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Virulence Properties of Shiga Toxin-Producing *Escherichia coli* (STEC) Strains of Serogroup O118, a Major Group of STEC Pathogens in Calves

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Shiga toxin-producing *Escherichia coli* (STEC) strains of serogroup O118 are the most prevalent group among STEC strains in diarrheic calves in Germany (L. H. Wieler, Ph.D. thesis, University of Giessen, 1997). To define their virulence properties, 42 O118 (O118:H16 [*n* = 38] and O118:H− [*n* = 4]) strains were characterized. The strains displayed three different Stx combinations (Stx1 [36 of 42], Stx1 and Stx2 [2 of 42], and Stx2 [4 of 42]). A total of 41 strains (97.6%) harbored a large virulence-associated plasmid containing hly_{EHEC} (*hly* from enterohemorrhagic *E. coli*). The strains' adhesive properties varied in relation to the eukaryotic cells tested. Only 28 of 42 strains (66.7%) showed localized adhesion (LA) in the human HEp-2 cell line. In contrast, in bovine fetal calf lung (FCL) cells, the number of LA-positive strains was much higher (37 of 42 [88.1%]). The locus of enterocyte effacement (LEE) was detected in 41 strains (97.6%). However, not all LEE-positive strains reacted positively in the fluorescence actin-staining (FAS) test, which indicated the attaching and effacing (AE) lesion. In HEp-2 cells, only 22 strains (52.4%) were FAS positive, while in FCL cells, the number of FAS-positive strains was significantly higher (38 of 42 [90.5%; $P < 0.001$]). In conclusion, the vast majority of the O118 STEC strains from calves (41 of 42 [97.6%]) have a high virulence potential (*stx*, hly_{EHEC}, and LEE). This virulence potential and the high prevalence of STEC O118 strains in calves suggest that these strains could be a major health threat for humans in the future. In addition, the poor association between results of the geno- and phenotypical tests to screen for the AE ability of STEC strains calls the diagnostic value of the FAS test into question.

Cattle are the main reservoir for Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains causing the hemolytic-uremic syndrome (HUS) in humans (2, 3, 14, 23, 31). In Germany, 94.0% of all HUS-associated STEC strains (O157:H7, O157:H−, O26:H11, O26:H−, O111:H−, O2:H6, O8:H21, O69:H−, O119:H5, and rough:H11) display three virulence features: production of Stx, the ability to cause the attaching and effacing (AE) lesion, and production of enterohemorrhagic *E. coli* hemolysin (Hly_{EHEC}) (4). The major route of STEC transmission is indirect, by contaminated food (4, 14, 31), but infections are also linked to direct transmission from animals to humans by contact (6, 24).

STEC strains cause diarrhea in calves, and this ability is mostly associated with strains that produce Stx1 only (STEC1) (12, 20, 26, 27, 30). Diarrhea is associated with the strains' ability to cause AE lesions in epithelial cells (9, 21, 30). This ability is conferred by the locus of enterocyte effacement (LEE) (17, 18). STEC1 strains from calves harbor the LEE significantly more often than do STEC2 strains ($P < 0.001$) (26, 30). Therefore, STEC1 strains are considered more virulent for calves than STEC2 strains.

E. coli strains of serogroup O118 are the most prevalent STEC strains in calves in Belgium (20) and Germany (26, 30). To further define the virulence properties of these strains, we characterized 42 O118 STEC strains from calves. Our data revealed that O118 STEC strains have a high virulence potential. To our knowledge, only one case of HUS has so far been reported to be associated with an O118 STEC strain (5). However, the sorbitol-fermenting O118 STEC strain in human infections is not detected when only sorbitol-MacConkey agar is used for diagnosis. Given the high virulence potential of these strains (Stx^+, LEE^+, and $Hly_{EHEC}{}^+$), we speculate that O118 will be an emerging health threat to humans in the future.

(The results of this study were presented in part at the 97th General Meeting of the American Society for Microbiology, Miami, Fla., 4 to 7 May 1997.)

MATERIALS AND METHODS

Bacterial strains. A total of 42 O118 STEC strains were isolated from 37 calves (32 diarrheic and 5 nondiarrheic) that were less than 16 weeks old from 30 farms in Germany during 1989 and 1996. Stool specimens were cultured on sorbitol-MacConkey, Gassner, and sheep blood agar (blood agar base supplemented with 10% defibrinated sheep blood [Merck]) (29). Hly_{EHEC} was detected on blood agar plates containing 10% washed sheep erythrocytes (29). Putative *E. coli* colonies (6 to 35 colonies/sample) were randomly selected, subcultured on nutrient agar slants, and biochemically confirmed to be *E. coli*. O serotyping of *E. coli* was performed according to standard methods (19).

