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Shiga Toxin-Producing *Escherichia coli* Strains from Bovines: Association of Adhesion with Carriage of *eae* and Other Genes

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Out of 174 bovine Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from diarrheic calves in Germany and Belgium, 122 strains (70.1%) were selected because of their reactivity with the *eae* (*E. coli* attaching and effacing gene) probe ECW1-ECW2. One hundred seven of these *eae*-positive strains (87.7%) harbored *stx1* genes, 13 strains (10.7%) had *stx2* genes, and 2 strains (1.6%) had both *stx* genes. The strains displayed 17 different O types, the majority (97 strains [79.5%]) belonging to O5 (5 strains), O26 (21 strains), O111 (13 strains) O118 (36 strains), O145 (9 strains), and O157 (13 strains). In the HEp-2 cell adhesion assay, 99 strains (81.1%) showed a localized adhesion, and 80 strains (65.6%) stimulated actin accumulation, as determined in the fluorescence actin staining test. None of the strains harbored genes coding for bundle-forming pili (*bfpA*), clearly differentiating them from enteropathogenic *E. coli*. *espB* gene sequences were only detectable in 23 (18.9%) of the *eae*-positive bovine STEC strains. Three different PCRs were established, differentiating between *eae* sequences of enteropathogenic *E. coli* strain E2348/69 (O127:H6) and STEC strain EDL933 (O157:H7). Primers matching in the more heterologous downstream *eae* sequences gave amplicons in only 8 of the 17 O types (O84:H−, O103:H2, O111:H−, O111:H2, O119:H25, O128:H−, O145:H28, and O157:H−). Only 15 STEC strains, belonging to serotypes O111:H−, O111:H2, O145:H28, and O157:H−, gave amplicons in all three *eae*-specific PCRs. These data demonstrate that bovine STEC strains are a heterogeneous group of pathogenic bacteria, a lot of which share virulence markers with STEC strains causing infections in humans. However, in contrast to human STEC strains, bovine *eae*-positive STEC strains are mainly restricted to the *stx1* genotype. The observation that *espB* sequences are not highly conserved might have consequences for the serological recognition of the ESPB protein in patients. Like in human STEC strains, *eae*-related sequences are closely associated with certain *E. coli* O groups; however, they are not serotype specific.

A major virulence factor of enteropathogenic *Escherichia coli* (EPEC) and Shiga toxin (STX)-producing *E. coli* (STEC [5]) in the pathogenesis of intestinal infections in humans and animals is their ability to cause the attaching and effacing (AE) lesion in enterocytes (6, 9, 11, 17, 28, 31). STEC strains produce an additional virulence factor, STX (5, 21, 23, 27, 33, 34).

Recently the 35-kbp chromosomal region locus of enterocyte effacement (LEE), which harbors the *sep*, *eae*, and *espB* genes, was described for EPEC and STEC. The LEE is a prerequisite for the AE lesion (26). The *E. coli* AE gene (*eae*) encodes a 94-kDa outer membrane protein, intimin, which is necessary but not sufficient for the production of the AE lesion (19). In EPEC strain E2348/69, the product of the *espB* gene, previously called the *eaeB* gene, is necessary for signal transduction in vitro (12). The LEE-encoded *sep* genes and a regulatory locus, *perA*, encoded on the large EPEC plasmid, were also shown to be necessary for full exploitation of the AE lesion (9, 12, 16, 18, 32). The recognition of LEE-encoded gene products by reconvalescent-phase patients' sera confirmes their role in pathogenicity (18).

The AE lesion caused by STEC is morphologically indistinguishable from that caused by EPEC. Although large STEC plasmids lack the *perA* locus, some authors have reported that adhesion of STEC strains to epithelial cells is mediated by large virulence-associated plasmids (13, 20, 35).

