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Genetic Diversity and Pathogenic Potential of Attaching and Effacing *Escherichia coli* O26:H11 Strains Recovered from Bovine Feces in the United States

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***Escherichia coli* O26 has been identified as the most common non-O157 Shiga toxin-producing *E. coli* (STEC) serogroup to cause human illnesses in the United States and has been implicated in outbreaks around the world. *E. coli* has high genomic plasticity, which facilitates the loss or acquisition of virulence genes. Attaching and effacing *E. coli* (AEEC) O26 strains have frequently been isolated from bovine feces, and there is a need to better characterize the relatedness of these strains to defined molecular pathotypes and to describe the extent of their genetic diversity. High-throughput real-time PCR was used to screen 178 *E. coli* O26 isolates from a single U.S. cattle feedlot, collected from May to July 2011, for the presence or absence of 25 O26 serogroup-specific and virulence-associated markers. The selected markers were capable of distinguishing these strains into molecularly defined groups (yielding 18 unique marker combinations). Analysis of the clustered regularly interspaced short palindromic repeat 1 (CRISPR1) and CRISPR2a loci further discriminated isolates into 24 CRISPR types. The combination of molecular markers and CRISPR typing provided 20.8% diversity. The recent CRISPR PCR target SP_O26-E, which was previously identified only in stx_2-positive O26:H11 human clinical strains, was identified in 96.4% (161/167 [95% confidence interval, 99.2 to 93.6%]) of the *stx*-negative AEEC O26:H11 bovine fecal strains. This supports that these *stx*-negative strains may have previously contained a prophage carrying *stx* or could acquire this prophage, thus possibly giving them the potential to become pathogenic to humans. These results show that investigation of specific genetic markers may further elucidate our understanding of the genetic diversity of AEEC O26 strains in bovine feces.**

Escherichia coli O26 has been identified as the most common non-O157 Shiga toxin-producing *E. coli* (STEC) serogroup to cause human illnesses resulting in hemorrhagic colitis and hemolytic-uremic syndrome (HUS) in the United States and in Europe (1–3). In an effort to ensure a safer food supply, in 2012, the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) expanded the list of serogroups designated adulterants in raw nonintact beef and products that will become nonintact beef to include non-O157 STEC serogroups O26, O45, O103, O111, O121, and O145 (4).

Cattle have been implicated as a reservoir for *E. coli* O26, and cattle likely serve as an important source of contamination of raw agriculture products that come into contact with contaminated manure, including beef, dairy, and produce (5, 6). Additionally, *E. coli* O26 is a highly diverse serogroup, and strains can vary from highly pathogenic to avirulent, nonpathogenic variants (7–10). Furthermore, this distribution of molecularly defined *E. coli* O26 pathogroups within bovine feces has not been fully described.

In *E. coli*, most virulence factors are located on potentially mobile elements (phages, plasmids, and pathogenicity islands), which can rapidly be exchanged, acquired, or lost, such as Shiga toxin phages (7–9, 11). This genetic exchange of virulence factors may have contributed to the genetic shift and clonal development observed within the *E. coli* O26 serogroup (12). Prior to the emergence of the new Stx2a-producing clone in the mid-1990s, the majority of *E. coli* O26:H11 isolates recovered from humans were primarily only stx_1 positive or, not as common, positive for stx_1 in combination with stx_2 but not stx_2 alone (13). Based on strains harboring these Shiga toxin combinations, pathogroups were distinguished by their pathogenicity, severity of disease, and select virulence factors. Enterohemorrhagic *E. coli* (EHEC) O26:H11 isolates were defined as being positive for *stx* and *eae* (beta variant) and also possibly possessing a specific allelic type of *arcA* (*arcA* allele 2) (14). This definition was further modified to include possible associations with other virulence factors such as *espK* and *espN* (7). Enteropathogenic *E. coli* (EPEC) O26 isolates were defined as being *eae* positive, *stx* negative, and *espK* negative, and EPEC O26 may be associated with a distinct allele of *arcA* (*arcA* allele 1) (7, 8).

The dissemination of a new highly virulent EHEC O26:H11 clone harboring only stx_{2a} across Europe (5, 12, 13, 15–22) and, more recently, other parts of the world, including the United States (1, 23), further expands the classification scheme and con-

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tinues to challenge the understanding of this diverse serotype. Bielaszewska et al. (12) used multilocus sequence typing to show that this new clone belongs primarily to sequence type 29 (ST29). Further investigation into this new highly pathogenic EHEC O26:H11 clone identified a second subpopulation of EHEC isolates carrying the stx_{2a} or stx_{2d} variant and lacking *espK* and *arcA* allele 2 but being positive for *eae*-beta and the SP_O26-E clustered regularly interspaced short palindromic repeat (CRISPR) PCR marker (19).

