Differences in Levels of Secreted Locus of Enterocyte Effacement Proteins between Human Disease-Associated and Bovine *Escherichia coli* O157

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Ongoing extensive epidemiological studies of verotoxin-carrying *Escherichia coli* O157 (stx^+ eae^+) have shown this bacterial pathogen to be common in cattle herds in the United States and the United Kingdom. However, the incidence of disease in humans due to this pathogen is still very low. This study set out to investigate if there is a difference between strains isolated from human disease cases and those isolated from asymptomatic cattle which would account for the low disease incidence of such a ubiquitous organism. The work presented here has compared human disease strains from both sporadic and outbreak cases with a cross-section, as defined by pulsed-field gel electrophoresis, of *E. coli* O157 strains from cattle. Human ($n = 22$) and bovine ($n = 31$) strains were genotyped for carriage of the genes for Shiga-like toxin types 1, 2, and 2c; *E. coli* secreted protein genes *espA*, *espB*, and *espP*; the enterohemolysin gene; *eae* (intimin); *ast* (enteroaggregative *E. coli* stable toxin [EAST]); and genes for common *E. coli* adhesins. Strains were also phenotyped for hemolysin, EspP, Tir, and EspD expression as well as production of actin and cytoskeletal rearrangement associated with attaching and effacing (A/E) lesions on HeLa cells. The genotyping confirmed that there was little difference between the two groups, including carriage of stx_2 and stx_{2c}, which was similar in both sets. *ast* alleles were confirmed to all contain mutations that would prevent EAST expression. *espP* mutations were found only in cattle strains (5 of 30). Clear differences were observed in the expression of locus of enterocyte effacement (LEE)-encoded factors between strains and in different media. EspD, as an indicator of LEE4 (*espA, -B,* and *-D*) expression, and Tir levels in supernatants were measured. Virtually all strains from both sources could produce EspD in Luria-Bertani broth, although at very different levels. Standard trichloroacetic acid precipitation of secreted proteins from tissue culture medium produced detectable levels of EspD from the majority of strains of human origin (15 of 20) compared with only a few (4 of 20) bovine strains ($P < 0.001$), which is indicative of much higher levels of protein secretion from the human strains. Addition of bovine serum albumin carrier protein before precipitation and enhanced detection techniques confirmed that EspD could be detected after growth in tissue culture medium for all strains, but levels from strains of human origin were on average 90-fold higher than those from strains of bovine origin. In general, levels of secretion also correlated with ability to form A/E lesions on HeLa cells, with only the high-level protein secretors in tissue culture medium exhibiting a localized adherence phenotype. This research shows significant differences between human- and bovine-derived *E. coli* O157 (stx^+ eae^+) strains and their production of certain LEE-encoded virulence factors. These data support the recent finding of Kim et al. (J. Kim, J. Nietfeldt, and A. K. Benson, Proc. Natl. Acad. Sci. USA 96:13288–13293, 1999) proposing different *E. coli* O157 lineages in cattle and humans and extend the differential to the regulation of virulence factors. Potentially only a subset of *E. coli* O157 isolates (stx^+ eae^+) in cattle may be capable of causing severe disease in humans.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a class of pathogenic *E. coli* strains that are associated with hemorrhagic colitis and hemolytic uremic syndrome in humans (34). There are several different serotypes of EHEC, with *E. coli* O157 being the major pathogen in humans, particularly in the United States and the United Kingdom. Recent epidemiological studies have shown a high prevalence of this organism in cattle and their environment (18, 40), but the level of human infection remains relatively low (2 to 6 of 100,000) (21, 36).

One possible explanation for this is a difference in the human-pathogenic potential between strains of bovine origin. A recent study has proposed different lineages for bovine and human strains based on octamer genome scanning (27), but this did not include analysis of virulence factors. EHEC strains isolated from human disease cases have been characterized for virulence determinants (3, 9, 45), as have bovine Shiga toxin-positive *E. coli* O157 strains (8, 14, 20, 41). The few studies that have compared human and bovine strains for carriage of virulence determinants have not found notable differences (7, 13, 33).

EHEC stains are similar to enteropathogenic *E. coli* (EPEC) strains in that they produce characteristic attaching and effacing (A/E) lesions in experimental infections and in vitro assays,

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the formation of which is dependent on the locus of enterocyte effacement (LEE) pathogenicity island (32). Genes found on the LEE include the genes encoding the adhesion molecule intimin (*eae*) and its receptor (*tir*), a type III secretion system, and the LEE effector molecules (*espA*, *espB*, and *espD*) (35). It is thought that after initial attachment to the host cell, type III secretion of Tir through an EspA filament and EspB-EspD pore (translocon) facilitates intimate attachment of the bacterium via intimin and Tir (23). Continued production and secretion of LEE-encoded proteins (including Tir, EspB, and EspD) leads to host cell actin rearrangement and the formation of A/E lesions (25, 30).

Other virulence factor genes found on the EHEC chromosome include those encoding the Shiga toxins Stx1 and Stx2 and variants (stx_1, stx_2, and stx_{2c}). For example, hemolytic uremic syndrome is associated with stx_2 and stx_{2c} carriage (8). *stx* genes are located on lysogenic phages. Their transmissibility has led to EHEC strains containing different combinations of Stx variants. Also found on the chromosome of EHEC strains are two copies of the *astA* gene (39), potentially encoding a small heat-stable enterotoxin known as EAST. Recent research has indicated that both copies of *astA* are unlikely to be expressed (43). EHEC strains also contain a 97-kb plasmid, pO157, that carries a number of genes, the products of which may be associated with virulence (12). These include an enterohemolysin gene (*ehx*), an immunomodulator gene (*lif*) (28), and *espP*, encoding a member of the SPATE (for serine protease autotransporters of *Enterobacteriaceae*) family (1). Consequently, the pathogenesis of EHEC strains is likely to be complex and multifactorial (37).