In calves, AE-positive *E. coli* and STEC of O groups O5, O26, and O111 cause diarrhea (6, 10, 28, 31). Epidemiological studies have outlined the importance of STX1-producing strains in neonatal calf diarrhea (30, 36). Besides their role in calf diarrhea, bovine STEC strains of O groups O26, O111, and O157 are considered to be the major source of infections in humans (21, 23). However, knowledge about the STEC virulence factors involved in the pathogenesis of calf diarrhea is still incomplete. The adhesive properties of bovine STEC especially need to be characterized in more detail. To improve our knowledge about the virulence properties of these strains, adhesion was compared with virulence genes in 122 *eae* gene-harboring bovine STEC strains isolated from diarrheic calves.

MATERIALS AND METHODS

Bacterial strains. One hundred four of the STEC strains were isolated from a total of 1,730 fecal samples from diarrheic calves (less than 16 weeks old) in Germany during 1989 and 1996. Each strain represents one animal. Stool specimens were cultured on Gassner, Sorbitol-MacConkey, BPLS (Merck, Darmstadt, Germany), and sheep blood agar (blood agar base supplemented with 10% [vol/vol] defibrinated sheep blood; Merck). Putative *E. coli* colonies (6 to 35 colonies per sample) were randomly selected, subcultured on nutrient agar slants, and biochemically confirmed to be *E. coli*. Seventy strains were isolated in Belgium, as published previously (30). These *stx*-positive strains had been iden-

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TABLE 1. Sequence and localization of primer pairs used for the detection of *eae*, *espB*, and *bfpA* genes

Primer[a]	Primer sequence (5′→3′)	Gene detected	Location within gene (nt)	PCR conditions (°C/s)		
				Denaturation	Annealing	Elongation
ECW1 (S)	TGC GGC ACA ACA GGC GGC GA	*eae*[b]	495–514[b]	94/60	62/90	72/90
ECW2 (AS)	CGG TCG CCG CAC CAG GAT TC	*eae*[b]	1004–1123[b]			
ECE1 (S)	CAA GCT GAT ATA TGA GCA GTA	*eae*[b]	1038–1058[b]	94/45	60/90	72/60
ECE2 (AS)	GCG TAC TCG ACT TCA ACG TT	*eae*[b]	1866–1885[b]			
ECE1 (S)	CAA GCT GAT ATA TGA GCA GTA	*eae*[c]	1038–1058[c]	94/45	60/90	72/60
ECE7 (AS)	CGA CAG AAC GGT AAT AGT AA	*eae*[c]	1631–1650[c]			
ECE18 (S)	CGG TGC GAC AGC CGC TCT GA	*espB*[d]	285–304[d]	94/60	62/90	72/90
ECE19 (AS)	CCG GAA ATC CTT CCG GCA GC	*espB*[d]	673–693[d]			
ECB1 (S)	GAT TGA ATC TGC AAT GGT GC	*bfpA*[e]	48–67[e]	94/60	57/60	72/80
ECB2 (AS)	GGA TTA CTG TCC TCA CAT AT	*bfpA*[e]	625–644[e]			

[a] S, sense; AS, antisense.
[b] EMBL/GenBank accession no. Z11541.
[c] EMBL/GenBank accession no. M58154.
[d] EMBL/GenBank accession no. Z21555.
[e] EMBL/GenBank accession no. Z12295.

tified from 3,753 strains isolated from 1967 to 1990 from the feces of diarrheic calves in Belgium. Reference strains elaborated for the HEp-2 cell and the fluorescence actin staining (FAS) test as well as for PCR were *E. coli* strains EDL933 (O157:H7) (38), CL-8 (O157:H7) (2), H19 (O26:H11) (33), E32511/0 (O157:H−) (33), and E2348/69 (O127:H6) (19).

Cell culture assays. To determine STX production, all bacterial strains were tested on Vero cells (ATCC CRL 1587) as described previously (36). Adhesion to HEp-2 cells (ATCC CCL23) was tested essentially as described previously (7). Briefly, 10 μl of overnight bacterial cultures in peptone water (containing 1% D-mannose) was inoculated into coverslip-containing 24-well plates, which had been seeded with 5×10^5 HEp-2 cells 48 h before. Cultures were incubated at 37°C for 3 h, the cells were washed, fresh RPMI 1640 was added, and then the cells were incubated for another 3 h. The cells were fixed with 3% formalin, and cultures were stained with Giemsa solution. The adhesion was monitored by light microscopy covering the whole slide. Bacteria were recorded as adhesive if a cluster of at least 10 bacteria adhered per HEp-2 cell.

