

Estimating the Prevalence of Potential Enteropathogenic *Escherichia coli* and Intimin Gene Diversity in a Human Community by Monitoring Sanitary Sewage

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Presently, the understanding of bacterial enteric diseases in the community and their virulence factors relies almost exclusively on clinical disease reporting and examination of clinical pathogen isolates. This study aimed to investigate the feasibility of an alternative approach that monitors potential enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) prevalence and intimin gene (*eae*) diversity in a community by directly quantifying and characterizing target virulence genes in the sanitary sewage. The quantitative PCR (qPCR) quantification of the *eae*, *stx*₁, and *stx*₂ genes in sanitary sewage samples collected over a 13-month period detected *eae* in all 13 monthly sewage samples at significantly higher abundance (93 to 7,240 calibrator cell equivalents [CCE]/100 ml) than *stx*₁ and *stx*₂, which were detected sporadically. The prevalence level of potential EPEC in the sanitary sewage was estimated by calculating the ratio of *eae* to *uidA*, which averaged 1.0% ($\sigma = 0.4\%$) over the 13-month period. Cloning and sequencing of the *eae* gene directly from the sewage samples covered the majority of the *eae* diversity in the sewage and detected 17 unique *eae* alleles belonging to 14 subtypes. Among them, *eae*- β 2 was identified to be the most prevalent subtype in the sewage, with the highest detection frequency in the clone libraries (41.2%) and within the different sampling months (85.7%). Additionally, sewage and environmental *E. coli* isolates were also obtained and used to determine the detection frequencies of the virulence genes as well as *eae* genetic diversity for comparison.

Although the majority of *Escherichia coli* strains are harmless commensal microorganisms in human intestine, numerous pathotypes have been identified to cause severe human diseases, including enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (1). EPEC is commonly associated with severe infant diarrhea; although large outbreaks of infant EPEC-caused diarrhea are rare in the developed world, EPEC-related diarrhea is still one of the most important causes of infant mortality in developing countries (2). EHEC strains, on the other hand, are frequently associated with food-borne outbreaks in the developed world, which are characterized by bloody diarrhea and hemolytic uremic syndrome (HUS) (2, 3). For instance, the EHEC strain O157:H7 has caused numerous food-borne outbreaks and more than 73,000 cases of disease in the United States (4–6), and the recent outbreak of EHEC O104:H4 strains in Europe was the deadliest ever recorded, infecting more than 3,900 people and causing 46 deaths (7).

As with many other pathogenic *E. coli* strains, the virulence factors of EPEC and EHEC are associated with mobile genetic elements (1). A common feature of EPEC and EHEC infection is the “attaching-and-effacing” (AE) histopathology, which is encoded by genes on a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) that was acquired through horizontal gene transfer (8–11). EHEC strains contain additional virulence factors, including the verocytotoxin Stx encoded by the *stx*₁ and *stx*₂ genes that are associated with bacteriophage (4). The *stx*₁ and *stx*₂ genes of EHEC strains confer severe disease symptoms in humans, whereas the LEE and its associated genes of EHEC and EPEC strains enable intimate attachment of *E. coli* cells to epithelial cells and the colonization of intestinal mucosa and, thus, are essential for the onset of diseases (8, 9, 12, 13). The parallel-evolution theory of pathogenic *E. coli* strains suggests that EHEC strains such as *E. coli* O157:H7 and O104:H4 evolved from

EPEC-like ancestors by sequentially acquiring molecular mechanisms through horizontal gene transfer that gradually conferred increased virulence (14–16).

The most studied LEE gene is the *eae* gene that encodes intimin, an outer membrane protein essential for the formation of the characteristic AE lesion of EPEC and EHEC strains (8, 9, 12, 13). The *eae* gene has been shown to be indispensable to the infectivity of EPEC and EHEC strains. An isogenic *eae* null mutant led to the loss of infectivity of an EPEC strain in human volunteers (12), and the deletion of the *eae* gene rendered an EHEC O157:H7 strain noninfective in animal models (8, 9). The importance of *eae* to pathogenicity is also illustrated by the observation that many Stx-producing *E. coli* strains are not pathogenic because of their lack of the *eae* gene.

Previous studies using clinical EPEC and EHEC isolates have revealed extraordinary genetic diversity in the *eae* gene. To date, at least 20 different *eae* subtypes, including *eae*- α 1, *eae*- α 2, *eae*- β , *eae*- β 2, *eae*- γ , *eae*- γ 3, *eae*- γ 6, *eae*- θ , *eae*- τ 1, *eae*- τ 2, *eae*- σ , *eae*- κ , *eae*- ϵ , *eae*- ϵ 2, *eae*- ϵ 8, *eae*- ζ , *eae*- η , *eae*- λ , *eae*- ρ , and *eae*- σ , have been reported in the literature and in the GenBank database (17–20). The different *eae* subtypes exhibit significant genetic variation among themselves (e.g., more than 15% of amino acid sequence difference was observed between the *eae*- α , *eae*- β , and *eae*- γ sub-

Received 15 August 2013 Accepted 8 October 2013

Published ahead of print 18 October 2013

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doi:10.1128/AEM.02747-13

types [21]), and the remarkable *eae* genetic diversity is believed to be involved in host and tissue tropism (20, 22). The majority of *eae* genetic variations are observed at the 3' end of the *eae* gene (23, 24), which encodes the highly variable, C-terminal extracellular domains responsible for receptor binding (25). The *eae* genetic diversity could also result from the variants of the *tir* gene, which is also located on the LEE and encodes the translocated intimin receptor (Tir) protein that gets anchored into epithelial cells for intimin binding (26).

