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Molecular Risk Assessment and Epidemiological Typing of Shiga Toxin-Producing *Escherichia coli* by Using a Novel PCR Binary Typing System▽

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Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen that causes diarrheal disease in humans and is of public health concern because of its ability to cause outbreaks and severe disease such as hemorrhagic colitis or hemolytic-uremic syndrome. More than 400 serotypes of STEC have been implicated in outbreaks and sporadic human disease. The aim of this study was to develop a PCR binary typing (P-BIT) system that could be used to aid in risk assessment and epidemiological studies of STEC by using gene targets that would represent a broad range of STEC virulence genes. We investigated the distribution of 41 gene targets in 75 O157 and non-O157 STEC isolates and found that P-BIT provided 100% typeability for isolates, gave a diversity index of 97.33% (compared with 99.28% for XbaI pulsed-field gel electrophoresis [PFGE] typing), and produced 100% discrimination for non-O157 STEC isolates. We identified 24 gene targets that conferred the same level of discrimination and produced the same cluster dendrogram as the 41 gene targets initially examined. P-BIT clustering identified O157 from non-O157 isolates and identified seropathotypes associated with outbreaks and severe disease. Numerical analysis of the P-BIT data identified several genes associated with human or nonhuman sources as well as high-risk seropathotypes. We conclude that P-BIT is a useful approach for subtyping, offering the advantage of speed, low cost, and potential for strain risk assessment that can be used in tandem with current molecular typing schema for STEC.

Shiga-toxin producing *Escherichia coli* (STEC) is a zoonotic pathogen that causes diarrheal disease in humans and is of public health concern because of its ability to cause outbreaks and severe disease such as hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) (42). *E. coli* O157:H7 is the most prevalent STEC serotype associated with STEC outbreaks, HC, and HUS (20, 30, 42); however, more than 400 serotypes of non-O157 STEC have also been implicated in outbreaks and sporadic human disease, with about 50 of these serotypes being associated with bloody diarrhea or HUS in humans (42, 56, 69). Internationally, the number of reported human diarrheal cases associated with non-O157 STEC (including those leading to HUS) is rising rapidly, mainly due to increased surveillance for these pathogens (13, 18, 27, 35, 61). There is currently no standard method for identifying non-O157 STEC of significance to human health from the over 500 STEC serotypes characterized to date; therefore, the World Health Organization has highlighted the development of such a method as a public health priority (68).

To this end, seropathotype (SPT) classification was developed to identify STEC serotypes linked to outbreaks and/or serious disease based on their relative incidences in human illness (31). Serotypes classified as SPT A (O157:H7 and O157:NM [nonmotile]) or SPT B (O26:H11/NM, O103:H2, O111:H8/NM, O121:H19, and O145:NM) have been associated with outbreaks and HUS; however, SPT A is more frequently reported. SPT C comprises serotypes (e.g., O5:NM, O91:H21, O113:H21, O121:NM, and O128:H2) that have been associated with sporadic cases of HUS but not with outbreaks. SPT D includes the remainder of the STEC serotypes that have been reported to cause sporadic disease and have been associated with diarrhea but not HUS, and serotypes classified as SPT E have not been associated with human illness.

Several studies have identified genetic elements associated with particular seropathotypes that appear to play a role in influencing bacterial virulence (7, 10, 19, 31). Bacterial virulence traits of STEC strains are governed by the dynamic exchange and loss of mobile genetic elements such as plasmids, transposons, insertion sequences, integrons, bacteriophages, and genomic islands (32). These genetic loci characterize the pathogenicity of bacterial species by determining what toxins are produced and how the bacterium attaches to and invades host cells, modulates the host cell cycle and immune responses, survives in stressful environments, and produces biofilms. Many virulence loci have been characterized for STEC, including a number of pathogenicity islands (e.g., the locus of enterocyte effacement [LEE], the locus of proteolysis activity [LPA], the high-pathogenicity island [HPI], the *E. coli* type III secretion apparatus [ETT2], the urease gene cluster, the long polar fimbrial operon, O-island 36 [OI-36], OI-57, OI-71, OI-122, OI-141, and OI-154), two key virulence plasmids (pO157 and pO113), and chromosomal lambdoid bacteriophage insertions that carry genes for two types of Shiga toxin (10, 14, 29, 31, 36, 38, 39, 42, 48, 59, 63). Variants of the genes encoding

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the Shiga toxins have been reported, and certain variants, including stx_2 and stx_{2c}, are more likely to be associated with HC and HUS (3, 15).

A growing body of studies have analyzed the association of many of these virulence genes with severity of disease, as well as the variability of these genes among STEC serotypes (5, 10, 15, 52). PCR-based methods can be used to detect a suite of virulence genes for an *E. coli* isolate. The presence or absence of these genes (binary typing) produces a genetic fingerprint for each isolate (11). The fingerprints can be used to identify strains that have a greater potential to cause harm using a process called molecular risk assessment (10).

We recently developed a PCR binary typing system (P-BIT) for subtyping *Campylobacter jejuni* based on 18 gene targets associated with epidemicity (11). The aim of the present study was to develop a similar P-BIT system that could be used to aid in risk assessment and epidemiological studies of STEC by using gene targets that would represent a broad range of STEC virulence genes. We examined the distribution of 41 virulence genes among STEC isolates to produce "virulence bar codes" for each isolate, which were then compared to typing by pulsed-field gel electrophoresis (PFGE), bacteriophage insertion site genotyping (BISG), seropathotype (SPT), and colonization potential as determined in a bovine *in vitro* organ culture (bIVOC) model infection system (S. M. Brandt and S. Paulin, unpublished data).

MATERIALS AND METHODS

Bacterial isolates. Seventy-five *E. coli* isolates (46 O157 and 29 non-O157) from a variety of human and nonhuman sources were used in this study and are summarized in Table 1.

DNA extraction. Isolates were grown on Luria broth (LB) (Invitrogen, Carlsbad, CA) agar for 18 to 24 h at 37°C. Three colonies were transferred into 10 ml LB and grown for 18 to 24 h at 37°C in a shaking water bath. DNA was extracted from 1 ml of broth culture using the DNeasy blood and tissue kit as per the manufacturer's instructions for Gram-negative bacteria (Qiagen, Hilden, Germany). DNA quantification was performed using a Nanodrop 1000 (Fisher Thermo Scientific, Waltham, MA). DNA was diluted to 50 ng μl^{-1} in sterile 1× Tris-EDTA (TE) buffer (pH 7.0) (Invitrogen) and stored at −20°C.

Binary typing of isolates. Forty-one gene targets for the P-BIT system were chosen based on published information regarding their role in STEC pathogenesis (Table 2). These included genes with an established role in facilitating infection and genes shown to be associated with human illness or severity of clinical infections. In many cases where a cluster of genes (e.g., a pathogenicity island or plasmid) had been implicated in disease, a single representative gene target was chosen. In cases where the variability of genes within a gene cluster had been associated with degrees of bacterial virulence, multiple gene targets from a particular loci were chosen. Detection of gene targets was performed using previously published PCR methods (Table 2).

One PCR per gene target was performed in a 25-μl volume containing 2.5 mM $MgCl_2$, 1× PCR buffer II (50 mM KCl, 10 mM Tris, pH 8.3) (Applied Biosystems, Foster City, CA), 250 μM each deoxynucleoside triphosphate (dNTP), 12.5 pmol each primer, 1.25 U AmpliTaq DNA polymerase (Applied Biosystems), and 50 ng extracted DNA. Thermocycling was performed in either an ABI 9700 or ABI Veriti PCR machine (Applied Biosystems) using the following conditions: 5 min at 95°C followed by 30 cycles of denaturation at 95°C for 30 s, annealing at various temperatures specific to the gene target (listed in Table 2) for 30 s, extension at 72°C for 30 s per 500 bp of amplicon size (listed in Table 2), and then a final extension at 72°C for 10 min.

