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Prevalence of Hemolysin Genes and Comparison of *ehxA* Subtype Patterns in Shiga Toxin-Producing *Escherichia coli* (STEC) and Non-STEC Strains from Clinical, Food, and Animal Sources

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Shiga toxin-producing *Escherichia coli* (STEC) belonging to certain serogroups (e.g., O157 and O26) can cause serious conditions like hemolytic-uremic syndrome (HUS), but other strains might be equally pathogenic. While virulence factors, like *stx* and *eae*, have been well studied, little is known about the prevalence of the *E. coli* hemolysin genes (*hlyA*, *ehxA*, *e-hlyA*, and *sheA*) in association with these factors. Hemolysins are potential virulence factors, and *ehxA* and *hlyA* have been associated with human illness, but the significance of *sheA* is unknown. Hence, 435 *E. coli* strains belonging to 62 different O serogroups were characterized to investigate gene presence and phenotypic expression of hemolysis. We further investigated *ehxA* subtype patterns in *E. coli* isolates from clinical, animal, and food sources. While *sheA* and *ehxA* were widely distributed, *e-hlyA* and *hlyA* were rarely found. Most strains (86.7%) were hemolytic, and significantly more hemolytic (95%) than nonhemolytic strains (49%) carried *stx* and/or *eae* ($P < 0.0001$). *ehxA* subtyping, as performed by using PCR in combination with restriction fragment length polymorphism analysis, resulted in six closely related subtypes (>94.2%), with subtypes A/D being *eae*-negative STECs and subtypes B, C, E, and F *eae* positive. Unexpectedly, *ehxA* subtype patterns differed significantly between isolates collected from different sources ($P < 0.0001$), suggesting that simple linear models of exposure and transmission need modification; animal isolates carried mostly subtypes A/C (39.3%/42.9%), food isolates carried mainly subtype A (81.9%), and clinical isolates carried mainly subtype C (66.4%). Certain O serogroups correlated with particular *ehxA* subtypes: subtype A with O104, O113, and O8; B exclusively with O157; C with O26, O111, and O121.

Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) became a focus of attention based on the continuously increasing numbers of non-O157-related illnesses reported worldwide (1–5). Over 100 different O serogroups are associated with Shiga toxin production, although not all STEC strains are pathogenic to humans (2, 6). However, some STEC strains are as virulent as O157:H7 and have caused outbreaks of severe illness (7, 8), including hemorrhagic colitis (HC), hemolytic-uremic syndrome (HUS), and even death (8–10). Relatively few organisms of these strains are needed to cause disease, and the consumption of contaminated foods or drinking water or close contact with STEC-infected animals have been shown to be important transmission routes for STEC infections in humans (11–13). Domestic and wild ruminants, especially cattle, are natural reservoirs of STEC that shed the bacteria with their feces in the environment (12). While STEC-infected animals usually do not show signs of disease and can be included in food production, STEC from these animals can be highly virulent to humans. Consumption of products of animal origin, such as meat, therefore presents a risk factor for STEC infection. Furthermore, most fresh produce is grown in close contact with the ground and is vulnerable to *E. coli* contamination from livestock area runoff, manure when used as fertilizer, and field intrusion of wildlife (5, 11, 14, 15).

While O157:H7 is well characterized and widely accepted as a human pathogen, non-O157 *E. coli* strains are very heterogenous in their virulence attributes. Certain serogroups are known to cause HC and HUS (e.g., O26 and O111), and O45 is associated with HC (1, 2); however, other serogroups (O91, O113, and O128) may cause HC and HUS but are less commonly isolated (1, 6). Specific virulence markers that could definitely characterize STEC isolates as pathogenic have not been universally agreed upon. Shiga toxin (stx_1 and stx_2), its variants, and the protein intimin (encoded by the *eae* allele), which is involved in attachment of the organisms to and effacing of gut mucosal cells, are only a few examples of established virulence factors (16–21). Also, several different types of hemolysin genes have been identified in various bacterial species and are often regarded as major virulence factors (22, 23). Four types of hemolysin have been identified in *E. coli*: alpha-hemolysin (*hlyA*), plasmid- and phage-carried enterohemolysin (*ehxA*, *e-hlyA*), and silent hemolysin (*sheA*). The plasmid-carried *ehxA* and *hlyA* belong to the RTX (repeat in toxin) family, which are widespread among Gram-negative pathogens and lyse erythrocytes from different mammalian species (24–26). The presence of enterohemolysin is only detected on washed sheep blood agar after overnight incubation at 37°C, as opposed to alpha-hemolysin, which is detectable after 3 to 6 h of incubation on standard blood agar plates (25, 27, 28). *hlyA*, found on the pathogenicity island (29), is produced by many *E. coli* strains that

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are associated with urinary tract infections (30–32). The *ehxA* gene, located on the large enterohemorrhagic *E. coli* virulence plasmid (28, 33), is frequently associated with diarrheal disease and HUS (34, 35). Since the detection of enterohemolysin is relatively easy due to its hemolytic activity, and its presence correlates with that of Shiga toxin, it has been suggested as an epidemiological marker for the rapid and simple detection of STEC strains (28, 36). Six genetically distinct *ehxA* subtypes (A to F) have been identified in *E. coli* by using PCR and restriction fragment length polymorphism (RFLP) analysis (37).

The bacteriophage-associated enterohemolysin (*e-hlyA*) is genetically unrelated to the plasmid-carried *ehxA* (38–40). It was suggested that the *e-hlyA* determinant does not encode a hemolysin, *per se*, as reported in earlier studies (38, 39), but instead causes the release of the silent hemolysin by lysis of the bacterial cells (40). The silent hemolysin (*sheA*), also called cytolysin A, encodes a pore-forming toxin (41, 42) not related to the RTX family (43, 44). It was first found in *E. coli* strain K-12 (45, 46), but it is also present in *E. coli* O157:H7 (47), as well as in other enteropathogenic *E. coli* strains (48). In cultured mammalian cells, cytotoxicity and inducement of macrophage apoptosis through the action of the silent hemolysin have been reported (49, 50). These findings indicate that *sheA* might contribute to the pathogenicity of *E. coli* strains, although its presence in correlation with the appearance of extraintestinal infections has yet not been clarified. Hemolytic activity due to the silent hemolysin can be only detected under certain conditions, for example, if *sheA* is present on high-copy-number plasmids or when certain regulator genes are overexpressed (51, 52).

The objective of this study was to investigate the prevalence of hemolytic phenotypes and/or genotypes (*ehxA*, *e-hlyA*, *sheA*, and *hlyA*) in correlation with the potential pathogenicity of various *E. coli* strains, as judged by the presence or absence of *stx* and *eae*. Furthermore, this study aimed to investigate the distribution of *ehxA* subtypes within and between isolates from clinical, food, and animal sources, as well as different O serogroups from different geographical areas. By including isolates from ground beef and fresh produce, food products which have been important transmission vehicles of *E. coli* infections in humans in the past (3–5, 11, 13, 53), we were able to investigate, by using *ehxA* subtype occurrence, a potential linear flow of infection originating from farm animals and food to ill individuals.