Previous studies have identified EHEC-like O26:H11 strains that express all of the genetic features of EHEC O26:H11 except for the presence of Shiga toxin-encoding genes (stx_1 and/or stx_2) (7, 24, 25). These strains might have lost their *stx* phage during culture, or alternatively, they might constitute a reservoir for EHEC O26 upon the subsequent acquisition of an *stx* phage. Furthermore, some Shiga toxin-negative strains have been identified to cause severe human illness (26–28). Although Shiga toxin-positive O26:H11 has been extensively studied, there is a lack of available literature on non-Shiga toxin variants. Based on molecular characteristics that have previously been described (8, 24, 29–31), we attempt to assess the relatedness of Shiga toxin-negative and *eae*-positive *E. coli* O26 strains from cattle to highly pathogenic O26 clones isolated in humans.

MATERIALS AND METHODS

***Escherichia coli* strains.** The strains investigated in this study ($n = 178$) were isolated from the feces of U.S. feedlot cattle. Strains originated from fecal samples collected between May and July 2011 from a study involving 17,148 cattle housed in 40 pens at a commercial feedlot in the Midwest (32). There is no known association of these strains with any human illness. *Escherichia coli* DNA was extracted by using an InstaGene matrix (Bio-Rad Laboratories, Marnes-La-Coquette, France) according to the manufacturer's guidelines. *Escherichia coli* strains, belonging to a collection at the French Agency for Food, Environmental and Occupational Health and Safety (Anses), that were used as PCR controls in this study included *E. coli* 07QMA 144.1 (avirulent O26:H11), ED21 (EHEC O26:H11; stx_1 positive [stx_1^+]), EH196 (EHEC O26:H11; stx_2^+), MB01 (EHEC-like O26:H11), EC293 (O26:H32), ED142 (O111:H8), and E2348/69 (EPEC O127:H6).

Molecular markers. PCR amplification was used to investigate 25 molecular markers for the characterization of the *E. coli* O26 strains in this study (see Table S1 in the supplemental material for oligonucleotides). Genetic markers included genes coding for the O26 antigen flippase (wzx_{O26}); Shiga toxins 1 and 2 (stx_1 and stx_2); adhesion factors (*eae*, *eae*-beta, and *iha*); the flagellar antigens H11 ($fliC_{H11}$), H32 ($fliC_{H32}$), and H8 ($fliC_{H8}$); effectors translocated by the type III secretion system (*espK*, *espV*, and *espN*); the EHEC hemolysin (*ehxA*); the long polar fimbriae [*lpfA*(O26)]; the bundle-forming pili (*bfpA*); the iron-repressible protein Irp2 (*irp2*); bacterial tellurite resistance (*terE*); urease (*ureD*); open reading frames of O island 57 (OI-57), Z2098 and Z2099; the CRISPR-associated markers SP_O26-C, SP_O26-D, and SP_O26-E; aerobic respiratory control protein A allele 2 (*arcA* allele 2); and the reference genetic marker for *E. coli* (*wecA*).

High-throughput real-time PCR. DNA amplifications were obtained through a combination of multiple high-throughput real-time PCR platforms. The PCR setup for the LightCycler 1536 system (Roche, Meylan, France) with a Bravo automated liquid dispenser (Agilent Technologies, Massy, France) was previously described (9, 29). Setup for the BioMark real-time PCR system (Fluidigm, San Francisco, CA) with the 192.24 Dynamic array (Fluidigm) was performed according to the manufacturer's recommendations, using 6-carboxyfluorescein (FAM)- and 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein succinimidyl ester (HEX)-labeled TaqMan probes and TaqMan gene expression PCR master mix (Applied Biosystems, Courtaboeuf, France). The thermal profile for the BioMark PCR was described previously (7). A LightCycler 480 (Roche, Meylan, France) or LightCycler Nano (Roche, Meylan, France) system was also used to perform additional real-time PCR amplifications.

CRISPR typing. The CRISPR1 and -2a loci were amplified by PCR as previously described (33). Double-stranded DNA sequencing of the CRISPR1 and CRISPR2a loci was performed by Eurofins MWG Operon (Courtaboeuf, France). Sequences were assembled and aligned by using the BioEdit biological sequence alignment editor (v7.2.5; BioEdit, Carlsbad, CA [http://www.mbio.ncsu.edu/Bioedit/bioedit.html]).