In this study human disease and bovine fecal *E. coli* O157 strains were screened for a range of virulence determinants, including toxins and adhesins. Secreted protein profiles of strains were analyzed, including specific detection of Tir, EspD, and EspP. Host cell actin rearrangement by fluorescent actin staining (FAS) was also investigated. The results showed that while genotypic heterogeneity was mainly confined to carriage of *stx* variants and *espP* alleles, there was considerable strain- and medium-dependent variation in EspD and Tir secretion levels. Esp secretion levels correlated with the extent of A/E lesion formation, including the localized adherence phenotype. Strains of human origin produced significantly higher levels of EspD in tissue culture medium than the majority of cattle strains. Therefore, the human-pathogenic potential of bovine *E. coli* O157 strains may be linked to not only Shiga-like toxin expression but also to variation in the regulation and expression of other virulence-associated factors.

MATERIALS AND METHODS

Bacterial strains. Thirty EHEC strains of serotype O157 and 1 of serotype O26 from human disease cases as well as 30 strains of serotype O157 isolated from asymptomatic cattle were obtained from the Scottish *E. coli* Reference Laboratory. ZAP001, -004, -008, -010, -026, -058 to -089, and -104 to -112 are human disease O157 strains. ZAP021 (bovine diarrhea) and ZAP078 (human disease) are O26 strains. ZAP028 to -057 are bovine *E. coli* O157 strains. The bovine strains used in this study represent all common variations in pulsed-field gel electrophoresis (PFGE) and phage type profiles found among *E. coli* O157 strains present in Scotland between 21 June 1999 and 16 November 1999. The PFGE methods used were described by Allison et al. (2). Their PFGE macrorestriction profiles differ by one to eight fragments; 24 of 30 bovine isolates differed by two or more PFGE fragments (two to eight fragment differences), and

16 of 30 differed by three or more fragments (data not shown). The human disease-associated strains were isolated from both major and sporadic outbreaks in Scotland over a 6-year period at the reference center. EPEC strain E2348/69 was supplied by Brendan Kenny, University of Bristol. All strains were stored on Protech beads at −70°C in Luria-Bertani (LB) broth with 20% glycerol. For all experiments the strains were initially plated out on Columbia agar with 10% horse blood (Oxoid, Basingstoke, United Kingdom).

PCR detection of genes encoding secreted proteins. All primer sequences used are shown in Table 1. For bacterial lysates, a colony was suspended in 100 μl of sterile phosphate-buffered saline (PBS) and boiled for 10 min. Lysates were stored at −20°C. All PCRs were carried out in 50-μl reaction volumes containing

TABLE 1. Oligonucleotide primers used in this study

Primer name	Primer sequence	Product size (bp)	Reference or source[a]
EASTa	5′-GAAGTTCTGGCTCAATGCGC-3′	172	This study
EASTb	5′GAAGTTCTGGCTCAATGCGC-3′		
LIFa	5′-GAGGCGCTGAAGATCAAACA-3′	330	This study
LIFb	5′-TGTATTATCAGGAGGTGCGC-3′		
ESPPa	5′-AAACAGCAGGCACTTGAACG-3′	301	This study
ESPPb	5′-AGACAGTTCCAGCGACAACC-3′		
EspP5′	5′GGTTGATTCCTGCGGGACG-3′		
EhxA	5′ACGATGTGGTTTATTCTGGA-3′	165	20
EhxB	5′CTTCACGTGACCATACATAT-3′		
StxA	5′GAAGGAAATAATTTATATGT-3′	Various	4
StxB	5′TTTGATTGTTACAGTCAT-3′		
EacAa	5′ACAAACTTTGGGGATGTTC-3′	550	22
EacAb	5′ACAAACTTTGGGATGTTC-3′		
EspAa	5′CACGTCTTGAGGAAGTTTGG-3′	299	This study
EspAb	5′CCGTTGTTAATGTGAGTGCG-3′		
EspBa	5′CGATGGTTAATTCCGCTTCG-3′	304	This study
EspBb	5′GCCTGCTGAATCTGATAGCT-3′		
FimA	5′GCCGGATTATGGGAAAGA-3′	600	31
FimB	5′GCCGCTGTAGAACTGAGGG-3′		
F4a	5′GGTGATTTCAATGGTTCGGTC-3′	764	22
F4b	5′AATGCTACGTTCAGCGGAGCG-3′		
F5a	5′TATTATCTTAGGTGGTATGG-3′	314	22
F5b	5′GGTATCCTTTAGCAGCAGTATTTC-3′		
F17A	5′GCAGAAAATTCAATTTATCCTTGG-3′	537	6
F17B	5′CTGATAAGCGATGGTGTAATTAAC-3′		
F41A	5′GCATCAGCGGCAGTATCT-3′	380	22
F41B	5′GTCCCTAGCTCAGTATTATGACCT-3′		
F107A	5′GTGAAAAGACTAGTGTTTATTTC-3′	510	22
F107B	5′CTTGTAAGTAACCGCGTAAGC-3′		
SfamA	5′CTCCGGAGAACTGGGTGCATCTTAC-3′	420	IMBID
SfamB	5′CGGAGGAGTAATTACAAACCTGGCA-3′		
pap1	5′GACGGCTGTACTGCAGGGTGTGGCG-3′	430	IMBID
pap2	5′ATATCCTTTCTGCAGGGATGCAATA-3′		

[a] IMBID, Institute for the Molecular Biology of Infectious Disease, Wurzburg, Germany.

