Escherichia coli H-Genotyping PCR: a Complete and Practical Platform for Molecular H Typing

Masaya Banjo,^a Atsushi Iguchi,^b Kazuko Seto,^c Taisei Kikuchi,^d Tetsuya Harada,^e Flemming Scheutz,^{f,g} Sunao Iyoda,^h
Pathogenic *E. coli* Working Group in Japan

^aInternational Course of Agriculture, Graduate School of Agriculture, University of Miyazaki, Miyazaki, Japan

^bDepartment of Animal and Grassland Sciences, Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan

^cDivision of Planning, Osaka Institute of Public Health, Osaka, Japan

^dDepartment of Infectious Diseases, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

^eDivision of Microbiology, Osaka Institute of Public Health, Osaka, Japan

^fDepartment of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark

^gThe International Centre for Reference and Research on *Escherichia* and *Klebsiella*, Statens Serum Institut, Copenhagen, Denmark

^hDepartment of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan

ABSTRACT In *Escherichia coli*, more than 180 O groups and 53 H types have been recognized. The O:H serotyping of *E. coli* strains is an effective method for identifying strains with pathogenic potential and classifying them into clonal groups. In particular, the serotyping of Shiga toxin-producing *E. coli* (STEC) strains provides valuable information to evaluate the routes, sources, and prevalence of agents in outbreak investigations and surveillance. Here, we present a complete and practical PCR-based H-typing system, *E. coli* H-genotyping PCR, consisting of 10 multiplex PCR kits with 51 single PCR primer pairs. Primers were designed based on a detailed comparative analysis of sequences from all H-antigen (flagellin)-encoding genes, *fliC* and its homologs. The specificity of this system was confirmed by using all H type reference strains. Additionally, 362 serotyped wild strains were also used to evaluate its practicality. All 277 H-type-identified isolates gave PCR products that corresponded to the results of serological H typing. Moreover, 76 nonmotile and nine untypeable strains could be successfully subtyped into any H type by the PCR system. The *E. coli* H-genotyping PCR developed here allows broader, rapid, and low-cost subtyping of H types and will assist epidemiological studies as well as surveillance of pathogenic *E. coli*.

KEYWORDS *Escherichia coli*, PCR, flagellin, serotyping

Serotyping of isolates with O (lipopolysaccharide) and H (flagellar) antigens is used as a conventional subtyping strategy in epidemiological studies of pathogenic *Escherichia coli* (1). The flagellar filament is composed of a single protein, flagellin, which is composed of four major domains: D0 and D1, forming the flagellar inner and outer tube, respectively, and D2 and D3, exposed on the surface of the flagellar filament (2). The D0 and D1 domains from both the N- and C-terminal ends are highly conserved within and between bacterial species, whereas the D2 and D3 domains from the middle region of the protein are hypervariable; this presumably is the cause of the H-antigen diversity (2). H antigens of motile isolates are identified by using H-specific antibodies in agglutination tests (1). Thus far, the International Centre for Reference and Research on *Escherichia* and *Klebsiella*, based at the Statens Serum Institut (SSI) in Denmark (<http://www.ssi.dk/English.aspx>), has recognized 53 *E. coli* H types, from H1 to H56, including three missing numbers: H13, H22, and H50 (3). Although the H typing of *E. coli* strains provides valuable information for subtyping of strains in outbreak investigations and surveillance, for the expression of flagellar proteins required to obtain a valid result,

Received 31 January 2018 Returned for
modification 5 March 2018 Accepted 12
March 2018

Accepted manuscript posted online 28
March 2018

Citation Banjo M, Iguchi A, Seto K, Kikuchi T,
Harada T, Scheutz F, Iyoda S, Pathogenic *E. coli*
Working Group in Japan. 2018. *Escherichia coli*
H-genotyping PCR: a complete and practical
platform for molecular H typing. J Clin
Microbiol 56:e00190-18. <https://doi.org/10.1128/JCM.00190-18>.

Editor Nathan A. Ledebor, Medical College of
Wisconsin

Copyright © 2018 American Society for
Microbiology. All Rights Reserved.

Address correspondence to Atsushi Iguchi,
iguchi@med.miyazaki-u.ac.jp.

strains need to be subcultured in semisolid agar several times. Full H serotyping is performed by agglutination with a pool of antisera followed by agglutination with the corresponding single antiserum. Thus, the H serotyping procedure is laborious and time-consuming. Furthermore, in an early stage of H typing, nonmotile isolates are classified as an H⁻ (minus) or HNM (nonmotile) group.

In *E. coli*, flagellin is encoded by the *fliC* gene on the chromosomal locus or its homologues (non-*fliC* flagellin-coding genes) at loci other than *fliC*, such as *flkA*, *fliA*, and *flmA* (4–8). When a mobile element carrying non-*fliC* and its attendant gene is inserted in the chromosome, these genes may mediate reversible flagellar phase variation. For example, Feng et al. (4) showed that the inserted *flkAB* operon expresses the FlkA flagellin and the repressor FlkB protein, which represses the expression of the existing *fliC*. Nine H types are encoded by non-*fliC*: *flkA* of H3, H35, H36, H47, and H53; *fliA* of H44 and H55; *fliA* of H17; and *flmA* of H54 (4–8).