MATERIALS AND METHODS

Bacterial strains. A total of 435 *E. coli* isolates from different sources (154 clinical, 200 food, 58 animal, nine environmental, and 14 unidentified source) and geographical areas were investigated. The strains belonged to 62 different O serogroups, including the 10 most clinically relevant STEC serogroups (O26, O103, O111, O145, O157, O91, O113, O128, O45, and O121). The 435 strains used in this study were kindly provided by the following (see also Table S1 in the supplemental material): 46 *E. coli* strains from various sources (water, animal, and clinical) from the *E. coli* Reference Center at Pennsylvania State University (54), 67 ground beef isolates from Mick Bosilevac (USDA U.S. Meat Animal Research Center, Clay Center, NE) (55), 101 agricultural food isolates from Shanker Reddy (USDA AMS Microbiological Data Program [MDP]), 32 food isolates from an FDA Center for Food Safety and Applied Nutrition (CFSAN)-sponsored produce survey, 99 isolates of different sources from the STEC Center at Michigan State University (www.shigatox.net), five strains of different sources obtained through Lydia V. Rump (University of Maryland, Department of Nutrition and Food Science, College Park, MD), and seven bovine isolates, five clinical strains, and strain AD4001-1B were provided by Andrew Lin (FDA San Francisco District Laboratory, Alameda, CA). Of the clinical isolates, 32 were obtained from Alifiya Ghadiali (FDA CFSAN, College Park, MD), and 40 were provided by Nancy Strockbine (Centers for Disease Control and Prevention, Atlanta, GA). Potential duplicate strains (e.g., strains originating from the same outbreak) were removed according to the available information, such as clinical outcome, host, geographical location, date of isolation, pulsed-field gel electrophoresis (PFGE) pattern, and the virulence gene pattern confirmed in this study.

Preparation of template DNA and culture conditions for hemolysin expression. Bacterial strains were grown aerobically for 18 to 24 h on tryptic soy agar (TSA) at 37°C. One colony was transferred into 1.5 ml of tryptic soy broth and incubated for another 18 to 24 h at 37°C in a shaking incubator. DNA was extracted using the DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions for Gram-negative bacteria. Template DNA was stored at −20°C until use.

Hemolytic activity was verified on STEC heart infusion washed blood agar with mitomycin C (SHIBAM) as described previously (56). A single colony of each strain was inoculated from TSA onto SHIBAM and incubated for 6 and 24 h at 37°C. The agar plates were examined after 6 h for indications of alpha-hemolysin effects and after 24 h for all hemolysin types.

Detection of established and putative virulence factors. Each strain was screened for the targets stx_1, stx_2, *eae*, *uidA*, *ehxA*, *hlyA*, *e-hlyA*, and *sheA* by using PCR (C1000 thermal cycler; Bio-Rad Laboratories Inc., Hercules, CA). One microliter of DNA template was added to a 24-μl PCR mixture containing 0.6 units HotStarTaq DNA polymerase (Qiagen Inc.), 2.5 μl 10× PCR buffer with 15 mM $MgCl_2$ (Qiagen Inc.), 0.2 mM deoxynucleoside triphosphates (Invitrogen Life Technologies, Grand Island, NY), and 4 μM of each primer (IDT, Inc., Coralville, IA, manufactured with standard desalting) (Table 1). The template was denatured for 15 min at 95°C, followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. All products were electrophoresed on precast 2% agarose gels containing ethidium bromide (E-Gel; Invitrogen Life Technologies) and visualized on a UV transilluminator (G:Box; Imgen Technologies, Alexandria, VA).

O serogroup identification. Serotype information for all 435 *E. coli* isolates used in this study was provided by the contributors of the strains. However, serogroups O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, and O157 were confirmed via a Luminex microbead-based suspension array with the Bio-Plex 200 instrument (Luminex Corporation, Austin, TX) as described previously (57). Antisera from Statens Serum Institut (MiraVista, Indianapolis, IN) were used to confirm the Bio-Plex results (58) for non-O157 *E. coli* (except serogroup O104), and the *E. coli* O157:H7 latex test kit (Remel, Lenexa, KS) was used to confirm O157.

Molecular characterization of *ehxA* subtypes. *ehxA* subtype identification via RFLP analysis was conducted for all *E. coli* strains that were *ehxA* PCR positive. PCR amplification of the complete *ehxA* gene was performed with 1 μl template DNA and 0.6 μM of each primer (ehxA ext. F/R) (Table 1). Amplification of the *ehxA* gene for *ehxA* subtype D strains did not result in PCR products; hence, the full-length *ehxA* gene of subtype D strains was amplified using the primers (ehxA RFLP F/R) described by Cookson et al. (37) (Table 1). Platinum PCR SuperMix High Fidelity (Invitrogen Life Technologies) was added to a total of 20 μl. Amplification and digestion with TaqI (Invitrogen Life Technologies) was conducted as described previously (37) with the following adjustments: an initial denaturing step at 95°C for 2 min and polymerization for 3.5 min at 72°C. Products were then electrophoresed and visualized on a UV transilluminator as described previously. For better amplicon separation and exact size determination, 1-μl aliquots of the digested PCR products were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

TABLE 1 PCR Primers used in this study for detection of target genes and sequencing analysis

Target	Primer name	Sequence (5′–3′)	Amplicon size (bp)	Reference or source
Shiga toxin 1	stx1 F	GACTTCTCGACTGCAAAGAC	306	Son et al.[a]
	stx1 R	TGTAACCGCTGTTGTACCTG		
Shiga toxin 2	stx2 F	CCCGGGAGTTTACGATAGAC	482	Son et al.
	stx2 R	ACGCAGAACTGCTCTGGATG		
Intimin[b]	eae F	GCGCGTTACATTGACTCCCG	245	Son et al.
	eae R	CCATTTGCTGGGCGCTCATC		
uidA	uidA F	GGTCACTCATTACGGCAAAG	379	Son et al.
	uidA R	CAGTTCAACGCTGACATCAC		
Enterohemolysin (plasmid)	ehxA F	TCTGTATCTGCGGGAGTTAG	136	Son et al.
	ehxA R	CAACGTGCTCAAACATAGCC		
Silent hemolysin	sheA F	GAGGCGAATGATTATGACTG	920	74
	sheA R	ACTTCAGGTACCTCAAAGAG		
Alpha-hemolysin	hlyA F	GTCTGCAAAGCAATCCGCTGCAAATAAA	561	74
	hlyA R	CTGTGTCCACGAGTTGGTTGATTAG		
Enterohemolysin (phage)	ehlyA F	TCGCAATCACATCACAACC	810	74
	ehlyA R	CCAGCAGTTCGTCATCATCTGAA		
Complete *ehxA* gene for RFLP and sequencing	ehxA ext. F	CAGGCAATACCATCATGAAC	3,166	Present study
	ehxA ext. R	GTGCATACAGACTATTATGAG		
Complete *ehxA* gene for subtype D	*ehxA*RFLP F	ATGACAGTAAATAAAATAAAGAAC	2,997	37
	*ehxA*RFLP R	TCAGACAGTTGTCGTTAAAGTTG		
Internal sequencing primers for complete *ehxA* gene	ehxA Seq R1	TGAGCCAAGCTGGTTAAGCT		Present study
	ehxA Seq F2	GGCTCTTGATGAATTGCTGAG	633	Present study
	ehxA Seq R2	ATTGTTGTCAGGGCTGCATC		
	ehxA Seq F3	GTCTGATCACATCGGCTGTT	642	Present study
	ehxA Seq R3	GTGTAAACTCCTTCGGTTGA		
	ehxA Seq F4	GGGATGAGAAGATCGGTGAAC	625	Present study
	ehxA Seq R4	CCTACTGACACCTCCTGTTC		
	ehxA Seq F5	CCGTATCTTATAATAAGACGG	670	Present study
	ehxA Seq R5	CCTCCTTCATCTGCAATTG		
	ehxA Seq F6	CTGACAGGAGGAAGCGGTAATG		Present study