Analysis of CRISPR sequences was performed by using a Python Script (2.7.6) developed in-house. This script uses a method developed by Yin et al. (34) to assign allele numbers for each strain. Briefly, each unique spacer and repeat are recorded in separate databases associated with a number and a letter, respectively. Each unique spacer combination within a CRISPR locus defines a CRISPR allele, the listing of which is also contained in a database. Each unique CRISPR1 and -2a allele combination was manually assigned an arbitrary CRISPR type (CT).

Descriptive analysis. The EHEC pathogroup is defined here as strains harboring stx_1 and/or stx_2, *eae*, *espK*, and *arcA* allele 2, as described previously by Bugarel et al. (7), or as stx_2-positive strains that tested negative for *espK* and *arcA* allele 2 but positive for *eae* and the SP_O26-E CRISPR PCR marker (35). EHEC-like strains are devoid of Shiga toxin genes but possess the above-mentioned virulence genes (*eae*, *espK*, and *arcA* allele 2) found in EHEC strains (7). Furthermore, EHEC-like strains derived from the newly described stx_2 clone would be expected to test negative for *espK* and *arcA* allele 2 but positive for *eae* and the SP_O26-E CRISPR PCR marker (35). EPEC isolates are defined here by the absence of Shiga toxin-encoding genes, *espK*, *arcA* allele 2, and SP_O26-E but the presence of the intimin gene (*eae*). The presence of an *E. coli* adherence factor plasmid carrying *bfpA* may or may not also be present in EPEC isolates and has been used to classify EPEC isolates as typical and atypical, with the latter not being associated with HUS or the bundle-forming pilus gene *bfpA* (7–9, 30, 36). Avirulent, nonpathogenic *E. coli* O26 isolates are distinguished based on the absence of Shiga toxin genes, *eae*, and all other virulence-associated markers (*espK*, *espV*, SP_O26-E, and *arcA* allele 2).

Relative frequencies were calculated for molecular markers among specified groups, and 95% confidence intervals (CIs) are reported (37). Combinations of various molecular markers were used to group strains into discrete marker types (MTs). Further combination of the MTs with the CTs allowed the definition of diversity types (DTs). Simpson's index of diversity ($1 - D$) was used to determine the genetic diversity of MTs and the discrimination power of the combined typing methods (DTs): the greater the value reported, the greater the sample diversity (38, 39). A hierarchical clustering dendrogram was constructed by using the molecular markers for each DT. The presence or absence of each marker was binomially coded as 1 or 0, respectively, and data were clustered by using Ward classification and Euclidean squared distances (STATGRAPHICS Centurion XV v15.2).

RESULTS

Molecular markers. The 178 *E. coli* strains of bovine fecal origin investigated were confirmed to be *E. coli* and to belong to the O26 serogroup by using the *wecA* and wzx_{O26} genetic markers, respectively. The flagellar antigen was identified as H11 ($fliC_{H11}$) for all strains, which consequently tested negative for $fliC_{H8}$ and $fliC_{H32}$. All strains also tested positive for both *eae* and *eae*-beta. The distributions and patterns of the 18 remaining genes are presented in Table 1. A hierarchical clustering dendrogram of the molecular markers is presented in Fig. 1. The combination of genetic markers present within a strain provided discrimination through the construction of unique MTs (Table 1). All strains tested positive for *irp2*, which encodes the iron-repressible protein. With the ex-

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TABLE 1 Marker types determined by genetic markers present within *Escherichia coli* O26:H11 strains[a]

MT[b]	No. of strains	Presence of marker																	
		stx_1	*eae*-beta	*espK*	*espV*	*ureD*	Z2098	Z2099	*iha*	*ehxA*	*espN*	*terE*	*irp2*	SP_O26-C	SP_O26-D	SP_O26-E	*bfpA*	*lpfA*(O26)	*arcA2*
1	1	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	−	+	−
2	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+
3	2	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+
4	5	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−	−	+	+
5	1	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
6	4	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
7	1	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−
8	7	−	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−
9	9	−	+	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+	−
10	70	−	+	−	−	−	−	−	−	−	−	+	+	−	−	+	−	+	−
11	2	−	+	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+	−
12	2	−	+	−	+	−	−	−	−	−	−	+	+	−	−	+	−	+	−
13	39	−	+	−	+	−	−	−	−	−	−	−	+	+	−	+	−	+	−
14	12	−	+	−	+	−	−	−	−	−	−	+	+	+	−	+	−	+	−
15	1	−	+	−	+	−	−	−	−	+	−	−	+	+	−	+	−	+	−
16	1	−	+	−	+	−	−	−	−	−	−	−	+	+	+	+	−	+	−
17	17	−	+	−	−	−	−	−	+	−	−	+	+	+	+	+	−	+	−
18	1	−	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	+	−
Total (%)[c]		3.4	100	5.6	34.3	6.2	6.2	6.2	15.7	6.7	2.8	65.7	100	50.6	10.1	91.0	0	99.4	5.6