Wang et al. (8), comparing *fliC* sequences from 43 H types, showed sequence properties with a highly variable central region between more conserved 5' (ca. 530 bp) and 3' (ca. 280 bp) regions. Recently, Joensen et al. (9) compared *fliC* and non-*fliC* sequences, covering all 53 H types, and showed that sequences within the same H type were highly conserved in similarity (at least $\geq 97\%$), whereas different H type sequences showed low sequence similarity (mostly $< 70\%$), except for pairs H1/H12 (97.4 to 94.7%), H5/H56 (92.0 to 92.7%), H8/H40 (94.5%), and H30/H32 (94.0%).

Based on these results, we believe that the remarkable sequence diversity and conservation in *fliC* and non-*fliC* genes is sufficient to make a molecular methodology for identifying *E. coli* H types. To date, researchers have developed various PCR-based methods for H typing. PCR-restriction fragment length polymorphism (RFLP) analysis of PCR products obtained by universal *fliC* primers is a useful technique for identifying broad H types (10, 11). However, this method does not cover non-*fliC* H types, and it occasionally fails to amplify *fliC* in some isolates. H type-specific PCR methods targeting the unique *fliC* sequences have also been reported (12–16). However, the typeable H types are limited, especially in Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) major serotypes. Broader, rapid, and low-cost methodologies for identifying H types may assist epidemiological studies as well as surveillance of pathogenic *E. coli*. Here, we present a basic set of 52 PCR primer pairs and a practical PCR-based H typing system comprising 10 multiplex PCR kits (*E. coli* H-genotyping PCR) for the comprehensive H typing of *E. coli*.

MATERIALS AND METHODS

Source sequences. Sequences of the flagellin-coding genes from H1 to H56 were obtained from the GenBank database, having been deposited there in a previous study (9) (see Table S1 in the supplemental material).

Sequence comparison. Multiple alignments of DNA sequences were constructed by using the CLUSTAL W program (17). Homology comparisons of paired sequences were performed using IMC (*in silico* molecular cloning) software (In Silico Biology, Yokohama, Japan). Pairwise distances between DNA sequences for symmetric matrices were calculated using ProtDist in the PHYLIP package (v3.696; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle, WA [<http://evolution.genetics.washington.edu/phylip.html>]) with the JTT model from DNA alignment generated by MAFFT (v7.164) with the L-INS-i option.

Bacterial strains. H type reference strains (from H1 to H56) from SSI were used to evaluate our PCR primers in simplex and multiplex PCR assays (Table S1). Additionally, 362 O:H serotyped wild *E. coli* isolates, including 277 H type-identified, 76 nonmotile, and nine H-untypeable isolates, were used for validation of the comprehensive multiplex PCR system. Serotyping of O and H antigens was performed according to the agglutination test using commercially available pooled and single antisera against all recognized *E. coli* O antigens (O1 to O188) and H antigens (H1 to H56) (SSI Diagnostica, Hillerød, Denmark). The motility was evaluated in a test tube with 0.3% semisolid agar of LB culture. The nonmotile isolates were uniformly classified as H⁻. Genomic DNAs were purified using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Simplex PCR. Simplex PCR was performed with the following protocol: each 30- μ l reaction mixture contained 2 μ l of genomic DNA, 6 μ l of 5 $\times$ Kapa Taq buffer, deoxynucleoside triphosphate (dNTP) mix (final concentration, 0.3 mM each), MgCl₂ (final concentration, 2.5 mM), primers (final concentration, 0.5 μ M each), and 0.8 U of Kapa Taq DNA polymerase (Kapa Biosystems, Woburn, MA, USA). The thermocycling conditions were 25 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. PCR products (2 μ l)

FIG 1 Sequence comparisons of *fliC* between three pairs. (A) Genetic organization of the *fliC* and its flanking genes. (B) Sequence comparisons of *fliC* between three pairs, H21 (1,476 bp) versus H40 (1,479 bp), H20 (1,731 bp) versus H46 (1,719 bp), and H1 (1,788 bp) versus H34 (1,638 bp). Line graphs show percent DNA sequence identity calculated with a 20-bp sliding window and a 5-bp step size. Gray boxes in the graphs indicate regions containing a gap(s) in comparative windows.

were electrophoresed in 1.5% agarose gels in 0.5× TBE (25 mM Tris-borate, 0.5 mM EDTA) and photographed under UV light after the gel was stained with ethidium bromide (1 mg/ml).

Multiplex PCR. Multiplex PCR was performed with the following protocol: each 30- μ l reaction mixture contained 2 μ l of genomic DNA, 6 μ l of 5× Kapa Taq buffer, dNTP mix (final concentration, 0.3 mM each), $MgCl_2$ (final concentration, 2.5 mM), primers (final concentration, 0.5 μ M each), and 0.8 U of Kapa Taq DNA polymerase. The thermocycling conditions included an initial denaturation at 94°C for 1 min, followed by 25 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and then a final extension at 72°C for 2 min. PCR products were separated and visualized by the same method as that described for simplex PCR.

