
2152-B	–	–	–	+	+	–	+	+	–	–	–	–
2194-B	–	–	–	+	+	–	–	+	–	+	–	–
2228-A	–	–	–	–	–	–	–	–	–	–	+	–
3674-A	–	–	–	+	+	–	+	+	–	–	–	–
4170	–	–	–	–	–	–	–	–	–	–	+	–
4196	–	–	–	–	–	–	–	–	–	–	+	–
4271-C	–	–	–	–	–	+	–	–	+	–	–	+
4277-H-A	–	–	–	–	–	–	–	–	–	–	+	–
4368-A	–	–	–	+	+	–	+	+	–	–	–	–
4435-B	–	–	–	–	–	–	–	–	–	–	+	–
4592-A	–	–	–	–	–	–	–	–	–	–	+	–
4730-C	–	–	–	+	+	–	–	+	–	+	–	–
4822-A	–	–	–	–	–	+	–	–	–	–	–	+
4848-A-B	–	–	–	–	–	+	–	–	+	–	–	+
4860-A	–	–	–	–	–	–	–	–	–	–	+	–
5019-A	–	–	–	–	–	–	–	–	–	–	+	–
5196-B	–	–	–	–	–	–	–	–	–	–	+	–
5583-H	–	–	–	+	+	–	–	–	–	+	–	–
5687-B	–	–	–	–	–	–	–	–	–	–	+	–
ATCC BAA-2196	–	–	–	+	+	–	–	+	–	–	–	–
CVM10026	–	–	–	+	+	+	–	+	–	+	–	–
CVM9942	+	–	–	–	–	–	–	–	–	+	–	–
CVM9952	–	–	–	–	–	–	–	–	–	+	–	–
2010C-4788	–	–	–	–	–	+	–	–	–	–	–	–
2011C-3274	–	–	–	–	–	–	–	–	+	–	–	–

^a +, present; –, absent.^b The strains sequenced in this study are shown in boldface. Only strains that carried antimicrobial resistance genes are shown.^c Aminoglycoside resistance.^d Beta-lactamase resistance.^e Phenicol resistance.^f Sulfonamide resistance.^g Tetracycline resistance.

MLST analysis using the core genes was highly discriminatory, with a discriminatory index of 0.997 (72 types/79 samples).

Lineage 1 was defined as the EHEC O26 lineage that comprised O26:H11 EHEC-like (ST29; *stx* negative) and O26:H11 EHEC (ST21) cattle strains, as well as most clinical O26:H11 *E. coli* GenBank genomes. Lineage 1 was composed of five different clades, with cattle O26:H11 strains belonging to clades C2 (ST21) and C5 (ST29). The ST29 EHEC-like cattle strains (lineage 1) clustered with the two O26:H11 *stx*⁺ strains isolated from clinical samples in 2009, showing nearly identical virulence profiles except for the *stx* gene, indicating the pathogenic potential of these strains. The other ST21 lineage 1 cattle strains clustered separately as a single clade and can be considered potentially pathogenic, as well, because they carried all the virulence genes known in an O26:H11 EHEC strain (6).

Lineage 2 was composed of EPEC O26 cattle strains (ST29) (Fig. 1) and was divided into three diverse clades, with different

virulence gene contents (Table 3). Interestingly, strain 21765, which was isolated from humans during the first raw milk cheese outbreak described in France in 2005 (5), belonged to lineage 2 (EPEC) but was described in the original genome publication as an EHEC strain. Most O26 EPEC strains from U.S. cattle (4/5) were negative for the alpha-hemolysin gene (indicative of the presence of the virulence plasmid), clustered in clade C3 of lineage 2, and might be considered less pathogenic than the EPEC strains from the other two clusters due to the absence of the virulence plasmid and other virulence genes present in the other two clusters (Fig. 1).