[a] The controls in this study belong to a collection at Anses and were used solely as PCR controls. Comparisons to the genotype profiles of these strains were not made. *Escherichia coli* control strains (and the corresponding genetic markers) were 07QMA 144.1 (*wecA* wzx_{O26} $fliC_{H11}$), ED21 (stx_1 *espV* Z2098 Z2099), EH196 [stx_2 *espK* SP_O26-C SP_O26-D *terE arcA2 lpfA*(O26) *ehxA*], MB01 (*espN eae eae*-beta *ureD iha irp2*), EC293 ($fliC_{H32}$), ED142 ($fliC_{H8}$), E2348/69 (*bfpA*), and 5292A (SP_O26-E) (this study). An exhaustive list of genetic markers present in each control strain is not included.

[b] All strains produced a positive result for *wecA*, wzx_{O26}, *eae*, and $fliC_{H11}$, and all strains produced a negative result for stx_2, $fliC_{H32}$, and $fliC_{H8}$.

[c] Total percentage of strains with the identified genetic marker.

ception of one strain, all strains contained the long polar fimbria gene *lpfA*(O26).

Discrimination of strains into their respective groups was performed based on the association of the virulence markers investigated. In this study, all strains tested negative for stx_2, and five strains were perfectly associated with the genetic markers stx_1, *eae*, *espK*, and *arcA* allele 2. The presence of these markers is in agreement with data for human strains classified in the EHEC pathogroup. Additionally, the presence of *espN*, which encodes an effector protein, translocated by the type III secretion system, was detected only in the stx_1^+ $espK^+$ strains. One strain, although it contained stx_1, lacked many other virulence-associated genes observed in the other Shiga toxin-positive strains, including *espK* and *arcA* allele 2. However, given that this strain contained intimin (*eae*) and SP_O26-E, with the latter marker not being observed in the previous five strains, it may have a different pathogenicity potential than the above-mentioned strains. Although negative for stx_2, this strain (MT1) displayed the characteristic pattern of the new virulent stx_{2a} clone (12, 15, 35).

In this study, five strains that displayed the characteristic profile of EHEC-like strains were identified, possessing the virulence-associated factors *eae*, *espK*, and *arcA* allele 2 and lacking *stx*. A further 161 *E. coli* O26:H11 strains were negative for *espK* and *arcA* allele 2 but were positive for *eae* and the SP_O26-E CRISPR marker. Given the profile of these strains, they might also be classified as EHEC-like strains, which may have derived from the new stx_2 clone. These strains were further divided into 11 MTs.

Given the absence of *bfpA*; which is located on the *E. coli* adherence factor plasmid; Shiga toxins; *espK*; *arcA* allele 2; and the CRISPR marker SP_O26-E, six O26:H11 strains represent three MTs (MTs 5 to 7) (Table 1) in this study and are most similar to human clinical isolates previously categorized as being atypical EPEC. No strains that lacked all the virulence-associated markers investigated were identified in this bank of strains.

The EHEC hemolysin gene *ehxA* was observed in five strains (MTs 2 and 3) and seven strains (MTs 4, 15, and 18) that displayed EHEC and EHEC-like profiles, respectively. An effector protein translocated by the type III secretion system, encoded by *espV*, was present in 33.5% (95% CI, 26.81 to 40.99%) of strains; positive strains belonged to MTs 2, 3, 12 to 16, and 18. The gene encoding urease production, *ureD*, was present in all but one of the strains with an EHEC profile (MT1) and in six strains exhibiting the EHEC-like profile (MTs 4 and 18). The gene encoding the adherence-conferring protein, *iha*, was present in MTs 2, 3, 4, 17, and 18, encompassing 28 strains.

According to Delannoy et al. (29), the presence of the open reading frames Z2098 and Z2099 carried on genomic OI-57 provided discrimination of EHEC and EHEC-like strains from strains belonging to other *E. coli* O26 pathogroups. Our strains that display profiles similar to those of these pathogroups are in accordance with these findings for only those strains that are positive for *arcA* allele 2, with the exception of one *arcA* allele 2-negative strain (MT18). This particular strain also carries many other virulence-associated markers (*espV*, *ureD*, *iha*, and *ehxA*), similar to those that have been found in the EHEC pathogroup (29). Although phenotypic expression was not investigated in this study, previously reported findings support that EHEC and atypical EPEC O26 expression of tellurite resistance correlates identically with the presence of *ter* genes (40–42). A large proportion of the strains (117/178; 65.7% [95% CI, 58.7 to 72.7%]), with the exception of 61 strains, contained *terE*.