[a] I. Son, R. Binet, A. Lin, T. S. Hammack, and J. A. Kase, submitted for publication.
[b] The *eae* primers were tested to include 15 *eae* variants.

Sequencing of the *ehxA* gene. The full-length *ehxA* gene of 12 *ehxA*-positive isolates—two of each of the six *ehxA* subtypes—was sequenced via standard DNA sequencing through a commercial facility (MCLAB, South San Francisco, CA). The *ehxA* gene was amplified using the primers described earlier (ehxA ext. F/R and ehxA RFLP F/R for subtype D strains) (Table 1). PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc.) as per the manufacturer's instruction using a microcentrifuge. Sequencing was performed with 10 internally designed primers (Table 1), as well as the two primers flanking the *ehxA* gene.

Sequencing and phylogenetic analysis. CLC Main Workbench 6.7.2 (CLC bio, Cambridge, MA) was used for editing DNA sequences. The phylogenetic tree was compiled using MEGA 4.0.2 (59, 60) with the neighbor-joining method (61) and the maximum composite likelihood method for determination of evolutionary distances (62). Sequences were aligned in MEGA 4.0.2 using ClustalW. The percent nucleotide sequence identity of the *ehxA* sequences was calculated using CLC Main Workbench 6.7.2.

Statistical analysis. The significance of observed differences in the pattern of *ehxA* subtypes among *ehxA*-positive isolates obtained from different sources (clinical, food, and animal), as well as observed differences in frequencies of isolates carrying one or more virulence factors (stx_1, stx_2, or *eae*) among hemolytic versus nonhemolytic isolates were evaluated by using Fischer's exact test. Differences in virulence factor carriage were evaluated both overall and with respect to each source type separately. All calculations were performed using the R programming language (63). An α level of 0.05 was used as the criterion for statistical significance.

TABLE 2 Prevalence of *ehxA* gene among *E. coli* strains from clinical, food, animal, and unidentified sources

ehxA genotype	Total no. (%) of strains with genotype	No. (%) of strains with *ehxA* genotype isolated from source			
		Clinical	Food	Animal	Unidentified
Positive	301 (69.2)	113 (73.4)	155 (77.5)	28 (48.3)	5 (35.7)
Negative	134 (30.8)[a]	41 (26.6)	45 (22.5)	30 (51.7)	9 (64.3)

[a] Nine *ehxA*-negative water isolates were included in the analysis, to represent the total of 435 *E. coli* isolates investigated in the study.

RESULTS

***ehxA* PCR-RFLP subtyping.** *ehxA*-positive *E. coli* strains were subtyped by PCR-RFLP. Overall, 301 (69.2%) of the 435 *E. coli* isolates investigated were *ehxA* positive (Table 2). The majority (85.0%; 96/113) of the *ehxA*-positive clinical isolates carried *stx* and *eae*, while almost the same proportion (84.5%; 131/155) of the *ehxA*-positive food isolates carried only *stx*. The majority of the *ehxA*-positive strains isolated from animal sources carried *stx* and *eae* (60.7%; 17/28) or *stx* only (35.7%; 10/28) (Table 3). The five *ehxA*-positive *E. coli* strains isolated from unidentified sources were all positive for *stx* and *eae*, except for one strain which was only *eae* positive.

For the PCR step, an attempt was made to use the primers described by Cookson and colleagues (37), but not all *ehxA*-positive isolates investigated in this study were detected. Hence, a new primer pair located just outside the *ehxA* gene was designed, yet the *ehxA* RFLP profiles obtained appeared as described previously (37). Six distinct (A, B, C, D, E, and F) *ehxA* subtypes were obtained by PCR-RFLP. Among a total of 301 *ehxA*-positive *E. coli* strains, the majority were represented by *ehxA* subtype A (50.8%), followed by subtypes C (36.2%) and F (8.0%). *ehxA* subtypes B (3.0%), D (1.3%), and E (0.7%) were detected less frequently (Table 3). The six *ehxA* subtypes were subdivided into *eae*-negative and *eae*-positive STEC strains; subtypes A and D were *eae* negative, except for one subtype A strain (an animal isolate) that was *eae* positive, and subtypes B, C, E, and F were *eae*-positive strains. Three subtype C strains (two clinical and one animal isolate) and one subtype B strain (unidentified source) were non-STEC strains but *eae* positive. *ehxA* subtype patterns differed significantly between isolates collected from different sources ($P < 0.0001$). While the animal isolates could be divided into subtypes A (39.3%; 11) and C (42.9%; 12) and the food isolates were mostly associated with subtype A (81.9%; 127), the majority of the *ehxA*-positive clinical isolates belonged to subtype C (66.4%; 75) (Table 3). Overall, all subtypes were found more or less in all categories of isolates, except for subtype D (2.6%, 4), which was exclusively found in food isolates (alfalfa sprouts [1], lettuce [2], and ground beef [1]), and subtype E (1.8%; 2), which was found only in *E. coli* strains isolated from ill individuals (Table 3). Furthermore, specific *ehxA* subtypes were not related to particular animal hosts, food products, or a specific clinical outcome (Fig. 1, 2, and 3).

Distribution of O serogroups and association with *ehxA* subtypes. Overall, 259 (59.5%) of the 435 *E. coli* isolates used in this study were serotyped as O26 (30 isolates), O45 (11), O91 (17), O103 (29), O104 (50), O111 (29), O113 (19), O121 (17), O128 (10), O145 (22), O157 (9) or O8 (16). The O serogroup for 62 (14.3%) strains was not determined or not provided. Of the remaining 114 (26.2%) strains, five each were of serogroups O1, O163, and O168, and six each were of serogroups O22, O116, and O174; the rest belonged to different serogroups associated with less than five strains. In this study, 130 (84.4%) of the 154 clinical and 52 (89.7%) of the 58 animal isolates, but only 40 (20.0%) of the 200 food isolates, were of the clinically important serogroups (O26, O45, etc.). Certain O serogroups were associated with particular *ehxA* subtypes. All *ehxA*-positive isolates of serogroups O26, O111, and O121 carried *ehxA* subtype C regardless of source, whereas all *ehxA*-positive isolates of serogroups O104, O113, and O8 were of *ehxA* subtype A. *ehxA* subtype B exclusively corresponded to *E. coli* strains of serogroup O157, and *ehxA* subtype F was found in all *ehxA*-positive isolates of serogroup O45. Other serogroups corresponded to different *ehxA* subtypes, with serogroup O91 being of *ehxA* subtypes A and C, serogroup O145 being of subtypes C and E, and O103 being of subtypes F and C.