Phylogenetic relationship of strains by targeted SNP analysis. To evaluate the results obtained by wgMLST and to determine the actual evolutionary phylogenetic relationships among the *E. coli* O26:H11/H[–] strains sequenced in this study and the genomes available at GenBank, a targeted SNP analysis was performed (Fig. 2). The SNP matrix consisted of 5,050 SNP positions in 2,965

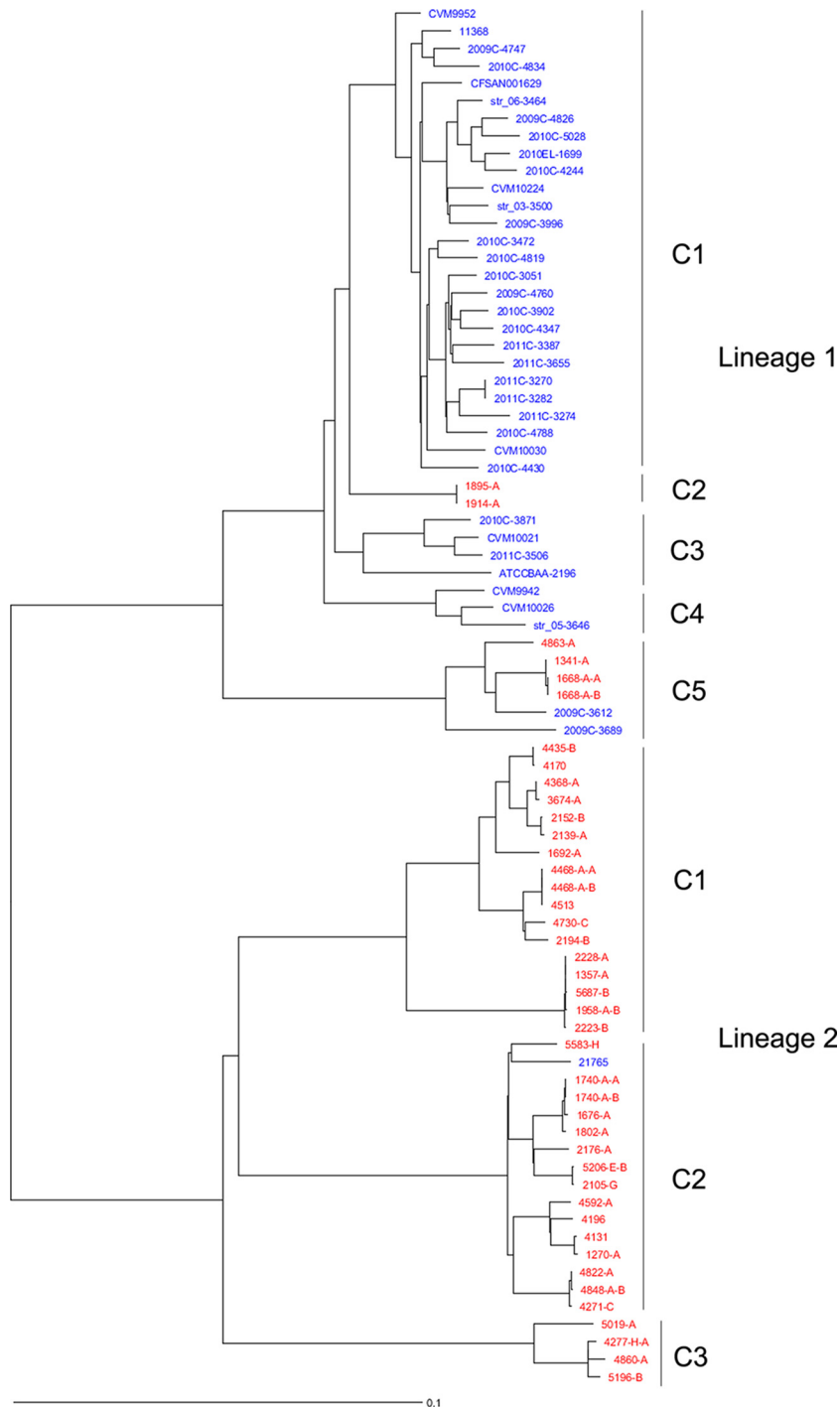


FIG 1 Phylogenetic analysis of the O26:H11/H⁻ *E. coli* strains sequenced in this study and the genomes available at GenBank by wgMLST. Ridom SeqSphere+ identified 2,965 core genes. The evolutionary history was inferred by using an NJ tree built using the genetic distance and showing the existence of two clear lineages within O26 strains, with lineage 1 composed of EHEC and EHEC-like O26:H11/H⁻ strains, while lineage 2 was composed of EPEC O26:H11/H strains. The *E. coli* strains sequenced in this study are in red, and strains from GenBank are in blue. C1 to C5, clades.

genes (identified previously by wgMLST) (see Table S3 in the supplemental material). A maximum-likelihood tree was generated, which agreed overall with the NJ tree generated with the wgMLST data with strong bootstrap support on each node (Fig. 2). Consistent with the wgMLST NJ tree, two main lineages with individual clades were observed in the SNP ML tree. This targeted SNP anal-

ysis demonstrated the robustness of the wgMLST analysis for determining lineages and clades within *E. coli* O26:H11/H⁻.