Presently, our understanding of bacterial enteric diseases in the community and of their virulence factors is based primarily on clinical disease reporting and examination of clinical pathogen isolates (27–29). This study aimed to investigate the feasibility of an alternative approach that monitors enteric disease prevalence and virulence factor diversity by directly quantifying and characterizing target virulence genes in the sanitary sewage of a human community. Specifically, the study used cultivation-independent quantitative PCR (qPCR) methods to quantify the concentrations of the *eae*, *stx*₁, and *stx*₂ genes in sanitary sewage over 13 months and estimated the prevalence levels of potential EPEC and EHEC strains in the community. The genetic diversity of the intimin gene *eae* in the community was estimated by constructing *eae* clone libraries for the sanitary sewage samples directly. Additionally, sewage and environmental *E. coli* isolates were also obtained and used to determine the detection frequencies of the virulence genes as well as *eae* genetic diversity, which was compared with the cultivation-independent DNA-based approaches.

MATERIALS AND METHODS

Sample collection. Sanitary sewage samples were collected at the Sand Island Wastewater Treatment Plant (SIWTP) in Honolulu, HI, over a 13-month period (April 2010 to April 2011). The SIWTP collects and treats approximately 60% of the sewage in the City of Honolulu. Raw sewage samples (1 liter) were collected hourly using an autosampler at a location immediately before the primary clarifier, and 40 ml of the completely mixed hourly samples was mixed to make daily composite samples. The daily composite samples were stored at 4°C in the dark until the end of the week, when 100 ml of completely mixed daily samples was pooled to make weekly composite samples. After mixing, the weekly composite samples (100 ml) were immediately centrifuged at 13,000 × *g* for 15 min at 4°C to pellet suspended solids and cells, and the supernatants were vacuum filtered through 0.45-μm-pore-size cellulose ester membrane filters. The pellets and cell-bearing membranes were subsequently pooled for each wastewater sample and stored at –80°C until required for DNA extraction and subsequent analysis. Two additional grab sewage samples were collected on 18 June and 2 July 2008 and used for the isolation of sewage *E. coli* strains.

***E. coli* isolation.** *E. coli* isolates were obtained from the grab sewage samples using the standard modified membrane-thermotolerant *E. coli* (mTEC) agar method (30). Briefly, the municipal wastewater samples were diluted in phosphate-buffered saline before being spread plated. Individual colonies with typical *E. coli* characteristics were purified by repetitive streaking on LB agar, followed by the IMViC (indole, methyl red, Voges-Proskauer, citrate) tests for *E. coli* verification. A total of 236 *E. coli* isolates were obtained from the sewage samples, which herein are referred to as sewage *E. coli* isolates. *E. coli* isolates from stream water and soil samples that were collected in 2009 from the Manoa watershed in a separate study (31), which herein are referred to as environmental *E. coli* isolates, were also used in this study for comparison. A total of 467 environmental *E. coli* isolates were selected from the original collection, which includes 288 isolates from soil and 179 isolates from stream water.

DNA extraction and qPCR quantification. The weekly wastewater samples were subjected to total genomic DNA extraction using an Ultra-

Clean Soil DNA Isolation Kit (MO Bio, Carlsbad, CA) according to the manufacturer's instructions. DNA extracts from the samples from the same month were pooled to make 12 monthly composite DNA samples, which were analyzed by qPCR to quantify *eae*, *stx*₁, *stx*₂, and *uidA* genes using target-specific primers and fluorescent probes (Table 1). The 20-μl qPCR mixtures contained 10 μl of 2× iTaq Universal Probe SuperMix (Bio-Rad, Hercules, CA), 0.25 μM each primer, 0.125 μM fluorescent probe, and 0.4 μg/μl bovine serum albumin (BSA). The qPCRs were performed on an ABI 7300 System (Applied Biosystem, Foster City, CA). The thermocycler program included 5 min of initial denaturation at 95°C and 45 cycles of amplification for 15 s at 95°C, followed by 1 min at 60°C. *E. coli* O157:H7 cells were used to construct calibration curves for the *uidA*, *eae*, *stx*₁, and *stx*₂ genes. Exponential-phase *E. coli* O157:H7 cells were serially diluted to make calibration standards of known numbers of cells (10¹ to 10⁶ CFU/ml), which were then subjected to DNA extraction using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Genomic DNA extracted from the sewage samples and the calibration standards were analyzed in the same batch of reactions.

Cloning of *eae* genes from sewage DNA extracts. Clone libraries of *eae* genes were constructed using PCR amplicons from the monthly sewage DNA extracts. Two rounds of PCR using the same PCR primers (*eae*-F1 and *escD*-R1) (Table 1) with an intermediate gel extraction step were carried out to enhance PCR amplification. The 25-μl PCR mixture contained 2.5 μl of 10× AmpliTaq Gold 360 buffer, 3 mM MgCl₂, 1 μl of 360 GC enhancer, 0.25 mM each deoxynucleoside triphosphate (dNTP), 0.1 μM each primer, 0.4 mg/ml BSA, 0.625 units of AmpliTaq Gold 360 DNA Polymerase (Invitrogen, Carlsbad, CA), and 1 μl of DNA template. The PCR was initialized at 95°C for 10 min, followed by 35 cycles of denaturing (95°C, 30s), annealing (57°C, 30s), and extension (72°C, 3 min), with a final extension at 72°C for 7 min. The first-round PCR amplicons were subjected to gel electrophoresis, and gel excision at the expected amplicon location was conducted regardless of the visibility of a DNA band. The excised gel blocks were extracted using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), and the extracts were used as DNA templates in the second round of PCR amplification. This procedure successfully amplified seven monthly composite DNA samples (August 2010, October 2010, November 2010, December 2010, February 2011, March 2011, and April 2011). The PCR amplicons of the second PCR amplification were gel purified and ligated into a pGEM T-easy cloning vector (Promega), according to the manufacturer's protocol, and then transformed into *E. coli* DH10B competent cells by electroporation.