Sequencing analysis and phylogenetic profiling of *ehxA* subtypes. Twelve *ehxA* genes, two of each of the six *ehxA* subtypes, were fully sequenced and aligned along with 11 *ehxA* sequences published by Cookson et al. (37). Based on the 23 *ehxA* sequences, a phylogenetic tree was generated using the neighbor-joining method with MEGA 4.0. Phylogenetic profiling showed a close relationship between *eae*-positive *ehxA* subtype B, C, and F strains, while *eae*-negative subtype A strains formed a distinct group with subtype E strains. The latter were associated with *eae*-positive STEC isolates in our study, but they can be *eae* negative as well (37). *ehxA* subtype D strains (*eae* negative) formed the most divergent subdivision, one well separated from the other subtypes (Fig. 4). Pairwise comparison analysis of the 23 *ehxA* sequences revealed a percent nucleotide sequence identity of 94.2% to 96.4% between *ehxA* subtype D and the remaining five *ehxA* subtypes.

TABLE 3 Virulence factors (*stx* only, *stx* and *eae*, or *eae* only) associated with *ehxA* subtypes of *E. coli* strains from clinical, food, animal, and unidentified sources

ehxA subtype	No. (%) of strains with subtype	No. of strains[a] with virulence factor(s) in isolates from source											
		Clinical			Food			Animal			Unidentified		
		stx	*stx*/*eae*	*eae*	*stx*	*stx*/*eae*	*eae*	*stx*	*stx*/*eae*	*eae*	*stx*	*stx*/*eae*	*eae*
A	153 (50.8)	15			**127**			**10**	1				
B	9 (3.0)		5			2			1				1
C	109 (36.2)		**73**	2		19			**11**	1		3	
D	4 (1.3)				**4**								
E	2 (0.7)		**2**										
F	24 (8.0)		16			3			4			1	

[a] The values listed in bold correspond to the predominant/unique *ehxA* subtype(s) in each category of isolate.

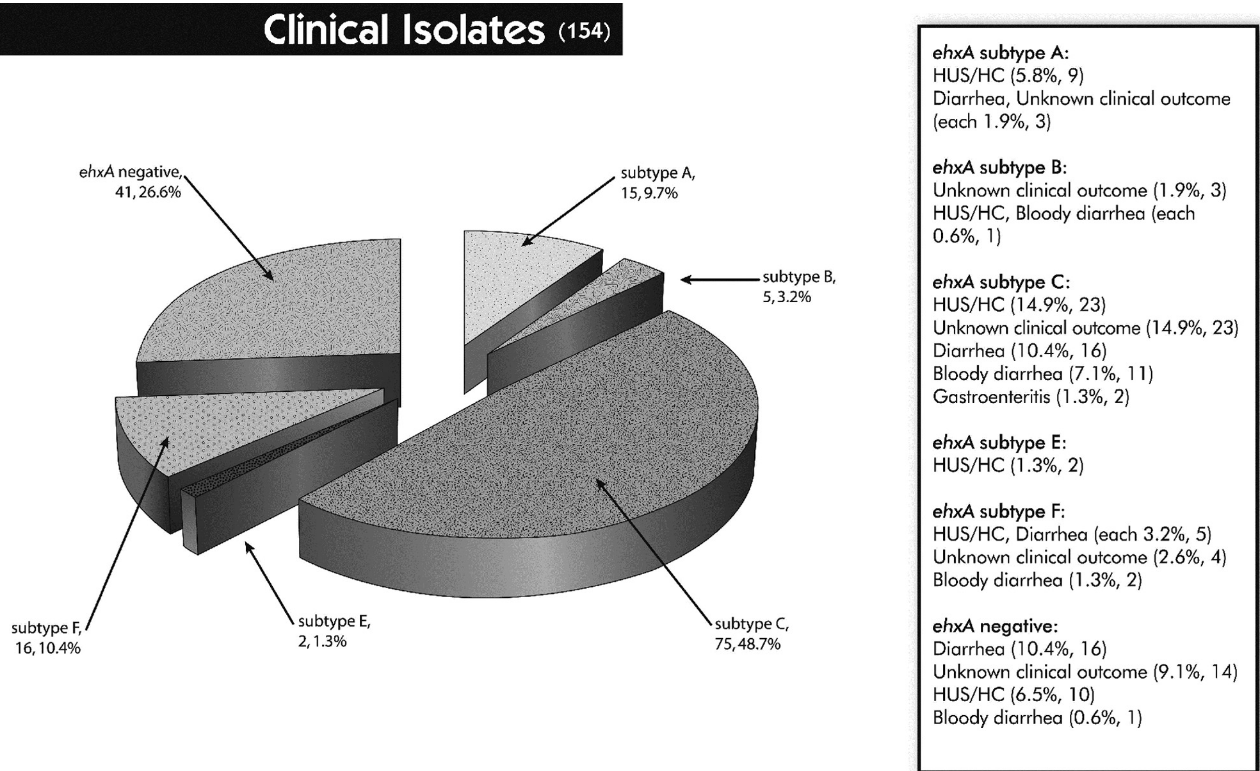

FIG 1 Distribution of clinical isolates to *ehxA* subtypes and *ehxA*-negative samples in association with the available clinical data. Values in parentheses are the total numbers of strains; percentages were calculated based on the total number of clinical isolates (n = 154).

The sequence similarity of non-subtype D *ehxA* subtypes was ≥97.3%.

Hemolysin gene prevalence and correlation with hemolytic expression. The ability of certain *E. coli* strains to lyse erythrocytes of mammalian species is termed hemolysis (27), and the use of SHIBAM proved to be an excellent medium for hemolysis detection for all types of hemolysins (56, 64). The degree of hemolysis varied by strain, from large and clear zones to turbid and smaller zones of hemolysis surrounding single colonies, as noted by others (25, 37, 65). Largely, the different degrees of hemolysis could not be linked to a particular hemolysin type or *ehxA* subtype, except for the alpha-hemolytic strains, which characteristically showed strong lysis and visible clearing on SHIBAM (27). Overall, 301 (69.2%) of the 435 *E. coli* strains were positive for *ehxA*, 408 (93.8%) were positive for *sheA*, 22 (5.1%) were positive for *e-hlyA*, and 12 (2.8%) were alpha-hemolytic. *sheA* was present mainly in correlation with other hemolysins, but 63 (16.7%) of the hemolytic strains carried *sheA* only. The phage-carried enterohemolysin (*e-hlyA*) was found in less than 6% of the strains and was mainly associated with the presence of *ehxA* and *sheA*. Generally, hemolytic activity could be attributed to the different types of hemolysins investigated in this study; all 377 hemolytic *E. coli* strains carried at least one hemolysin gene. Of the 57 nonhemolytic strains, two carried *ehxA*, but no hemolytic phenotype was detected.