The CRISPR-associated markers SP_O26-C and SP_O26-D were previously developed and identified, which, when present alone or in combination, allowed 100% sensitivity and 97.5%

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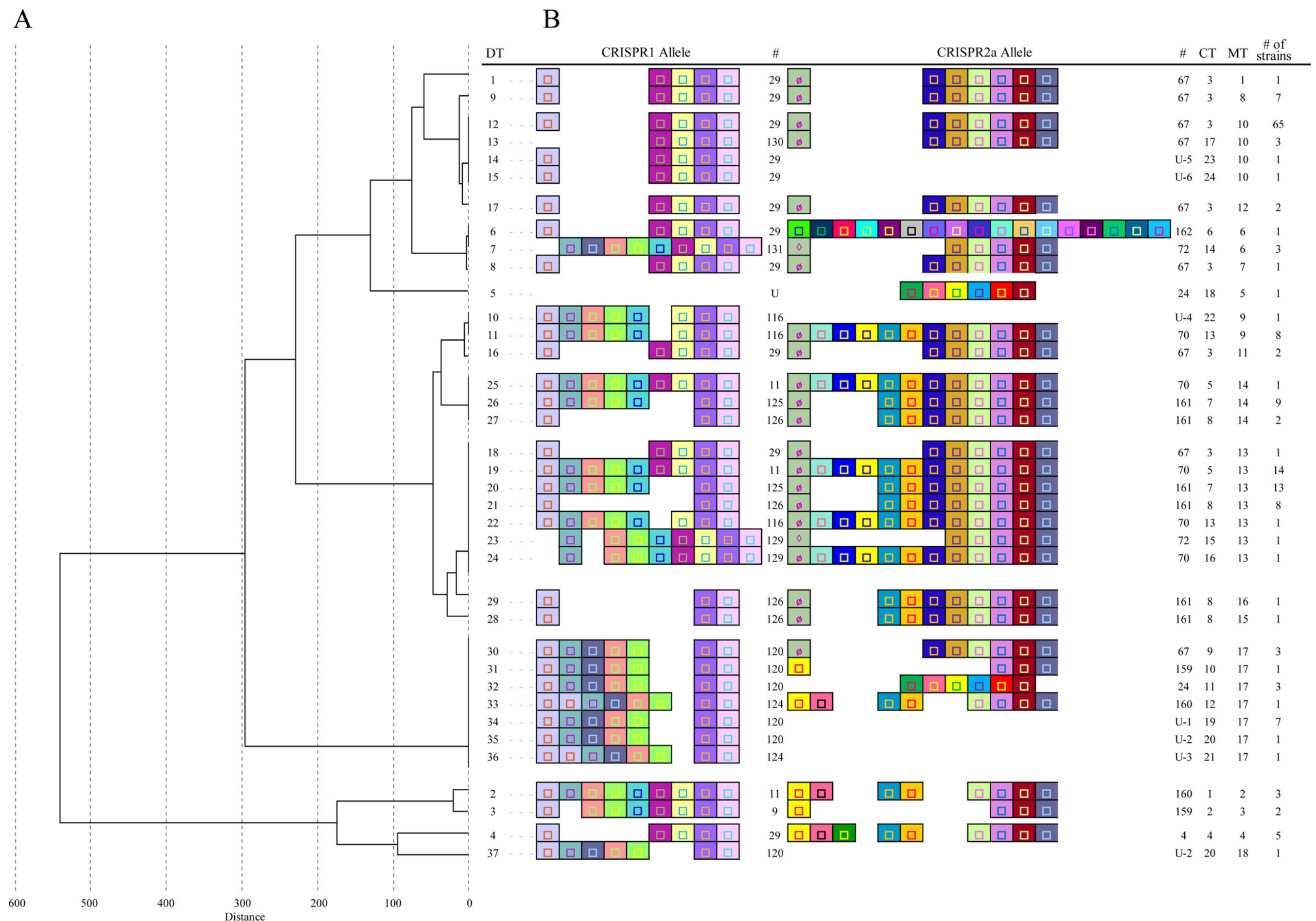