PCR products were separated by electrophoresis on 2% SeaKem LE agarose gels (Lonza, Rockland, ME) with 1× Tris-borate-EDTA (TBE) (USB Corporation, Cleveland, OH) running buffer for 70 min at 110 V, and amplicons were visualized using 0.5 μg ml^{-1} ethidium bromide. The presence or absence of amplicons of the expected size (Table 2) was loaded into a BioNumerics version 5.10 (Applied Maths, Ghent, Belgium) database. Interstrain relationships were assessed by numerical analysis of the P-BIT data using the simple matching coefficient and Ward's clustering.

P-BIT bar codes were generated as previously published (11). Briefly, genes were divided into groups of three in the order in which they are shown in Table 3. The PCR result for each gene was recorded as 1 if an amplicon of correct size was present and as 0 for a negative result. The result for the first gene in the group was multiplied by 1, the second by 2, and the third by 4. The results were then added together to yield a number from 0 to 7 representing the results from the three genes. The results were sequentially combined by concatenation to form a bar code.

Statistical analysis of P-BIT data. Based on the frequency of detection of genes by PCR in isolates, an association of each P-BIT target gene with O157 or non-O157 STEC isolates, human or nonhuman sources, or dendrogram clusters was determined using the chi-square (χ^2) test. A P value of <0.05 was considered significant. Our results were then compared to expected associations of genes with O157, non-O157, or both O157 and non-O157 STEC isolates based on previously published studies (Table 2). The total numbers of 24-gene P-BIT targets present in isolates from each SPT group, with HUS- or non-HUS-associated serotypes, and with outbreak- or non-outbreak-associated serotypes were compared using a Mann-Whitney U test; this statistical test was chosen because of the variable number of isolates contained in the groups being tested and because the distribution of the data was not always normal.

PFGE typing of isolates. All isolates were analyzed by PFGE using the standardized PulseNet protocol with *Salmonella enterica* serovar Braenderup H9812 digested with XbaI as a sizing standard (21, 54). DNA samples were digested with XbaI and separated by electrophoresis at 6.0 V cm^{-1} for 20 h on a 1% (wt/vol) SeaKem Gold agarose gel (Lonza, Rockland, ME) using initial and final switch times of 2.2 and 54.2 s, respectively. PFGE profiles were analyzed and compared using BioNumerics version 5.10 and submitted to the PulseNet Aotearoa (New Zealand) *E. coli* database, where XbaI pattern designations were assigned.

Shiga toxin-encoding bacteriophage insertion site genotyping. A subset of 25 isolates were analyzed by a genotyping system based on the diversity of insertion sites of the *stx*-carrying bacteriophages (60). A multiplex PCR was performed as previously published using DNA extracted by the method described above (67).

RESULTS

Binary typing. Forty-one gene targets were chosen from published methods to create a PCR-based typing system that would distinguish between O157 and non-O157 STEC isolates and provide information based on virulence gene content (Table 2). Gene targets were chosen based on published information regarding a role in STEC pathogenesis or an association with human disease or severity of disease sequelae. The system included gene targets from a range of known pathogenicity islands, virulence plasmids, and gene clusters so as to provide a broad overview of the virulence gene content of an isolate of interest. Additional gene targets shown to be variable among STEC isolates were also included to provide the system with discriminatory power.

The STEC P-BIT system produced 58 types from 75 STEC isolates, including 29 O157 types from 46 isolates and 29 non-O157 types from 29 isolates. The system had an overall diversity index of 97.33% ± 2.4%, with 100% discriminatory power for non-O157 isolates. The diversity index for O157 isolates was slightly lower, at 92.85% ± 5.87%.

Numerical analysis of P-BIT data. Statistical analysis of the P-BIT results using the χ^2 test was performed to identify genes associated with O157 STEC, with non-O157 STEC, or with both O157 and non-O157 STEC (Table 3). All of the genes that would be expected to be associated with O157 STEC, based on previous studies (Table 2), were significantly associated with O157 STEC isolates ($P < 0.05$), except for stx_{2c} due to the low prevalence of this gene among the O157 isolates that were used in this study. However, stx_{2c} was present mainly in

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TABLE 1. STEC isolates used in this study