Hemolytic expression in association with virulence factors. Overall, 377 (86.7%) of the 435 *E. coli* strains investigated in this study showed hemolytic activity on washed sheep blood agar after 24 h of incubation at 37°C. Significantly more hemolytic strains (95.0%; 358/377) were associated with *stx* and/or *eae* positivity than among nonhemolytic strains (49.1%; 28/57) ($P < 0.0001$) (Table 4; Fig. 5). Of the 19 hemolytic strains (5.0%) that lacked the presence of Shiga toxin and/or intimin, two strains were alpha-hemolytic and *sheA* positive, 16 carried only *sheA*, and one carried *sheA* and *e-hlyA*. Also noteworthy was one strain, isolated from food, that did not grow on SHIBAM but carried the stx_1, stx_2, *eae*, *ehxA*, and *sheA* genes. Of the 154 clinical isolates, 95.7% (133/139) of the hemolytic strains carried *stx* and/or *eae*, as opposed to 53.3% (8/15) of the nonhemolytic strains ($P < 0.0001$). Of the 200 food isolates, 98.9% (180/182) of the hemolytic strains were *stx* and/or *eae* positive, while 76.5% (13/17) of the nonhemolytic strains harbored *stx* only ($P < 0.001$). About 86% (38/44) of the hemolytic strains and only 24% (6/14) of the nonhemolytic strains isolated from animals were associated with *stx* and/or *eae* ($P < 0.05$). The nine *E. coli* strains isolated from water were all positive for *sheA* only, and four strains showed hemolytic activity. Fourteen strains were isolated from unidentified sources, and seven of the eight hemolytic strains were positive for *stx* and/or *eae*. Of the six nonhemolytic strains, one strain carried stx_2 (Table 4; Fig. 5).

DISCUSSION

Certain *E. coli* virulence factors, such as Shiga toxin and intimin, have been well investigated, but the role of hemolysin genes (i.e., *hlyA*, *ehxA*, and *sheA*) in *E. coli* pathogenicity and the association between these potential virulence factors and *stx* and *eae* are largely unknown. Others have noted that the presence of enterohemolysin (*ehxA*) is a good epidemiological marker for the presence of Shiga toxin (28, 36) and that at least six different genetic

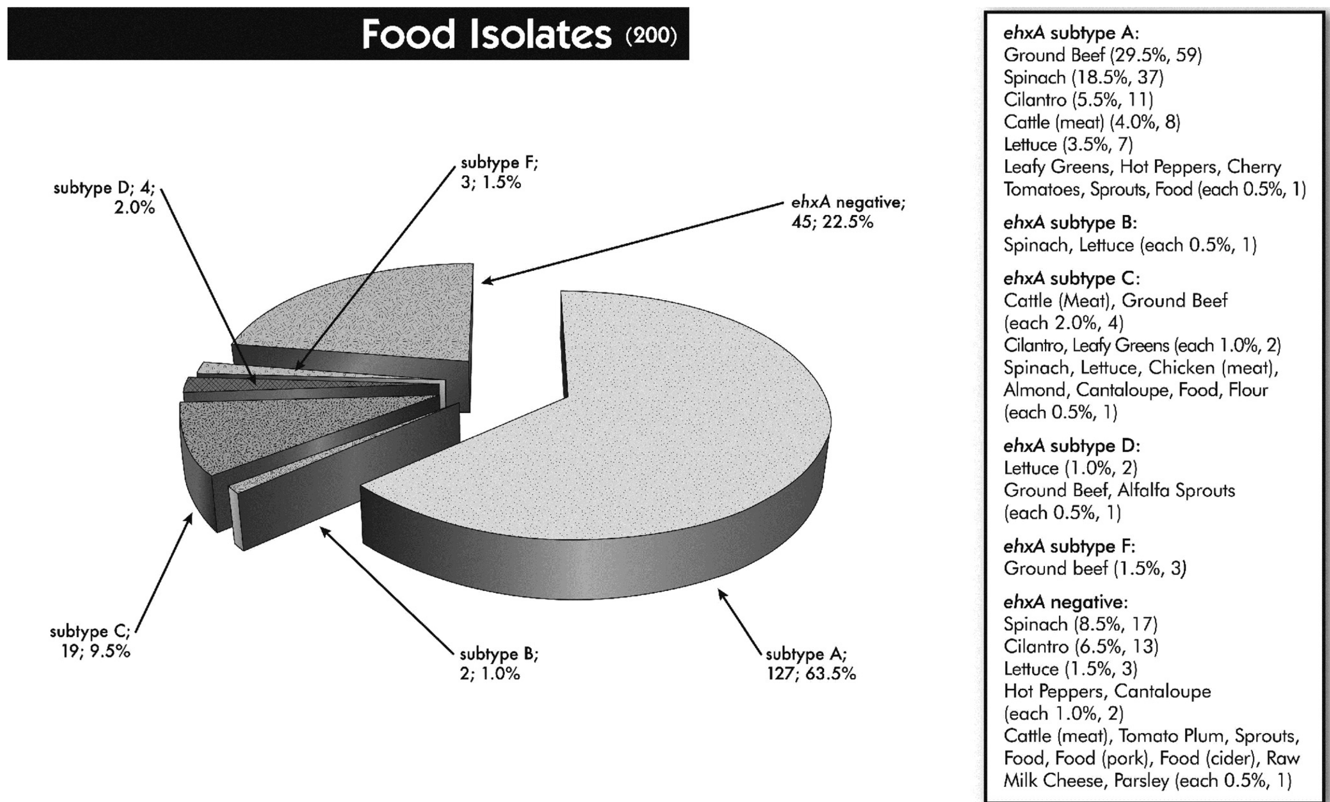

FIG 2 Distribution of food isolates to *ehxA* subtypes and *ehxA*-negative samples in association with different food products. Values in parentheses are the total numbers of strains; percentages were calculated based on the total number of food isolates ($n = 200$).

ehxA subtypes exist in *E. coli* (37). To our knowledge, the investigation of hemolysin gene prevalence, as well as *ehxA* subtype distribution in food isolates compared to clinical and animal isolates from various geographical locations, has not been previously documented.

One interesting finding from the current study was the variation in *ehxA* subtype patterns observed between the different categories of isolates ($P < 0.0001$). The majority of food isolates contained *ehxA* subtype A (64%); however, approximately 20% did not carry the *ehxA* gene. Nearly 10% of the food isolates were associated with *ehxA* subtype C. In contrast, the clinical isolates more often carried *ehxA* subtype C (50%) versus A (10%), while approximately 25% were *ehxA* negative. Alternately, the animal isolates were nearly equally related to *ehxA* subtypes A and C (each 20%), but about 50% lacked *ehxA* (Fig. 1 to 3). Importantly, 60% of the *ehxA*-negative animal isolates were non-STEC.

