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# Expression of Putative Virulence Factors of *Escherichia coli* O157:H7 Differs in Bovine and Human Infections

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***Escherichia coli* O157:H7 is a commensal organism in cattle, but it is a pathogen in humans. This differential expression of virulence suggests that specific virulence factors are regulated differently in human and bovine hosts. To test this hypothesis, relative real-time reverse transcription-PCR was used to relate the expression of several putative virulence genes (*eae*, *espA*, *stx*$_2$, *rfbE*, *ehxA*, and *iha*) to that of the "housekeeping" gene *gnd* during natural human and experimental bovine infection with *E. coli* O157:H7. We examined these genes in fecal samples from eight humans and four calves. *iha* and *espA* were significantly more expressed in bovine infections. *rfbE* and *ehxA* appeared to be more highly expressed in human infections, though these differences did not achieve statistical significance. Our results support the hypothesis that some virulence-associated genes of O157:H7 are differentially expressed in a host-specific manner.**

*Escherichia coli* O157:H7, a human pathogen that causes diarrhea, bloody diarrhea, and the hemolytic uremic syndrome (HUS), has acquired multiple virulence factors that might aid its pathogenicity. Principal among these are the phage-encoded Shiga toxins (Stx proteins), which inhibit protein synthesis in eukaryotic cells (41), and the *LEE* pathogenicity island, which encodes products associated with formation of attaching-and-effacing lesions at the host surface (reviewed in reference 13). *E. coli* O157:H7 also has a 92-kb nonconjugative plasmid that encodes several putative virulence factors, including enterohemolysin (encoded by *ehxA*, also termed *hlyA* [1, 5]), a member of the RTX family of exoproteins. *E. coli* O157:H7 also expresses the IrgA homologue adhesin (Iha), which was recently identified as a virulence factor in uropathogenic *E. coli* (16, 38).

Some of these factors can affect animal hosts. For example, *E. coli* O157:H7 causes attaching-and-effacing lesions on cultured bovine cells (31) and in neonatal calves (8). Despite this, *E. coli* O157:H7 is not known to cause natural disease in any host except humans. This discrepancy suggests the hypothesis that specific virulence factors of *E. coli* O157:H7 are regulated differently when the bacteria are in the human rather than the bovine gut. To test this hypothesis, real-time reverse transcription-PCR (RT-PCR) was employed to examine gene expression in fecal samples from children infected with *E. coli* O157:H7 and from experimentally infected calves. The resulting data support the hypothesis that virulence genes are differentially expressed in the human and bovine hosts.

## MATERIALS AND METHODS

**Strains and media.** Bacteria were grown in a shaking incubator at 37°C in Luria-Bertani (LB) medium. When appropriate, ampicillin (100 μg/ml) or nalidixic acid (32 μg/ml) was added. Strain 86-24 is a well-studied clinical isolate of *E. coli* O157:H7 (39). Strain 86-24$^{\text{nalR}}$ (3) was used to infect calves. ORN172(pIHA) contains *iha* on a high copy plasmid (38). Shiga toxin-producing *E. coli* (STEC) O103:H2 expresses enterohemolysin at high levels (34).

**Clinical samples.** Fecal specimens from children infected with *E. coli* O157:H7 were obtained from the Pacific Northwest. The Institutional Review Boards of the Children's Hospital and Regional Medical Center, Seattle, Wash., approved the use of these specimens, which were frozen immediately after collection and stored at −70°C until use.

Six conventional Holstein calves were housed in isolation units and prescreened for health status and freedom from fecal STEC/enterohemorrhagic *E. coli*. Half of the calves were obtained at 1 day of age, screened during week 1, inoculated on day 8, monitored for fecal shedding for 7 days, and euthanized and necropsied at 14 to 16 days of age. The remaining calves were obtained at 6 weeks of age, screened for 1 week, challenged at 7 weeks of age, monitored for fecal shedding for 7 days, and euthanized and necropsied between 54 and 58 days of age. Calves were orally challenged with $10^9$ to $10^{10}$ CFU of *E. coli* O157:H7 strain 86-24$^{\text{nalR}}$ and monitored for 6 to 8 days for appetite, demeanor, diarrhea, and Nal$^r$ fecal *E. coli* O157:H7. At necropsy, approximately 50 g of fecal sample from the rectum was mixed in a stomacher bag, aliquoted, and frozen. The Washington State University Institutional Animal Care and Use Committee approved this research.