FIG 1 Molecular marker hierarchical clustering dendrogram compared to CRISPR allele profiles. (A) Hierarchical clustering dendrogram of the molecular markers for each diversity type (DT). (B) Graphic representation of the virulence gene profile and spacer arrangements in CRISPR1 and CRISPR2a of the 178 O26:H11 *E. coli* strains. Each unique spacer is represented by a unique color combination of the center shape and background. The shape in the center indicates the spacer length (○, 32 bp; ◇, 1,262 bp; ∅, 1,293 bp). Gaps were introduced to improve the alignment of similar CRISPR arrays. CRISPR1 and -2a allele numbers (#) are shown in their respective columns to the right of the CRISPR arrays. Each unique molecular gene profile was assigned a marker type (MT) number. Each unique combination of CRISPR1 and CRISPR2a alleles was assigned a CRISPR type (CT) number. CRISPR alleles denoted with a U indicate that the CRISPR amplicon was unable to be amplified. The individual combination of an MT and a CT was assigned a DT.

specificity for the detection of EHEC isolates (33). Of the O26:H11 strains investigated in this study, these CRISPR-associated markers were absent in 50.0% (3/6 [95% CI, 9.9 to 90.0%]) of the strains that displayed an EHEC profile. The presence of both the SP_O26-C and SP_O26-D CRISPR-associated markers was observed in 14.5% (24/166 [95% CI, 9.2 to 19.8%]) of strains with an EHEC-like profile. Furthermore, one strain (MT18) contained all of the virulence-associated markers analogous to those of EHEC strains with the exception of *stx*, *espK*, *espN*, and *arcA* allele 2. Additionally, detection of the combination of the CRISPR PCR markers SP_O26-C and SP_O26-D in 23 of these strains suggests their close relatedness to EHEC-like strains (33).

The CRISPR-associated marker SP_O26-E was previously identified only in stx_2-positive O26:H11 human clinical isolates that resulted in HUS (35). Unexpectedly, this CRISPR PCR test was positive for a large number of strains (162/178; 91.0% [95% CI, 86.8 to 95.2%]).

CRISPR typing. The O26:H11 strains were investigated for their CRISPR1 and CRISPR2a loci. The observed CRISPR array profiles are depicted in Fig. 1. Eleven alleles were identified in the CRISPR1 loci (6.2% allele diversity; 11 alleles/178 strains) constructed from combinations of three direct repeats, 10 spacers, and one untypeable allele (see Table S2 in the supplemental material). Many spacers in the CRISPR1 locus were shared among strains. Similar to previously reported findings, the CRISPR2a locus contributes to a greater diversity between alleles than does CRISPR1 (34, 35, 43). Indicative of this are the six direct repeats, 40 unique spacers, and six untypeable allele sequences that made up 15 unique CRISPR2a alleles in the O26:H11 bovine strains (8.4% allele diversity; 15 alleles/178 strains) (see Table S2 in the supplemental material). The combination of CRISPR1 and CRISPR2a alleles constituted 24 CRISPR types (CT), which represent 13.5% allele diversity (24 CTs/178 strains). CT3 was dominant among the strains (79/178), characterizing primarily those strains that were also positive for the CRISPR PCR marker SP_O26-E.

All identified spacers, in both CRISPR loci, were 32 bp in length, with the exception of the presence of two similar trans-

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posons, of 1,261 and 1,262 bp, in two CRISPR2a spacers that shared an identity of 1,256 bp (see Table S2 in the supplemental material). The 1,261-bp transposon is inserted just upstream of spacer 7 and generates a 1,293-bp spacer. The 1,262-bp transposon does not include a leftover sequence from a known spacer and generates a 1,262-bp spacer. Interestingly, the 1,261-bp transposon has been previously observed in highly virulent stx_{2a}- and stx_{2d}-positive O26:H11 human clinical isolates belonging to multilocus sequence type 29 (ST29) (35).

Within CRISPR1 and CRISPR2a were one and six untypeable alleles, respectively. These alleles were unable to be amplified by PCR in their entirety, and therefore, confirmation of the complete CRISPR allele could not be achieved. Incomplete CRISPR arrays may have been observed due to the CRISPR alleles being too long to generate a complete amplicon during PCR, or they may have lost this CRISPR locus. Conversely, the other CRISPR locus could be amplified and a CRISPR allele number could be identified, respectively, for these strains (Fig. 1).

Strain diversity. Within the strains investigated, 18 MTs were observed (Table 1). Of the MTs identified, strains exhibiting an EHEC-like profile contributed to 12 MTs. However, 42.2% (70/166) of strains belonged to MT10, carrying the profile of EHEC-like strains belonging to the new stx_2-positive O26 clone. Further discrimination among these O26:H11 bovine strains was achieved through a combination of the MT and CT, allowing 37 diversity types (DTs), with 20.8% diversity (37 DTs/178 strains), to be recognized (Fig. 1). Using Simpson's index of diversity, the genetic diversities within the MTs and DTs are 0.78 and 0.84, respectively.