Isolate	Serotype	Country	Source	41-gene P-BIT code	SPT	Reference(s)
ERL05-1904	O5:NM	New Zealand	Lamb, minced	611-00000100-1040	C	19
ERL04-2204	O6(rel):NM	New Zealand	Lamb, diced	651-00000140-1441	D	19
ERL05-0344	O8:H25	New Zealand	Lamb, diced	611-00000040-4000	E	22
ERL97-0595	O8:H25	New Zealand	Meat	670-00000000-4040	E	22
NZRM4160	O26:H11	New Zealand	Human	673-17645330-6362	B	31
NZRM4155	O26:NM	New Zealand	Human	663-17645330-2362	B	19
ERL05-1307	O38:H26	New Zealand	Beef, minced	673-00100100-1040	E	22
ERL05-0411	O68:H4	New Zealand	Lamb, minced	711-04122100-1340	D	15
ERL05-1850	O75:H8	New Zealand	Beef, minced	433-04100140-5341	E	22
ERL02-2853	O84:H2	New Zealand	Human	673-55645160-7042	C	15
ERL03-0954	O84:H2	New Zealand	Bovine	653-44644120-7062	C	15
NZRM4163	O84:NM	New Zealand	Human	673-55244160-7240	C	22
NZRM4153	O91:H21	New Zealand	Human	673-00010160-6010	C	31
ERL97-3923	O91:NM	New Zealand	Ovine meat	453-00010050-7043	C	19
ERL04-0388	O107:H51	New Zealand	Human	023-00010000-2342	D	This study
ERL05-1848	O113:H21	New Zealand	Beef, minced	673-65746770-5453	C	31
NZRM3616	O113:H21	New Zealand	Human	673-00004160-4410	C	31
ERL04-2941	O117:H7	New Zealand	Human	043-00000000-2362	D	33, 71
ERL05-1308	O123:H10	New Zealand	Lamb, minced	613-00000140-5341	E	2
ERL05-0622	O128:H2	New Zealand	Lamb, minced	611-00000120-7042	C	19
ERL06-2014	O128:H2	New Zealand	Human	673-00010040-7003	C	19
NZRM4157	O128:NM	New Zealand	Human	673-00010140-7543	D	15
NZRM4162	O130:H11	New Zealand	Human	672-00000140-6000	C	13
ERL99-1671	O145:NM	New Zealand	Human	010-55605340-5000	B	33
NZRM4166	O153:H25	New Zealand	Human	601-04004040-4043	C	22
96/2998	O157:[H7]	Australia	Human	762-77737751-0040	A	31
ACC 3634	O157:H7	Australia	Human	772-77737751-0040	A	31
CDC 16-98	O157	USA	Human	773-77777770-0000	A	31
CDC 20-98	O157	USA	Human	773-77777770-0040	A	31
E27	O157:H7	New Zealand	Animal	771-77777770-0000	A	31
E47	O157:H7	New Zealand	Animal	773-77777770-0000	A	31
E56	O157:H7	New Zealand	Human	773-77777770-0040	A	31
ERL01-4131	O157:H7	New Zealand	Milk	713-77777770-0000	A	31
ERL02-0927	O157:H7	New Zealand	Human	773-77777370-0000	A	31
ERL02-1097	O157:H7	New Zealand	Food	773-77776770-0000	A	31
ERL02-2190	O157:H7	New Zealand	Human	773-77777770-0000	A	31
ERL02-2447	O157:H7	New Zealand	Human	773-77777770-0000	A	31
ERL02-2841	O157:H7	New Zealand	Human	773-77777770-0000	A	31
ERL02-3939	O157:H7	New Zealand	Human	773-77777770-0000	A	31
ERL02-4897	O157:H7	New Zealand	Human	763-77777770-0000	A	31
ERL03-1416	O157:H7	New Zealand	Water	711-76556770-0000	A	31
ERL03-4262	O157:H7	New Zealand	Venison, minced	773-77777751-0000	A	31
ERL04-3476	O157:H7	New Zealand	New Zealand pork	773-77777751-0002	A	31
ERL05-0623	O157:H7	New Zealand	Bobby veal	771-73777770-0041	A	31
ERL05-1306	O157:[H7]	New Zealand	Lamb, diced	771-77777771-0040	A	31
ERL05-1784	O157:H7	New Zealand	Australia pork	733-77777751-0040	A	31
ERL06-2084	O157:[H7]	New Zealand	Water	753-77777750-0000	A	31
ERL06-2442	O157:H7	New Zealand	Bovine	713-77777770-0020	A	31
ERL06-2448	O157:[H7]	New Zealand	Bovine	713-77777770-0040	A	31
ERL06-2456	O157:H7	New Zealand	Bovine	733-77777770-0040	A	31
ERL06-2495	O157:H7	New Zealand	Bovine	713-77777770-0000	A	31
ERL06-2497	O157:H7	New Zealand	Bovine	773-77577760-0040	A	31
ERL06-2503	O157:[H7]	New Zealand	Bovine	773-77777770-0000	A	31
ERL06-2505	O157:H7	New Zealand	Bovine	733-77777770-0000	A	31
ERL06-2517	O157:H7	New Zealand	Bovine	733-77777770-0000	A	31
ERL06-2532	O157:[H7]	New Zealand	Bovine	733-77777770-0000	A	31
ERL08-1225	O157:H7	New Zealand	Spring water	753-77777771-0000	A	31
ERL99-3094	O157:H7	New Zealand	Water	713-77777750-0000	A	31
ERL99-3231	O157:H7	New Zealand	Spring water	773-77777770-0000	A	31
ERL99-4475	O157:H7	New Zealand	Bovine	773-77777770-0000	A	31
NZRM3614	O157:H7	Australia	Human	773-73277730-0000	A	31
NZRM3647	O157:H7	Australia	Human	773-77777730-0000	A	31
NZRM4156	O157:H7	New Zealand	Spring water	773-77777730-0000	A	31
NZRM4159	O157:H7	New Zealand	Bovine	773-77777770-0000	A	31
NZRM4164	O157:H7	New Zealand	Human	773-77777770-0040	A	31
NZRM4168	O157:H7	New Zealand	Human	773-77777770-0000	A	31
NZRM4169	O157:H7	New Zealand	Human	773-77377771-0000	A	31
ERL02-3185	O157:NM	New Zealand	Human	773-77777770-0000	A	31
NZRM4150	O157:NM	New Zealand	Human	773-77777771-0000	A	31
NZRM4165	O157:NM	New Zealand	Human	771-77777370-6641	A	31
ERL04-0301	O157:H16	New Zealand	Human	651-10100040-0000	E	7
ERL05-1845	O176:H4	New Zealand	Pork, minced	713-00022000-1340	E	This study
ERL05-0346	O176:NM	New Zealand	Human	771-00022100-1340	D	This study
ERL04-2759	O177:NM	New Zealand	Human	473-55245360-5042	C	22
ERL98-3865	Ont:H18[a]	New Zealand	Human	611-00002041-4302	D	15

[a] Ont, not O-serotypeable.

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TABLE 2. Primers and PCR conditions for gene targets of the STEC binary typing system

Genes[a]							PCR conditions			
Gene name	Locus	Virulence function	Associated with diarrhea	Associated with HUS and/or outbreaks	Variable among STEC isolates	Reference(s)	Primers (forward/reverse)	Annealing temp (°C)	Product size (bp)	Reference(s)
agn43	Chromosome	Biofilms	×			53	CTGGAAACCGGTCTGCCCTT/CCTGAACGCCCAGGGTGATA	55	433	53
agn43$_{EDL933}$	Chromosome	Biofilms	×			53	CGTATCGCTGTGCCCGATAAC/CCGTATACGAGTTGTCAGAATCA	55	707	46, 53
chuA	Chromosome	Iron uptake	×			44	GACGAACCAACGGTCAGGAT/TGCCGCCAGTACCAAAGACA	55	279	9
cif	Chromosome	Cell cycle control				37	AACAGATGGCAACAGACTGG/AGTCAATGCTTTATGCGTCAT	50	383	4
espC	Chromosome	Hemolysis	×		×	41	TAGTGCAGTGCAGAAAGCAGTT/AGTTTTCCTGTTGCTGTATGCC	55	301	53
iutA	Chromosome	Iron uptake			×	34	GGCTGGACATCATGGGAACTGG/CGTCGGGAACGGGTAGAATCG	60	301	43
paa	Chromosome	Colonization	×			1, 4	ATGAGGAACATAATGGCAGG/TCTGGTCAGGTCGTCAATAC	50	350	4
pic	Chromosome	Hemolysis	×			50	ACTGGATCTTAAGGCTCAGGAT/GACTTAATGTCACTGTTCAGCG	48	572	53
stx$_1$	Chromosome	Toxin	×			15	ATAAATCGCCATTCGTTGACTAC/AGAACGCCCACTGAGATCATC	65→60	180	47
stx$_2$	Chromosome	Toxin	×	×		15	GGCACTGTCTGAAACTGCTCC/TCGCCAGTTATCTGACATTCTG	65→60	255	47
stx$_{2c}$	Chromosome	Toxin	×	×		15	GCGGTTTTATTTGCATTAGT/AGTACTCTTTTCCGGCCACT	50	124	65
stx$_{2d}$	Chromosome	Toxin	×			15	GGTAAAATTGAGTTCTCTAAGTAT/CAGCAAATCCTGAACCTGACG	52	175	65
ECs3737	ETT2	TTSA			×	52	GGAAATCTGCATTAACTCTTCTGC/CGGGAATACCATCCAGTCG	50	530	51
eivF	ETT2	TTSA			×	52	ATTACTGCTCATTGACCGAAGC/GATTTCCAACACGGCTCTGG	60	457	51
etrA	ETT2	TTSA			×	52	CTTCTTCCTAACGAAACATCATTA/TGACATATCAACTTTCTCTTACGC	52	914	51
yqeH	ETT2	TTSA			×	52	CATGCAATAGTTGCTCAATGC/CCCATTCTCTTTTCGATTCG	55	553	51
fyuA	HPI	Iron uptake			×	29	TGATTAACCCCGCGACGGGAA/CGCAGTAGGCACGATGTTGTA	60	784	26
irp2	HPI	Iron uptake			×	29	AAGGATTCGCTGTTACCGGAC/TCGTCGGGCAGCGTTTCTTCT	60	280	29
eaeA	LEE	Attachment	×			25	GACCCGGCACAAGCATAAGC/CCACCTGCAGCAACAAGAGG	65→60	384	47
espA-γ1	LEE	Unknown	×			17	GCGAGTTCTTCGACATC/ACGAATACCAGTTACACTT	55	333	17
espI[b]	LPA	Mucosal			×	59	ATGGACAGAGTGGAGACAG/GCCACCTTTATTCTCACCA	50	560	59
iha	LPA	Attachment			×	59	CAGTTCAGTTTCGCATTCACC/GTATGGCTCTGATGCGATG	50	1,305	59
lpfA$_{O26}$	LPF operon	Attachment			×	62	GTTCTGTTTGCCTTATCTGC/TAAGTCAGGTTGAAGTCGAC	55	509	62
lpfA$_{O113}$	LPF operon	Attachment			×	62	ATGAAGCGTAATATTATAG/TTATTTCTTATATTCGAC	50	573	12