Another remarkable finding was the stark contrast in intimin prevalence rates between food and clinical isolates; the majority of the food isolates (85%) were associated with *eae*-negative STEC strains, while nearly the same proportion (78%) of clinical isolates carried *eae* (Table 3). The association of *eae*-positive clinical STEC isolates and severe diarrhea and HUS has been noted (2, 19, 66–68). However, several *eae*-negative non-O157 STEC strains have been associated with severe human disease and HUS outbreaks, e.g., O104:H21 (USA, 1994), O113:H21 (Australia, 1998), and O104:H4 (Germany, 2011). It is surprising that the overall prevalence patterns for *eae* and *ehxA* subtypes obtained from the food isolates were generally not reflected in the examined clinical isolates. Although the food isolates investigated in this study did encompass a variety of fresh produce types (e.g., spinach, lettuce, sprouts) as well as ground beef, they did not represent a survey of all food products available. In the United States, leafy greens (e.g., lettuce and spinach) and ground beef have been important transmission vehicles for human *E. coli* infections involving *E. coli* O157:H7, as well as non-O157 STEC (3–5, 13, 53). While the USDA recommends that ground beef be cooked to 160°F to destroy pathogens that might be present, including harmful *E. coli*, leafy greens are usually consumed raw and possibly with little cleaning. Thus, any enteric pathogens acquired during growth in an open agricultural environment might be still present and infectious (11, 14). However, the observation of mostly *eae*-positive clinical isolates and *eae*-negative food isolates has been noted in several studies and therefore seems to be a general trend (18, 75–78). It might be that STEC strains carrying *ehxA* subtype A, strains which lack the *eae* gene, generally do not cause severe illness in infected patients or are mainly associated with sporadic cases; thus, we have not found this subtype as frequently in clinical isolates, when often a patient is not ill enough to seek medical care.

Another reason for the minority of *ehxA* subtypes C and F strains among isolates from food might be the limitation of the isolation method used. For example, the 67 ground beef isolates used in this study were isolated by the use of phenotypic characteristics (55). As shown in this study and supported by others, hemolytic activity as observed on washed sheep blood agar is a good indicator to identify *ehxA*-positive STEC (28, 35, 36, 64). However, in our study, two *ehxA*-positive isolates, one of which

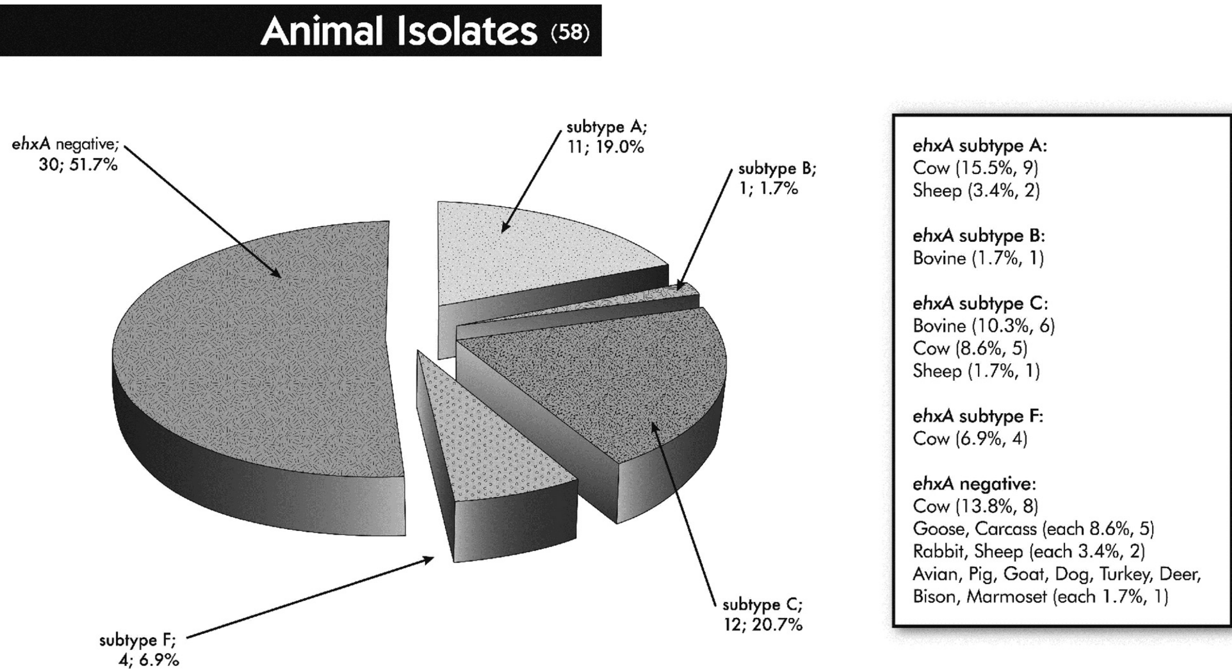

FIG 3 Distribution of animal isolates to *ehxA* subtypes and *ehxA*-negative samples in association with different animal hosts. Values in parentheses are the total numbers of strains; percentages were calculated based on the total number of animal isolates ($n = 58$).

carried *ehxA* subtype C and one that carried *ehxA* subtype A, did not express hemolytic activity on SHIBAM. In addition, one *eae*-, *ehxA*-positive STEC isolate of *ehxA* subtype C isolated from food failed to grow on SHIBAM. Because of their nonhemolytic activity, such colonies might have gone unrecognized in the Bosilevac et al. study (55). In fact, Bosilevac and colleagues noted an inconsistency between the screening of the enrichment and the culture results; significantly fewer STEC isolates were obtained from *eae*- and *stx*-positive samples than from samples that were only *stx* positive (69). This result may explain the higher frequency of *eae*-negative isolates in food samples. Based on these findings, it is possible that more *eae*-positive STEC isolates and correspondingly more subtype C and F strains will be identified in food samples, as improved isolation methods and selective agars for non-O157 STEC are developed.

Three previous studies investigated *ehxA* subtypes. The study by Boerlin et al. mainly focused on the evolutionary lineage of *ehxA* and noted that *ehxA* sequences cluster into two main groups corresponding to the presence or absence of *eae*, suggesting that this is due to the evolution of two different virulence plasmids associated with these strains (70). Newton et al. confirmed the *ehxA* phylogeny division into two major clusters and showed that, in fact, *eae*-negative strains carry quite a different virulence plasmid than that found in many *eae*-positive strains (71). We intend to investigate these findings further by using *ehxA*-positive isolates identified in this study. Along with elucidating the finer structure in the *ehxA* phylogeny by using RFLP, Cookson and colleagues identified six *ehxA* subtypes that clustered into groups of *eae*-negative (subtype A) and *eae*-positive (subtype E) strains, *eae*-positive strains (subtypes B, C, and F), and a third cluster of *eae*-negative subtype D strains (37). Sequencing analysis and phylogenetic and pairwise comparison of the *ehxA* gene in the present study confirmed the Cookson et al. findings (Fig. 4).