DISCUSSION

E. coli O26:H11/H⁻ is the second most frequent EHEC serotype, causing diarrhea and HUS in the United States (3, 29) and world-

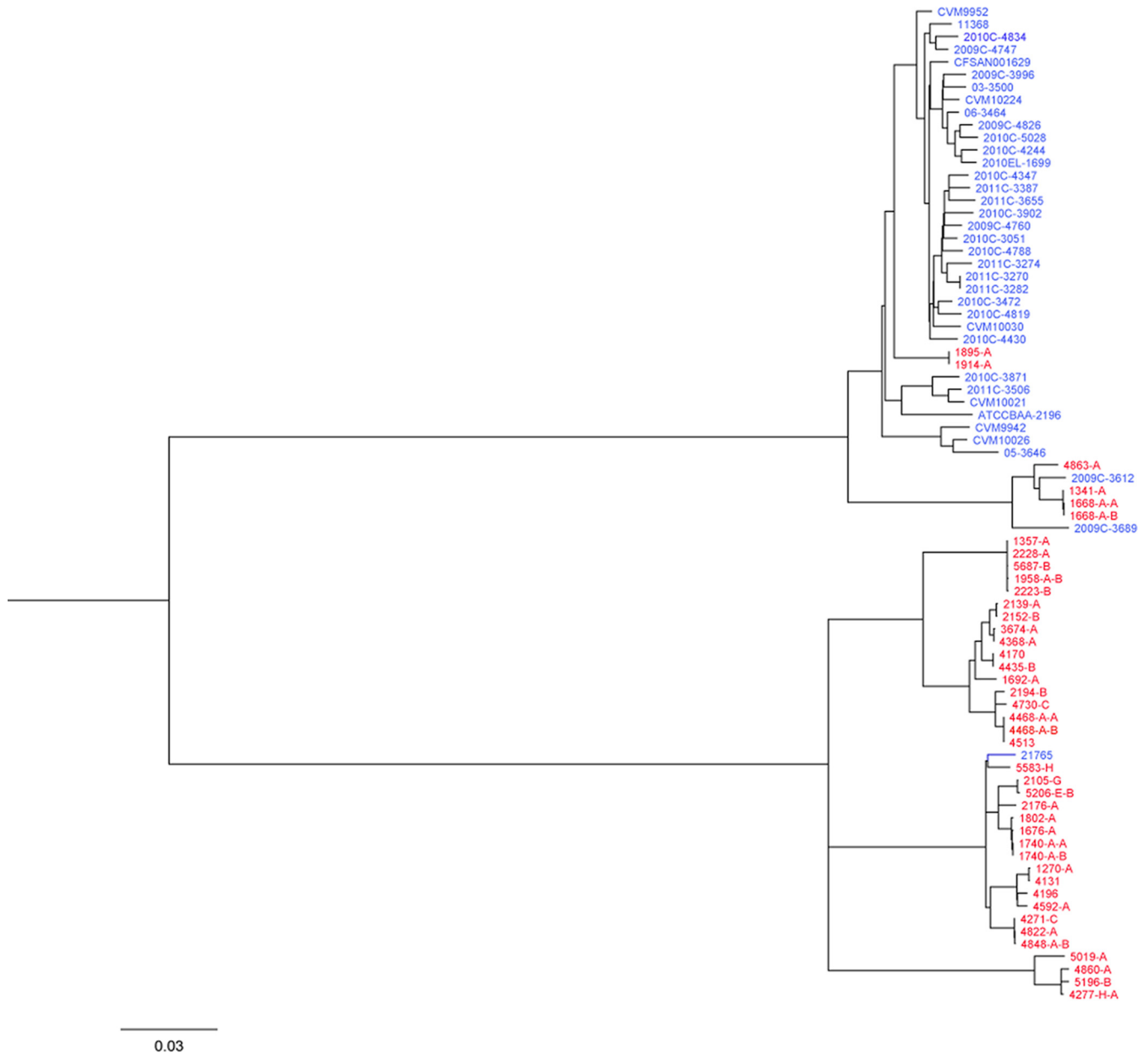


FIG 2 Molecular phylogenetic analysis of the O26:H11/H⁻ *E. coli* strains sequenced in this study and the genomes available at GenBank by the maximum-likelihood method. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura 2-parameter model (55). The tree is drawn to scale, with branch lengths measured in substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 4,916 positions in the final data set. The bootstrap values are not shown for visualization purposes. The lineages and clades are the same as in Fig. 1. The *E. coli* strains sequenced in this study are in red, and strains from GenBank are in blue.

wide (4, 6, 9, 30). In recent years there has been an increase in the occurrence of this serotype and other non-O157 serotypes in humans associated with consumption of contaminated food, mainly beef and dairy products. A new O26:H11/H⁻ clone (ST29; *stx*_a⁺, *eae*⁺, plasmid gene profile *ehxA*⁺, and *etpD*⁺) has been recently reported to be distributed all over Europe (30) and has been found in cattle (31). Few studies have been conducted on O26:H11/H⁻ in the United States (17, 18), and none has investigated if this new emerging clone exists in the United States. Our preliminary analysis of O26:H11/H⁻ strains from the United States showed the presence of different virulence plasmids and suggested that these

strains could be divided further into different lineages (unpublished results). In the present study, we performed an in-depth analysis of 42 O26:H11/H⁻ strains isolated from cattle in 2011 using whole-genome sequence phylogenetic analysis by wgMLST and targeted SNPs. We compared them to the genomes of other O26:H11 strains available at GenBank (37 genomes).