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TABLE 2—*Continued*

Genes[a]							PCR conditions			
Gene name	Locus	Virulence function	Associated with diarrhea	Associated with HUS and/or outbreaks	Variable among STEC isolates	Reference(s)	Primers (forward/reverse)	Annealing temp (°C)	Product size (bp)	Reference(s)
nleC	OI-36	Unknown	×	×		10	ACAGTCCAACTTCAACTT TTCC/ATCGTACCCAGC CTTTCG	55	777	10
nleG2-3	OI-57	Unknown	×	×		10	GGATGGAACCATACCTG G/CGCAATCAATTGCTA ATGC	55	551	10
nleG	OI-71	Unknown	×	×		10	ATGTTATCGCCCTCTTCT ATAAAT/ACTTAATACT ACACTAATAAGATCCA	55	902	10
efa1a	OI-112	Colonization	×	×	×	7, 31, 70	TATCAGGCCAATCAAAAC AG/AGACACTGGTAAA TTTCGC	48	974	24
nleB	OI-112	Colonization	×	×	×	7, 31, 70	GGAAGTTTGTTTACAGA GACG/AAAATGCCGCTT GATACC	55	297	10
pagC	OI-112	Immune evasion	×	×	×	7, 31, 70	ATGAGTGGTTCAAGACT GG/CCAACTCCAACAGT AAATCC	55	521	31
sen	OI-112	Enterotoxin	×	×	×	7, 31, 70	GGATGGAACCATACCTG G/CGCAATCAATTGCTA ATGC	55	551	31
$lpfA_{O157/OI-141}$	OI-141	Attachment			×	62	CTGCGCATTGCCGTAAC/ ATTTACAGGCGAGAT CGTG	55	412	62
$lpfA_{O157/OI-154}$	OI-154	Attachment			×	62	GCAGGTCACCTACAGGC GGC/CTGCGAGTCGGC GTTAGCTG	55	525	62
saa	pO113	Hemolysis			×	8, 49	CGTGATGAACAGGCTATT GC/ATGGACATGCCTGT GGCAAC	60	119	8
subA	pO113	Toxin			×	8, 49	TATGGCTTCCCTCATTGC/ TATAGCTGTTGCTTCT GACG	50	556	49
ehxA	pO157	Hemolysis	×		×	5, 6, 41, 57, 58	GCATCATCAAGCGTACGT TCC/AATGAGCCAAGCT GGTTAAGCT	65→60	534	47
espP	pO157	Mucosal damage	×		×	5, 6, 41, 57, 58	AAACAGCAGGCACTTGA ACG/GGAGTCGTCAGTC AGTAGAT	50	1,830	5
etpD	pO157	Type II secretion			×	5, 6, 57, 58	CGTCAGGAGGATGTTCA G/CGACTGCACCTGTTC CTGATTA	48	1,062	58
katP	pO157	Catalase peroxidase			×	5, 6, 57, 58	TGCATCCGTTGATGATGT TT/TTTCAGGAACGGTG AGATCC	55	720	5
toxB	pO157	Toxin			×	5, 6, 57, 58	ATACCTACCTGCTCTGGA TTGA/TTCTTACCTGAT CTGATGCAGC	55	599	64
ureC	Urease cluster	Ureolysis			×	16, 45	TCTAACGCCACAACCTGT AC/GAGGAAGGCAGAA TATTGGG	50	397	41

[a] ETT2, *E. coli* type III secretion apparatus; HPI, high-pathogenicity island; LEE, locus of enterocyte effacement; LPA, locus of proteolysis activity; LPF, long polar fimbriae; OI, O island; TTSA, type III secretion apparatus.
[b] Also known as *nleA*.

O157 isolates ($n = 9$) in comparison to non-O157 STEC isolates ($n = 1$). Eight of 11 genes that would be expected to be associated with non-O157 STEC, based on previous studies (Table 2), were associated with non-O157 STEC isolates ($P < 0.05$), except for the genes *subA*, *saa*, and *cif* due to the low prevalence of these genes in the non-O157 isolates that were chosen for this study; this was not unexpected for the genes *subA* and *saa*, as they are located on a virulence plasmid associated with certain non-O157 serotypes (8, 49). Seven of the eight genes that would be expected to be found frequently in both O157 and non-O157 STEC were prevalent (>50%) in both O157 and non-O157 STEC isolates; however, three of these genes (*eivF*, *etrA*, and *pic*) were significantly associated with the O157 STEC isolates used in this study.

Statistical analysis of the P-BIT results using the χ^2 test was also performed to identify genes associated with human versus nonhuman sources. Three genes (*pic*, *espC*, and $lpfA_{O26}$) were associated with human isolates. *pic* and *espC* were associated with both O157 and non-O157 human isolates; $lpfA_{O26}$ was associated with non-O157 human isolates. *ECs3737* was the only gene associated with nonhuman isolates.

Cluster analysis of P-BIT data. Interstrain relationships were assessed by preparing a cluster dendrogram of P-BIT data using the simple matching coefficient and Ward's cluster-

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TABLE 3. Association of P-BIT gene targets with O157, non-O157, human, and nonhuman STEC isolates