Reference strains utilized for the adhesion and the fluorescence actin-staining (FAS) test as well as for DNA hybridization were *E. coli* EDL933 (O157:H7), CL-8 (O157:H7), H19 (O26:H11), E32511/0 (O157:H−), and E2348/69 (O127:H6) (30).

DNA hybridization and PCR. *stx* genes were detected by colony blot hybridization experiments with oligonucleotide probes 772 (stx_1) and 849 (stx_2) as previously described (27). Possession of the LEE was determined with LEE probes A to D as described by McDaniel et al. (17). The plasmid-encoded hly_{EHEC} was detected by PCR with oligonucleotide primers Ehly1 and Ehly5 and by Southern blot hybridization with DNA probe Ehly1-5 (29).

Cell culture assays. To determine Stx production, all bacterial strains were tested on Vero cells (ATCC CRL 1587) as described previously (27). Adhesion

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TABLE 1. Serovars, *stx* genotypes, LEE-associated genes, and adhesive and hemolytic properties of bovine Stx-producing *E. coli* of serogroup O118 ($n = 42$)

Serovar and strain	Clinical status of calf	*stx* gene	hly_{EHEC}	Hly_{EHEC}	Hybridization with LEE probes A to D	Type of adhesion on:		Intensity of FAS reactivity on[a]:	
						HEp-2 cells	FCL cells	HEp-2 cells	FCL cells
O118:H16									
GS1236-1[b]	No diarrhea	stx_1	+	+	+	LA	LA	++++	++
GS1232-1	No diarrhea	stx_1	+	+	+	LA	LA	+++	−
GS1161-1[b]	No diarrhea	stx_1	+	+	+	LA	LA	++	++
GS1180-1	No diarrhea	stx_1	+	+	+	LA	LA	++	++
GS1205-1	No diarrhea	stx_1	+	+	+	LA	LA	++	+
RW2303[c]	Diarrhea	stx_1	+	+	+	LA	LA	++++	+++
RW2126[c]	Diarrhea	stx_1	+	+	+	LA	LA	+++	+++
RW2125[d]	Diarrhea	stx_1	+	+	+	LA	LA	+++	++
RW2173	Diarrhea	stx_1	+	+	+	LA	LA	+++	+
RW0136	Diarrhea	stx_1	+	+	+	LA	LA	++	++++
RW0203	Diarrhea	stx_1	+	+	+	LA	LA	++	+++
GS1181-1	Diarrhea	stx_1	+	+	+	LA	LA	++	++
AS2169	Diarrhea	stx_1	+	+	+	LA	LA	+	+++
340	Diarrhea	stx_1	+	+	+	LA	LA	+	++
RW2199	Diarrhea	stx_1	+	+	+	LA	LA	+	++
666/89	Diarrhea	stx_1	+	+	+	LA	LA	+	+
RW2022	Diarrhea	stx_1	+	+	+	LA	LA	+	+
RW2266	Diarrhea	stx_1	+	+	+	LA	LA	−	+++
RW1306[e]	Diarrhea	stx_1	+	+	+	LA	LA	−	++
RW1912	Diarrhea	stx_1	+	+	+	LA	LA	−	+
RW1362	Diarrhea	stx_1	+	−	+	Negative	LA	−	+
RW2121	Diarrhea	stx_1	−	−	−	Negative	LA	−	−
RW1303[f]	Diarrhea	stx_1	+	+	+	Negative	LA	−	+
RW1366	Diarrhea	stx_1	+	+	+	Negative	LA	++	++
RW1319	Diarrhea	stx_1	+	+	+	Negative	LA	+	++
RW1305	Diarrhea	stx_1	+	+	+	Negative	LA	−	+
RW1817	Diarrhea	stx_1	+	+	+	Negative	LA	−	+
RW1307[e]	Diarrhea	stx_1	+[g]	+	+	Negative	LA	−	+
RW1318	Diarrhea	stx_1	+	+	+	Negative	Negative	++	+
RW1299	Diarrhea	stx_1	+	+	+	Negative	Negative	−	+
RW1304[f]	Diarrhea	stx_1	+	+	+	Negative	Negative	−	+
RW2017	Diarrhea	stx_1	+	+	+	Negative	Negative	−	−
RW1365	Diarrhea	stx_1	+	−	+	Negative	Negative	−	++
RW2116	Diarrhea	stx_2	+	+	+	LA	LA	−	+++
RW2039	Diarrhea	stx_2	+	+	+	LA	LA	−	+
RW2136	Diarrhea	stx_2	+	+	+	LA	LA	−	+
RW2297[d]	Diarrhea	$stx_{1/2}$	+	+	+	LA	LA	++	+++
RW1911	Diarrhea	$stx_{1/2}$	+	+	+	LA	LA	−	++
O118:H−									
557/89	Diarrhea	stx_1	+	+	+	LA	LA	+	+
RW2311	Diarrhea	stx_1	+	+	+	LA	LA	−	−
RW1302	Diarrhea	stx_1	+	+	+	Negative	LA	−	+++
RW2030	Diarrhea	stx_2	+	+	+	LA	LA	−	++