Accumulation of actin filaments was detected by the FAS test (24). The bacteria were incubated on HEp-2 cells for a total of 6 h. The cells were then washed three times, fixed with 3% formalin, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min. After three further washes, coverslips were incubated with fluorescein isothiocyanate-phalloidin (5 μg/ml; Sigma Chemicals, Deisenhofen, Germany). The coverslips were examined by fluorescence microscopy for actin accumulation.

Each strain was tested at least three times in each assay. The reaction intensity was determined as the number of infected cells, scored as follows: +, ≤1% (of cells infected); ++, >1 to ≤10%; +++, >10 to ≤50%; and ++++, >50 to ≤100%.

PCR. Primers used for PCR and generation of DNA probes are shown in Table 1. Synthetic oligonucleotide primer pairs ECW1-ECW2, ECE1-ECE7, and ECE1-ECE2 were chosen to amplify *eae* gene-related fragments. Primer pair ECE18-ECE19 was specific for *espB* gene sequences. To detect *bfpA*-related genes (8), primer pair ECB1-ECB2 was used. PCR mixtures contained 5.0 μl of template DNA (50 μl of overnight bouillon plus 150 μl of A.dest. at 100°C for 10 min), 2.5 μl of 10× PCR buffer (TFL Puffer; Biozym), 1 μl (1 μM) of both primers, 200 μM deoxynucleoside triphosphates, and 2.5 U of DNA polymerase (TFL; Biozym). Amplification was performed on a thermal cycler (Eppendorf) in 30 cycles, utilizing temperature profiles as described in Table 1. Each amplification reaction was preceded by an initial denaturation step (94°C for 5 min) and followed by a final elongation step (72°C for 5 min). PCR results were analyzed by separation of 8 μl of each PCR product by agarose gel electrophoresis.

Probe preparation and Southern blot hybridization. DNA probes ECW1-ECW2, ECE18-ECE19, and ECB1-ECB2 were labeled during PCR amplification with digoxigenin-11-dUTP. Southern blotting and hybridization were performed according to the recommendations of the vendor (Boehringer, Mannheim, Germany).

DNA sequence analysis. The identity of the PCR-amplified probes was proven by automated DNA sequence analysis with the prism ready reaction dye-deoxy terminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. PCR products were purified for sequence analysis with the Qiaquick-spin PCR purification kit (Qiagen, Hilden, Germany). For each sequencing reaction, 100 ng of purified DNA was used.

Statistics. The frequencies analyzed were compared by using the Pearson product-moment coefficient.

RESULTS

General properties of the strains. A total of 174 bovine STEC strains were isolated from diarrheic calves. Polymyxin B lysates of all STEC strains were cytotoxic for Vero cells in the Vero cell cytotoxicity assay. A 629-bp *eae* gene sequence of strain 570/89 (O111:H−; *stx1*) was digoxigenin labeled during the PCR, yielding DNA probe ECW1-ECW2. DNA sequence analysis revealed this probe to be 100% identical to the respective nucleotide sequences of *eae* from strains EDL933 and E2348/89 (data not shown). By colony blot hybridization with probe ECW1-ECW2, 122 strains (70.1%) were identified as carrying *eae* genes. Of these 122 STEC strains, 107 strains harbored *stx1* genes (87.7%), 13 (10.7%) yielded *stx2* genes, and 2 (1.6%) harbored both *stx* genes. The STEC strains belonged to a total of 17 different O groups (Table 2). However, the majority of the strains (97 [79.5%]) were members of only six O types, in particular groups O5 (5 strains), O26 (21 strains), O111 (13 strains), O118 (36 strains), O145 (9 strains), and O157 (13 strains).