2 μl of bacterial lysate, 200 μmol of deoxynucleotide mix (dATP, dTTP, dCTP, and dGTP), 100 pmol of each primer, 5 μl of 10× PCR buffer with 1.5 mM MgCl$_2$, and 2.5 U of *Taq* DNA polymerase (Roche Diagnostics). *stx* PCR and restriction fragment length polymorphism (RFLP) were carried out as previously described using the Lin-all system (4), as was the *ehx* PCR (20). The *eae* PCR was performed as described previously (22). The primers for detection of *espA* and *espB* (Table 1) were designed from the sequence of the *E. coli* O157 EDL933 LEE (35). Cycling conditions were denaturation at 94°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 90 s for 30 cycles, with a final extension at 72°C for 10 min. Primers for detection of the *astA* gene were designed from the *astA* gene sequenced from enteroaggregative *E. coli* (42). EASTa and EASTb were designed to amplify a 172-bp region encompassing the entire coding sequence plus adjacent sequence. Cycling conditions were denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s for 30 cycles, with a final extension at 72°C for 10 min. Primers for detection of the *lif* gene and the *espP* gene were designed from the entire pO157 sequence (12). LIFa and LIFb were designed to amplify a 330-bp region of the N-terminal conserved region of the projected Lif protein. Cycling conditions were denaturation at 94°C for 45 s, annealing at 51°C for 45 s, and extension at 72°C for 90 s for 30 cycles, with a final extension at 72°C for 10 min. ESPPa and ESPPb were designed to amplify a 301-bp N-terminal region of the *espP* gene. Cycling conditions were denaturation at 94°C for 45 s, annealing at 59°C for 45 s, and extension at 72°C for 90 s for 30 cycles, with a final extension at 72°C for 10 min.

PCR detection of genes encoding *E. coli* adhesins. A range of *E. coli* adhesins were screened for by PCR (Table 1). The adhesins were as follows: type 1 pili, which are found in approximately 90% of all *E. coli* strains; K88 (F4) antigen, which is found in *E. coli* serotypes associated with disease in pigs; K99 (F5) antigen, which is found in calf enterotoxigenic *E. coli* strains; the F17 fimbrial family, including F17a, F17b, F17c, and F111 fimbriae produced by bovine *E. coli* strains; the F41 fimbrial family, which are also found in calf enterotoxigenic *E. coli* strains; F107 adhesins, which have been reported as porcine colonization factors; and S and P family adhesins. These were detected using primers designed at the Institute for the Molecular Biology of Infectious Disease, Wurzburg, Germany (Table 1).

Plasmid extraction and Southern blotting. The large virulence plasmid pO157 was isolated with a Midi-prep spin kit (Qiagen) using the manufacturer's protocol for low-copy-number plasmids.

Plasmids were digested with *Bam*HI (New England Biolabs), and the digested DNA was separated in a 0.7% agarose gel. The DNA was transferred to a nylon membrane and UV cross-linked. Hybridization was performed using the digoxigenin-11-deoxyuridine triphosphate (DIG) DNA labeling and detection kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, an *espP* gene probe was prepared by incorporating DIG during the *espP* PCR described above. The PCR product was purified with the QIAquick PCR purification kit (Qiagen), and the probe was hybridized to a membrane at 68°C. The hybridized probe was detected using alkaline phosphatase-labeled anti-DIG antibody according to the manufacturer's instructions.

Sequence analysis. For sequencing, the PCR products were purified using a Qiagen PCR purification kit and cloned into pCR4-TOPO using the TOPO-TA cloning kit (Invitrogen BV, Groningen, The Netherlands) according to the manufacturer's instructions. Sequence analysis and sequence alignments were carried out using the Vector NTi and AlignX programs (Informax, Oxford, United Kingdom). Sequence analysis of the PCR products was performed in each case using two clones.

Protein precipitation. Bacteria were grown in LB broth or minimum essential medium (MEM) with 25 mM HEPES (Sigma-Aldrich, Dorset, United Kingdom) to an optical density at 600 nm of 0.9. Suspensions were centrifuged at 4,000 × *g* for 10 min. The supernatants were filtered, and the proteins were precipitated by the addition of 10% (vol/vol) trichloroacetic acid (TCA) with overnight incubation at 4°C. Precipitates underwent subsequent centrifugation at 4,000 × *g* for 30 min at 4°C. The protein pellet was dried and suspended in 1.5 M Tris (pH 8.8), and after addition of sample buffer, the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. In order to compare protein levels in supernatants and to rule out the precipitation procedure as a source of variation, bovine serum albumin (BSA) (1 μg/ml) was added in certain cases to supernatants prior to precipitation.

Detection of protein expression. Protein samples were separated by SDS-PAGE and blotted onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Bucks, United Kingdom) using a mini Trans-blot electrophoretic transfer cell (Bio-Rad, Herts, United Kingdom). After blocking overnight, membranes were incubated for 3 h with monoclonal antibody (anti-EspD, -P, and -Tir) at a dilution of 1:75 (14, 15). Membranes were washed three times and then incubated for 2 h with horseradish peroxidase-conjugated goat anti-

mouse immunoglobulin secondary antibody (Dako, Cambridge, United Kingdom) at a dilution of 1:750. Bound antibody was visualized after 5 min of incubation with a 1:1 mixture of Pierce (Rockford, Ill.) Luminol enhancer solution and Pierce Stable Peroxide solution. Relative levels of EspD were determined by quantifying luminescence using a Flowgen MultiImage cabinet and MultiImage Spot densitometer software (Flowgen, Staffs, United Kingdom).