We showed that these 42 O26:H11/H⁻ strains were highly diverse, as previously described (17), with most of them belonging to ST29 ($n = 40$), either EHEC-like ($n = 4$) or EPEC ($n = 36$). The remaining two strains were ST21 and formed an independent, unique clade within lineage 1. In contrast to the cattle strains, the

majority of the O26 genomes retrieved from GenBank were ST21 (92%). By wgMLST and targeted SNP phylogeny, we were able to distinguish two clearly distinct lineages among the *E. coli* O26 strains analyzed, with EHEC-like strains clustering together and differing by 76 (1668-A-A, 1668-A-B, and 1341-A) and 160 (4863-A) SNPs (out of 5,050 SNPs) from two clinical EHEC strains, 2009C-3612 and 2009C-3689, respectively. Therefore, EHEC-like O26:H11 (*stx*-negative) strains seem to be EHEC strains that have lost or could acquire the Shiga toxin prophage, confirming a previously suggested hypothesis (6, 18). A search for possible known insertion sites for *stx*₂ phages (*wrbA*, *sbcB*, *yehV*, *yecE*, and Z2577) (32) found that all the sites were intact in the four *stx*-negative ST29 EHEC strains and that the two ST21 O26 *E. coli* cattle strains have *wrbA* and *yehV* sites occupied by a phage. These phylogenetic analyses confirmed that hypothesis and that these *stx*-negative O26:H11/H⁻ “EHEC-like” strains are not EPEC but can be grouped in O26 lineage 1 and therefore can be called EHEC O26. These EHEC O26 (*stx*-negative) strains can be distinguished from pathogenic EPEC O26 by more than 689 alleles and more than 1,900 SNPs, plus the presence of the EHEC virulence plasmid. Furthermore, within the two lineages, at least five and three different clades could be distinguished, respectively, showing the high genomic diversity among the genomes analyzed.