RESULTS

Sequence comparison. To confirm the sequence conservation of 5′ and 3′ regions as well as the variation of the central region of *fliC*, three pairs of *fliC* genes, H21 (1,476 bp) versus H40 (1,479 bp), H20 (1,731 bp) versus H46 (1,719 bp), and H1 (1,788 bp) versus H34 (1,638 bp), were probatively compared.

The 5′ 500- to 530-bp regions and 3′ 240- to 270-bp regions were highly (mostly >90%) conserved between *fliC* sequences, whereas central regions contained several gaps and sequence homologies were mostly less than 90% (Fig. 1). Subsequently, sequences separated into three regions from *fliC* genes of all 53 H types were compared in each region (Fig. 2). The sequences of the 5′ region (519 bp) and 3′ region (267 to 270 bp) were divided into two groups. The composition of the groups was the same in

FIG 2 Symmetric matrices of pairwise distances and those clustering trees within 5', central, and 3' regions of *fltC* and non-*fltC* genes. DNA sequences from all 53 H types were used. Heatmap boxes indicated in red represent high homology between DNA sequences.

FIG 3 Location of SNPs on *fliC* between four pairs. A vertical line in gene illustrations indicates an SNP between H1 versus H12, H30 versus H32, H5 versus H56, and H8 versus H40. The locations of PCR primers designed on each *fliC* gene are shown above the gene illustrations.

each region. The smaller group contained seven *fliC* and six non-*fliC* genes, and the larger group contained 37 *fliC* and three non-*fliC* genes; sequences were mostly conserved (more than 90% among each group). On the other hand, the central region (259 to 1,222 bp) between 5' and 3' regions was highly diversified, and all sequences showed less than 90% sequence homologies between nearest sequences, except for four pairs: H1/H12 (98%), H30/H32 (93%), H5/H56 (91%), and H8/H40 (90%) (Fig. 2). The highly conserved central region between H1 and H12 (997 bp) contained only 18 single-nucleotide polymorphism (SNP) sites (Fig. 3), of which nine were nonsynonymous changes.

PCR primers for H typing. Based on the results of sequence comparisons described above, a total of 52 PCR primer pairs were designed to identify 51 individual H types and one pair (H1/H12) with very similar *fliC* sequences (Fig. 3 and Table 1; see also Table S2 in the supplemental material). All primer pairs targeted unique sequences of the central region of *fliC* and non-*fliC*, and primer length and G+C content were kept at 20 to 21 bp and 45 to 60%, respectively. The PCR product sizes ranged from 150 bp (H4) to 774 bp (H1/H12) (Table 1).

Evaluation with reference strains. All 53 H type reference strains from H1 to H56 were used to evaluate the specificity of our PCR primers in simplex PCR assays. Clear PCR products of the expected sizes were obtained from the corresponding strain(s), except for the H17 reference strain, and no extra products were produced in the size range of 100 to 1,000 bp. PCR products were obtained only from the strains using primers that were appropriately targeted to amplify each strains' respective *fliC*, with the exception of nine H type reference strains carrying non-*fliC* (Table 2). We confirmed that reference strain H3 carried *flkA*-H3 and *fliC*-H16, H35 carried *flkA*-H35 and *fliC*-H11, H36 carried *flkA*-H36 and *fliC*-H11, H44 carried *fliA*-H44 and *fliC*-H4, H47 carried *flkA*-H47 and *fliC*-H21, H53 carried *flkA*-H53 and *fliC*-H40, H54 carried *fliA*-H54 and *fliC*-H21, and H55 carried *fliA*-H55 and *fliC*-H38 (Table 2). Using the H17 (*fliA*)-targeting primer pair, PCR products were not obtained from H17 reference strain P12b, which was thought to carry *fliA*. We determined this strain carries *fliC*-H4 (Table 2).

Multiplex PCR system. Subsequently, 10 multiplex PCR sets (MP-A to MP-J) were produced that included 51 primer pairs, not containing H17 (Fig. 4). Each reaction set contained four to six primer pairs whose PCR products were of different sizes and made step-like patterns on agarose gels (Fig. 4). Eleven H types associated with STEC major serotypes were assembled into MP-A (H2, H7, H11, H25, H28, and H34) and MP-B (H8, H10, H14, H19, and H21) (Fig. 4). Others were randomly assembled into eight reaction