A/E lesion formation assay. Bacterial adhesion and fluorescence staining of actin were performed as previously described (29). Briefly, subconfluent cultures of HeLa cells on glass coverslips were placed in the wells of a multiwell tissue culture plate with 1 ml of MEM and 10% fetal calf serum. Bacteria were grown in LB broth overnight at 37°C, and then a 1:50 dilution was made of the culture in MEM, which was incubated at 37°C with shaking to an optical density at 600 nm of 0.5. MEM was removed from tissue culture cells, and 1 ml of a 1:500 dilution of the bacterial culture was added to the cells. The mixture was centrifuged at 2,000 × *g* for 5 min and incubated at 37°C in 5% CO$_2$ for 5 h. Fresh medium was added after 3 h. After incubation, cells were washed three times in PBS to remove nonadherent bacteria, fixed in 4% formalin for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. After three washes in PBS, coverslips were treated with a 5-μg/ml solution of fluorescein isothiocyanate-phalloidin (Sigma-Aldrich) in PBS to specifically stain actin filaments. Bacteria were counterstained using anti-O157/O26 primary antibody (MAST Diagnostics, Merseyside, United Kingdom) and a tetramethyl rhodamine isocyanate-labeled anti-rabbit immunoglobulin secondary antibody (Dako). Coverslips were washed three times in PBS and examined by fluorescence and phase-contrast microscopy using a Leica DMLB fluorescence microscope. HeLa cells with no bacteria added were fixed, permeabilized, and stained as a negative control for each assay.

RESULTS

Adhesin profiling. A broad range of common adhesins associated with pathogenic *E. coli* were screened for by PCR in the two sets of strains. All strains tested negative for all of the adhesin genes assayed (F4, F5, F17, F41, F107, and P and S fimbriae), with the exception of the *fim* switch region (type 1 fimbriae). All *E. coli* O157 strains contained the *fim* switch, but with a 16-bp deletion in the switch region (38). A primer bridging this deletion has been previously identified as very effective in defining the presence of *E. coli* O157 by PCR (31).

LEE gene carriage. The presence of three of the genes found on the LEE pathogenicity island, *eae, espA,* and *espB,* was assayed by PCR. Fifty of 53 strains tested positive for all three, indicating that these strains carry the LEE pathogenicity island. All strains tested were positive for *eae*, with only three strains being negative for both *espA* and *espB*. All negative tests were repeated for confirmation.

***stx* genotype.** The *stx* genotypes of 51 EHEC O157 strains were investigated by PCR and RFLP (4). Results were based on duplicate reactions. Seventeen out of 21 human EHEC O157 strains carried a stx_2 or stx_{2c} gene (or both). Of the 30 bovine EHEC O157 strains, 29 carried a stx_2 or stx_{2c} gene (or both). Six of 21 human strains contained stx_1, a total similar to that for the bovine strains (8 of 30) (Table 2). This is in contrast to a similar study in Germany of *E. coli* O157 strains, which showed a correlation between bovine strains and carriage of stx_1 and *espP* (11). When carriage of various combinations of the stx_1, stx_2, and stx_{2c} genes was examined, there appeared to be no clear differences between bovine and human strains (Table 2).

Analysis of *espP, ehx, lif,* and *astA*. All human EHEC strains (21 serotype O157 strains and 1 serotype O26 strain) carried *espP*, *ehx*, *lif*, and *astA*. Examples of the agarose gels obtained for these PCRs are shown in Fig. 1. Certain genes were not detected in some strains: five of the bovine strains tested negative for *espP*, three tested negative for *ehx*, and one tested

TABLE 2. *stx* gene carriage in *E. coli* O157 strains

Source	% of strains positive by PCR (no. positive/total)							
	stx_1	stx_2	stx_{2c}	$stx_1\ stx_2$	$stx_1\ stx_{2c}$	$stx_2\ stx_{2c}$	$stx_1\ stx_2\ stx_{2c}$	No *stx* genes
Bovine	3.3 (1/30)[b]	3.3 (1/30)	0	10.0 (3/30)	0	60.0 (18/30)	13.3 (4/30)	10.0 (3/30)
Human	10.0 (4/21)	33.3 (7/21)	9.5 (2/21)	0	0	28.6 (6/21)	9.5 (2/21)	0

negative for *astA*. All negative PCRs were confirmed. All 31 bovine strains (30 serotype O157 strains and 1 serotype O26 strain) carried *lif*. Of the 53 strains assayed, only the 3 strains negative by PCR for *ehx* did not exhibit hemolysis on Columbia agar plates containing 10% horse blood.

Using primers EASTa and EASTb (Table 1), *astA* was amplified and sequenced from two human EHEC O157 strains (ZAP001 and ZAP026), three bovine EHEC O157 strains (ZAP029, ZAP041, and ZAP049) and one bovine O26 strain
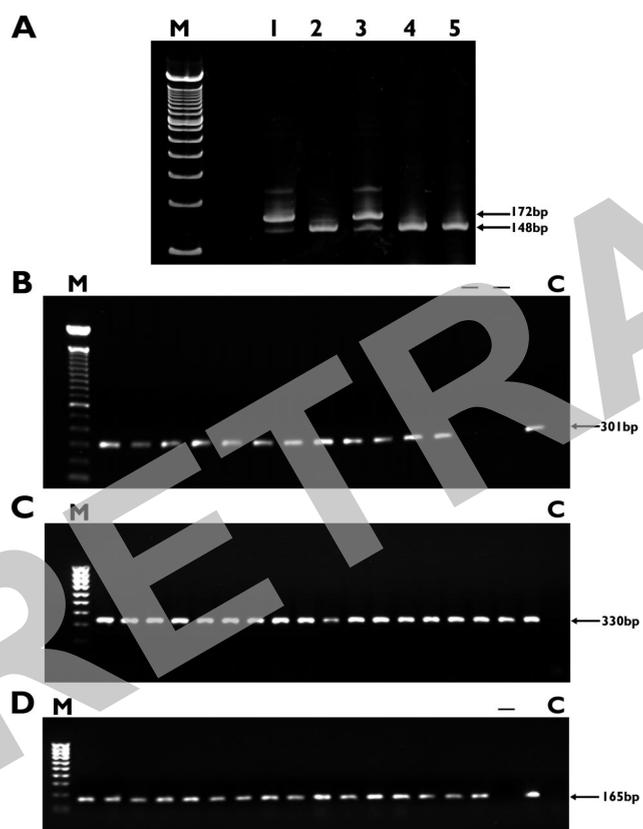