**Positive controls.** To create stool with known amounts of *E. coli* O157:H7, bacteria were grown in LB broth to an optical density at 600 nm of 0.6 and $10^8$ CFU were added per gram of stool donated by a healthy volunteer. To examine the sensitivity of the assay, stool was spiked with broth-grown *E. coli* O157:H7 diluted in phosphate-buffered saline.

**RNA extraction from stool.** RNA was extracted using a modification of the silica-binding method (2, 4). Buffers L6, L11, L10, and L2 and silica were prepared as described previously (2, 4). Guanidine isothiocyanate (GITC)-containing buffers were stored in the dark and used within 3 weeks after preparation. The silica pH was adjusted to exactly 2.0 using 32% HCl. Approximately 0.2 g stool was mixed with 5 ml L6 containing 0.2 g polyvinylpyrrolidone (PVP-40). For watery human samples and all bovine samples, the amount of stool was increased to 0.4 g. The mixture was vortexed thoroughly and centrifuged for 5 min at 4,300 × *g* and 4°C. The supernatant was transferred to fresh tubes containing 2.5 ml GITC-phenol (7.5 M GITC, 0.5% sodium dodecyl sulfate, 1 mM EDTA, 50 mM sodium acetate [NaOAc] [pH 4.0], 50% $H_2O$-saturated phenol) and 2.5 ml chloroform. The solution was vortexed and centrifuged (5 min, 4,300 × *g*, 4°C). The aqueous phase was transferred to a fresh tube containing 10 ml ethanol (EtOH) and 400 μl 3 M NaOAc, incubated for 30 min at −20°C, and centrifuged (20 min, 4,300 × *g*, 4°C). The precipitated nucleic acids were vacuum dried, resuspended in 3 ml L11, and split into 1-ml aliquots in microcentrifuge tubes, each containing 300 μl silica (pH 2.0) to bind DNA. The tubes were vortexed thoroughly, shaken (10 min, 4°C), and centrifuged (2 min, 2,000 × *g*), and the

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TABLE 1. Primers and probes used in this study

| Primer or probe[a] | Sequence (5′→3′) | $T_m$ (°C)[b] | Gene detected | Size (bp) of PCR amplicon | Reference |
|---|---|---|---|---|---|
| eaeL.2004RT (forward) | GTCTCAAACGCAAGCAACCA | 59 | *eae* | 101 | This work |
| eae-probe.2004RT (probe) | TCGTGCGACGATAAC | 69.4 | | | This work |
| eaeR.2004RT (reverse) | CATCACTGACTGTCGCACTAACAGT | 59 | | | This work |
| espAL-RT (forward) | GCCAAACTTCCTCAAGACGTG | 58.4 | *espA* | 100 | This work |
| espA-probe (probe) | CATAAGTGTAACTGGTATTC | 67.7 | | | This work |
| espAR-RT (reverse) | CACCAGCGCTTAAATCACCAC | 59.2 | | | This work |
| gnd-L-TT (forward) | GGTAATACCTTCTTCCAGGACACC | 58 | *gnd* | 105 | This work |
| gnd-probe (probe) | CCGTGAGCTTTCTG | 68.9 | | | This work |
| gnd-R-TAT (reverse) | TAGTGCGCCCTCCTCACC | 58.3 | | | This work |
| ehxAL-RT (forward) | GATATATTCCATGGCGCAGATG | 58.1 | *ehxA* | 77 | This work |
| ehxA-probe (probe) | TCGAAGGTAATTATGGT | 63.4 | | | This work |
| ehxAR-RT (reverse) | CCATCATCGCCGTATAGTCG | 57.7 | | | 27 |
| IhaL.2004RT (forward) | GCATCTCACGGATGCACTTG | 58.8 | *iha* | 91 | This work |
| iha-probe.2004RT (probe) | CCGCTATGAACATCATG | 70.2 | | | This work |
| ihaR.2004RT (reverse) | CAGATATGCACGCGGACTGA | 59.4 | | | This work |
| rfbEL-RT (forward) | CAAGTCCACAAGGAAAGTAAAGATG | 57.2 | *rfbE* | 85 | This work |
| rfbE-probe (probe) | CACTTATTGGATGGTCT | 65 | | | This work |
| rfbER-RT (reverse) | ATTCCTCTCTTTCCTCTGCGG | 58.5 | | | 44 |
| stxIIL.June (forward) | GATGTTTATGGCGGTTTTATTTGC | 58.4 | $stx_2$ | 83 | 14 |
| stxII-probe.June (probe) | TCTGTTAATGCAATGGC | 69.6 | | | This work |
| stxIIR.June (reverse) | TGGAAAACTCAATTTTACCTTTAGCA | 59.6 | | | 14 |

[a] Probes were labeled with 6FAM at the 5′ end and minor groove binder/nonfluorescent quencher at the 3′ end.