Detection of *eae*, *stx*₁, and *stx*₂ in *E. coli* isolates. Fresh single colonies of the sewage and environmental *E. coli* isolates were grown overnight in LB broth at 37°C and with constant shaking (200 rpm). One milliliter of the cell cultures was centrifuged at 10,000 × *g* for 5 min, and the cell pellets were resuspended in 50 mM NaOH solution and boiled for 10 min to release genomic DNA. After centrifugation at 10,000 × *g* for 10 min, the supernatants were used as DNA templates to amplify the *eae*, *stx*₁, and *stx*₂ genes using multiplex PCR with target-specific primers (Table 1). The 25-μl PCR mixture contained 1 mM dNTPs, 0.1 μM each primer, 2.1 mM MgCl₂, 1× reaction buffer (10 mM Tris-HCl and 50 mM KCl), 0.8 μg/μl BSA, 1 unit of *Taq* DNA polymerase, and 1 μl of template DNA. The hot-start technique was used to minimize nonspecific amplification. The thermocycler program included a 5-min initial denaturing step at 95°C, followed by 35 cycles of amplification (95°C for 30 s, 60°C for 30 s, and 72°C for 90 s) and a final extension step (72°C for 5 min). The PCR amplicons were then subjected to gel electrophoresis to detect the presence of target genes.

The *eae* genes from the *E. coli* isolates were PCR amplified and then grouped by restriction fragment length polymorphism (RFLP) to identify the isolates carrying unique *eae* subtypes. Total genomic DNA of the *eae*-positive *E. coli* isolates was used to amplify the variable region of the *eae* gene with primers *eae*-F1 and *escD*-R1 (Table 1) using a previously described procedure (18). The PCR amplicons were digested using three

TABLE 1 Primer pairs and probes used in qPCR, multiplex PCR, PCR-RFLP, and sequencing

Assay	Target	Primer name or probe ^a	Sequence (5'–3')	Size (bp)	Reference(s) or source
qPCR	<i>stx</i> ₁	Forward	TTTGTACTGTSACAGCWGAAGCYTTACG	132	45, 46
		Reverse	CCCCAGTTCARWGTRAGRTCMACRTC		
		Probe	CTGGATGATCTCAGTGGGCGTCTTATGTAA		
	<i>stx</i> ₂	Forward	TTTGTACTGTSACAGCWGAAGCYTTACG	128	45, 46
		Reverse	CCCCAGTTCARWGTRAGRTCMACRTC		
		Probe	TCGTCAGGCACTGTCTGAAACTGCTCC		
	<i>eae</i>	Forward	CATTGATCAGGATTTTCTGGTGATA	102	45, 47
		Reverse	CTCATGCGGAAATAGCCGTTA		
		Probe	ATAGTCTCGCCAGTATTCGCCACCAATACC		
	<i>uidA</i>	Forward	GTGTGATATCTACCCGCTTCGC	83	33, 48
Reverse		AGAACGGTTTGTGGTTAATCAGGA			
Probe		TCGGCATCCGGTCAGTGGCAGT			
Multiplex-PCR	<i>stx</i> ₁	Forward	CAGTTAATGTGGTGGCGAAGG	348	49
		Reverse	CACCAGACAATGTAACCGCTG		
	<i>stx</i> ₂	Forward	ATCCTATTCGCCGGAGTTTACG	584	49
		Reverse	GCGTCATCGTATACACAGGAGC		
	<i>eae</i>	Forward	TCAATGCAGTTCGGTTATCAGTT	482	50
		Reverse	GTAAGTCCGTTACCCCAACCTG		
RFLP	<i>eae</i>	eae-F1 escD-R1	ACTCCGATTCTCTGGTGAC GTATCAACATCTCCGCCCA	1,800–2,100 ^b	18
Sequencing ^c	<i>eae</i>	cesT-F9	TCAGGGAATAACATTAGAAA		18
		eae-R3	TCTTGTGCGCTTTGGCTT		
		eae-seq	GMWKMRGWTGTKTAATCCAAG		
		M2eae	GTCGACCAGTTGGGGTAA		

^a All TaqMan probes all use 6-carboxyfluorescein (FAM) as the reporter dye and 6-carboxy-tetramethylrhodamine (TAMRA) as the quencher dye.

^b The size of amplicon depends on the allele.

^c Sequencing primers also include the two used in RFLP.

different restriction enzymes (AluI, HhaI, and RsaI) at 37°C for 8 h, and the digestion products were visualized via gel electrophoresis.

Sequencing. Plasmid extractions were conducted on seven clone libraries using the alkaline lysis method, and the inserts were sequenced using the vector primers M13F and M13R. Both were conducted by the Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB) sequencing facility at the University of Hawaii at Manoa, Honolulu, HI. The 3' highly variable regions of the inserts were compared within the clone libraries to identify the unique *eae* gene inserts. The number of clones sequenced for the individual clone libraries was adjusted based on the sequencing rarefaction curves in order to exhaust the *eae* diversity in the libraries and to maximize the recovery of unique *eae* sequences. As a result, a total of 328 clones were sequenced, with some clone libraries sequenced more extensively than others. For the identified unique *eae* gene inserts, full-length sequences were then determined using the additional sequencing primers eae-seq and M2eae (Table 1). For the *eae*-positive *E. coli* isolates, the unique *eae* subtypes determined by PCR-RFLP were sequenced by amplifying the whole length of the *eae* gene with primers cesT-F9 and escD-R1 (Table 1). Similarly, additional sequencing primers, including eae-F1, eae-R3, and eae-seq, were used to assemble the full sequence length. Contigs were constructed from the sequences using SeqMan (DNASTAR, Madison, WI) until full-length *eae* genes were obtained.