Isolates in which gene is frequently found[a]	Locus	Gene	O157/non-O157 isolate comparison			Human/nonhuman isolate comparison		
			Prevalence			Prevalence		
			No. (%) O157 (n = 46)	No. (%) non-O157 (n = 29)	χ^2 P value	No. (%) human (n = 36)	No. (%) nonhuman (n = 39)	χ^2 P value
O157 and non-O157	ETT2	*eivF*	45 (98)	3 (10)	<0.001	20 (56)	28 (72)	NS[b]
		etrA	46 (100)	23 (79)	<0.01	32 (89)	37 (95)	NS
		yqeH	46 (100)	26 (90)	NS	33 (92)	39 (100)	NS
		ECs3737	44 (96)	25 (86)	NS	30 (83)	39 (100)	<0.05
	Chromosome	*pic*	37 (80)	16 (55)	<0.05	31 (86)	22 (56)	<0.025
	Chromosome	*espC*	35 (76)	18 (62)	NS	32 (89)	21 (54)	<0.005
	Chromosome	*agn43*	44 (96)	26 (90)	NS	32 (89)	38 (97)	NS
	LPA	*iha*	40 (87)	19 (61)	NS	30 (83)	29 (74)	NS
O157	LEE	*eaeA*	46 (100)	6 (21)	<0.001	26 (72)	26 (67)	NS
		espA-g1	45 (98)	1 (3)	<0.001	19 (53)	27 (69)	NS
	OI-122	*pagC*	45 (98)	6 (21)	<0.001	23 (64)	28 (72)	NS
		sen	44 (96)	7 (21)	<0.001	25 (69)	26 (67)	NS
		efa1a	45 (98)	2 (7)	<0.001	21 (58)	26 (67)	NS
		nleB	43 (93)	11 (38)	<0.001	25 (69)	29 (74)	NS
	OI-36	*nleC*	45 (98)	4 (17)	<0.001	19 (53)	30 (77)	NS
	OI-57	*nleG2-3*	43 (93)	8 (25)	<0.001	25 (69)	26 (67)	NS
	OI-71	*nleG*	43 (93)	6 (31)	<0.001	21 (58)	28 (72)	NS
	OI-154	$lpfA_{O157/OI-154}$	45 (98)	5 (17)	<0.001	23 (64)	27 (69)	NS
	OI-141	$lpfA_{O157/OI-141}$	44 (96)	3 (10)	<0.001	20 (56)	27 (69)	NS
	Chromosome	$agn43_{EDL933}$	43 (93)	7 (24)	<0.001	22 (61)	28 (72)	NS
	Chromosome	*paa*	43 (93)	5 (17)	<0.001	24 (67)	24 (62)	NS
	Chromosome	*chuA*	45 (98)	5 (17)	<0.001	21 (58)	29 (74)	NS
	Chromosome	*ureC*	45 (98)	10 (34)	<0.001	27 (75)	28 (72)	NS
	pO157	*ehxA*	45 (98)	20 (69)	<0.005	30 (83)	35 (90)	NS
		toxB	45 (98)	5 (17)	<0.001	23 (64)	27 (69)	NS
		etpD	43 (93)	1 (3)	<0.001	17 (47)	27 (69)	NS
		katP	44 (96)	4 (14)	<0.001	21 (58)	27 (69)	NS
		espP	38 (83)	10 (34)	<0.001	24 (67)	24 (62)	NS
	Chromosomal	stx_2	43 (93)	17 (59)	<0.001	29 (81)	31 (79)	NS
		stx_{2c}	9 (20)	1 (3)	NS	5 (14)	5 (13)	NS
Non-O157	LPA	*espI*	0 (0)	18 (62)	<0.001	7 (19)	11 (28)	NS
	LPF	$lpfA_{O26}$	1 (2)	13 (45)	<0.001	11 (31)	3 (8)	<0.025
	LPF	$lpfA_{O113}$	1 (2)	20 (69)	<0.001	13 (36)	8 (21)	NS
	HPI	*irp2*	0 (0)	11 (38)	<0.001	7 (19)	4 (10)	NS
		fyuA	1 (2)	11 (38)	<0.001	8 (22)	4 (10)	NS
	pO113	*subA*	1 (2)	4 (14)	NS	3 (83)	2 (5)	NS
		saa	0 (0)	3 (10)	NS	2 (6)	1 (3)	NS
	Chromosome	*cif*	1 (2)	4 (14)	NS	3 (8)	2 (5)	NS
	Chromosome	stx_1	12 (26)	22 (76)	<0.001	16 (44)	18 (46)	NS
	Chromosome	stx_{2d}	2 (4)	8 (28)	<0.025	4 (11)	6 (15)	NS
	Chromosome	*iutA*	1 (2)	14 (48)	<0.001	10 (28)	5 (13)	NS

[a] Based on previously published studies outlined in Table 2.
[b] NS, not significant.

ing (Fig. 1). The 75 isolates were separated into two main branches; branch 1 comprised O157:H7 and O157:NM isolates, and branch 2 comprised all other serotypes. The O157 branch was further segregated into four clusters (clusters 1 to 4) at the 72% similarity level. The non-O157 branch was also segregated into four clusters (clusters 5 to 8) at the 42% similarity level.

Non-O157 serotypes that have been associated with severe disease and outbreaks were found in clusters 7 and 8. These included serotypes commonly associated with HUS (e.g., O26:H11 and O145:NM) and less commonly associated with HUS (e.g., O84:H2, O91:H21, O113:H21, O128:H2, O130:H11, and O177:NM) (13, 19, 22, 31, 33). These isolates were PCR positive for many of the genes previously shown to be associated with diarrheal disease, including many associated with HUS and/or outbreaks (Table 2). The gene targets associated with clusters 7 and 8, in comparison to clusters 5 and 6, by the χ^2 test ($P < 0.05$) included *pic*, *espC*, *iha* (LPA), *eaeA* (LEE), *pagC* (OI-122), *sen* (OI-122), *nleG2-3* (OI-57), *nleG* (OI-71), $agn43_{EDL933}$, *paa*, *ureC*, *toxB* (pO157), *espP* (pO157), $lpfA_{O26}$, and $lpfA_{O113}$.

Human and nonhuman isolates were distributed among all of the dendrogram clusters except cluster 1, which contained only nonhuman O157:H7 STEC isolates (n = 9) from water, bovine, and milk sources. Cluster 6 also contained a subcluster comprised of nonhuman non-O157 STEC isolates (n = 6) from

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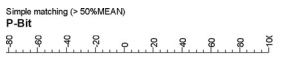

Strain	Serotype	Source	*Xba*I Type	BISG	bIVOC colonisation	
ERL01-4131	O157:H7	Milk	Xb0272			
ERL06-2495	**O157:H7**	**Bovine**	**Xb0040**			
ERL06-2442	O157:H7	Bovine	Xb0164		High	
ERL06-2084	**O157:[H7]**	**Water**	***Xb0070***			
ERL99-3094	O157:H7	Water	Xb0271			Cluster 1
ERL06-2505	**O157:H7**	**Bovine**	***Xb0137***	1		
ERL06-2517	**O157:H7**	**Bovine**	**Xb0040**	1		
ERL06-2532	O157:[H7]	Bovine	Xb0154	1		
ERL03-1416	O157:H7	Water	Xb0179		High	
NZRM3647	O157:H7	Human	Xb0244	11		
NZRM4156	**O157:H7**	**Water**	***Xb0223***	1		
NZRM3614	O157:H7	Human	Xb0197	17		
E47	O157:H7	Animal	Xb0138			
ERL02-2190	O157:H7	Human	Xb0034	1		
ERL02-2447	**O157:H7**	**Human**	***Xb0137***	1		
ERL02-2841	O157:H7	Human	Xb0138	1		
ERL02-3185	O157:NM	Human	Xb0037	1		
ERL02-3939	O157:H7	Human	Xb0047	1		
ERL06-2503	**O157:[H7]**	**Bovine**	**Xb0040**	1	High	Cluster 2
ERL99-3231	**O157:H7**	**Water**	***Xb0223***			
ERL99-4475	**O157:H7**	**Bovine**	**Xb0040**			
NZRM4159	**O157:H7**	**Bovine**	**Xb0040**			
NZRM4168	O157:H7	Human	Xb0156	1		
CDC 16-98	O157	Human	Xb0158	1		
ERL02-0927	O157:H7	Human	Xb0021	1		
ERL02-1097	O157:H7	Food	Xb0045			
ERL02-4897	**O157:H7**	**Human**	***Xb0018***	1		
ERL06-2448	O157:[H7]	Bovine	Xb0144			
ERL06-2456	O157:H7	Bovine	Xb0207			
CDC 20-98	O157	Human	Xb0208	3		
E56	**O157:H7**	**Human**	**Xb0168**	3		
NZRM4164	**O157:H7**	**Human**	**Xb0168**	3		
ERL06-2497	O157:H7	Bovine	Xb0095		Low	Cluster 3
ERL05-1306	O157:[H7]	Lamb	Xb0191		High	
E27	O157:H7	Animal	Xb0138			
ERL05-0623	O157:H7	Veal	Xb0092		Low	
NZRM4165	O157:NM	Human	Xb0048	5	Low	
ERL03-4262	O157:H7	Venison	Xb0120			
ERL04-3476	**O157:H7**	**Pork**	**Xb0070**		High	
ERL05-1784	**O157:H7**	**Pork**	**Xb0070**			
ERL08-1225	O157:H7	Water	Xb0060	5		Cluster 4
NZRM4150	O157:NM	Human	Xb0054	5		
NZRM4169	O157:H7	Human	Xb0041	1	High	
ACC 3634	**O157:H7**	**Human**	**Xb0018**			
96/2998	**O157:[H7]**	**Human**	**Xb0018**	NT		
ERL04-0388	O107:H51	Human	Xb0081			
ERL04-2941	O117:H7	Human	Xb0103			Cluster 5
ERL05-0411	O68:H4	Lamb	Xb0145			
ERL05-1845	O176:H4	Pork	Xb0147			
ERL05-0346	O176:NM	Human	Xb0084			
ERL04-2204	O6(rel):NM	Lamb	Xb0131			
ERL05-1904	O5:NM	Lamb	Xb0088			
ERL05-1307	O38:H26	Beef	Xb0114			
ERL05-0622	O128:H2	Lamb	Xb0177			
ERL05-1308	O123:H10	Lamb	Xb0143			Cluster 6
ERL05-1850	O75:H8	Beef	Xb0001			
ERL05-0344	O8:H25	Meat	Xb0273			
ERL04-0301	O157:H16	Human	Xb0270	5		
ERL98-3865	Ont:H18	Human	Xb0080			
NZRM4166	O153:H25	Human	Xb0198			
ERL97-0595	O8:H25	Lamb	Xb0134			
NZRM4162	O130:H11	Human	Xb0101			
NZRM3616	O113:H21	Human	Xb0213			
NZRM4153	O91:H21	Human	Xb0130			Cluster 7
ERL06-2014	O128:H2	Human	Xb0055			
ERL97-3929	O91:NM	Ovine	Xb0002			
NZRM4157	O128:NM	Human	Xb0180			
ERL02-2853	O84:H2	Human	Xb0083			
ERL04-2759	O177:NM	Human	Xb0076			
NZRM4163	O84:NM	Human	Xb0217			
ERL03-0954	O84:H2	Bovine	Xb0274			Cluster 8
ERL05-1848	O113:H21	Beef	Xb0219			
ERL99-1671	O145:NM	Human	Xb0076			
NZRM4155	O26:NM	Human	Xb0025			
NZRM4160	O26:H11	Human	Xb0141			