Adhesive properties and ability to induce actin accumulation in HEp-2 cells. The results reflecting adhesive properties and the ability to induce actin accumulation in HEp-2 cells are shown in Table 2. None of the strains adhered in a diffusive or aggregative manner. Ninety-nine (81.1%) of the 122 *eae*-positive strains showed a localized adhesion (LA) to HEp-2 cells, defined as a clustered adhesion of at least 10 *E. coli* cells to one HEp-2 cell. Even fewer strains (80 [65.6%]) stimulated actin accumulation (FAS positive). Seventy-two STEC strains (59.0%) were both FAS and LA positive. Hence, 34.4% of the *eae*-positive bovine STEC strains were not able to induce actin accumulation in HEp-2 cells. A total of 26 strains belonged to O groups O111 and O157; 7 of these strains were also FAS negative.

The LA-negative and FAS-negative phenotype did not correlate well. Only 15 strains (12.3%) were both LA and FAS negative. Eight strains (6.6%) that did not show LAs were positive in the FAS test, and 27 FAS-negative strains (22.1%) showed an LA. The intensities of the FAS reactivity and of the LA correlated significantly ($P < 0.001$ [data not shown]).

Analysis of *eae* and *espB* genes. To further analyze *eae*-specific sequences, the bovine STEC strains were tested in three different PCRs. Primers were selected which specifically

TABLE 2. Serotypes, adhesive properties, FAS reactivity, and genotypes of 122 bovine STEC strains[a] found *eae* positive by hybridization with *eae* probe ECW1-ECW2

Serotype	No. of strains	Gene	Type of adhesion	FAS reactivity	*eae* PCR with primer pair:			Hybridization with *espB* probe ECE18-ECE19
					ECW1-ECW2	ECE1-ECE7	ECE1-ECE2	
O4:H−	1	*stx2*	LA	Negative	+	−	−	−
O5:H−	5	*stx1*	LA	Positive	+	−	−	−
O15:H11	1	*stx1*	LA	Positive	+	−	−	−
O17,77:H18	1	*stx1*	LA	Positive	+	−	−	−
O26:H−	4	*stx1*	LA	Positive	+	−	−	−
O26:H+	5	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Negative	+	−	−	−
	1	*stx1*	Negative	Positive	+	−	−	−
O26:H?	1	*stx1*	LA	Positive	+	−	−	−
O26:H11	5	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Negative	+	−	−	−
	1	*stx1*	Negative	Negative	+	−	−	−
	2	*stx2*	LA	Positive	+	−	−	−
O53:H2	1	*stx1*	Negative	Negative	+	−	−	−
O80:H−	1	*stx1*	LA	Negative	+	−	−	−
O84:H−	1	*stx1*	LA	Negative	+	+	−	+
O84:H?	1	*stx1*	Negative	Negative	+	−	−	+
O92:H−	1	*stx1*	Negative	Positive	+	−	−	−
O103:H2	1	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Positive	+	−	+	−
	1	*stx1*	Negative	Positive	+	−	+	−
	1	*stx1*	Negative	Negative	+	−	+	−
O111:H−	3	*stx1*	LA	Positive	+	+	+	+
	4	*stx1*	LA	Negative	+	+	+	+
	1	*stx1*	LA	Negative	+	−	+	+
	2	*stx1*	Negative	Positive	+	+	+	+
	2	*stx2*	LA	Positive	+	−	+	+
O111:H2	1	*stx1*	LA	Positive	+	+	+	+
O118:H−	1	*stx1*	LA	Negative	+	−	−	−
	1	*stx1*	Negative	Negative	+	−	−	−
	1	*stx1*	LA	Positive	+	−	−	−
	1	*stx2*	LA	Negative	+	−	−	−
O118:H16	11	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Positive	+	−	−	+
	4	*stx1*	LA	Negative	+	−	−	−
	3	*stx1*	Negative	Positive	+	−	−	−
	8	*stx1*	Negative	Negative	+	−	−	−
	3	*stx2*	LA	Negative	+	−	−	−
	1	*stx1/2*	LA	Positive	+	−	−	−
	1	*stx1/2*	LA	Negative	+	−	−	−
O119:H25	1	*stx1*	Negative	Negative	+	+	−	+
O128:H−	1	*stx1*	LA	Negative	+	−	+	−
O145:H+	6	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Negative	+	−	−	−
O145:H28	2	*stx2*	LA	Positive	+	+	+	−
O153:H+	1	*stx1*	LA	Positive	+	−	−	−
O157:H−	3	*stx1*	LA	Positive	+	+	+	+
	1	*stx1*	LA	Positive	+	−	+	+
	2	*stx1*	LA	Positive	+	−	+	−
	4	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Negative	+	−	+	−
O157:H+	1	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	Negative	Negative	+	−	−	−
Nontypeable	7	*stx1*	LA	Positive	+	−	−	−
	1	*stx1*	LA	Positive	+	−	+	−
	2	*stx2*	LA	Negative	+	−	−	+