Data analysis. The concentrations of *uidA*, *eae*, *stx*₁, and *stx*₂ are expressed as calibrator cell equivalents (CCE) per 100 ml of sewage sample. The calibration curves for *uidA*, *eae*, *stx*₁, and *stx*₂ using *E. coli* O157:H7 cells as calibrator cells all showed an *R*² value larger than 0.98. In calculating geometric means, 0.9 was used to mathematically represent samples with no detection of target genes (i.e., below the detection limit). All gel images of PCR-RFLP were processed using GelCompar II (Applied Maths, Austin, TX). The RFLP banding pattern for each isolate was nor-

malized with an external DNA size marker (DNA Hyperladder I; Bioline, Taunton, MA) that was loaded into the first and last lanes of each gel. Dendrograms were created based on Pearson's correlation and the unweighted-pair group method with arithmetic mean (UPGMA). Rarefaction curves were calculated using the Analytical Rarefaction software package available from the University of Georgia Stratigraphy Lab (<http://www.uga.edu/strata/software/index.html>). The closest matches of the unique *eae* sequences were obtained by comparison with *eae* gene entries in the GenBank database using BLASTN. The phylogenetic relationships between the *eae* clone sequences and the *eae* genes in sewage and environmental *E. coli* isolates of this study and representative *eae* subtypes from the GenBank database were analyzed using MEGA 5 (32), where a phylogenetic tree was constructed using the maximum-likelihood method of tree inference and the Tamura-Nei nucleotide substitution model for sequence alignment.

Nucleotide sequence accession numbers. The *eae* gene sequences obtained in this study have been deposited in the GenBank database under accession numbers KF771362 to KF771382.

RESULTS AND DISCUSSION

***E. coli* virulence genes in sanitary sewage.** Concentrations of *eae*, *stx*₁, and *stx*₂ genes in the municipal wastewater samples over 13 months were quantified using qPCR (Fig. 1A). The *eae* gene was detected in all 13 (100%) sewage DNA samples, while *stx*₁ and *stx*₂ were detected in only 2 (15.4%) and 3 (23.1%) of the 13 samples, respectively. The geometric mean concentrations (\pm geometric standard deviation) of *eae*, *stx*₁, and *stx*₂ during the sampling period were 399 (\pm 3.1), 1.5 (\pm 3.5), and 2.1 (\pm 5.3) CCE/100 ml, respectively, which corresponded to an *eae* gene abundance 266

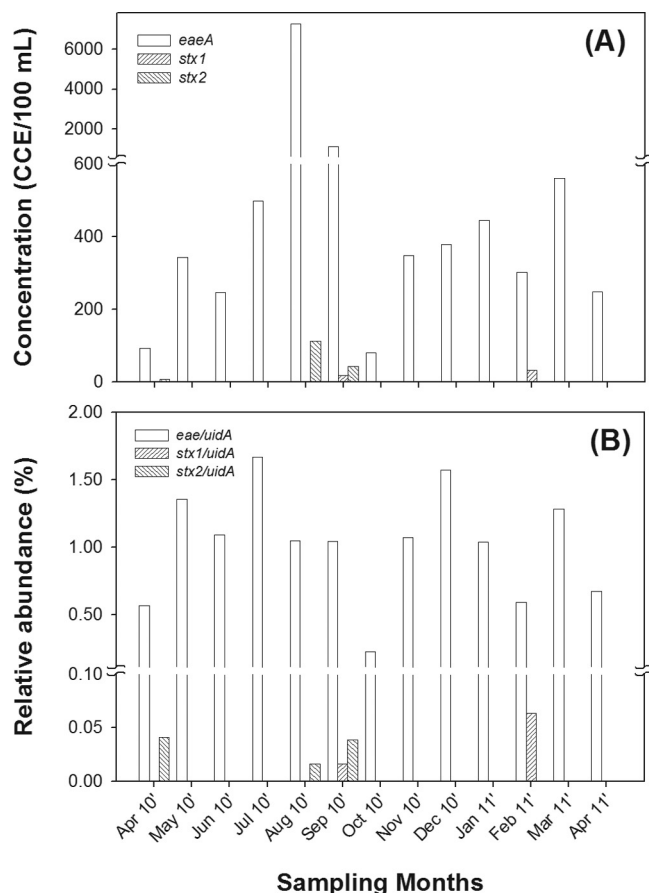


FIG 1 Concentrations of *eae*, *stx*₁, and *stx*₂ determined by qPCR (A) and relative abundances as indicated by the ratios of *eae* to *uidA*, *stx*₁ to *uidA*, and *stx*₂ to *uidA* (B) in the SIWTP sewage over 13 consecutive months.

and 190 times the abundances of *stx*₁ and *stx*₂, respectively. This indicated that the majority of *eae*-bearing *E. coli* in sewage was potential EPEC, which is defined herein as *E. coli* cells with the *eae* gene and without the *stx*₁ and *stx*₂ genes, while the abundance levels of EHEC and/or Shiga toxin-producing *E. coli* (STEC) cells were significantly lower and negligible in comparison to the potential EPEC cells. The abundance of potential EPEC cells fluctuated considerably over the sampling period (93 to 7,240 CCE/100 ml), with the two highest concentrations detected in the summer months (August and September 2010). The percentage of potential EPEC within the total *E. coli* population was estimated by calculating the ratio of *eae* to *uidA* gene copy numbers in the wastewater samples (Fig. 1B). This ratio represents the prevalence of potential EPEC in the sanitary sewage and, hence, in the human community. The ratio of *eae* abundance to *uidA* abundance over the 13-month sampling period ranged from 0.2% to 1.7% and averaged 1.0% ($\sigma = 0.4\%$). The abundance ratios of *stx*₁ to *uidA* and of *stx*₂ to *uidA* were very small in comparison (Fig. 1B), which is similar to the pattern of their qPCR-determined concentrations (Fig. 1A).