TABLE 1 H-genotyping PCR primers^a

H type	Flagellin-coding gene	Hg type	Primer sequence		Product size (bp)
			Forward	Reverse	
H1/H12	<i>fliC</i>	Hg1/12	ATGCGCTGACTGCATCAAAG	CCTTGCCGTTGTTAGCATCG	774
H2	<i>fliC</i>	Hg2	TGATCCGACATCTCCTGATG	CCGTCATCACCAATCAACGC	228
H3	<i>flkA</i>	Hg3	CTACATCAGGAGCGGTTGCA	CAGCGTCCAGAACCTGCTTA	504
H4	<i>fliC</i>	Hg4	GATTTCAGCGCGGCGAAACT	GGTTGCAGAATCAACGACCG	150
H5	<i>fliC</i>	Hg5	CGCGTCGATTAATCATACAG	GTTGCTTTTGCCGAGTATT	225
H6	<i>fliC</i>	Hg6	TGCTGGCGCAGATGGAAATA	GCCGCATCCTCAGTAGTTGT	624
H7	<i>fliC</i>	Hg7	CCCTTCATGCTGATGTGGGT	ATACCCGGCAACAGTCACAG	548
H8	<i>fliC</i>	Hg8	CGGCGCGGTTAAGAATGATG	CCGTTTTACCATCTGCGCTG	467
H9	<i>fliC</i>	Hg9	TCCAAACACCGGTGATTCTG	ACCGGTAGAATCGCTGTTGG	208
H10	<i>fliC</i>	Hg10	GCTTCAGGATCGTTAGGTAC	CGTCGCTGCAGTAGGAGTAG	165
H11	<i>fliC</i>	Hg11	AACAACAACCTGCAGCGGATG	TCGGGCTACCACCTTCTGAT	341
H14	<i>fliC</i>	Hg14	TGCAACTGTGGTAGGCAACA	ACGCCCCGGGTCAAATATTT	234
H15	<i>fliC</i>	Hg15	AGTGCTCCGACTTCTGCAAT	GTCCCGCCGATAGTCACTTT	554
H16	<i>fliC</i>	Hg16	GTACAGGTGGAAGGGCAGAC	CACGCATAATTGCCTGTGCA	463
H17	<i>flnA</i>	Hg17	CACTCCAGTCGCTGTAACCG	GCGTTGGTGCCGTCATAATC	180
H18	<i>fliC</i>	Hg18	ACGGAGGCAGATGTTGGTTT	TGAGTATCAGTGCCTGCGTT	305
H19	<i>fliC</i>	Hg19	GCTGGCGATACATTACCGC	CGCCGCTGTCATCAATGTTT	592
H20	<i>fliC</i>	Hg20	CTGTCTAGCCTCGCTGATGG	AGACCGCTTTACCTGAACCG	727
H21	<i>fliC</i>	Hg21	GCAACTAAGCTTGCAAGTGGC	TCCTGGCAGCGTTCAGATCA	304
H23	<i>fliC</i>	Hg23	ACATGCTGAGCCGGGTAAAA	CGCAGCATAATCAGCAACGG	547
H24	<i>fliC</i>	Hg24	CTGTCCGACTTCGCAACAACC	CATCAACGTTGGCGGCATAC	399
H25	<i>fliC</i>	Hg25	AGCCTGTGCTGCACAACAT	CTGTTTGCTTGGCATCGTCAAC	181
H26	<i>fliC</i>	Hg26	ACAGCGGCTGATGTATTGGG	AGTGCGCCAGTGGTATCTTT	596
H27	<i>fliC</i>	Hg27	ACAAAAGTCGGTGGTACAGGT	AATGTTGCGTCTTCCCTGT	212
H28	<i>fliC</i>	Hg28	CTGGCATAACAACAGGCACAC	TCAGCTTTGGTGTAAGCGTC	285
H29	<i>fliC</i>	Hg29	TGCACCAATCGCTGTGAAGA	TCCCTGTCACGGTAACAAAA	305
H30	<i>fliC</i>	Hg30	CTATTAGCGGTTATACCGGT	TTCGCATCAACTGCTGCATT	379
H31	<i>fliC</i>	Hg31	GGCCTGTGCAAAATCAACTGC	GCCCCAGCAGTTTTTGTGT	485
H32	<i>fliC</i>	Hg32	CTATTAGCGGTTATGCTGGC	TTCGCATCAACTGCATCATG	379
H33	<i>fliC</i>	Hg33	TCTGCACAACGTACAGACCC	GGAAACCGGTAGTAACGCCAT	166
H34	<i>fliC</i>	Hg34	GGGAAAACGACAGCTGCATC	CGGCTTGCGAGTAGTAGGTT	439
H35	<i>flkA</i>	Hg35	ACTACTGAACTAGTCCGGC	GCCATCAGCAGCAACTTTTG	645
H36	<i>flkA</i>	Hg36	GACCTATACTGGCTCTGCAGG	GCAACCTTACCAGAACCGTT	732
H37	<i>fliC</i>	Hg37	CGGATGCAAAATCTGACTACC	GGCCTTACCTGTCAACTCAT	605
H38	<i>fliC</i>	Hg38	CTGACCGATGCTTCAGGGTT	CTAACTGGCCTGACTGCGTT	202
H39	<i>fliC</i>	Hg39	GAACACCACTGACGTACCT	GTTGCGCAGAACGTCAACAC	172
H40	<i>fliC</i>	Hg40	GGTGCAGTCAAGGATAAAGA	CATCAATGCAGTACCACTC	202
H41	<i>fliC</i>	Hg41	TTAACGGGGGCTGGTTTCTC	GGCACTCGTCGTTAATTTACCG	705
H42	<i>fliC</i>	Hg42	CTGCTTTGGGGCAAAGGTT	CTTACCCTGAACGGTAGCGA	269
H43	<i>fliC</i>	Hg43	TGCTGATGCTGCGTTAGGAA	TGGTTTTGCCATCAGAGCCA	497
H44	<i>flIA</i>	Hg44	AACACAACACTCTGCGGC	CTGTGTTGTGCGAGTTGTG	187
H45	<i>fliC</i>	Hg45	GCAACGGGAACAGGTCCTTA	CCTGCGCCATTGAAGCTAC	749
H46	<i>fliC</i>	Hg46	CGTAGACGCAGGGCTTAACA	TGGTTACGCTGCCATCAGTT	728
H47	<i>flkA</i>	Hg47	GCCACCACGCTTAAAGGAGA	GTTTTGGTTGCTGCTGTTGC	281
H48	<i>fliC</i>	Hg48	ACCGGTGGTGATAACGATGG	TCATTACATCCGAGCGTA	293
H49	<i>fliC</i>	Hg49	CACTATTGGCGCGGAAAGG	CTTACAGCCCCGTGCTCTT	432
H51	<i>fliC</i>	Hg51	ACCGGTACTTACAAGGCTGC	CAGTATCACCGTCGGTAGCG	382
H52	<i>fliC</i>	Hg52	CTTCCGTTAATGCTGGTGGC	CATTTGTTGACGCGTCAACA	225
H53	<i>flkA</i>	Hg53	AAGACGGTGATATCACGCT	GCCATCAGCGTTTTCAACCA	233
H54	<i>flmA</i>	Hg54	AGTGACGCAATTCAGGTGA	CCGTTGCTATCGGTGTAGGT	347
H55	<i>flIA</i>	Hg55	GCATCTGTTTCTATTGGCGG	GTCGCGGCAGGATCTGTATC	560
H56	<i>fliC</i>	Hg56	CGCGTCCATTGACCATGCAA	GTGGCTGTTGCCGTAGTGTC	225