One interesting finding of this study was that the French O26:H11 strain 21765, isolated in 2005 during a milk cheese outbreak (5), belonged to one of the three clades within O26 lineage 2 (EPEC) and not to the EHEC lineage, as originally described (5). This strain carried all the virulence genes described in O26 EPECs but differed from the other members of that clade by also carrying a *stx*_{2a} phage. The strain was isolated prior to the *E. coli* O104:H4 strain that caused a large outbreak in Germany in 2011 (33). The O104:H4 strain was characterized as a new pathotype or atypical *E. coli*, being phylogenetically close to enteroaggregative *E. coli* (EAEC) and carrying virulence genes often observed in those strains, but carrying a *stx*_{2a} phage, as well (34, 35). These O104:H4 strains caused a large number of disease cases, with 22.3% of the patients developing HUS (36). Several authors called for a new pathotype definition for this strain as enteroaggregative-hemorrhagic *E. coli* (EAHEC) (37), with numerous researchers attempting to explain its origin (38). Our phylogenetic analysis showed that other *E. coli* strains from a different pathogenic background (EPEC) can also acquire a *stx* phage and cause disease in humans, which may be a more common trait in *E. coli* strains than previously thought. Our research clearly showed that WGS reveals more about strains that were previously incorrectly or not entirely characterized. For example, by wgMLST analysis, we were also able to show that some O26:H11 strains isolated at the Gastrointestinal Bacteria Reference Unit in the United Kingdom from 2009 to 2013, whose genomes were reported (9) and recently available at GenBank (only Sequence Read Archive [SRA]), were in fact EPEC strains: 670/13, 2290-502/12, and 680/13 (results not shown). These three strains were *stx* negative and clustered separately from the remaining EHEC O26 strains on the phylogenetic tree (9). However, in that publication, the actual nature of these strains (EHEC, EHEC-like, or EPEC) was not stated.

Some of the U.S. cattle strains ($n = 3$) were untypeable for their H antigen, resulting in O26:H⁻ serotypes using conventional serotyping techniques. After *in silico* serotyping analysis, we found that the H antigen gene was fragmented; further analysis of the *fliC* genes of these three strains showed that the gene was disrupted by

an undetermined insertion sequence (IS). However, analysis of the short sequences at either end of the disruption showed that the IS present might be IS2. The lack of H antigen production was described previously for O157:H⁻ German strains (39), though in that case, there was a deletion of 12 bp in the *fliC* H7 gene. The evolution of pathogens is largely influenced by IS elements, which have contributed to the diversification of EHEC strains, specifically O157 (40–43). Recently, an IS excision enhancer (IEE) element was discovered in EHEC O157 promoting the excision and deletion of IS3 family members, resulting in genomic rearrangements and strain diversification (44, 45). In the strains analyzed, the IEE element (IEE cluster I) was present solely in O26 lineage 1, suggesting that these strains have a higher potential to increase their diversity and virulence than those of lineage 2 (46). IEE cluster I has been found in different pathogenic strains from diverse serotypes (O157:H7, O26:H11, O111:H11, O118:NM, O145:H28, and O139:NM/H38) that are frequently involved in serious outbreaks (2, 47).

Analysis of known *E. coli* virulence genes (see Table S1 in the supplemental material) in O26 strains sequenced in this study and GenBank O26 strains showed they were very similar, with some exceptions. The main difference was in the presence of *stx*, *efa1*, *espK*, *katP*, *espP*, alpha-hemolysin (α -*hlyA*), and *ehxA* genes (Tables 3 and 4). The lack of observation of some of these genes may be due to the technique implemented to generate the genomes (e.g., 454, which generates insertions/deletions in homopolymer tracks), which did not allow optimal assembly. The *stx* genes were found exclusively in strains belonging to O26 lineage 1 (the EHEC lineage), regardless of their origins, except for strain 21765 (*stx*⁺, O26 lineage 2 [EPEC], clade C2 [this study]), a clinical strain isolated during the French outbreak (5). The *efa1* gene, encoding an *E. coli* factor for adherence, was present in most EHEC lineage 1 strains, except ST29 strains. Some of the virulence genes carried by plasmids in EHEC strains are *espK*, *katP*, *espP*, *ehxA*, *etp*, and *toxB*. *espK* was present in most O26 EHEC lineage 1 strains (90%), except four strains, while the O26 lineage 2 strains were all negative for *espK*. Strain 21765 was negative for *espK*, consistent with its being an EPEC O26 strain. Therefore, the *espK* gene is still a good marker for differentiating O26 EHEC strains from O26 EPEC strains, as proposed previously (6). The *katP* (catalase/peroxidase) gene was not present in any of the U.S. cattle strains analyzed and in only one of the GenBank cattle genomes (CVM10021), while it was present in 27 of the remaining 36 O26 *E. coli* GenBank genomes. The *katP* gene is a plasmid-borne gene and protects *E. coli* against peroxide damage (48). The gene is absent in the new ST29 clone that recently emerged in Europe (30, 49), showing that the majority of the clinical U.S. strains for which genomes are available in GenBank do not belong to this newly described clone. The *espP* (serine protease) gene was absent in all cattle strains from O26 lineage 2 (EPEC) and present in the majority (76%) of O26 lineage 1 (EHEC) strains. *espP* is one of the virulence genes located on the plasmid that participate actively in the colonization of the gut by EHEC strains by downregulating complement (50). The other two important virulence plasmid genes, *ehxA* and *toxB*, were present only in O26 lineage 1 (EHEC) strains but were not found in 6 and 9 of them, respectively. This indicates diverse plasmid contents in these strains. *toxB* was missing in the new European O26:H11/H⁻ clone (30, 49). The *etp* genes were missing in all EHEC O26 genomes analyzed and have been reported to be present in the newly emerged O26:H11/H⁻