The detection frequencies and the average concentrations of *eae*, *stx*₁, and *stx*₂ indicated significantly different prevalence levels in the sewage and, hence, in the community. Since the concentrations of *stx*₁ and *stx*₂ genes were negligible compared to the con-

centration of the *eae* gene, the prevalence of potential EHEC and/or STEC was expected to be very low, while potential EPEC was likely to be the dominant group of diarrheagenic *E. coli* strains in sanitary sewage. The different abundance levels of EPEC and EHEC in sanitary sewage was expected since EPEC generally causes chronic and mild diarrhea while EHEC is associated with acute and severe disease (1).

Although the *eae* gene was consistently detected in all sewage samples, its absolute concentrations determined by qPCR exhibited huge variation during the sampling months. For instance, the largest *eae* concentration was 77.8 times that of the lowest *eae* concentration (Fig. 1A). Apart from the temporal variation of potential EPEC abundance in the community, various environmental factors, such as sewage dilution by heavy rainfall, sample heterogeneity, and method limitations such as PCR inhibition, could all have contributed to the observed variation. To counter some of the variations caused by these factors, the concentration of the *uidA* gene in the sewage samples, which quantifies the overall *E. coli* population (33), was used as a common denominator. The ratio of *eae* to *uidA* exhibited much less temporal variation and gave a normalized estimation of the prevalence of potential EPEC in the sanitary sewage.

Virulence gene detection in *E. coli* isolates. The relative prevalence levels of potential EPEC and EHEC cells in sanitary sewage were also tested using sewage *E. coli* isolates. A total of 236 sewage *E. coli* isolates obtained from two sampling events were analyzed using multiplex PCR assays that detect the presence of the *eae*, *stx*₁, and *stx*₂ genes (Table 2). The *stx*₁ and *stx*₂ genes were not detected in any of the 236 sewage *E. coli* isolates, while an average of 26.3% of *E. coli* isolates were *eae* positive. Additionally, a total of 467 *E. coli* isolates that were previously obtained from soil and water samples from the Manoa watershed (31) were also analyzed for the presence of *eae*, *stx*₁, and *stx*₂. None of the environmental *E. coli* isolates contained the *stx*₁ and *stx*₂ genes, while 1.7% and 1.1% of the *E. coli* isolates from the Manoa soil and water samples were *eae* positive, respectively.

Although the detection frequencies of *eae*, *stx*₁, and *stx*₂ in the sewage isolates support the observation made by qPCR that potential EPEC strains were the dominant *eae*-bearing *E. coli* cells in sanitary sewage, the ratio of potential EPEC to the general *E. coli* population exhibited a large variation between the two sampling events, with one being 0% and the other being 44.3% (Table 2). This large variation suggests that the sample sizes (up to 140 isolates per sampling event) were still too small to be representative. The requirement of large numbers of *E. coli* isolates to achieve acceptable levels of representativeness in sewage can be attributed to both the aggregative form of microbial cells in sewage and the large concentration of *E. coli* cells in sanitary sewage (up to 10⁶ cells/ml). Previous studies using limited numbers of *E. coli* isolates also reported large variations in the detection frequencies of the *eae* gene, ranging from 0.03% in a beach sand *E. coli* population (34) to 10.4% in an *E. coli* population from wildlife feces (35). In contrast, experiments using high-throughput approaches reported more modest *eae* prevalence in environmental *E. coli* populations; for example, Hamilton et al. (36) found 3.6% of 24,493 *E. coli* isolates from beach water to be *eae* positive. The qPCR approach can largely circumvent the sample size issue by using relatively large volumes of sewage samples (100 ml in this study) in the analysis, which are more likely to provide a reasonable representation of the sewage microbial community.

TABLE 2 Multiplex PCR detection of *eae*, *stx*₁, and *stx*₂ in *E. coli* isolates from sewage and environmental samples

Source	Sampling date(s) (mo/day/yr)	No. of isolates	Gene frequency (no. of positive isolates [%])		
			<i>eae</i>	<i>stx</i> ₁	<i>stx</i> ₂
Sewage	06/08/2008	96	0 (0)	0 (0)	0 (0)
	07/02/2008	140	62 (44.3)	0 (0)	0 (0)
Soil	Various ^a	288	5 (1.7)	0 (0)	0 (0)
Water	Various	179	2 (1.1)	0 (0)	0 (0)

^a Multiple sampling sites and dates were used.

Intimin gene (*eae*) diversity. The intimin gene (*eae*) diversity in municipal wastewater was investigated by constructing *eae* clone libraries for the monthly composite sewage samples. Seven clone libraries were successfully constructed, from which a total of 328 *eae* gene clones were sequenced. The overall sequencing effort detected 17 unique *eae* sequences, which covered the majority of the *eae* diversity in the wastewater samples, as indicated by the overall rarefaction curve approaching an asymptotic state (Fig. 2A). Rarefaction analysis for the individual monthly clone libraries indicated that the sequencing effort recovered the most dominant *eae* genes within the individual monthly libraries (Fig. 2B).