DISCUSSION

In *E. coli*, the O26 serogroup is one of the main causative agents of HUS worldwide, second only to the O157 serogroup (1–3). EHEC O26 is considered to have evolved from attaching and effacing *E. coli* (AEEC) O26 strains through the acquisition of Shiga toxin (*stx*)-encoding genes (28). AEEC strains, which cause typical attaching and effacing (A/E) lesions in the intestinal mucosa, are characterized by the presence of the *eae* (intimin) gene carried on LEE (locus of enterocyte effacement). The *eae* gene is responsible for the intimate attachment of the bacteria to the cell wall (28, 44). The aim of this work was to investigate the distribution of *E. coli* O26 genotypes within bovine feces and identify the pathogenic potential of Shiga toxin-negative and *eae*-positive strains that belong to the AEEC pathogroup. Recent findings in Brazil of *E. coli* O26 containing the H8 antigen belonging to EHEC and *stx*-negative strains led to the inclusion of this antigen in this study (45). Furthermore, antigen H11 and nonpathogenic antigen H32, commonly isolated in food products, were also tested (18, 45–47). Investigation of the H antigen revealed that all strains were positive for $fliC_{H11}$.

The *eae* gene was detected in all strains investigated in this study and was further confirmed to be of the *eae*-beta subtype. This subtype was previously observed in diarrheagenic clones of EHEC (44) and EPEC O26:H11 (48). A portion of the strains investigated here ($n = 6$) display the genetic characteristics to be classified as an atypical EPEC isolate due to the absence of the bundle-forming pilus-encoding gene, *bfpA*, contained in the enteroadherent factor (EAF) virulence plasmid (7–9, 36). In contrast, typical EPEC isolates that contain *bfpA*, which allows *E. coli* to locally adhere to cultured epithelial cells (36, 49), were absent from the strains sampled.

In concordance with FSIS guidelines, six of the O26:H11 strains, belonging to MTs 1 to 3, would be classified as adulterants if present in raw beef products because they were found to harbor both *stx* and *eae*. Furthermore, these strains, along with seven strains belonging to MTs 4, 15, and 18, displaying the EHEC-like profile contained EHEC hemolysin, of which two strains displayed the EHEC-like profile of the new O26 clone. The absence of *ehxA* in all other strains suggests the absence of the large EHEC plasmid carrying this gene (41, 50).

The investigation of molecular markers allowed a high level of genetic diversity to be observed, with 18 MTs being identified in these O26:H11 strains (Simpson's index of diversity = 0.78). Furthermore, this observed high level of diversity is interesting given that the strains investigated here originated from a limited population of cattle and were isolated over a short period of time (32). This high level of genetic diversity contrasts with data for O157:H7, in which more homogeneity was previously observed with regard to virulence profiles within this *E. coli* serotype (34, 51). The absence of agreement in the level of diversity between O26 and O157 is further supported by a study by Yin et al. (34) in which only 3 CRISPR sequence types were identified in 57 O157 isolates, compared to the 13 CRISPR sequence types among 48 O26 isolates and the 24 CRISPR types identified in this study. Whittam et al. (52) also confirmed the high degree of diversity among O26 strains, demonstrated by 20 electrophoretic types created by distinctive multilocus genotypes in 93 O26 isolates.

The commonalities among *stx*-negative and *stx*-positive bovine O26:H11 strains are of particular interest. Zhang et al. (13) determined a commonality among all STEC O26 isolates with the presence of a high-pathogenicity island (HPI) characterized by the presence of *irp2*. Those authors further concluded that this HPI is genetically stable and a common genome component of the STEC O26 clonal lineage (13). All O26 strains investigated in this study were positive for *irp2*, indicating the presence of this HPI and suggesting the close relatedness of these strains, including those that are Shiga toxin negative, to the STEC clonal lineage.

The EHEC pathogroup was previously defined by Bugarel et al. (7) as harboring stx_1 and/or stx_2, *eae*, *espK*, and *arcA* allele 2. The emergence of a new stx_2 clone expanded that definition to include stx_2-positive strains that tested negative for *espK* and *arcA* allele 2 but positive for *eae* and the SP_O26-E CRISPR PCR marker (12, 35). Furthermore, EHEC strains can be converted to EHEC-like strains, and vice versa, by transduction with Shiga toxin phages (27). Using these definitions, 172 strains could be grouped here as EHEC and EHEC-like strains. The variability of the virulence-associated genes *espV*, *iha*, *terE*, *lpfA*(O26), and *ehxA* present among the strains illustrates the diversity of this O26 pathogroup. One strain with an EHEC-like profile (MT18) in particular contained 13 virulence- and CRISPR-associated markers. Interestingly, only four of these markers [*eae*-beta, *terE*, *irp2*, and *lpfA*(O26)] were shared among all strains displaying the EHEC profile.