^aSee Table S2 in the supplemental material for this same list in Excel format.

kits. In multiple PCR assays, the same results as those for simplex PCR assays were obtained from all reference strains.

Validation with wild strains. By using the multiplex PCR kits, we also assayed 362 O:H-serotyped wild-type *E. coli* isolates, including 277 H type-identified isolates, nine H-untypeable isolates, and 76 nonmotile isolates (Table 3). All 277 H type-identified isolates belonging to all H types (except for H3, H46, H53, and H56) gave PCR products (Hg type, H genotype) that corresponded to the results obtained by serological *E. coli* H typing. Additionally, all strains that were positive for a non-*fliC* Hg type were also

TABLE 2 PCR results using H type reference strains^a

Primer and target gene	Reference strain and marker gene																													
	H1 (<i>fliC</i>)	H12 (<i>fliC</i>)	H2 (<i>fliC</i>)	H4 (<i>fliC</i>)	H5 (<i>fliC</i>)	H6 (<i>fliC</i>)	H7 (<i>fliC</i>)	H8 (<i>fliC</i>)	H9 (<i>fliC</i>)	H10 (<i>fliC</i>)	H11 (<i>fliC</i>)	H14 (<i>fliC</i>)	H15 (<i>fliC</i>)	H16 (<i>fliC</i>)	H18 (<i>fliC</i>)	H19 (<i>fliC</i>)	H20 (<i>fliC</i>)	H21 (<i>fliC</i>)	H23 (<i>fliC</i>)	H24 (<i>fliC</i>)	H25 (<i>fliC</i>)	H26 (<i>fliC</i>)	H27 (<i>fliC</i>)	H28 (<i>fliC</i>)	H29 (<i>fliC</i>)	H30 (<i>fliC</i>)				
Hg1/12 (<i>fliC</i>)																														
Hg2 (<i>fliC</i>)																														
Hg4 (<i>fliC</i>)																														
Hg5 (<i>fliC</i>)																														
Hg6 (<i>fliC</i>)																														
Hg7 (<i>fliC</i>)																														
Hg8 (<i>fliC</i>)																														
Hg9 (<i>fliC</i>)																														
Hg10 (<i>fliC</i>)																														
Hg11 (<i>fliC</i>)																														
Hg14 (<i>fliC</i>)																														
Hg15 (<i>fliC</i>)																														
Hg16 (<i>fliC</i>)																														
Hg18 (<i>fliC</i>)																														
Hg19 (<i>fliC</i>)																														
Hg20 (<i>fliC</i>)																														
Hg21 (<i>fliC</i>)																														
Hg23 (<i>fliC</i>)																														
Hg24 (<i>fliC</i>)																														
Hg25 (<i>fliC</i>)																														
Hg26 (<i>fliC</i>)																														
Hg27 (<i>fliC</i>)																														
Hg28 (<i>fliC</i>)																														
Hg29 (<i>fliC</i>)																														
Hg30 (<i>fliC</i>)																														
Hg31 (<i>fliC</i>)																														
Hg32 (<i>fliC</i>)																														
Hg33 (<i>fliC</i>)																														
Hg34 (<i>fliC</i>)																														
Hg37 (<i>fliC</i>)																														
Hg38 (<i>fliC</i>)																														
Hg39 (<i>fliC</i>)																														
Hg40 (<i>fliC</i>)																														
Hg41 (<i>fliC</i>)																														
Hg42 (<i>fliC</i>)																														
Hg43 (<i>fliC</i>)																														
Hg45 (<i>fliC</i>)																														
Hg46 (<i>fliC</i>)																														
Hg48 (<i>fliC</i>)																														

^aGray shading indicates a positive result from a combination of the primer and the reference strain.

positive for one of the PCR primer sets for *fliC* Hg types: O128:H35 carried *flkA*-H35 and *fliC*-H2, O172:H36 carried *flkA*-H36 and *fliC*-H16, O26:H36 carried *flkA*-H36 and *fliC*-Hg27, O128abc:H47 carried *flkA*-H47 and *fliC*-H2, O156:H47 carried *flkA*-H47 and *fliC*-H11, O161:H54 carried *flmA*-H54 and *fliC*-H21, and O75:H55 carried *flIA*-H55 and *fliC*-H38.