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beef and lamb sources. All but one isolate in both of these clusters was negative for one or both of the *pic* and *espC* genes. Statistical analysis by the χ^2 test confirmed that these two genes were not associated with isolates in these clusters ($P < 0.005$).

Comparison with other typing methods. Pulsed-field gel electrophoresis (PFGE) typing of isolates using XbaI macrorestriction of genomic DNA was more discriminatory than P-BIT typing (Fig. 1). PFGE typing produced diversity indexes of 99.28% ± 0.66% (in comparison to 97.33% for P-BIT) when comparing all 75 STEC isolates and 98.07% ± 1.63% (in comparison to 92.85% for P-BIT) when comparing O157 STEC isolates only. Both P-BIT and PFGE XbaI typing were completely discriminatory for non-O157 STEC. There was good correspondence between isolates that shared PFGE XbaI types (shown in bold in Fig. 1) and clustering produced by P-BIT. Although PFGE XbaI typing was more discriminatory overall, P-BIT could discriminate most isolates that PFGE XbaI typing could not, except for two isolates that shared type Xb0168 and three isolates that shared type Xb0040.

Bacteriophage insertion site genotyping (BISG) also corresponded well with clusters formed by PBIT typing (Fig. 1). BISG type 1 was mostly present in clusters 1 and 2. BISG types 3 and 5 were contained in clusters 3 and 4.

The colonization potentials of nine O157 STEC isolates had been previously characterized using a bovine *in vitro* organ culture (bIVOC) system (Brandt and Paulin, unpublished data). The bIVOC model infection system identified low- and high-colonizing strains using bovine colonic tissue. High-colonizing strains identified by this study were present in P-BIT clusters 1, 2, and 4 (Fig. 1). All of the low-colonizing strains were present in cluster 3. One high-colonizing strain was present in cluster 3; however, this strain was PCR positive for stx_{2c}, a defining genetic feature of isolates contained in cluster 4 and a gene that has been shown to be associated with HUS (15).

Minimum STEC P-BIT set. A subset of 24 gene targets could discriminate O157 from non-O157 isolates and generated the same cluster groups as the full set of 41 gene targets (Fig. 2). These cluster groupings correlated well with seropathotype classification, with SPT A located in clusters 1 to 4, SPT B and C mainly in clusters 7 and 8, and SPT D and E mainly in clusters 5 and 6.

The 24-gene target P-BIT system produced an 8-digit bar code that could be used for strain typing and risk assessment. High numbers, with 7 being the maximum, indicated PCR-positive results for many target virulence genes. Isolates classified as SPT A to C contained bar codes with high numbers at most positions, indicating potential to cause severe disease, whereas isolates classified as SPT D or SPT E had low numbers at most positions, indicating low potential to cause severe disease.

The total number of positive PCR results using the 24-gene target P-BIT system correlated with predicted STEC virulence based on the seropathotyping scheme (Fig. 3). Serotypes classified as SPT A or SPT B had the most P-BIT targets present (averages of 16.5 and 15.3 genes, respectively), followed by SPT C (average of 10.3 genes), then SPT D (average of 6.6 genes), and lastly SPT E (average of 5.6 genes). There was no significant difference between the number of P-BIT targets present in serotypes classified as SPT A or SPT B or between serotypes classified as SPT D or SPT E using a two-tailed Mann-Whitney U test; however, there were significantly greater numbers of P-BIT targets present in serotypes associated with HUS or outbreaks (averages of 15.1 and 16.4 genes, respectively) in comparison to serotypes not associated with HUS or outbreaks (averages of 6.1 and 8.1 genes, respectively). These results established that that the STEC P-BIT system can identify high-risk serotypes of importance to public health; however, it does not discriminate between serotypes that cause sporadic cases of diarrhea and serotypes that are not associated with disease in humans.

DISCUSSION

The aim of this study was to develop a novel subtyping method for STEC that could also provide information about the potential for a given isolate to cause disease in humans. We have chosen to develop a PCR-based approach because this platform is commonly available to research and reference laboratories, does not require expensive equipment or consumables, and is not labor-intensive and because methods can easily be standardized across laboratories. By producing a simple bar code derived from the presence or absence of virulence genes as detected by PCR, P-BIT results can easily be exchanged via e-mail, collected by commonly used database programs, and compared without a need for specialized software or training. In addition, the STEC P-BIT system was built with gene targets from an array of known genomic and extrachromosomal virulence loci that are known to be distributed among O157 and non-O157 STEC isolates so as to offer complete typeability.

Phenotypic methods for typing STEC, such as serotyping and phage typing, offer limited applicability, as few laboratories are equipped to carry out the full range of O- and H-antigen serotyping and phage typing has not yet been developed for non-O157 STEC (28). Octamer-based genome scanning (OBGS), lineage-specific polymorphism assay (LSPA), single nucleotide polymorphisms (SNPs), and microarray analysis are molecular methods with the potential for high discriminatory power; however, protocols have not been developed for application of these methods to non-O157 STEC, and expensive sequencing or microarray scanning equipment is required (28). Random amplification of polymorphic DNA (RAPD) typing is a simple and inexpensive PCR-based method that has been developed for both O157 and

FIG. 1. Cluster dendrogram of 41-gene P-BIT data in comparison with PFGE typing using XbaI macrorestriction of genomic DNA (54), bacteriophage insertion site genotyping (BISG) (67), and colonization potential (high or low) of bovine *in vitro* organ culture (Brandt and Paulin, unpublished data). Strains that share the same PFGE XbaI profile with another strain and that also cluster closely based on P-BIT type are highlighted in bold. Strains that share the same PFGE XbaI profile with another strain but do not cluster closely based on P-BIT type are highlighted in bold and italic. Ont, not O-serotypeable.