[b] The melting temperature ($T_m$) was calculated in Primer Express.

RNA-containing supernatants were transferred to a tube containing 700 μl L10 and 200 μl silica (pH 2.0) to bind RNA. Again, the tubes were vortexed thoroughly, shaken, and centrifuged under the same conditions, and the supernatants were discarded. The RNA-containing pellets were washed twice with 1 ml L2, twice with 1 ml 70% EtOH, and once with 1 ml acetone. The pellets were dried by incubating the tubes with open lids at 56°C for 5 min. To elute RNA, 200 μl $H_2O$ was added to the tubes, which were then vortexed thoroughly and incubated (56°C for 10 min with lids closed). RNA was concentrated by adding 3 volumes EtOH, 1 volume 3 M NaOAc, and 1 μl glycogen (Roche), freezing at −80°C overnight, and centrifuging (20 min, 11,000 × *g*, 4°C). Pelleted RNA was washed with 70% EtOH, dried, and resuspended in a 1:1 solution of formamide and $H_2O$.

DNA was eliminated using Ambion's (Austin, TX) "DNA-free" DNase. Samples were treated twice following the recommendations of the manufacturer, using 1 unit of DNase per microgram of nucleic acid. RNA integrity after the DNase treatment was confirmed by visualizing rRNA after electrophoretic separation in a 0.8% agarose gel.

**Primers and probes.** Primers and probes were designed using Primer Express 1.5 (PE Applied Biosystems) and modified to allow approximately 10 bp between primer and probe binding sites. When possible, we used previously published primers and probes (Table 1). TaqMan probes were ordered from Applied Biosystems (Foster City, CA) and fluorescently labeled with 6-carboxyfluorescein at the 5′ end and minor groove binder/nonfluorescent quencher at the 3′ end. Primers and probes were tested using positive control (spiked) stools (described above). Real time RT-PCR was also performed on normal stool without added O157:H7 to confirm that amplification did not occur in the absence of *E. coli* O157:H7. To determine the sensitivity of mRNA detection, real-time RT-PCR was performed for each gene on stool from a healthy individual spiked with dilutions of broth-grown bacteria.

**Real-time RT-PCR.** Real-time RT-PCR was performed on 50 ng fecal RNA by using the Invitrogen SuperScript III Platinum one-step quantitative RT-PCR system. TaqMan probes (described above) were used so that the reaction contained two levels of specificity: (i) the annealing of specific primers and (ii) hybridization of the probe. For each gene, amounts of primer, probe, and $MgSO_4$ were optimized. These were used at the concentrations detailed in Table 2. All reactions using clinical samples were performed in triplicate using RNA from a single extraction. Reactions were run on a Rotor-Gene 2000 real-time cycler: program parameters were 55°C for 25 min, 95°C for 2 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s. To confirm that RNA was free of DNA, an additional reaction was included that was identical to the RT-PCR but substituted platinum *Taq* for the RT enzyme mix provided in the kit. If a clinical specimen yielded no amplicon for a particular gene, the reaction was repeated. If only two of the three reactions yielded a product, these two were included in the analysis.

**Standard curves.** RNA was extracted from bacteria grown with shaking at 37°C to logarithmic phase (12). For each gene, real-time RT-PCR was performed on 10, 5, 1, 0.5, and 0.1 ng of RNA. Conditions were identical to those used for the clinical samples. A standard curve was generated by plotting log 10 ng RNA on the *x* axis and the cycle threshold ($C_T$) value on the *y* axis. The equation of the slope of the best-fit line was used to determine relative amounts for each gene for the clinical samples.

**Determination of ratios of genes of interest to control gene.** For each clinical sample, real time RT-PCR was performed for each gene in triplicate in a single experiment. The relative amounts of RNA were determined from the $C_T$ values by reference to the standard curves. The average relative amount of the transcript of interest was then divided by the average relative amount of *gnd* to determine the ratio for each gene.