As a rare case, O40:H40 (in which the H type was encoded by *fliC*-H40) was also positive for a non-*fliC* (*flkA*-H47). The product sizes from all nine H34 wild strains were slightly larger than that of the H34 reference strain (439 bp) (data not shown). Sequences of the larger product revealed that the 45-bp fragment was deleted from the *fliC* central region of the H34 reference strain.

By using the H-typing PCR, all 76 nonmotile and nine H-untypeable strains were classified into any of 18 Hg types, including Hg2, Hg4, Hg7, Hg8, Hg11, Hg14, Hg16, Hg19, Hg21, Hg23, Hg25, Hg26, Hg27, Hg28, Hg32, Hg33, Hg45, and Hg56. Unusually, an O111:H⁻ strain was positive for two Hg types of *fliC*, Hg27 and Hg33.

DISCUSSION

This is the first report to show a PCR-based complete H-typing platform. Although serotyping is a standard method for subtyping of pathogenic *E. coli* isolates, agglutination reactions with specific antisera are laborious and time-consuming. In this study,

TABLE 2 (Continued)

Primer and target gene	Reference strain and marker gene																											
	H31 (<i>fliC</i>)	H32 (<i>fliC</i>)	H33 (<i>fliC</i>)	H34 (<i>fliC</i>)	H37 (<i>fliC</i>)	H38 (<i>fliC</i>)	H39 (<i>fliC</i>)	H40 (<i>fliC</i>)	H41 (<i>fliC</i>)	H42 (<i>fliC</i>)	H43 (<i>fliC</i>)	H45 (<i>fliC</i>)	H46 (<i>fliC</i>)	H48 (<i>fliC</i>)	H49 (<i>fliC</i>)	H51 (<i>fliC</i>)	H52 (<i>fliC</i>)	H56 (<i>fliC</i>)	H3 (<i>fliA</i>)	H17 (<i>fliA</i>)	H35 (<i>fliA</i>)	H36 (<i>fliA</i>)	H44 (<i>fliA</i>)	H47 (<i>fliA</i>)	H53 (<i>fliA</i>)	H54 (<i>fliA</i>)	H55 (<i>fliA</i>)	
Hg1/12 (<i>fliC</i>)																												
Hg2 (<i>fliC</i>)																												
Hg4 (<i>fliC</i>)																												
Hg5 (<i>fliC</i>)																												
Hg6 (<i>fliC</i>)																												
Hg7 (<i>fliC</i>)																												
Hg8 (<i>fliC</i>)																												
Hg9 (<i>fliC</i>)																												
Hg10 (<i>fliC</i>)																												
Hg11 (<i>fliC</i>)																												
Hg14 (<i>fliC</i>)																												
Hg15 (<i>fliC</i>)																												
Hg16 (<i>fliC</i>)																												
Hg18 (<i>fliC</i>)																												
Hg19 (<i>fliC</i>)																												
Hg20 (<i>fliC</i>)																												
Hg21 (<i>fliC</i>)																												
Hg23 (<i>fliC</i>)																												
Hg24 (<i>fliC</i>)																												
Hg25 (<i>fliC</i>)																												
Hg26 (<i>fliC</i>)																												
Hg27 (<i>fliC</i>)																												
Hg28 (<i>fliC</i>)																												
Hg29 (<i>fliC</i>)																												
Hg30 (<i>fliC</i>)																												
Hg31 (<i>fliC</i>)																												
Hg32 (<i>fliC</i>)																												
Hg33 (<i>fliC</i>)																												
Hg34 (<i>fliC</i>)																												
Hg37 (<i>fliC</i>)																												
Hg38 (<i>fliC</i>)																												
Hg39 (<i>fliC</i>)																												
Hg40 (<i>fliC</i>)																												
Hg41 (<i>fliC</i>)																												
Hg42 (<i>fliC</i>)																												
Hg43 (<i>fliC</i>)																												
Hg45 (<i>fliC</i>)																												
Hg46 (<i>fliC</i>)																												
Hg48 (<i>fliC</i>)																												
Hg49 (<i>fliC</i>)																												
Hg51 (<i>fliC</i>)																												
Hg52 (<i>fliC</i>)																												
Hg56 (<i>fliC</i>)																												
Hg3 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg17 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg35 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg36 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg44 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg47 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg53 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg54 (non- <i>fliC</i>) [<i>fliA</i>]																												
Hg55 (non- <i>fliC</i>) [<i>fliA</i>]																												

to efficiently screen H types, 10 multiplex PCR sets containing 51 primer pairs, termed *E. coli* H-genotyping PCR, were developed. The PCR method chosen here is the most frequently and widely used molecular technique for the investigation and surveillance of pathogenic bacteria. The optimized and unified PCR conditions, which were the same as those of the PCR-based O-typing system, termed *E. coli* O-genotyping PCR and reported in our previous study (18), dramatically enhance work efficiency in subtyping *E. coli* isolates.