FIG. 1. PCR amplification and detection of *astA* (A), *espP* (B), *lif* (C), and *ehx* (D). Samples were run on 1% agarose gels as described in Materials and Methods. Lanes M, 100-bp ladder; lanes C, negative controls. Numbers to the right of the gels indicate the sizes of the relevant amplicons. (A) *astA* PCR. Lanes 1 and 3, amplified products from ZAP021 and ZAP049 showing both alleles of the gene (148 and 172 bp); lanes 2, 4, and 5, reactions which have amplified the allele containing just the point mutation (see Results). (B) *espP* PCR, showing a selection of human and bovine EHEC strains, all of which are positive except for two bovine strains (lanes −) which are negative for carriage of the gene. (C) *lif* PCR. All human and bovine strains were positive. (D) *ehx* PCR, showing a selection of human and bovine strains, all of which are positive for *ehx* except for one bovine strain (lane −).

(ZAPO21). These data confirmed that only two mutant alleles (172 and 148 bp) of the gene could be detected (Fig. 1A) (43). Both alleles are present in the same EHEC strains (e.g., ZAP021 and ZAP049), presumably representing the two copies detected on the *E. coli* O157 chromosome previously by Southern blotting (39). Our data also confirmed the presence of the 24-bp deletion allele (43) in the prototypic EPEC strain E2348/69 (data not shown).

Forty-eight of 53 EHEC strains were positive for *espP* by PCR, with the remaining 5 strains testing positive by Southern blotting. These five strains were also negative for EspP by Western blotting when probed with a monoclonal antibody raised against the EspP produced by *E. coli* O26, termed PssA (14, 15). The *Bam*HI digest of pO157 produces a range of fragments, including a 8.9-kb fragment known to contain the *espP* gene (9). In the strains that tested negative for *espP* by PCR, the *espP*-containing restriction digest product was approximately 1 kb smaller as determined by Southern blotting (data not shown). Primers EspP5′ and EspPb were used to amplify a 2-kb DNA fragment, sequencing of which confirmed a deletion covering positions 138 to 1359 of the *espP* gene. This *espP* allele is identical to one identified in German human EHEC strains (10). In our study this deletion was detected only among bovine *E. coli* O157 (*stx*⁺) strains.

LEE protein secretion. Proteins present in the supernatants of 30 EHEC strains, 15 human and 15 bovine, were TCA precipitated and visualized by SDS-PAGE on a 12.5% polyacrylamide gel by silver staining. There was considerable variation in the levels of protein present, both between strains and when the same strains were cultured in different media, i.e., LB broth and MEM (data not shown). Detection of specific factors (EspP, EspD, and Tir) was carried out by Western blotting on 24 of these strains (11 human *E. coli* O157, 1 human *E. coli* O26, 11 bovine *E. coli* O157, and 1 bovine *E. coli* O26). Importantly, both human and cattle strains secreted similar amounts of EspP when cultured in LB broth (Fig. 2). This demonstrated that the preparation and loading of the secreted protein samples allowed for valid comparison of protein levels in the LB supernatants between strains (Fig. 2). In LB broth virtually all the strains analyzed demonstrated secretion of EspD by Western blotting, although at very different levels. Tir could also be detected from the majority of strains in LB broth, although again at very different levels, with overexposure of blots being required to detect its expression in lanes apparently negative in Fig. 2 (data not shown).

The secretion pattern changed dramatically when the bacteria were cultured in MEM, which was previously shown to produce high-level secretion (5, 17). TCA precipitation produced markedly different levels of secreted protein in strains grown to the same optical density in MEM. From these preparations, EspD could be detected in only 12 of the 22 *E. coli*
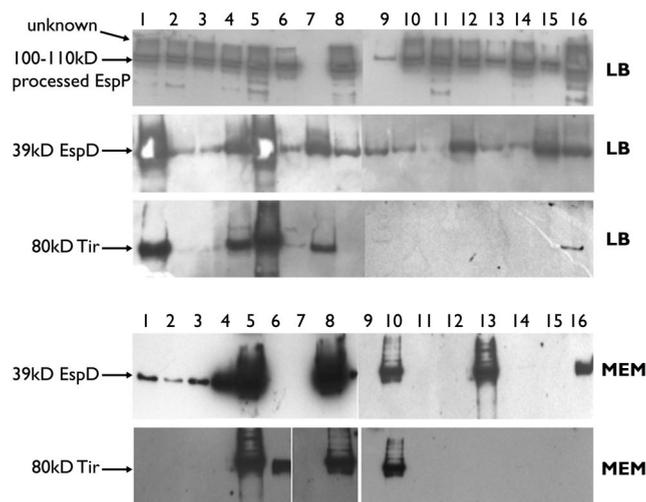
FIG. 2. Western blot detection of EspP, EspD, and Tir in supernatants of 16 *E. coli* O157 strains cultured in LB broth and MEM. Lanes 1 to 4 (ZAP001, -004, -008, and -010, respectively) show human disease O157 outbreak strains. Lanes 8 (ZAP058), 11 (ZAP065), and 16 (ZAP088) show human O157 strains associated with sporadic disease cases. Lanes 6 (ZAP032), 7 (ZAP038), 9 (ZAP041), and 12 to 15 (ZAP052, -054, -034, and -028, respectively) show bovine *E. coli* O157 strains. Lane 5 contains a bovine O26 strain, and lane 10 contains a human O26 strain associated with a sporadic disease case. The expected sizes of the products detected are shown. A range of sizes is given for EspP, as these are most likely degradation products of the fully processed protein (16). The unknown band could be preprocessed EspP or the product of a cross-reaction with a related protein (16). The medium in which the bacteria were cultured before precipitation of supernatants (LB broth or MEM) is indicated to the right of the blots.