TABLE 2. Optimized conditions for amplification of each gene

| Gene | Probe (nM) | Primer (nM) | $MgSO_4$ (mM) | Detection[a] |
|---|---|---|---|---|
| *eae* | 300 | 600 | 5 | $10^6$ |
| *espA* | 300 | 400 | 5 | $10^6$ |
| *ehxA* | 300 | 200 | 5 | $10^6$ |
| *gnd* | 300 | 500 | 5 | $10^5$ |
| *iha* | 300 | 400 | 5 | $10^5$ |
| *rfbE* | 400 | 400 | 6 | $10^5$ |
| $stx_2$ | 300 | 300 | 6 | $10^5$ |

[a] CFU *E. coli* O157:H7/g of positive control stool containing smallest amount of *E. coli* O157:H7 detectable by this primer set.

**Statistical analysis.** For each putative virulence gene, the ratio of mRNA to *gnd* mRNA for human samples was compared to that for bovine samples by using the two-tailed Mann-Whitney test. Significance was set at 5%.

## RESULTS

**Selection of genes for study.** To determine if virulence genes are expressed differently when *E. coli* O157:H7 is excreted from humans compared to cattle, we selected several known or candidate virulence loci for expression analysis. These included LEE pathogenicity island genes *eae* and *espA*, as well as the gene encoding Shiga toxin 2 ($stx_2$). $stx_2$ was selected because, unlike $stx_1$ and other Shiga toxin variant genes, it is present in nearly all North American isolates (26, 39). We also included genes for which a role in human infection is uncertain: *rfbE*, which is involved in the synthesis of the O antigen of lipopolysaccharide (LPS), and *ehxA*, which encodes enterohemolysin. Finally, we included *iha*, which encodes Iha, an adherence-conferring molecule that is a virulence factor in uropathogenic *E. coli* (16, 38). Levels of expression of these genes were related to that of a constitutively expressed housekeeping gene, *gnd*. *gnd* was selected as a reference locus because it is unusually polymorphic among *E. coli* housekeeping genes (25), enabling the specific analysis of expression of the O157:H7 gene against a background of commensal *E. coli*. Moreover, it differs from the *gnd* genes of related *E. coli* O55:H7 and *E. coli* with the O157 *rfb* region, which are only distantly related to *E. coli* O157:H7 and are not pathogens. Thus, $gnd_{\mathrm{O157:H7}}$ provides a target that is theoretically quite specific for the identification of pathogenic *E. coli* O157:H7.

**RNA extraction from fecal samples.** We developed an extraction method to isolate sufficiently pure RNA, free of RNases and inhibitors of enzymatic reactions. First, we employed a silica-binding method (2, 4) to isolate intact RNA as determined by separation on a 0.8% agarose gel (data not shown). However, we were unable to amplify fecal RNA obtained by this method, and we could not amplify RNA from broth-grown bacteria mixed 1:1 with fecal RNA extracted using this method. We suspected that inhibition of amplification reactions was at least in part the result of polyphenolics, which inhibit PCR (19). Polyphenolics are found in the leaves, bark, and fruit of most plants and reportedly copurify with nucleic acids during extraction from plant material and sludge (23, 37). PVP binds polyphenolics, and adding PVP-40 reduces inhibition of amplification by polyphenolics when included in an RNA extraction (15, 36, 37). Therefore, additional steps to optimize the RNA extraction protocol included adding PVP-40 in the lysis buffer, and the resulting procedure (see Materials and Methods) enabled the isolation of RNA suitable for RT-PCR from control (uninfected) human stool samples to which O157:H7 was added (data not shown).

**Selection and optimization of primers and probes.** Primers and TaqMan probes designed for real-time RT-PCR are shown in Table 1. Optimum concentrations of primer, probe, and $MgSO_4$ were determined (Table 2) using positive control stools (described in Materials and Methods). For each gene, an increase in fluorescence during real-time RT-PCR was observed for RNA from human stool spiked with *E. coli* O157:H7 but not for RNA from unspiked stool containing commensal *E. coli* (data not shown). Furthermore, gene expression was not detected in RNA from a bovine fecal sample taken before infection (data not shown), nor was it detected in bovine samples with low numbers of *E. coli* O157:H7 (see below). Thus, the primers and probes are specific for *E. coli* O157:H7 and do not amplify codefecated flora.

The sensitivity of the assay was determined by performing real-time RT-PCR on control stool spiked with serial dilutions of broth-grown *E. coli* O157:H7, ORN172(pIHA) (for *iha*), or STEC O103:H2 (for *ehxA*). ORN172(pIHA) and STEC O103:H2 highly express *iha* and *ehxA*, respectively, and were used because LB-grown O157:H7 expressed these genes at very low levels. The lowest dilution for which an increase in fluorescence was observed for each gene is listed in Table 2.