Most STEC outbreaks and sporadic cases have been attributed to O157:H7, O26:H11, O111:H8, O45:H2, O103:H2, O145:H28, and O121:H19 (or the nonmotile isolates) (14, 19–25). Additionally, other STEC serotypes, such as O6:H10, O91:H10/H14/H21, O103:H11/H25, O111:H11/H21, O145:H25/H34, O165:H25, and O178:H19, from foods, animals, and humans, have also been reported (14, 22, 24–31). Two multiplex PCR kits developed in this study, MP-A and MP-B, are particularly useful for distinguishing these H types.

E. coli strains with different serotypes usually show different evolutionary lineages and groups. In particular, the identification of strains of the same serotype is essential in outbreak investigations and surveillance. There are different H types even in the same O group, and detailed subtyping is important for investigation and surveillance.

MP-A

Lane	Strain	Hg type	Gene	Product size (bp)
1	H25	Hg25	<i>fliC</i>	181
2	H2	Hg2	<i>fliC</i>	228
3	H28	Hg28	<i>fliC</i>	285
4	H11	Hg11	<i>fliC</i>	341
5	H34	Hg34	<i>fliC</i>	439
6	H7	Hg7	<i>fliC</i>	548

MP-F

Lane	Strain	Hg type	Gene	Product size (bp)
1	H40	Hg40	<i>fliC</i>	202
2	H47	Hg47	<i>fliA</i>	281
3	H32	Hg32	<i>fliC</i>	379
4	H15	Hg15	<i>fliC</i>	554
5	H36	Hg36	<i>fliA</i>	732

MP-B

Lane	Strain	Hg type	Gene	Product size (bp)
1	H10	Hg10	<i>fliC</i>	165
2	H14	Hg14	<i>fliC</i>	234
3	H21	Hg21	<i>fliC</i>	304
4	H8	Hg8	<i>fliC</i>	467
5	H19	Hg19	<i>fliC</i>	592

MP-G

Lane	Strain	Hg type	Gene	Product size (bp)
1	H38	Hg38	<i>fliC</i>	202
2	H42	Hg42	<i>fliC</i>	269
3	H51	Hg51	<i>fliC</i>	382
4	H43	Hg43	<i>fliC</i>	497
5	H37	Hg37	<i>fliC</i>	605
6	H45	Hg45	<i>fliC</i>	749

MP-C

Lane	Strain	Hg type	Gene	Product size (bp)
1	H44	Hg44	<i>fliA</i>	187
2	H53	Hg53	<i>fliA</i>	233
3	H30	Hg30	<i>fliC</i>	379
4	H31	Hg31	<i>fliC</i>	485
5	H55	Hg55	<i>fliA</i>	560
6	H20	Hg20	<i>fliC</i>	727

MP-H

Lane	Strain	Hg type	Gene	Product size (bp)
1	H39	Hg39	<i>fliC</i>	172
2	H52	Hg52	<i>fliC</i>	225
3	H29	Hg29	<i>fliC</i>	305
4	H49	Hg49	<i>fliC</i>	432
5	H26	Hg26	<i>fliC</i>	596
6	H46	Hg46	<i>fliC</i>	728

MP-D

Lane	Strain	Hg type	Gene	Product size (bp)
1	H33	Hg33	<i>fliC</i>	166
2	H5	Hg5	<i>fliC</i>	225
3	H24	Hg24	<i>fliC</i>	399
4	H3	Hg3	<i>fliA</i>	504
5	H6	Hg6	<i>fliC</i>	624

MP-I

Lane	Strain	Hg type	Gene	Product size (bp)
1	H56	Hg56	<i>fliC</i>	225
2	H54	Hg54	<i>fliA</i>	347
3	H16	Hg16	<i>fliC</i>	463
4	H41	Hg41	<i>fliC</i>	705

MP-E

Lane	Strain	Hg type	Gene	Product size (bp)
1	H27	Hg27	<i>fliC</i>	212
2	H18	Hg18	<i>fliC</i>	305
3	H35	Hg35	<i>fliA</i>	645
4	H1	Hg1/12	<i>fliC</i>	774
5	H12			

MP-J

Lane	Strain	Hg type	Gene	Product size (bp)
1	H4	Hg4	<i>fliC</i>	150
2	H9	Hg9	<i>fliC</i>	208
3	H48	Hg48	<i>fliC</i>	293
4	H23	Hg23	<i>fliC</i>	547

FIG 4 Primer combination for multiplex H-genotyping PCR and gel images of PCR products in each multiplex PCR kit from MP-A to MP-J. Lanes at both ends are size markers (100-bp DNA ladder; GeneDirex).