[a] Numbers of infected cells with actin accumulation. −, FAS negative; +, ≤1% of infected cells positive; ++, >1 to ≤10%; +++, >10 to ≤50%; and ++++, >50 to ≤100%.
[b] Strains GS1236-1 and GS1161-1 were isolated from the same animal.
[c] Strains RW2303 and RW2126 were isolated from the same animal.
[d] Strains RW2125 and RW2297 were isolated from the same animal.
[e] Strains RW1306 and RW1307 were isolated from the same animal.
[f] Strains RW1303 and RW1304 were isolated from the same animal.
[g] Strain RW1307 reacted negatively with PCR probe Ehly1-5 but positively with DNA probe Ehly1-Ehly5.

to HEp-2 cells (ATCC CCL23) or fetal calf lung (FCL) cells was tested essentially as described previously (8, 30). FCL cells were prepared by trypsinization of lungs from 4- to 5-month-old bovine fetuses. Bacteria were recorded as adhesive when a cluster of at least 10 bacteria adhered per HEp-2 or FCL cell (30).

Accumulation of actin filaments was detected by the FAS test with a 6-h incubation (15, 30). Each strain was tested at least three times in each assay. The reaction intensity was rated by determination of the number of infected cells (as percentages). Any cell that showed at least one actin accumulation associated with bacteria was considered FAS positive (+, ++, +++, and ++++, ≤1%, >1 to ≤10%, >10 to ≤50%, and >50 to ≤100%, respectively).

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

RESULTS

Serovars. The 42 STEC strains of serogroup O118 displayed two different serovars. Most strains (38 of 42) were of serovar O118:H16; 4 strains were typed O118:H− (Table 1).

Toxins. The vast majority (40 of 42) of the strains harbored only a single *stx* gene (Table 1). A total of 36 strains were positive for stx_1, 4 strains were stx_2 positive, and only 2 strains harbored both toxin genes. Stx was always detected in the Vero cell assay. In addition, 41 strains harbored hly_{EHEC}, but the Hly_{EHEC} phenotype was detected in only 39 of these 41 strains.

Adhesive properties. The strains' adhesive properties varied in relation to the eukaryotic cells tested. Only 28 strains (66.7%) showed localized adhesion (LA) in the human HEp-2 cell line. In contrast, in bovine FCL cells, the percentage of LA-positive strains was much higher (37 strains [88.1%]; Table 1).

With the exception of one strain (RW2121 [O118:H16, Stx1]), the strains reacted positively with all four LEE probes (LEE probes A to D). In contrast to these genotypical results, the number of strains that actually caused a positive FAS reaction was considerably lower. Only 22 (53.7%) of the 41 LEE-positive strains were FAS positive in HEp-2 cells. However, the number of FAS-positive strains increased remarkably ($P < 0.001$) when bovine FCL cells were used (38 strains [92.7%]; Table 1).

Most of the strains induced the AE lesion on a small percentage of the cells. An AE lesion on more than 50% of the cells was induced by only two strains on HEp-2 cells and by just one strain on FCL cells. This higher number of AE-positive bacterial strains in FCL cells was due mostly to an increase in strains that infected less than 10% of the cells. Thus, there was no difference in adhesion intensity between the two cell types ($P > 0.05$).