**Analysis of human and bovine specimens.** RNA was extracted from eight human and four bovine specimens. Six bovine specimens were available, but we could detect *E. coli* O157:H7 gene expression in only four. The remaining two specimens contained $<10^4$ CFU *E. coli* O157:H7/g. Each gene was amplified from each RNA preparation in triplicate. For the majority of the >80 reactions performed in triplicate, the $C_T$ values within the triplicates varied by <10%. For four reactions, the $C_T$ values of the triplicates varied by <35%. These four were cases in which the $C_T$ value was >30, i.e., near the limit of detection. The relative amounts of RNA were determined from the experimental $C_T$ values by reference to the standard curves.

Average relative amounts of mRNA for each gene were then normalized to the average relative amount of *gnd* mRNA of the same specimen. The resulting ratios were compared between human and bovine specimens by using the two-tailed Mann-Whitney test independently for each gene. For calf no. 3, in which expression of only some genes was detected, the sample was excluded from analysis for the genes whose expression was not detected. We elected to exclude these samples rather than assign an arbitrary low number because our statistical analysis used a nonparametric method that requires that the data be rank ordered. Assigning an artificial value had the potential to denote that data point with an incorrect rank and might have invalidated the results. Table 3 indicates the ratios of expression relative to *gnd*.

*iha* and *espA* exhibited statistically significant differences in expression between human and bovine hosts (Fig. 1). In both cases, the genes were more highly expressed in cattle feces. In contrast, *ehxA* (Fig. 2) ($P = 0.073$) and *rfbE* ($P = 0.11$) appeared to be more highly expressed in human stool, although these results failed to achieve statistical significance.

## DISCUSSION

In addition to providing insight into host-specific virulence factor expression, we believe this study to be the first description of quantitative determination of specific bacterial mRNA levels in feces containing commensal flora. Preparation of RNA from cholera patient stools has previously been described (20, 22); however, these analytes were described as having a rice-water appearance characteristic of *Vibrio cholerae* stool, and microscopically, they contained few nonvibrios. Those authors did not mention any problems with inhibition and were able to extract RNA by using Trizol reagent, a phenol-chloroform based method. This suggests that cholera pa-

TABLE 3. Ratios of gene expression to that of *gnd*[a]

| Sample no. | Type[b] | Patient outcome[c] | Specimen status[d] | Specimen consistency | CFU O157:H7/g | Ratio of transcript to that of *gnd*[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *eae* | *espA* | *ehxA* | *gnd* | *iha* | *rfbE* | $stx_2$ |
| 1 | Bovine | Normal | Normal | Normal | $4.9 \times 10^6$ | 18 | 19 | 0.6 | 1 | 0.09 | 0.048 | 15 |
| 2 | Bovine | Normal | Normal | Normal | $9.4 \times 10^5$ | 3.5 | 5 | 0.41 | 1 | 0.57 | 0.024 | 1.3 |
| 7 | Bovine | Normal | Normal | Normal | $1.5 \times 10^4$ | 36 | 11 | 0.27 | 1 | 1.8 | 0.026 | 0.97 |
| 3 | Bovine | Normal | Normal | Normal | $2.5 \times 10^7$ | | | 0.01 | 1 | 0.04 | 0.0001 | 0.05 |
| A | Human | Diarrhea | Uncomplicated | Watery | $2 \times 10^9$ | 15 | 1.8 | 2 | 1 | 0.04 | 0.11 | 0.63 |
| B | Human | Diarrhea | Uncomplicated | Solid | $1.2 \times 10^8$ | 13 | 15 | 3.6 | 1 | 0.03 | 0.11 | 0.55 |
| C | Human | Bloody diarrhea, HUS | Pre-HUS | Watery | $1.1 \times 10^8$ | 12 | 3.3 | 5.1 | 1 | 0.05 | 0.28 | 9.1 |
| D | Human | Bloody diarrhea | Uncomplicated | Solid | $1 \times 10^8$ | 0.3 | 0.13 | 0.46 | 1 | 0.03 | 0.02 | 0.48 |
| E | Human | Bloody diarrhea | Uncomplicated | Solid | $1 \times 10^8$ | 0.7 | 0.28 | 0.09 | 1 | 0.01 | 0.0032 | 0.47 |
| F | Human | Diarrhea | Uncomplicated | Soft | $6 \times 10^7$ | 3.6 | 0.93 | 4.2 | 1 | 0.02 | 0.23 | 1.4 |
| G | Human | Bloody diarrhea, HUS | HUS | Watery | $3 \times 10^7$ | 3 | 1 | 0.56 | 1 | 0.02 | 0.066 | 0.29 |
| H | Human | Bloody diarrhea | Uncomplicated | Watery | $1.8 \times 10^7$ | 5.3 | 1.6 | 3.4 | 1 | 0.03 | 0.38 | 1 |

[a] The relative amount of each transcript was determined using standard curves, following which transcripts of the genes of interest were normalized to *gnd*.