O157 strains, 9 of which were associated with human disease (9 of 11) and 3 of which were bovine *E. coli* O157 strains (3 of 11). To confirm this difference, a further 18 strains (9 bovine and 9 human) were analyzed for EspD precipitation after growth in MEM. In total, 15 of 20 human strains were positive, in comparison to only 4 of 20 bovine strains ($P < 0.001$). The higher level of protein secreted into MEM by the human strains facilitates their precipitation by TCA. Strains initially designated EspD negative are likely to be secreting proteins but at levels not high enough to promote efficient precipitation with 10% TCA. To confirm this, carrier BSA (1 µg/ml) was added to all supernatants before precipitation. EspD could now be detected in all supernatants using sensitive detection techniques (Supersignal; Pierce) but at markedly different levels for human and bovine strains. The addition of BSA as an internal standard allowed quantitative comparison between the two sets of strains (Fig. 3). The mean level of EspD produced by 10 human strains was 90-fold higher than the mean produced by 10 bovine strains analyzed.

As it was in LB broth, Tir secretion was heterogeneous among strains, with only two *E. coli* O157 strains giving clearly detectable levels (lanes 6 and 8 in Fig. 2 [ZAP032 and ZAPO58, respectively]), and these strains differed from the high Tir producers in LB medium, which were biased towards human disease-associated strains. Both *E. coli* O26 strains produced high levels of both EspD and Tir in MEM (lanes 5 and 10 in Fig. 2) (17). Production of EspP was lost in all strains upon growth in MEM, as has been reported previously (17).
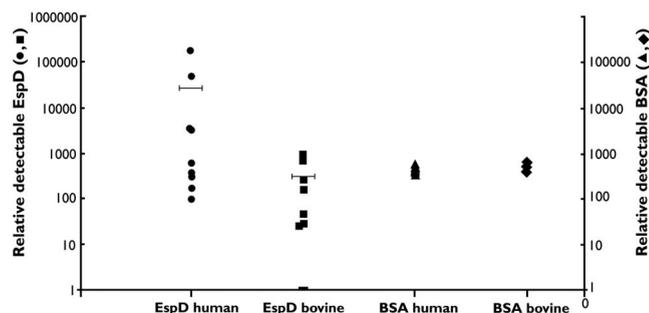

FIG. 3. Relative levels of EspD secreted in MEM from 10 human strains (EspD human) and 10 bovine strains (EspD bovine). Horizontal bars represent the mean value for each data set. Relative EspD levels were determined as described in Materials and Methods. BSA (1 µg/ml) was added to all supernatants before precipitation. The relative levels of BSA precipitated from each sample are represented by the BSA human values and BSA bovine values. The levels of BSA were detected after Coomassie blue staining.

These results clearly demonstrate a marked heterogeneity in secretion of LEE effector proteins between different *E. coli* O157 strains and in different media. Moreover, strains of human disease origin generally produce considerably higher levels of secreted proteins than bovine strains (EspD in MEM and Tir in LB medium).

A/E lesion formation. A subset of 16 strains (8 bovine and 8 human) from the 24 examined initially for EspD and Tir secretion were also analyzed for their ability to induce actin and cytoskeletal rearrangements in HeLa cell monolayers. Actin rearrangement was determined by FAS, and colocalization of bacteria was carried out via anti-LPS (O157 or O26 as appropriate) primary and tetramethyl rhodamine isocyanate secondary antibody binding (29). The strains were compared by determining the number of HeLa cells displaying actin aggregation at sites of bacterial attachment 5 h postinoculation (Table 3; Fig. 4). All 16 strains assayed were able to stimulate some degree of actin aggregation in comparison to the negative control. However, the strains varied considerably in the number of foci and the level to which they stimulated these rearrangements, i.e., in localized adherence (LA) versus diffuse adherence (DA) (Table 3; Fig. 4). The strains that formed detectable lesions in the highest percentage of host cells also could display the LA phenotype (Table 3). In turn, this activity correlated well with high-level EspD secretion in MEM.