Cookson et al. also investigated the *ehxA* subtype prevalence mainly in sheep and cattle isolates, as well as in 25 clinical isolates, all of which originated from New Zealand (37). Comparably, we investigated the *ehxA* subtype prevalence in a much larger data set: 154 clinical isolates, 200 food isolates, and 58 animal isolates from various U.S. geographical areas and beyond. While the study by Cookson and colleagues revealed that clinical isolates carry subtypes C, A, and B in decreasing numbers, the present study additionally identified subtypes E and F, which had not been previously reported (37). The overall predominance of *ehxA* subtypes A and C, as well as the strong link between subtypes and *eae* presence or absence, were confirmed in the current study (Table 3). We furthermore validated that *ehxA* subtypes were closely tied to particular O serogroups (37). For example, *ehxA* subtype A strains were closely associated with O8, O22, O88, O91, O113, O116, O163, and O174 strains, which are less commonly isolated during outbreaks (1, 6) but represented the most frequently isolated STEC isolates from food samples in our study. Importantly, O serogroups O91 and O113 have been linked to human illness in the past (1, 6, 7, 72), and their presence in the U.S. food supply is of concern. In contrast, the six serogroups (O26, O45, O103, O111, O121, and O145) that are most frequently isolated due to outbreaks (1, 2, 73) and accounted for the majority of the clinical isolates (64%) in our study were closely associated with *ehxA* subtypes C and F. Subtype B was exclusively associated with strains of O serogroup O157. These findings confirmed associations that may help with the identification of clinically relevant STEC without the need for laborious and expensive serotyping methods. Of note, Cookson at al. identified a single O121 strain of *ehxA* subtype B, indicating that subtype B might not be exclusively associated with O157 STEC. This was not confirmed in our study; all O121 strains were of subtype C.

Most of the strains (87%) analyzed in this study were hemo-

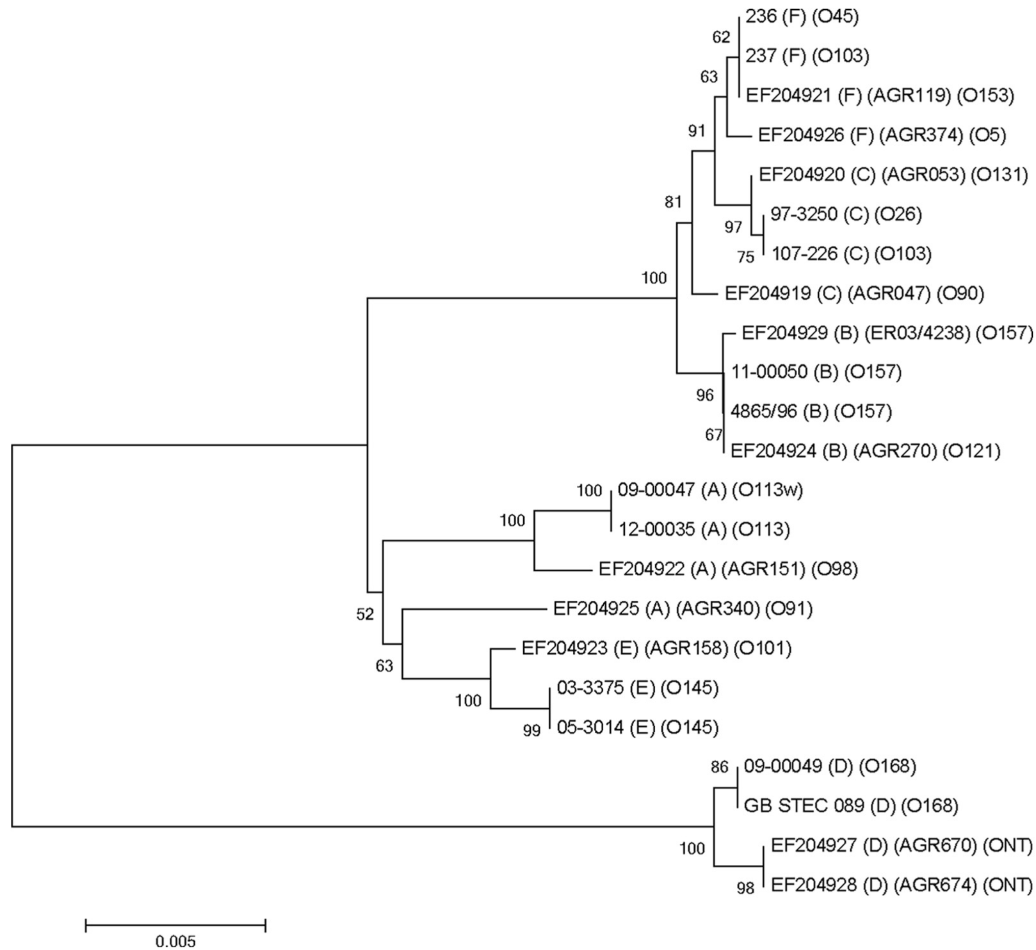

FIG 4 Phylogenetic relationships of *E. coli ehxA* sequences from this study, in comparison with sequencing results reported by Cookson et al. (EF204919 to EF204929) (37). The phylogenetic tree was generated using the neighbor-joining method with MEGA 4.0. Bootstrap values of ≥50% are shown at branch points.

lytic, as observed on SHIBAM, and almost all (95%) carried *stx* and/or *eae*. Hemolytic strains carried at least one hemolysin gene; while *e-hlyA* (5%) and *hlyA* (3%) were found less frequently, the silent hemolysin was widely distributed (94%) among *E. coli* isolates, as noted previously (48, 74). The majority of the *sheA*-positive strains carried other hemolysin genes, mainly *ehxA*; however, 63 hemolytic strains carried only *sheA*, suggesting that *sheA* might be responsible for hemolytic expression. In fact, previous studies have revealed that *sheA* is not totally silent in some *E. coli* strains, and construction of *sheA* deletion mutants uncovered a complete loss of hemolytic activity, indicating a *sheA*-dependent hemolytic phenotype (22, 40, 48, 74). The addition of mitomycin C into the SHIBAM agar used in this study (56) may have further contributed to an increased release of *sheA*, presumably resulting in detectable levels of hemolytic activity (40). Additionally, anaerobic conditions during incubation of blood agar plates resulted in increased hemolytic expression due to *sheA*, and strains that were nonhemolytic under aerobic conditions expressed hemolytic activity when incubated anaerobically (22). These findings were not confirmed in our study; most of the strains either did not grow

TABLE 4 Virulence factors in hemolytic and nonhemolytic *E. coli* strains from clinical, food, animal, and unidentified sources

	No. of H and NH strains[a] associated with virulence factor(s) that were isolated from indicated source							
	Clinical (n = 154)		Food (n = 199)[b]		Animal (n = 58)		Unidentified (n = 14)	
Virulence factor(s)	H	NH	H	NH	H	NH	H	NH
stx only	19	3	157	13	16	4	1	1
stx and *eae*	103	3	23	0	18	1	5	0
eae only	11	2	0	0	4	1	1	0
stx and *eae* negative	6	7	2	4	6	8	1	5

[a] H, hemolytic; NH, nonhemolytic.
[b] One strain failed to grow on SHIBAM.