[b] All bovine samples were from calves obtained at 1 day of age except no. 7, which was obtained at 6 weeks of age.

[c] Refers to ultimate outcome.

[d] Refers to patient status at time sample was taken.

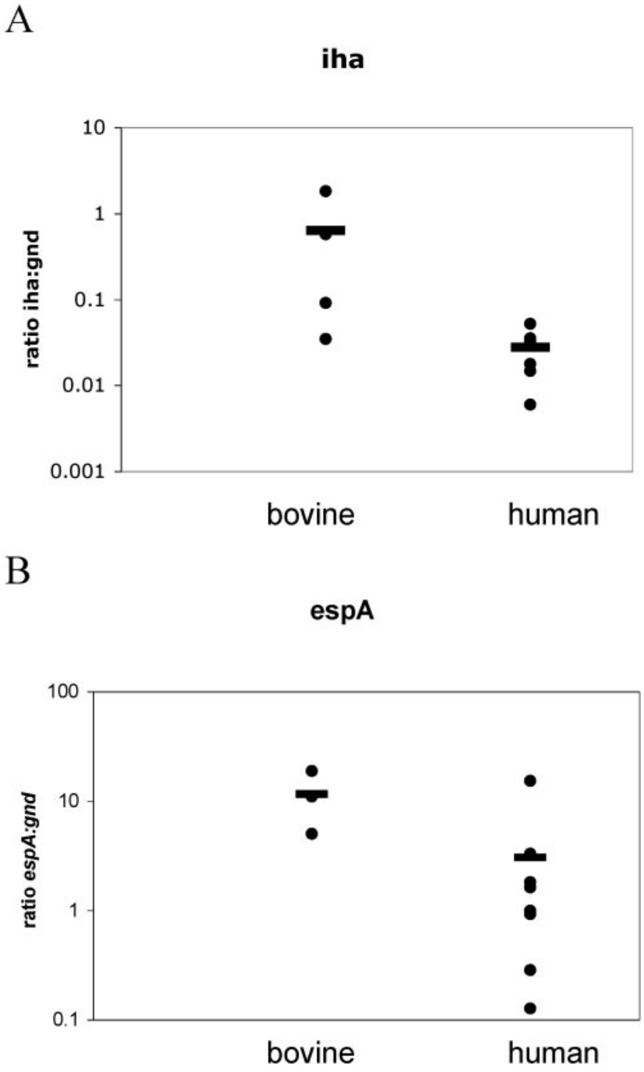

FIG. 1. Genes differentially expressed in human and bovine hosts. *iha* (A) or *espA* (B) expression normalized to *gnd* expression is shown with specimens grouped by source. The bars represent the means for human and bovine specimens ($P < 0.05$).

tient stools are relatively pure cultures of *V. cholerae* that possess few inhibitors. In contrast, our specimens were rarely aqueous, contained abundant commensal *E. coli* and other bacterial flora, and sometimes also contained blood or plant material which can inhibit PCR. We therefore believe that our RNA extraction technique can be applied to study gene expression of other bacteria that reside in the microbially complex milieu of the vertebrate intestine.

Because our specimens contained variable numbers of *E. coli* O157:H7, it was necessary to normalize virulence gene expression to an O157-specific control gene. *gnd* encodes the third enzyme of the pentose-phosphate pathway and is adjacent to the *rfb* cluster on the chromosome. Selective pressure for O-antigen diversity has led to a high mobility rate for the *rfb* region, and this recombination has also involved the neighboring gene *gnd*, which cotransfers with *rfb* (25, 40), generating variation in *gnd*. Therefore, though it is a housekeeping gene,