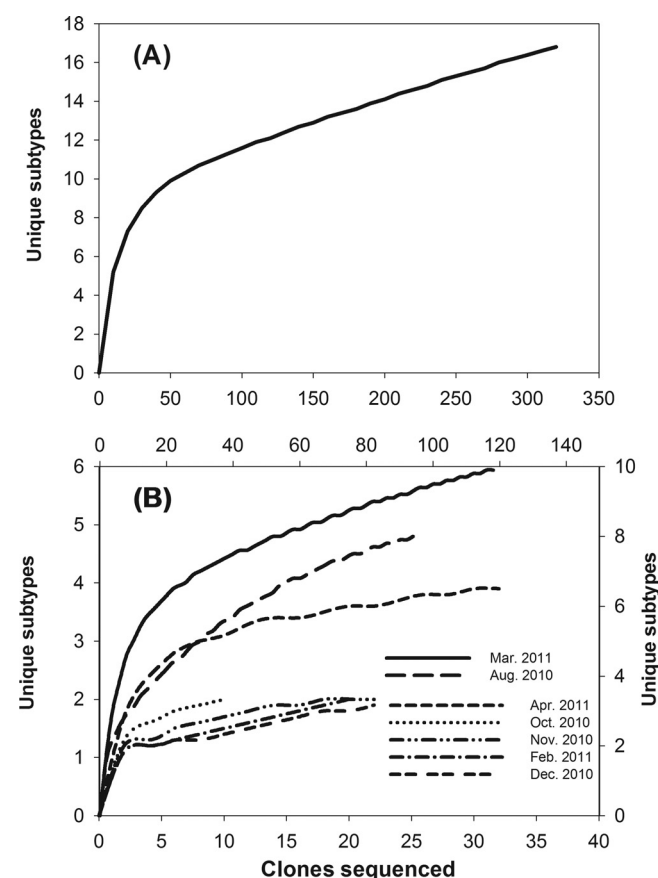


FIG 2 Rarefaction curves of *eae* sequencing in all clone libraries combined (A) and in individual monthly clone libraries (B). In panel B, rarefaction curves for the March 2011 and August 2010 clone libraries use the top and right coordinates, while rarefaction curves for other clone libraries use the bottom and left coordinates.

TABLE 3 Unique *eae* sequences and their accession numbers and closest matches in the GenBank database

Clone no.	Accession no.	Identity (no. of shared bases/ total no. of bases [%])	Closest match in GenBank	
			Subtype	Reference
1A1	AJ271407	1,678/1,695 (99)	ζ	20
1A2	DQ523605	1,919/1,919 (100)	β2	18
1A7	AJ633130	1,244/1,249 (99)	ι2	51
1A9	AP010960	2,001/2,011 (99)	θ	43
1C6	FM180568	1,941/1,952 (99)	α1	52
1D3	AJ308551	1,823/1,824 (99)	ι1	20
1E9	AY696838	1,995/2,001 (99)	ο	42
1F1	DQ523607	1,860/1,880 (99)	γ3	18
1F2	DQ523600	1,925/1,932 (99)	α2	18
1F6	AJ271407	1,901/1,948 (98)	ζ	20
1F9	AJ271407	1,926/1,946 (99)	ζ	20
1G4	CP003109	1,948/1,984 (98)	γ	53
1G8	AP010958	2,023/2,037 (99)	ε	43
2A9	AJ308552	1,926/2,011 (99)	κ	20
2D3	AB647569	1,702/1,710 (99)	ε8	54
2F7	DQ523607	1,678/1,695 (99)	γ3	18
3C11	DQ523613	1,989/1,993 (99)	ρ	18

Different levels of *eae* gene diversity were observed among the different monthly samples; samples for August 2010, March 2011, and April 2011 contained higher levels of *eae* diversity than samples from other months, as indicated by steeper rarefaction curves of these three months.

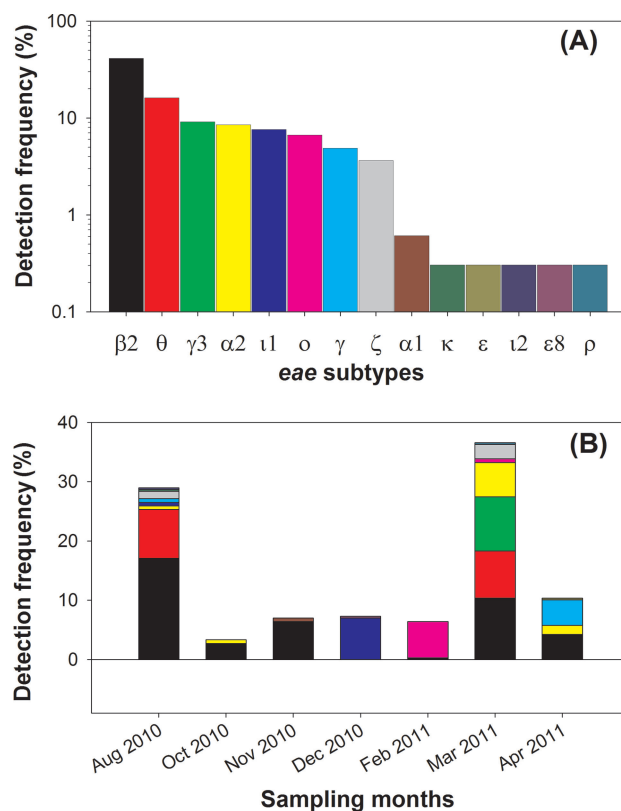


FIG 3 Frequencies of the 13 *eae* subtypes detected in the SIWTP sewage in the clones sequenced (A) and their distribution in the different monthly *eae* clone libraries (B). The same color codes are used for the two panels.