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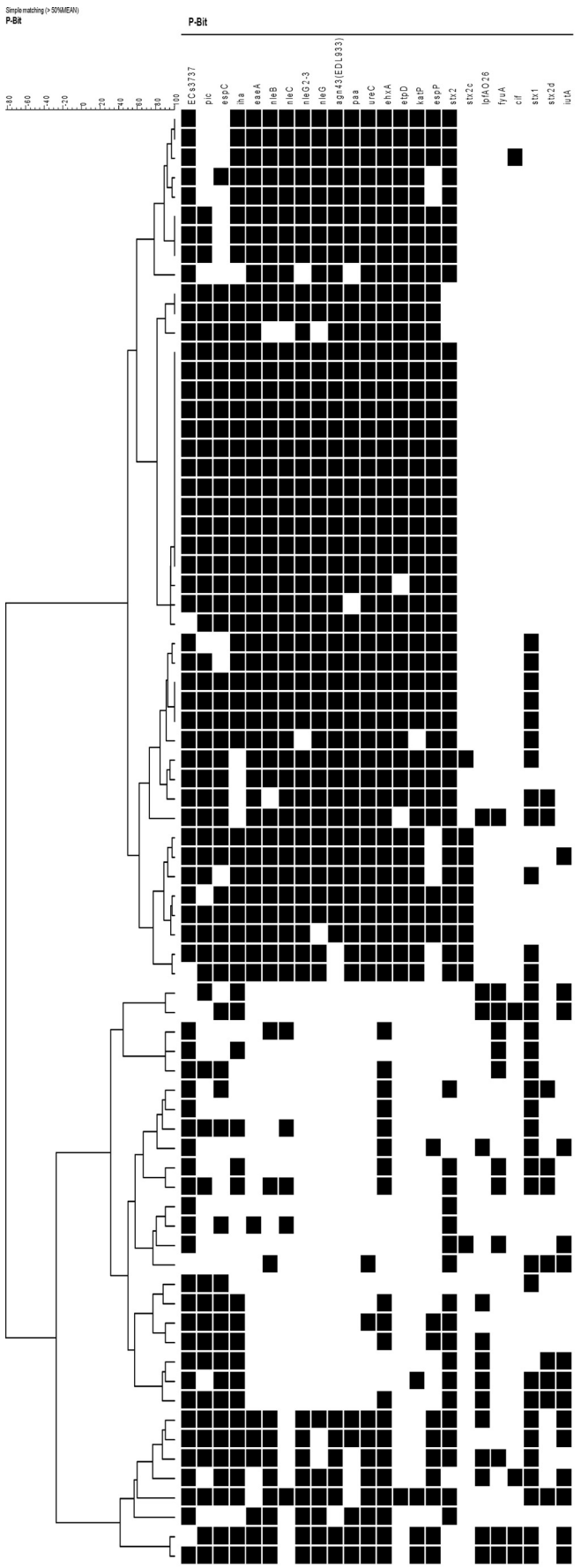

Strain	Serotype	24 P-BIT Code	SPT
ERL01-4131	O157:H7	17777300	A
ERL06-2495	O157:H7	17777300	A
ERL06-2442	O157:H7	17777340	A
ERL06-2084	O157:[H7]	57777200	A
ERL99-3094	O157:H7	17777200	A
ERL06-2505	O157:H7	37777300	A
ERL06-2517	O157:H7	37777300	A
ERL06-2532	O157:[H7]	37777300	A
ERL03-1416	O157:H7	16557300	A
NZRM3647	O157:H7	77777100	A
NZRM4156	O157:H7	77777100	A
NZRM3614	O157:H7	73277100	A
E47	O157:H7	77777300	A
ERL02-2190	O157:H7	77777300	A
ERL02-2447	O157:H7	77777300	A
ERL02-2841	O157:H7	77777300	A
ERL02-3185	O157:NM	77777300	A
ERL02-3939	O157:H7	77777300	A
ERL06-2503	O157:[H7]	77777300	A
ERL99-3231	O157:H7	77777300	A
ERL99-4475	O157:H7	77777300	A
NZRM4159	O157:H7	77777300	A
NZRM4168	O157:H7	77777300	A
CDC 16-98	O157	77777300	A
ERL02-0927	O157:H7	77775300	A
ERL02-1097	O157:H7	77757300	A
ERL02-4897	O157:H7	67777300	A
ERL06-2448	O157:[H7]	17777301	A
ERL06-2456	O157:H7	37777301	A
CDC 20-98	O157	77777301	A
E56	O157:H7	77777301	A
NZRM4164	O157:H7	77777301	A
ERL06-2497	O157:H7	77573301	A
ERL05-1306	O157:[H7]	76777701	A
E27	O157:H7	76777300	A
ERL05-0623	O157:H7	72777303	A
NZRM4165	O157:NM	76775333	A
ERL03-4262	O157:H7	77777600	A
ERL04-3476	O157:H7	77777604	A
ERL05-1784	O157:H7	37777601	A
ERL08-1225	O157:H7	57777700	A
NZRM4150	O157:NM	77777700	A
NZRM4169	O157:H7	77377700	A
ACC 3634	O157:H7	77767601	A
96/2998	O157:[H7]	67767601	A
ERL04-0388	O107:H51	21000035	D
ERL04-2941	O117:H7	41000075	D
ERL05-0411	O68:H4	14101021	D
ERL05-1845	O176:H4	11000021	E
ERL05-0346	O176:NM	70001021	D
ERL04-2204	O6(rel):NM	50001203	D
ERL05-1904	O5:NM	10001001	C
ERL05-1307	O38:H26	71101001	E
ERL05-0622	O128:H2	10001115	C
ERL05-1308	O123:H10	11001223	E
ERL05-1850	O75:H8	35101223	E
ERL05-0344	O8:H25	10000200	E
ERL04-0301	O157:H16	52100200	E
ERL98-3865	Ont:H18	10000624	D
NZRM4166	O153:H25	04040207	C
ERL97-0595	O8:H25	70000001	E
NZRM4162	O130:H11	71001210	C
NZRM3616	O113:H21	71041300	C
NZRM4153	O91:H21	71001310	C
ERL06-2014	O128:H2	71000216	C
ERL97-3929	O91:NM	51004217	C
NZRM4157	O128:NM	71001217	D
ERL02-2853	O84:H2	77671315	C
ERL04-2759	O177:NM	77271305	C
NZRM4163	O84:NM	77251331	C
ERL03-0954	O84:H2	55651155	C
ERL05-1848	O113:H21	75757307	C
ERL99-1671	O145:NM	16661200	B
NZRM4155	O26:NM	67675175	B
NZRM4160	O26:H11	77675175	B

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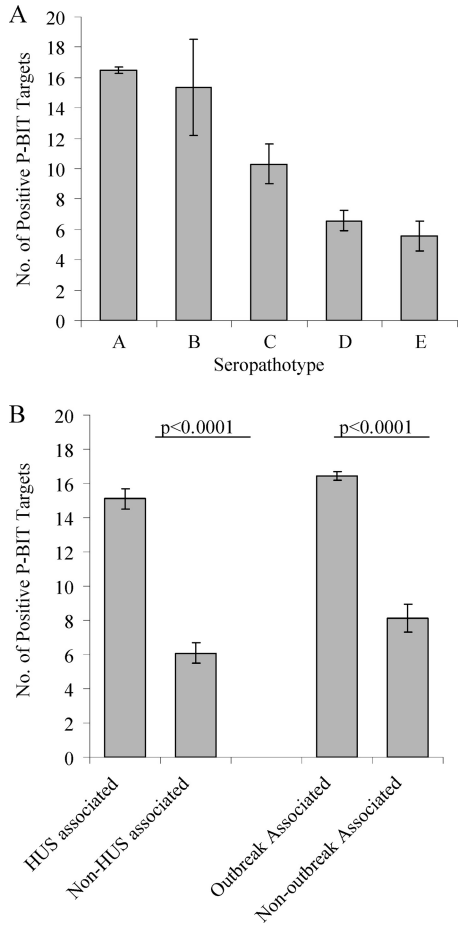