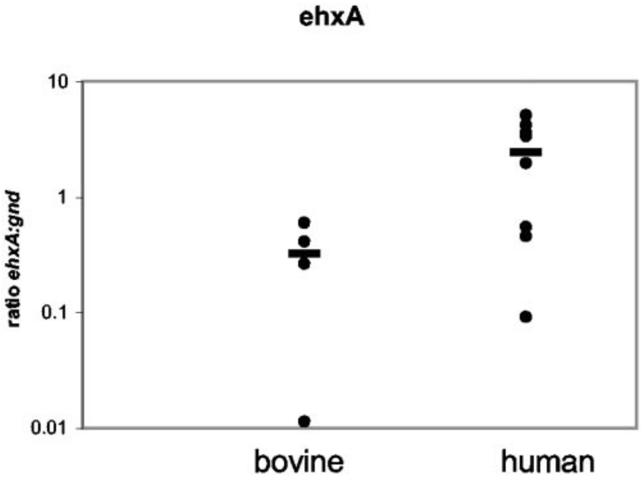

FIG. 2. *ehxA* exhibits a trend towards differential expression. *ehxA* expression normalized to *gnd* expression is shown with specimens grouped by source. The bars represent the means for human and bovine specimens ($P = 0.073$).

*gnd* is sufficiently polymorphic that we could design primers and probes specific for *gnd* of *E. coli* O157:H7. *gnd* mRNA abundance is regulated by the growth rate and fluctuates by 2.5-fold when grown on acetate versus glucose (29). This regulation is the result of growth rate rather than carbon source (45), and further regulation of 6-phophogluconate dehydrogenase levels is achieved by posttranscriptional regulation via blocking of translation initiation (6). rRNA is often used as a constitutively expressed control, and its synthesis is also regulated according to growth rate (reviewed in reference 28). Therefore, *gnd* provides a comparable, O157-specific control for our specimens. It might also be a useful target for identifying *E. coli* O157:H7 in other microbially complex substances such as food or environmental samples.

In our samples, *gnd* expression roughly correlated with CFU/g of *E. coli* O157:H7, especially for the bovine samples (see supplementary Table 1 at http://faculty.washington.edu/moseley/rashidsup.pdf). For human fecal samples with a solid consistency we detected lower abundances of all genes, including *gnd*, than in human fecal samples with a liquid consistency. We speculate that this is the result of additional inhibitors from food being present in solid fecal samples, thus reducing detection of all messages. In support of this, samples B and C both contain approximately $10^8$ CFU/g, but sample B is of solid consistency and C is of watery consistency (Table 3; see supplementary Table 1 at http://faculty.washington.edu/moseley/rashidsup.pdf). Supplementary Table 1 shows the raw data for all samples, and values for all genes are lower in the solid sample B. This suggests that inhibitors present in this and other solid samples affect all genes equally and further illustrates the need to normalize data internally prior to comparing across samples. In human samples, we observed the highest levels of *gnd* mRNA in the samples with the highest CFU per gram, which were of a liquid consistency. We did not detect *gnd* mRNA in fecal samples from a healthy human or a calf prior to infection, nor were we able to detect *gnd* in specimens from two calves with low CFU per gram. Thus, we conclude that detection of the O157:H7 *gnd* allele message by our primers specifies the presence of *E. coli* O157:H7.

We were able to detect gene expression in bovine and human clinical specimens at concentrations lower than those found to be the limit of detection in positive control stool (Table 2). We believe that this reflects a combination of differences in the inhibitors present in these specimens and differences in gene expression between broth-grown and feces-grown bacteria.

We found *iha* and *espA* to be more highly expressed in cattle than in humans. Iha, encoded by *iha*, is an adherence factor found among several pathotypes of *E. coli* (38, 42). *espA* is located in the LEE pathogenicity island, and EspA is important in forming a filament that bridges the bacterium and host cell and may be involved in protein translocation (17). Both Iha and EspA might play a role during colonization of the bovine intestine. In fact, EspA has been shown to be a colonization factor of STEC O26:H− during bovine colonization (43), and the LEE pathogenicity island was demonstrated to be important for bovine colonization of O157:H7 (10). Our data suggest that Iha might also act as a colonization factor in cattle. Host species specificity of colonization factors is well known. For example, different virulence factors of *Salmonella enterica* serovar Typhimurium are required for colonization of cattle compared to chickens (24). Similarly, Iha may play a more important role in bovine colonization than in human diarrheal disease. Such a role may allow Iha-expressing STEC to persist in their animal reservoirs prior to transmission to humans.

Another possible explanation for increased detection of *iha* and *espA* in cattle could be the presence of non-O157:H7 *E. coli* that contains these genes. However, in two specimens containing few O157:H7 CFU per gram of stool, we were unable to amplify any of the genes of interest, including *iha* and *espA*, and in a third we were unable to amplify *espA*. Additionally, we did not observe expression of any of these loci in a fecal sample from calf no. 7 prior to infection with *E. coli* O157:H7. This argues against the possibility that detection of these genes was the result of non-O157:H7 *E. coli*. Increased expression of *iha* and *espA* in O157:H7 remains the simplest and most likely explanation for detection of those genes in the bovine specimens.