clone in Europe (30). On the other hand, the α -*hlyA* gene, another plasmid-borne virulence gene described for EPEC strains, was mostly present in O26 lineage 2 (EPEC), except in five strains, with four of them forming an independent clade within lineage 2 and appearing to lack a virulence plasmid; therefore, they may be considered less pathogenic.

The prevalence of antimicrobial resistance genes in the U.S. cattle O26 strains was higher (70%) than in the clinical O26 *E. coli* GenBank genomes (16%). Of the EHEC strains from U.S. cattle, only ST21 (*stx*₁⁺) carried antibiotic resistance genes. Some cattle strains carried multiple antimicrobial resistance genes (31%), while only 9% of the clinical GenBank strains carried more than one antibiotic resistance gene. Resistance to antibiotics has been described in other O26 strains from cattle (Scotland; 2004), although the percent resistance to one or several antibiotics was below 2% (51). The mechanism for resistance to multiple antibiotics in O26:H11 strains is not new, and antibiotic resistance has been described previously in a *stx*⁺ O26:H11 strain (O6877) isolated from a clinical case in Australia as part of an integron element (52). The integron was located in the 111-kb virulence plasmid (pO26-CRL) (52). The plasmid is similar to another virulence plasmid in O26:H11 strain H30 (165 kb) (53) based on its virulence gene content (*espP*, *exhA*, *katP*, and *toxB*). These differences in virulence plasmid contents between the two EHEC O26 strains reaffirm the high diversity inside EHEC lineage O26:H11. A BLAST search for integron class 1, 2, and 3 integrase genes (*intI1*, *intI2*, and *intI3*) showed that they were absent among the cattle strains analyzed, and only one strain was positive for the *intI1* gene (CVM9942, a strain isolated from cattle that carried *aad* and *tetB* genes) among the GenBank strains. Interestingly, some of the O26 genomes analyzed carried the microcin H47 system (*mchB*, *mchC*, *mchF*, and *mcmA* genes). This system has been reported to produce microcin H47, which acts as a bactericidal antibiotic and is produced by naturally occurring *E. coli* (54). The presence of this system in some of the O26 strains analyzed highlights the plasticity of O26 genomes in the environment.

Overall, the results presented here showed that cattle in the United States can be a reservoir of two different O26:H11 phylogenetic lineages: O26 lineage 1—EHEC O26:H11/H⁻ (either *stx*⁺ or *stx* negative, but with the potential to acquire a *stx* prophage)—and O26 lineage 2—EPEC O26:H11/H⁻. The wgMLST analysis allowed the differentiation of O26 strains into two different lineages, with O26 EHEC-like strains grouping with EHEC and therefore being EHEC strains that have either lost or not gained the *stx* prophage. The wgMLST also allowed us to correctly identify strains that were misidentified as EHEC (strain 21765) or not categorized at all (strains 670/13, 2290-502/12, and 680/13). We have also provided a description of their virulence and antimicrobial resistance gene profiles. The strains from these two observed lineages are potentially pathogenic and produce different outcomes of the illnesses (EPEC produces watery diarrhea, while EHEC produces bloody diarrhea), with lineage 1 infection potentially progressing to HUS. Most U.S. cattle strains also carried multiple antibiotic resistance genes. Finally, this study showed that most O26:H11 clinical strains from the United States (2009 to 2011) belonged to an O26:H11 EHEC clone (ST21 or ST29; *stx*₁⁺ *ehxA*⁺ *toxB*⁺) different from the newly described O26:H11 EHEC European clone (ST29; *stx*₁⁺ and/or *stx*₂⁺ *ehxA*⁺ *etpD*⁺).

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