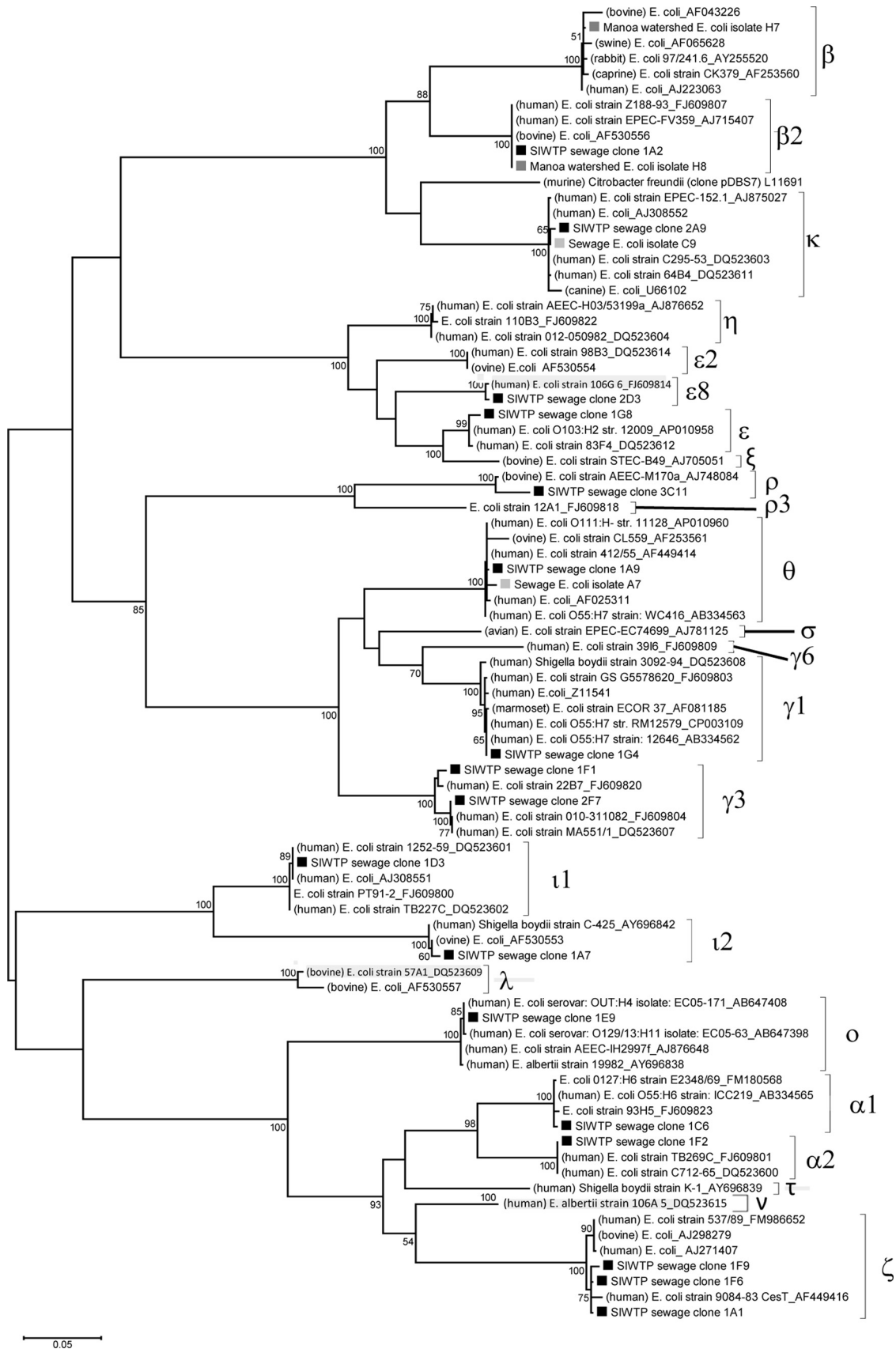


FIG 4 Phylogenetic relationship between the unique SIWTP sewage *eae* clones, unique *eae* genes from sewage and environmental *E. coli* isolates, and known *eae* subtypes from the GenBank database. Entries labeled with black and gray squares represent sequences obtained from clone library sequences and from isolated *E. coli* strains in this study, respectively. The host sources of known *eae* subtypes are provided in parentheses.

The 17 unique *eae* gene sequences were compared with entries from the GenBank database to identify their closest matches and determine their subtypes. All 17 sequences had high identity scores (98 to 100%) to known *eae* gene entries in the database and were subsequently classified to 14 different *eae* subtypes (Table 3). Most of the unique *eae* gene sequences belonged to different subtypes, except for clones 1F1 and 2F3, which were classified as *eae*- γ 3, and clones 1A1, 1F6, and 1F9, which were classified as *eae*- ζ .

The highly polymorphic intimin protein corresponds to the extraordinarily high *eae* genetic diversity, which is indicated by the 27 different *eae* alleles deposited in the GenBank database (18). Previous efforts using *E. coli* isolates from various host sources, including humans (17, 19, 20, 37) and ruminants (19, 22, 38), have detected at least 20 different *eae* subtypes. Fourteen of them, including *eae*- β 2, *eae*- ν 1, *eae*- θ , *eae*- α 2, *eae*- γ , *eae*- θ , *eae*- α 1, *eae*- γ 3, *eae*- κ , *eae*- ϵ , *eae*- ϵ 8, *eae*- ζ , *eae*- ν 2, and *eae*- ρ , were detected in this study by cloning the *eae* gene directly from the sanitary sewage samples. Six *eae* subtypes that were previously reported in the literature, including *eae*- β , *eae*- η , *eae*- ϵ 2, *eae*- λ , *eae*- σ , and *eae*- γ 6, were not detected in the SIWTP sewage samples. With more extensive sequencing efforts, these known *eae* subtypes and even new subtypes could be detected in the sanitary sewage.