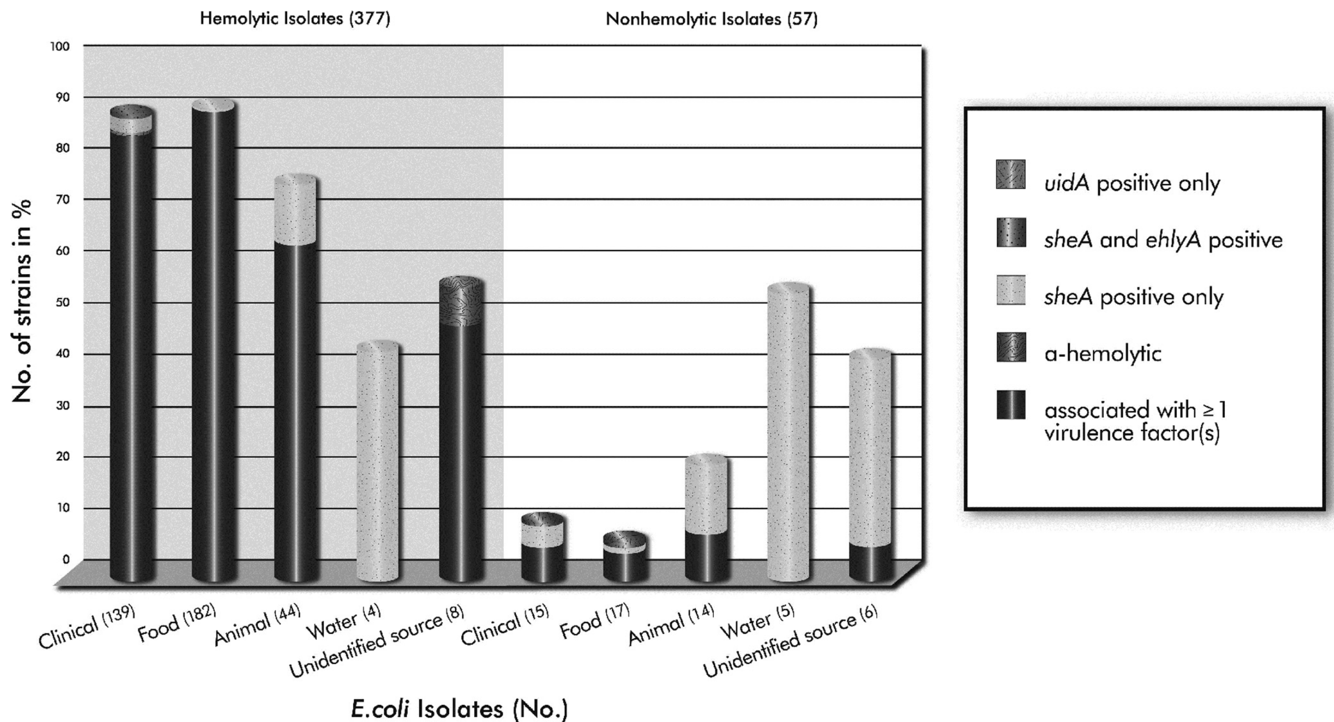

FIG 5 Proportional distribution of hemolytic and nonhemolytic phenotypes within the different categories of isolates, in relation to the presence of at least one/or no virulence factor (stx_1, stx_2, *eae*). Values in parentheses are the total numbers of isolates investigated. (Note: One food isolate failed to grow on SHIBAM.)

anaerobically, or hemolytic activity was decreased or completely absent compared to aerobic conditions (unpublished data). The significance of *sheA* in pathogenicity of *E. coli* strains remains largely unclear. Although *sheA* is present in highly pathogenic *E. coli* strains (e.g., O157:H7 and O104:H4 [Germany, 2011]) and previous studies have shown the ability of *sheA* to lyse erythrocytes and to exhibit cytotoxic and apoptotic activities toward cultured mammalian cells (41, 42, 46, 49, 50), *sheA* is also widely spread among many nonpathogenic *E. coli* strains. But as shown by Ludwig et al. (48), many strains harbor only mutant *sheA* derivatives or fragments therefore of nonfunctional *sheA* genes. Overall, more study is necessary to identify the exact role of the silent hemolysin in *E. coli* strains. Importantly, data generated in this study established that strains that express hemolytic activity, whether due to *sheA* or other hemolysin genes, are significantly more likely (95%) to be associated with highly deleterious virulence factors (*stx*, *eae*) than nonhemolytic strains (49%) ($P < 0.0001$) (Table 4; Fig. 5).

In summary, no apparent association was found between specific *ehxA* subtypes and a particular clinical outcome or food/animal source (Fig. 1 to 3). The widely different *ehxA* subtype prevalence rates between clinical, food, and animal isolates were surprising, given that farm animals are natural reservoirs of STEC and that food, especially fresh produce and ground beef, is an important transmission vehicle for *E. coli* infections in humans (3–5, 11, 12, 13, 53). One would expect similar *ehxA* subtype patterns between isolates from clinical, animal, and food sources. However, a linear flow of infection originating from farm animals and food to ill individuals persists, since we did find all *ehxA* subtypes more or less in all categories of isolates; also, not all isolates carried *ehxA* (Tables 2 and 3). Based on our findings, *ehxA* subtype C and F strains (*eae* positive), which were predominant in the clinical isolates (80%) and are associated with O serogroups such as O26, O45, O103, O111, O121, and O145, are possibly more frequently linked to severe clinical outcomes than *ehxA* subtype A strains (14%; *eae* negative). Overall, more clinical samples associated with bloody diarrhea, HC, and HUS, as well as a greater variety of food products, are needed to draw more exact conclusions.

The strong link between *ehxA* subtypes and *eae* in STEC makes it largely possible to distinguish between *eae*-positive/negative STEC/*E. coli* strains by *ehxA* subtype. Additionally, the close association between particular *ehxA* subtypes and certain O serogroups, especially O157 and O serogroups that are frequently isolated due to outbreaks, might further contribute to the detection of clinically important STEC. But further work is needed to refine *ehxA* RFLP primers in order to detect all subtypes reliably. Hemolytic activity, as observed on SHIBAM, shows that this medium generally represents an excellent medium for the isolation of *stx*- and/or *eae*-positive *E. coli* strains, regardless of the type of hemolysin gene expressed. In fact, significantly more hemolytic strains were associated with virulence factors (*stx*, *eae*) than nonhemolytic strains ($P < 0.0001$) (Table 4; Fig. 5). Overall, *sheA* and *ehxA* were widely distributed among the *E. coli* isolates tested, while *e-hlyA* and *hlyA* were rarely identified. Further studies are required to investigate the lack of hemolytic expression for the few *ehxA*-positive *E. coli* strains identified in this study.

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