Additional genes analyzed demonstrated trends towards higher expression in one species of host versus the other. We expect that this may have reached statistical significance had more specimens been available for analysis. Consistent with higher expression of LEE pathogenicity island gene *espA* in calves, *eae* also demonstrated a trend towards higher expression in calves. *rfbE* demonstrated a trend towards higher expression in the human, as did *ehxA*. *rfbE*, also called *per*, is necessary for expression of the O157 antigen on LPS (3). It is unclear why the bacterium would benefit from increased O-antigen expression in the human host compared to the bovine host. Perhaps the LPS provides a protective benefit to the bacterium. Although enterohemolysin has been demonstrated to have pore-forming activity (35), a defined role for this toxin during pathogenesis has been unclear. Our data support the possibility that enterohemolysin plays a role during human pathogenesis by *E. coli* O157:H7.

Iha is repressed by iron (R. A. Rashid, P. I. Tarr, and S. L. Moseley, submitted for publication). Our observation that *iha* is more highly expressed in cattle could indicate that the bovine host is more iron limiting than the human host. However, enterohemolysin expression is increased when cells are grown in iron-restricting medium (7), and *ehxA* showed a trend towards higher expression in specimens of human origin. This suggests that iron levels alone are unlikely to account for differences in expression of these genes.

Interestingly, not all genes demonstrated a trend towards host-specific expression: $stx_2$ was not apparently differentially expressed. This observation could be due to a slight difference in expression that was undetected among the few specimens analyzed. Alternatively, the possibility exists that $stx_2$ is equally expressed in humans and bovines. Indeed, $stx_2$ is a late gene product of the temperate bacteriophage 933W (32). It has previously been noted that cattle may lack vascular Shiga toxin receptors (33) and that some Shiga toxins are activated by a compound present in mucus (18, 21). In conjunction with our data, these studies suggest the possibility that Stx-mediated pathogenesis may in part be regulated at the host level via receptors, activation of toxin, and possibly other factors.

We excluded from analysis those samples for which we did not detect expression, rather than assigning an arbitrary number. For calf no. 3, *gnd* levels were high. If *espA* is truly expressed below the limit of detection, then it might be valid to assign a low value for the *espA* ratio. If *espA* expression from this animal is assigned the lowest rank, statistical significance of the difference between *espA* expression in humans and cattle is lost. However, we do not know that our inability to detect *espA* in this sample is the result of low gene expression. Other possibilities include the loss or mutation of the LEE pathogenicity island during the course of infection or unexplained technical problems with the assay of this specimen. For these reasons, we elected to include in the analysis only samples for which we were able to detect message.

We observed wide variation of expression for a given gene within a host species. We do not believe this reflects detection of gene expression of normal flora for reasons described above. However, we are unable to explain the variation by correlating increased gene expression with disease status of the donor. It may be that fecal samples represent gene expression from a variety of locations within the intestine, including both adherent and nonadherent bacteria, which may have different gene expression profiles. Variability of gene expression from sample to sample remains a limitation of these data, and assessment of biological significance must be approached with caution.

Another limitation of our data is that the transcriptome of *E. coli* O157:H7 in feces may differ from that in organisms adhering to the mucosal surface in the intestines. Several studies have demonstrated in vitro the influence of epithelial adherence on bacterial gene expression (11, 30). Analysis of expression levels of bacteria at this site during the course of human infection would require biopsy material that would be difficult to obtain in sufficient quantity for analysis. Therefore, examination of fecal bacteria seems a practical alternative. In support of this method, Larocque et al. detected differential gene expression between early and late stages of infection with *V. cholerae* by measuring gene expression in vomitus and feces (20).

It has previously been observed that *E. coli* O157:H7 regulates virulence gene expression in a lineage-specific manner (9). Our data suggest that this organism additionally regulates virulence gene expression in a host-specific manner.

In conclusion, our data support the hypothesis that at least a subset of *E. coli* O157:H7 virulence gene expression alters according to the host species. Additionally, our study provides a method of bacterial RNA isolation from mixed-flora fecal specimens that contain inhibitors, which may prove useful in the study of other intestinal tract pathogens and of the human enteric transcriptome in various additional states.

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