The presence of the CRISPR-associated marker SP_O26-E in a large proportion of the strains (91%) was unexpected. Indeed, this marker was recently designed to target new highly pathogenic O26:H11 strains carrying an stx_2 gene (including strains carrying an stx_{2d} gene) and CRISPR2a alleles 67 and 71 (35). Of these strains, 51.8% (84/162) displayed CRISPR2a allele 67. The similar CRISPR profiles and virulence genes suggest that these Shiga toxin-negative strains could have the potential to become highly

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pathogenic to humans through the acquisition of a Shiga toxin phage. The loss of Shiga toxin genes in *E. coli* has been observed in samples from HUS patients that initially excreted *stx*$^+$ *eae*$^+$ strains and subsequently produced *stx*-negative *eae*$^+$ strains (26, 27). In accordance with these observations, one strain screened in this study, belonging to DT10, tested positive for stx_2 in the initial study where it was isolated (32) and subsequently tested negative for a Shiga toxin in this study; however, it cannot be determined when this strain may have lost this Shiga toxin. Therefore, it is possible that the Shiga toxin-negative strains in this study contained a Shiga toxin at some point that was lost upon initial or subsequent microbial culturing from bovine feces (27). These strains could also constitute a reservoir for a new highly pathogenic O26:H11 clone.

To further investigate the Shiga toxin-negative strains and their relatedness to human-pathogenic strains, multilocus sequence typing was employed on a few strains with various genotypic characteristics (35). Two strains belonging to the most dominant genotypic profile (DT12), containing SP_O26-E, as well as a strain with an EHEC-like profile (DT4; SP_O26-E negative) and a strain with an *espK*- and *arcA* allele 2-negative EHEC profile (DT1; SP_O26-E positive) were identified as ST29 strains belonging to ST clonal complex 29 (CC29), while a strain with an *espK*$^+$ *arcA* allele 2$^+$ EHEC profile (DT2; SP_O26-E negative) was determined to being an ST21 strain belonging to CC29 (data not shown). Furthermore, one SP_O26-E-negative strain belonging to DT5 (EPEC profile) was determined to belong to ST548 and CC10. ST29, found in the new clone, is associated with severe human illness, while ST21 is associated with less severe disease (15, 28, 35). Some strains belonging to ST29 were previously identified in bovine feces in Scotland and Switzerland (5, 15) although in much smaller proportions and with the majority of these strains being *stx* positive (5). It is acknowledged that only two DT12 strains were tested; however, given the same molecular and CRISPR profiles of all strains in this DT, these results suggest that the Shiga toxin-negative strains in our study, which are also SP_O26-E positive, may belong to this clonal complex and have the potential to become highly pathogenic through the acquisition of a Shiga toxin gene, supporting the development of new highly pathogenic O26:H11 clones similar to those observed in Europe (12). Further investigation into the linkage of these Shiga toxin-negative strains to the stx_{2a} clone is warranted. In particular, it would be interesting to conduct a retrospective study on O26 isolates recovered from bovine feces to substantiate this hypothesis. CC10 was previously identified in *E. coli* O26 isolates from cattle and humans in Scotland and in *E. coli* O111 isolates from humans in Germany (2, 5). Given the genetic relatedness of these serogroups, the strain identified in this study could potentially cluster closely with O111:H8 given that this CC10 strain was also the only strain negative for the long polar fimbria gene *lpfA*(O26) (52, 53). Further investigation employing whole-genome sequencing will allow this phylogeny to be determined.

To our knowledge, the distribution of molecularly defined *E. coli* O26 pathotypes within U.S. bovine feces has not been previously described. *E. coli* O26 is a diverse serogroup, as demonstrated in this work, with 37 diversity types. Given that we cannot know the human disease outcome of these bovine fecal strains, classification into distinct pathogroups is not possible. However, the array of diversity and pathogenic characteristics observed for the Shiga toxin-negative strains supports the objective of this study, through their potential ability to become pathogenic due to their high degree of similarity with Shiga toxin-positive strains. These data suggest that high-throughput PCR of select genetic markers in combination with the use of CRISPR loci as a typing method can provide a high level of discrimination of *E. coli* O26:H11 strains isolated from bovine feces (Simpson's index of diversity = 0.84), thus building upon the development of CRISPR typing as an efficient and definitive method for establishing cases and sources of foodborne outbreaks associated with products of bovine origin.

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