DISCUSSION

The aim of this study was to establish whether differences in virulence determinant carriage or expression could be detected between *E. coli* O157 strains isolated from cattle and those from outbreaks of human disease. The 53 bovine and human strains analyzed were selected because they encompass the major variations in PFGE profiles observed in Scotland as determined by the Scottish *E. coli* O157 reference laboratory. Obvious differences were detected in the level of secretion of LEE-encoded factors between strains and in different media. Human strains produced significantly higher levels of secreted proteins in MEM, as measured by blotting of EspD. A/E lesion formation and actin rearrangement potential correlated with

TABLE 3. Analysis of EHEC strains for A/E lesion formation

Strain, serotype, source	A/E lesions after 6 h[a]	Adherence phenotype[b]	EspD secretion (MEM)[c]
ZAP001, O157, human	5	LA	++
ZAP004, O157, human	3	DA	++
ZAP008, O157, human	3	DA	++
ZAP010, O157, human	4	LA	+++
ZAP058, O157, human	9	LA	+++
ZAP065, O157, human	2	DA	+/−
ZAP088, O157, human	5	LA	++
ZAP028, O157, bovine	3	DA	+/−
ZAP032, O157, bovine	2	DA	+
ZAP034, O157, bovine	3	DA	+/−
ZAP038, O157, bovine	2	DA	+/−
ZAP041, O157, bovine	2	DA	+/−
ZAP052, O157, bovine	4	LA	++
ZAP054, O157, bovine	1	DA	+/−
ZAP021, O26, bovine	8	LA	+++
ZAP078, O26, human	7	LA	+++

[a] Actin rearrangement around the site of attached bacteria was detected by FAS as described in Materials and Methods and previously (26, 31). Results are representative of monolayers and are shown as the number showing actin rearrangement per 10 HeLa cells when viewed at a magnification of ×1,000.

[b] Only strains exhibiting LA can be considered as forming microcolonies (29).

[c] Ability of strains to secrete EspD in MEM, as detected by Western blotting (Fig. 2 and 3).

this finding. If A/E lesion formation is an important part of the virulence of EHEC O157, then bovine strains may differ in their human-pathogenic potential.

The majority of our strains produced high levels of supernatant proteins when grown in LB broth; however, there was considerable variation between the strains in levels of LEE-secreted protein production in this medium. This was determined by Western blotting of EspD and Tir. SepL and EspA, -B, and -D are transcribed from the same promoter, and therefore EspD expression may reflect that of EspA and EspB. Tir is transcribed with intimin and a protein of unknown function, so Tir secretion may also be indicative of intimin production (5, 24). In LB broth, while there was considerable variation in secretion levels between strains, the strain-specific levels of EspD and Tir were always related, and all strains except ZAP034 and ZAP041 could produce both proteins, although Tir production from the majority of bovine strains examined was only just detectable. Technical considerations were ruled out as an explanation of these differences, as the EspP levels detectable in the secreted protein preparations were very consistent.

The pattern of EspD and Tir secretion changed significantly when the strains were cultured in MEM, which has been shown to lead to maximal LEE protein secretion (5, 17). Standard TCA precipitation and Western blotting detected EspD from 15 of 20 human *E. coli* O157 strains compared to 4 of 20 bovine *E. coli* O157 strains ($P < 0.001$) (Fig. 2). The levels of secreted proteins in the supernatants of negative strains were so low that it could prevent effective precipitation. Only through addition of BSA carrier protein and use of a more sensitive detection technique could EspD be detected in all supernatants. Comparison of EspD levels from the two sets of strains confirmed much higher expression levels (average of 90-fold) from the human strains. This difference could also apply to other proteins expressed from the same operon (EspA and EspB). Also, in MEM, Tir could not usually be detected, with
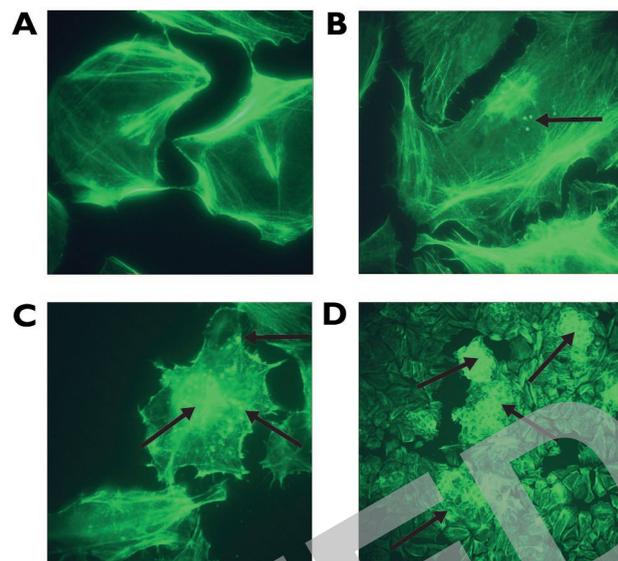

FIG. 4. FAS of HeLa cells following addition of different *E. coli* O157 strains. (A) Negative control, with unchallenged HeLa cells stained with fluorescein isothiocyanate-phalloidin after 5 h of incubation in 5% CO_2. Magnification, ×1,000. (B) HeLa cells challenged with ZAP028 and stained after 5 h of incubation. Magnification, ×1,000. This strain exhibits the DA phenotype (arrows indicate the areas of actin rearrangement correlating with diffusely adhered bacteria). (C) HeLa cells challenged with ZAP058 and stained after 5 h of incubation. Magnification, ×1,000. The image shows bacteria demonstrating the LA phenotype and microcolony formation (arrows indicate the areas of actin rearrangement indicative of localized adherent bacteria and microcolonies). (D) HeLa cells challenged with ZAP021 and stained after 5 h of incubation. Magnification, ×100. The image shows macrocolony formation on cells, indicated by profuse actin rearrangement around the periphery of the cells (arrows show actin rearrangement indicating large colony formation). Images were acquired using a Leica fluorescent DMLB microscope and Leica image capture software.

only 4 of 22 strains (2 *E. coli* O157 and 2 *E. coli* O26 strains) giving detectable levels of Tir, and those strains producing high levels of EspD in MEM did not necessarily produce detectable Tir. Therefore, with two exceptions (one O157 strain and one O26 strain), Tir secretion did not appear to be enhanced by growth in MEM rather than LB medium as reported previously (15); however, shaking as opposed to static cultures were used in our study. These results demonstrate that there are marked differences in production of EHEC LEE proteins between EHEC O157 strains and in different media. In the case of EspD secretion in MEM, there was a positive correlation between this ability and isolation of strains from cases of human disease.