[a] All strains were negative for *bfpA* sequences.

amplify homologous and heterologous *eae* sequences of EPEC strain E2348/69 (O127:H6) and STEC strain EDL933 (O157:H7), respectively. All 122 strains yielded positive results with primer pair ECW1-ECW2, confirming the hybridization results with probe ECW1-ECW2. In contrast, only 17 strains gave amplicons with primer pair ECE1-ECE7. Twenty-seven strains reacted positive in the ECE1-ECE2 PCR. A total of 15 strains, exclusively belonging to serotypes O111:H− (9 strains), O111:H2 (1 strain), O145:H28 (2 strains), and O157:H− (3 strains), gave amplicons in all three *eae* PCRs (Table 2).

Only 23 of the 122 bovine *eae*-positive strains (18.9%) reacted positively in the *espB*-specific PCR. To confirm these

results, all 122 strains were additionally tested by colony blot hybridization with DNA probe ECE18-ECE19 generated from bovine STEC strain 570/89 (O111:H−). DNA sequencing showed that probe ECE18-ECE19 was 100% identical to the *espB* of EPEC strain E2348/69. Even under low-stringency conditions, only the 23 *espB* PCR-positive strains gave hybridization signals. All STEC strains of O group O111 (13 strains), but only 4 of 13 strains of O group O157, were positive for *espB* (Table 2). Only 13 (56.5%) of the *espB*-positive strains were FAS positive.

Of these 23 *espB*-positive strains, 13 strains additionally gave positive results in all three *eae* PCRs. Those 13 strains were either of serotype O111:H− (9 strains), serotype O111:H2 (1 strain), or serotype O157:H− (3 strains).

None of the 122 strains reacted positively in the *bfpA*-specific PCR with elaboration by the E2348/69-specific primer pair ECB1-ECB2.

DISCUSSION

The large number (70.1%) of *eae*-positive STEC found in diarrheic calves underlines the importance of this attribute for the pathogenesis of calf diarrhea and further strengthens the function of cattle as a reservoir for human infections. In human disease, *eae*-positive strains are particularly virulent (1, 25). Furthermore, our findings remarkably extend the list of *eae*-positive STEC strains known to date (14, 25, 37) by adding serotypes O4:H−, O15:H11, O53:H2, O80:H−, O84:H−, O92:H−, O118:H−, O118:H1, O119:H25, O128:H−, O145:H28, and O153:H+.

The results of this study, revealing that 122 of 174 (70.1%) STEC strains isolated from diarrheic calves harbor *eae* genes, confirm that the *eae* gene is a virulence marker for bovine STEC strains. All STEC infections in calves studied so far were shown to cause AE lesions (6, 10, 17, 28, 31). However, our finding that only 65.6% of these *eae*-positive strains caused a positive FAS reaction in HEp2 cells raises the question of whether screening for the *eae* gene is an adequate method for the identification of AE-positive STEC strains.

The use of different primers to amplify *eae*-related sequences revealed that the *eae* alleles are highly heterogeneous. This finding extends the results of others (14, 25). However, in contrast to these previous results, our data do not confirm a serotype specificity of the *eae* alleles. These contrasting results may be explained by the larger number of strains elaborated in this study. Furthermore, the number of STEC clones in the United States could be more limited than it is in Europe, since the strains included in this study were isolated exclusively in Belgium and Germany. Our results revealed a significant association between the intensity of the FAS reactivity and the intensity with which the strains adhered in a localized manner (LA). Since we were not able to detect *bfpA*-related genes which confer LA to EPEC (8, 15), these data underline the important role of the *eae* gene product intimin for adhesion.