FIG. 3. Correlation between the presence of P-BIT gene targets (based on the 24-gene scheme), seropathotype classification, and a serotype's reported association with HUS or outbreaks. (A) The greatest number of P-BIT gene targets were present in serotypes associated with outbreaks and HUS (SPT A and SPT B), followed by strains associated with HUS only (SPT C), strains associated with diarrhea (SPT D), and lastly strains not associated with human disease (SPT E). (B) HUS (SPT A-C)- and outbreak (SPT A and B)-associated serotypes were positive for a significantly higher number of P-BIT gene targets than were non-HUS (SPT D and E)- and non-outbreak (SPT C-E)-associated serotypes. Bars indicate the average number of positive PCR results, and error bars indicate the standard error of the mean. Statistical significance was determined by a two-tailed Mann-Whitney U test.

non-O157 STEC; its main disadvantage lies in difficulties with method standardization and reproducibility (28). Pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) are highly discriminatory typing methods that are suitable for subtyping O157 and non-O157 STEC; however, these methods are labor-intensive and require expensive equipment and software to facilitate comparisons of strain types (28). Multiple-locus variable-number tandem-repeats analysis (MLVA) is highly discriminatory, rapid, and low-cost; however, standard protocols for O157 STEC have not yet been agreed upon, and only one protocol has been developed for a non-O157 serotype, O26 (28).

Of these methods, PFGE is the most commonly used STEC subtyping method and has provided essential strain identification for disease surveillance and outbreak investigations (28). Our results indicate that PFGE typing using XbaI macrorestriction of genomic DNA is more discriminatory for O157 STEC than P-BIT; however, the two typing methods had equal discriminatory power for non-O157 STEC. Some correspondence was observed between PFGE XbaI type relatedness and P-BIT profile relatedness, which is not unexpected as both methods are dependent on the genomic content of strains. P-BIT was able to discriminate some strains that PFGE was not and vice versa, suggesting that the two subtyping methods may be suitable to use in tandem, especially given that although PFGE is more discriminatory, P-BIT results can be obtained faster and indicate seropathotype along with virulence potential.

The strength of P-BIT is the ability to subtype in conjunction with a broad assessment of STEC pathogenicity that can be used for all STEC isolates. P-BIT had a high discriminatory power for STEC, including O157 STEC, which is a challenging organism to subtype due to the clonal relatedness of strains (66). Cluster analysis of P-BIT results identified clusters of strains that based on SPT classification have been more frequently associated with outbreaks and severe disease (clusters 1 to 4, 7, and 8) and clusters of strains that have been reported less frequently or have not been involved in human disease (clusters 5 and 6). In addition, statistical analysis of P-BIT results identified genes associated with human or nonhuman isolates. This information was used to build a minimum P-BIT system comprised of 24 gene targets that was as discriminatory as the full gene system and generated the same cluster dendrogram.

The 24-gene target P-BIT system produced a genetic fingerprint of STEC virulence by detecting genes that have been associated with outbreaks and severe disease, including the LEE, OI-36, OI-57, OI-71, OI-122, pO157, the long polar fimbrial operon, the urease gene cluster, stx_1, and stx_2. We recognize that P-BIT data provide indicative but not absolute data regarding the presence or absence of a gene, as sequence variation of target genes at the site of primer annealing might result in a false-negative PCR result. This is a compromise to build a rapid, low-cost system for assessing virulence and strain type.

Molecular risk assessment for non-O157 STEC has greatly advanced due to a number of recent studies that found a strong association between the presence of non-LEE effectors (*nle*) located in STEC O islands with serotypes classified as SPT A to C, and thereby also associated with outbreaks and HUS (7, 10, 19, 23, 31). Our results supported these findings, and there-

FIG. 2. Cluster dendrogram of 24-gene P-BIT data in comparison with seropathotype (SPT). Serotypes classified as SPT A and B have been associated with HUS and outbreaks. Serotypes classified as SPT C have been associated with sporadic HUS but not outbreaks. Serotypes classified as SPT D have been associated with diarrhea. Serotypes classified as SPT E have not been associated with human disease. P-BIT bar codes were generated as previously published (11).

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fore gene targets to detect OI-36, OI-57, OI-71, and OI-122 were included in the minimum P-BIT system. These gene targets were necessary for generating the clusters that corresponded well with SPT classification but were not sufficient; our work also identified other gene targets (*pic*, *espC*, and *iha*) that were important for separating serotypes classified as SPT C from serotypes classified as SPT D and SPT E. Previous studies have shown that other methods used to form *E. coli* phylogenetic groupings, such as multilocus sequence typing (MLST) and multilocus enzyme electrophoresis (MLEE), do not correlate with SPT classification and are not useful for assessing the public health risk of isolates (71). Because P-BIT analyzes the virulence gene content of isolates, it is a much better predictor of SPT classification and thereby the potential for a strain to cause serious illness. P-BIT clustering and SPT classification were not in complete agreement; however, serotyping is not an absolute predictor of strain virulence, and we would argue that examining the virulence gene content of a strain and how this compares to those of other known highly pathogenic strains is more objective and potentially a more accurate predictor of risk to human health. This is supported by Scheutz, who argued that a classification system based on a virulence profile, which could be fluid as more information regarding pathogenic mechanisms became available, would be better suited for grouping isolates into those capable of causing severe disease (outbreaks/HUS), those that are likely to cause sporadic diarrheal disease, and those that are associated only with animals [F. Scheutz, presented at the Public Health Significance of Non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Public Meeting, Arlington, VA, 17 October 2007].

To this end, P-BIT bar codes used in combination with P-BIT clustering can, in principle, be used for risk assessment of isolates based on P-BIT type and on whether the isolate clusters with other isolate types known to be associated with outbreaks or severe disease. We have demonstrated that 24-gene P-BIT clustering corresponds well with SPT classification. The true test for the P-BIT typing system as a risk assessment tool will come when it is routinely used side by side with other established typing methods, such as PFGE, to analyze clinical isolates, investigate outbreaks, and attempt to establish attribution.

In addition to comparison with epidemiological data, molecular risk assessment will be improved through more work comparing genetic fingerprints with phenotypic characteristics of isolates, especially traits involved in bacterial pathogenesis. P-BIT clustering was compared with a recent study characterizing the colonization potentials of a small number of O157 STEC isolates in a model bovine infection system (Brandt and Paulin, unpublished data). Isolates with low potential for colonizing bovine tissue were all located in cluster 3. This suggests that genetic determinants specific to cluster 3 may be associated with reduced colonization of cattle tissues. Interestingly, isolates in cluster 3 were of BISG types 3 ($n = 3$) and 5 ($n = 1$). These BISG types are frequently found in cattle around the world, apart from Australia and Germany (67). The prevalence of these BISG types in New Zealand cattle is unknown. Also, since certain O157 phage types have been found more frequently in animals than in humans (40, 55), further evaluation of P-BIT clustering with O157 phage typing could reveal that cluster 3 and the nonhuman cluster 1 correspond to particular O157 phage types associated with animals.

In conclusion, the STEC P-BIT typing system offers subtyping in a rapid, low-cost format and enables STEC strains to be assessed for the potential risk to public health. Molecular risk assessment is a useful tool to aid in public health surveillance and outbreak investigations; such uses could include providing information to help public health officials gauge the level of mobilization of resources required to address a potential threat, as well as a tool to monitor over time the emergence of new potentially dangerous STEC strains. Molecular risk assessment can also be used for source attribution and can provide important information for targeted intervention along the farm-to-fork continuum. Such tools are of paramount importance to address the increasing public health concern about non-O157 STEC.

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