DISCUSSION

The finding that O118 strains are the most prevalent STEC strains in calves (26) and that 97.6% of the bovine strains of serogroup O118 possess a combination of all three STEC virulence factors (Stx, LEE, and hly_{EHEC}) which are associated with 94% of all HUS cases in Germany (4) has at least two implications for the epidemiology of STEC infections. Bovine STEC strains of serogroup O118 have to be considered (i) highly virulent for both humans and calves and (ii) a possible emerging health threat to humans in the future. Thus, the surveillance of food from bovine origin for STEC contamination in Germany should not only focus on the currently most relevant serovars (O157:H7, O157:H−, O26:H11, O26:H−, O111:H−, O2:H6, O8:H21, O69:H−, O119:H5, and rough:H11) but should include strains of serogroup O118.

Since AE-positive *E. coli* strains have been shown to cause diarrhea in calves (7, 9, 21, 30), it is tempting to speculate that the reason for the epidemiological association between isolation of O118 STEC strains and diarrhea (26, 30) is the strains' ability to cause the AE lesion. Consistently, we recommend that the bacteriological diagnosis of neonatal calf diarrhea due to *E. coli* should focus not only on ETEC strains (13) but on Stx- and AE-positive *E. coli* strains additionally.

Although in calves a high percentage of STEC strains harbor the LEE-encoded *eae* (23, 30), only a small percentage of STEC isolates from adult cattle show this genotype (22, 23). The reason for this phenomenon is currently unknown. Presumably, the physiological differences in the intestinal environments between suckling calves and ruminating cattle play an important role. Before rumination, AE-positive STEC strains should have a colonization advantage in the intestinal tract. After rumination, AE ability may no longer be an advantage, since in the rumen a mucous membrane-specific adhesion is no longer necessary. Thus, the percentage of *eae*-positive STEC strains can decrease with age, although the total number of STEC strains shed may not change substantially. This assumption is supported by the fact that the number of *eae*-positive STEC strains peaks at an age of 3 to 4 weeks, while *eae*-negative STEC strains are increasingly shed at an age of 8 to 10 weeks (28).

Humans develop HC and HUS after infections with STEC-contaminated food mostly of bovine origin (14). The most important STEC serotype, O157:H7 (1, 14), is associated with large food-borne outbreaks. However, direct transmission from animals to humans is another mode of infection with STEC strains (6, 24). Outbreaks can occur when groups of children visit farms and are encouraged to touch the animals. In this respect, at least in Germany, the high virulence potential of O118 STEC strains could be a threat to children in the future but has not heretofore been recognized. To date, we are only aware of one case of O118-associated HUS (5). Future case control studies on the direct transmission mode must include all STEC strains and should not be limited to O157:H7 only.

The findings that only 53.7% of the LEE-positive bovine O118 STEC strains were AE positive when tested in the human HEp-2 cell line but that 92.7% were positive when bovine FCL cells were used indicate that the in vitro AE ability of bovine STEC strains is highly dependent on the particular cell used for testing. The weak correlation between possession of the LEE and AE phenotype in vitro is probably due to differences in gene expression. So far, the regulation of LEE-associated genes in STEC strains is only poorly understood (10). In our FAS test comparing human HEp-2 and bovine FCL cells, incubation temperature, time interval, and medium utilized were identical. Our results on the quantity of adhesion give no indication for a species-specific adhesion, since the intensities with which the bovine STEC strains adhered to the human and the bovine cells tested did not vary significantly ($P > 0.005$). Furthermore, it is not known to date whether bovine STEC strains harbor specific adhesins, i.e., fimbriae, that confer the first step in adhesion. The previously published differences in the C-terminal region of the adhesin intimin also do not hint at a species-specific adhesion mechanism (11, 16, 25, 30). In addition, the worldwide epidemiological finding that the vast majority of STEC serovars detected in bovines are also found in STEC-associated human diseases (31) argues against a species-specific adhesion mechanism.

The most important practical implication of the weak association between possession of the LEE and the in vitro AE ability of STEC strains is the idea that the FAS test with HEp-2 cells is not a suitable test to screen for the AE ability of these strains. An antibody-based assay (enzyme-linked immunosorbent assay and slide agglutination) of, e.g., intimin expression would be a more sensitive diagnostic procedure. Another practical aspect is the detection of Hly_{EHEC}. Two hly_{EHEC}-positive strains did not show the hemolytic phenotype. This already previously observed weak phenotype (30) may be due to a low level of secretion of the hemolysin and warrants a highly critical screening in the diagnostic lab.

To this end, we are currently testing the clonal relationship of these O118 STEC strains to better understand whether they have emerged recently or whether they are endemic in calves.

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