Furthermore, our data suggest that the LEE of STEC strains of serovars O84:H−, O111:H−, O111:H2, O119:H25, and O157:H− may evolutionarily be more closely related to the LEE of EPEC strain E2348/69 O127:H6 than to the LEE of the other STEC serovars examined. This can be concluded from the corresponding results of the *espB* and *eae* PCR and the DNA hybridization experiments, respectively.

In human EPEC strains, the *eae* gene is not the only gene involved in the AE lesion. The transfer of the *eae* gene into laboratory AE lesion-negative *E. coli* strains confers only a so-called "shadow type" (9). At least in EPEC, other gene loci, like the *espB* locus and the *sep* loci, are needed to reconstitute a full phenotype (12, 18, 22, 32). Only recently, a gene cluster, the LEE, has been identified which is a prerequisite for the ability of EPEC and STEC O157:H7 to cause the AE lesion (26). Besides the *eae* gene, the function of the gene product of another gene that is located in the LEE, *espB* (22), previously called *eaeB* (12), has been well characterized in EPEC. In vitro, the *espB* gene was shown to be necessary for signal transduction of EPEC strain E2348/69 (12). Consequently, we tested the STEC strains for the presence of the *espB* gene. Of the 122 *eae*-positive bovine STEC strains, only 23 (18.8%) harbored the *espB* gene, and even 10 of these strains reacted negatively in the FAS test. We screened for *espB* by utilizing a gene probe that is 100% identical to the central region of the published *espB* gene of EPEC strain E2348/69 under low-stringency conditions. The missing association between possession of *espB* and a positive FAS reactivity suggests that bovine STEC strains harbor genes that are structurally different from the EPEC *espB* gene but functionally identical. A similar finding was reported for other type III secretion proteins of members of the family *Enterobacteriaceae* (39). To our knowledge, this is the first report of *espB* genes in bovine STEC. Whether the data about the less-conserved regions in the *espB* also hold for human STEC strains has to be determined. If so, this should have a profound impact on patients' antibody responses. Sera of hemolytic-uremic syndrome patients were shown to strongly recognize the *espB* gene product (18). If *espB* gene products vary in protein structures, infection with different STEC serotypes should lead to the recognition of different ESPB epitopes.

The remarkably large number of strains with O type O118 (29.5%) reveals these strains as a potential new emerging pathogen of diarrhea in calves. Some recent epidemiological studies from Europe did not recognize any bovine strains with O group O118 (3, 4). STEC strains of this serotype have so far only been isolated sporadically from human patients (25, 37). Further studies should focus on the role of O118 strains in calves and in humans. To date, it is uncertain whether these strains are specific pathogens for bovines. However, since cattle are the major reservoir for STEC infections in humans, O118 strains could cause future infections in humans.

Interestingly, the vast majority of the strains (107 of 122 [87.7%]) harbored *stx1* genes only. A similar finding was recently reported by Barrett et al. (1). All bovine STEC strains elaborated in this study were isolated from diarrheic calves. Thus, it can be assumed that STX1-positive strains are more virulent for calves than strains harboring other *stx* genes. The virulence factors detected in STX1-positive strains confirm recent epidemiological results, which revealed an association between isolation of STX1-positive strains and diarrhea in calves (36). This also explains the significantly higher percentage of cattle showing STX1-neutralizing antibodies (29).

In conclusion, a high percentage of STEC strains from diarrheic calves in Germany and Belgium, most of which produce STX1 only, harbor *eae* sequences. The *eae* genes are associated with certain serotypes, but are not serotype specific. In calves, systemic diseases attributable to an effect of STXs, such as hemolytic-uremic syndrome in humans or edema disease in piglets, are not known. Thus, a more important virulence factor for pathogenicity of bovine STEC strains seems to be their AE ability. AE-positive STEC strains are particularly virulent in humans. The high percentage of bovine STEC strains with the AE ability further strengthens the zoonotic importance of these bacteria.

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