Prevalence of *eae* subtypes in sanitary sewage. The prevalence of *eae* subtypes in sanitary sewage was determined by their overall detection frequencies (Fig. 3A) and their temporal variation in the monthly sewage samples (Fig. 3B). Six subtypes, including *eae*- β 2 (41.2%), *eae*- θ (16.2%), *eae*- γ (9.1%), *eae*- α 2 (8.5%), *eae*- ν 1 (7.6%), and *eae*- θ (6.7%), were present in the clone libraries with a detection frequency of >5% and hence were considered to be the dominant subtypes in this study, while the remaining seven subtypes (*eae*- α 1, *eae*- γ 3, *eae*- κ , *eae*- ϵ , *eae*- ϵ 8, *eae*- ζ , *eae*- ν 2, and *eae*- ρ) were considered to be rare subtypes (i.e., detection frequency of <5%).

The overall detection frequencies of the *eae* subtypes (Fig. 3A) were further analyzed in the context of their temporal variation (Fig. 3B). The most prevalent subtype was *eae*- β 2, which was present in six out of seven clone libraries (0.31% to 17.1%) and was represented by more than 50% of the clones in three clone libraries (August, October, and November 2010). The second most frequently detected subtype was *eae*- α 2, which was detected in four of the seven clone libraries with a significantly smaller relative abundance (1.2% to 4.2%) than that of *eae*- β 2. The remaining subtypes, including both the dominant and the rare ones, were detected at much lower frequencies, which indicated a strong temporal fluctuation of these serotypes in municipal sewage. In terms of the *eae* genetic diversity (i.e., the total number of different *eae* subtypes detected) in each month, the sewage samples collected in August 2010, March 2011, and April 2011 contained eight, seven, and four different *eae* subtypes, respectively, while samples from all of the other months contained just two *eae* gene subtypes each, further indicating the temporal component of *eae* genetic diversity in sanitary sewage.

The *eae*- β and *eae*- γ subtypes were the two most frequently detected subtypes in clinical isolates associated with human diarrheal diseases (17, 20, 38, 39). For example, Zhang et al. (20) found *eae*- β and *eae*- γ to be present in 34.2% and 31.5% of the EPEC strains from patients in Germany, respectively (20), while Blanco et al. (17) reported *eae*- β and *eae*- γ to be present in 28.6% and 38.6% of EHEC isolates from patients in Spain (17). However, in the sanitary sewage samples in our study, the prevalence of *eae*- γ

was only 8.3%, and *eae*- β was not detected at all in the sanitary sewage samples. The low prevalence of *eae*- γ in sanitary sewage could be attributed to the low prevalence of EHEC, as observed by qPCR indicating the infrequency and low abundances of *stx*₁ and *stx*₂ genes (Fig. 1), because *eae*- γ is more likely to be associated with EHEC strains such as O157:H7 and O55:H7 (37, 40). The lack of *eae*- β in the SIWTP sanitary sewage was surprising, given that *eae*- β was frequently detected in both diarrhea-related clinical isolates (17, 20, 38, 39) and in environmental samples (35, 36). Instead, *eae*- β 2 was the most dominant *eae* subtype in the SIWTP sanitary sewage. The different *eae* subtype prevalences in clinical isolates and in sanitary sewage were further illustrated by the observation that four of the five major *eae* subtypes detected in the sanitary sewage were infrequently detected in clinical *E. coli* isolates, including *eae*- β 2 (17, 37, 41), *eae*- ν 1 (20), *eae*- θ (42), *eae*- α 2 (18, 19), and *eae*- θ (43).

The *eae* subtypes in sewage and environmental isolates. The 69 *eae*-positive *E. coli* isolates (62 from sanitary sewage and 7 from the Manoa watershed) were first screened using a PCR-RFLP procedure (data not shown), followed by sequencing to determine the *eae* subtypes. The *eae* subtypes from the sewage and environmental *E. coli* isolates were then compared with those detected by *eae* cloning and the known subtypes in the GenBank database by constructing a phylogenetic tree (Fig. 4). Nearly all of the sewage *E. coli* isolates (61/62) exhibited the same RFLP as that of *eae*- κ , which was subsequently confirmed by sequencing of the *eae* gene in the representative strain C9. The other sewage *E. coli* isolate, A7, contained the *eae*- θ gene. Two isolates from the Manoa watershed exhibited the same RFLP as *eae*- β , which was confirmed by sequencing the *eae* gene in the representative strain H7. The other five isolates from the Manoa watershed showed the same RFLP as *eae*- β 2, which was also confirmed by sequencing the *eae* gene in the representative strain H8.

The sewage *eae*-positive *E. coli* isolates contained only two *eae* subtypes, κ and θ , with *eae*- κ being more dominant (61/62). Cloning methods indicated that *eae*- θ was a dominant subtype, while *eae*- κ was a rare subtype (Fig. 3). This discrepancy could be attributable to the temporal variation as the culture-dependent and culture-independent approaches were conducted on sewage samples collected in different years. However, given the large variation in *eae* detection frequencies among the sewage *E. coli* isolates (Table 2), a more probable explanation would be that the culture-based approach, due to its limited sample size, introduced significant bias in examining the *eae* diversity in sanitary sewage. Although only a very small number of *eae*-positive environmental *E. coli* isolates were examined, the detection of *eae*- β and *eae*- β 2 was interesting, given the prevalence of *eae*- β 2 in sanitary sewage (Fig. 3) and the frequent detection of *eae*- β in wildlife feces (19, 44) and in environmental waters (36).

ACKNOWLEDGMENTS

We acknowledge Ken Tenno of the Water Quality Laboratory, Department of Environmental Services of the City and County of Honolulu, for collecting sanitary sewage samples. We also thank Yong Li for preparing *E. coli* O157:H7 cells as qPCR calibration standards.

This material is based upon work partially supported by the National Science Foundation under grant number 0964260 and by the U.S. Environmental Protection Agency under grant number R834871 to T.Y.

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