In order to examine whether these protein secretion differences affect the strains' ability to induce cytoskeletal rearrangements, 16 strains were tested for A/E lesion formation with HeLa cells. The strains could be grouped into two categories: those that could form obvious microcolonies with evident actin rearrangement (LA) or those that adhered diffusely (DA) but still induced rearrangement (Fig. 4). The LA phenotype correlated with those strains that also induced A/E lesions in the highest proportion of host cells (Table 3). Furthermore, these strains (with one exception, ZAP032) were also high-level producers of EspD in MEM. Presumably these strains would also

express EspA and -B at higher levels. Therefore, the bovine strains examined, while filling the usual criteria for pathogenic potential (*stx* and *eae* positive), demonstrated marked variation in LEE-encoded factor expression that correlated with the potential to form A/E lesions. While the majority of strains analyzed could express LEE-encoded factors under certain conditions, the regulation of these factors differed between strains. These regulatory differences, highlighted by EspD expression in MEM, may indicate the human-pathogenic potential of a strain. Recent work (19) has shown that a pivotal regulator of LEE-encoded factors is Ler (LEE-encoded regulator), and our ongoing work is assessing whether changes in the activity of this regulator could account for the different expression profiles detected in this study.

In contrast to the results of the phenotypic analyses, there appeared to be very few genotypic differences between these two subsets which could account for pathogenic potential. Most of the human disease strains (except serotype O26) carried a stx_2 or stx_{2c} gene, as has been reported previously (9). The percentages of bovine strains carrying either of these genes was also very high (Table 2), with only one bovine strain being negative for a stx_2 variant. The percentage of strains carrying stx_1 was also similar for bovine and human strains (Table 2), in contrast to the case in other countries, where EHEC strains in cattle tend to carry stx_1 (9).

There was little or no heterogeneity in carriage of the other toxins or virulence-associated determinants. All strains were negative for genes encoding F4, F5, F17, F41, F107, and P and S fimbriae. *fim* (type 1 fimbriae) was detected by PCR in all strains but is not expressed by EHEC O157 (31, 38). Almost all strains tested carried genes encoding enterohemolysin, EAST, EspP, Lif, intimin, EspA, and EspB, consistent with the presence of the LEE and pO157. *astA* (EAST) has been detected in different types of diarrheagenic *E. coli* (44), including on the chromosome of *E. coli* O157 in two copies (39). Recent work has identified two types of mutated *astA* genes in *E. coli* O157, a 24-bp deletion of the first 8 codons of the gene coding sequence and a single base substitution in the ATG start codon, distributed among strains (43). In this study, the presence of one or both mutated alleles was confirmed in a number of strains, including O157, O26, and the prototype EPEC strain E2348/69. Most EHEC strains appear to contain two copies of the gene, as previously documented (39). Therefore EAST will not be produced by EHEC O157 and is not a functional toxin in EHEC pathogenesis.

All of the human strains produced EspP, whereas six of the bovine strains did not. Five of these were also negative by PCR, and while they were confirmed as possessing *espP* sequence on pO157 by Southern blotting, their RFLP patterns showed a deletion in the *espP* region. These five strains contain a 1.4-kb deletion occurring at the 5′ end of the gene that has been previously reported (10). It is of interest that in our study, all of the strains containing a mutated *espP* gene were bovine strains (5 of 30). Therefore, in this study, expression of this factor does appear to correlate with human disease. The role of EspP in EHEC pathogenesis and production of the protein in human disease and during bovine colonization merits further investigation. Indeed, recent work has shown that mutation in a SPATE-encoding gene led to decreased virulence in

animal models by interfering with colonization of the intestine (see Discussion in reference 26).

Recent studies have shown that *E. coli* O157 is prevalent in cattle and their environment (18, 40). However, the incidence of human infection with this organism is still relatively low. This study has identified for the first time clear differences between *E. coli* O157 (stx^+ eae^+) strains in their ability to express virulence-associated factors. In addition, there is a positive correlation between strains of human origin and high-level expression of EspD in tissue culture medium. This expression pattern was observed in only a minority of bovine strains and may be an indicator of human-pathogenic potential.

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RETRACTION

Differences in Levels of Secreted Locus of Enterocyte Effacement Proteins between Human Disease-Associated and Bovine *Escherichia coli* O157

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Volume 69, no. 8, p. 5107–5114, 2001: The first author of this article informed the coauthors that he manipulated the final panel of Fig. 2 to provide an image of sufficient quality for publication. The coauthors were unaware of the manipulation. Although his action does not call into question the validity of the results, we prefer to retract the article. In doing so, however, we note that subsequent research over the last 3 years, including two published papers (A. J. Roe, H. Yull, S. W. Naylor, M. J. Woodward, D. G. E. Smith, and D. L. Gally, Infect. Immun. **71:**5900–5909, 2003, and A. J. Roe, S. W. Naylor, K. J. Spears, H. M. Yull, T. A. Dransfield, M. Oxford, I. J. Kendrick, M. Porter, M. J. Woodward, D. G. Smith, and D. L. Gally, Mol. Microbiol. **54:**337–352, 2004), has validated the original findings of the article, in particular the heterogeneity in EspD and Tir expression demonstrated in Fig. 2.