Although O157:H7 is a leading STEC serotype associated with hemorrhagic colitis and hemolytic-uremic syndrome (HUS), EPEC O157:H16, O157:H39, and O157:H45 were isolated from human and environmental samples (32, 33). Although serotype O103:H2 is one of the most frequently isolated non-O157 STEC, O103:H11 and O103:H25 STEC strains were also isolated from patients (14, 34). STEC O145 has emerged as one of the major STEC O groups, which encompasses motile and nonmotile strains of serotypes O145:H25 and O145:H28 (16, 35). Phylogenetic analyses of these strains clearly revealed that different serotype isolates belonged to different lineages and may have different potential risks for humans.

A point to note when using the *E. coli* H-genotyping PCR is that two Hg types of results are obtained from a strain carrying non-*fliC*, because non-*fliC* is additionally inserted into the genome carrying *fliC* on the chromosome locus (4). In this case, because non-*fliC* usually represses the expression of the existing *fliC*, the phenotypic H

TABLE 3 List of 362 wild strains used for validation by using a full set of *E. coli* H-genotyping PCRs

Hg type	Gene	No. of strains tested	Serotype (no. of isolates; <i>n</i> = 277 H type-identified isolates)	Subtotal	Serotype (no. of isolates; <i>n</i> = 76 nonmotile isolates, <i>n</i> = 9 motile but H-untypable isolates)	Subtotal
Hg1/12	<i>flhC</i>	12	O22:H1 (1), O85:H1 (1), O9:H12 (1), O139:H1 (3), O170:H1 (1), O25:H12 (1), O49:H12 (1), O71:H12 (1), O87:H12 (1), O104:H12 (1)	12		0
Hg2	<i>flhC</i>	12	O3:H2 (1), O10:H2 (1), O45:H2 (1), O73:H2 (1), O80:H2 (1), O103:H2 (3), O128:H2 (1), O144:H2 (1), O180:H2 (1)	11	O103:H ⁻ (1)	1
Hg3	<i>flhA</i>	0		0		0
Hg4	<i>flhC</i>	16	O15:H4 (1), O19:H4 (1), O99:H4 (1), O104:H4 (2), O113:H4 (1), O120:H4 (1), O131:H4 (1), O154:H4 (1), O161:H4 (1)	10	O2:H ⁻ (1), O9:H ⁻ (1), O113:HUT (1), O119:H ⁻ (1), O119:HUT (1), OUT:H ⁻ (1)	6
Hg5	<i>flhC</i>	4	O20:H5 (1), O75:H5 (1), O167:H5 (1), OUT:H5 (1)	4		0
Hg6	<i>flhC</i>	8	O27:H6 (1), O33:H6 (1), O81:H6 (1), O102:H6 (1), O120:H6 (1), O125ac:H6 (1), O142:H6 (1), O150:H6 (1)	8		0
Hg7	<i>flhC</i>	18	O1:H7 (1), O18ac:H7 (1), O55:H7 (1), O74:H7 (1), O110:H7 (1), O147:H7 (1), O153:H7 (1), O157:H7 (3)	10	O157:H ⁻ (8)	8
Hg8	<i>flhC</i>	15	O23:H8 (1), O39:H8 (1), O111:H8 (1), O168:H8 (1), O174:H8 (1), O179:H8 (1)	6	O111:H ⁻ (9)	9
Hg9	<i>flhC</i>	9	O28ab:H9 (1), O36:H9 (1), O101:H9 (1), O116:H9 (2), O130:H9 (1), O155:H9 (2), O169:H9 (1)	9		0
Hg10	<i>flhC</i>	10	O40:H10 (1), O49:H10 (1), O52:H10 (1), O95:H10 (1), O114:H10 (1), O152:H10 (1), O149:H10 (1), O162:H10 (2), O184:H10 (1)	10		0
Hg11	<i>flhC</i>	17	O26:H11 (3), O69:H11 (1), O103:H11 (2), O111:H11 (2), O130:H11 (1), O151:H11 (1)	10	O26:H ⁻ (1), O103:H ⁻ (5), O157:HUT (1)	7
Hg14	<i>flhC</i>	10	O51:H14 (1), O91:H14 (4), O138:H14 (1)	6	O91:H ⁻ (4)	4
Hg15	<i>flhC</i>	4	O23:H15 (1), O166:H15 (2), OUT:H15 (1)	4		0
Hg16	<i>flhC</i>	17	O6:H16 (1), O46:H16 (1), O89:H16 (1), O112ac:H16 (1), O118:H16 (2), O123:H16 (1), O136:H16 (1), O168:H16 (1), O175:H16 (1)	10	O6:H ⁻ (4), O25:H ⁻ (2), O157:H ⁻ (1)	7
Hg17	<i>flhA</i>	0		0		0
Hg18	<i>flhC</i>	9	O17:H18 (1), O44:H18 (3), O73:H18 (1), O112ab:H18 (1), O148:H18 (2), O183:H18 (1)	9		0
Hg19	<i>flhC</i>	14	O20:H19 (1), O35:H19 (1), O36:H19 (1), O59:H19 (1), O76:H19 (1), O112ab:H19 (1), O121:H19 (3), O160:H19 (1), O163:H19 (1)	11	OUT:H ⁻ (3)	3
Hg20	<i>flhC</i>	5	O1:H20 (1), O74:H20 (1), O155:H20 (1), O159:H20 (2)	5		0
Hg21	<i>flhC</i>	12	O21:H21 (1), O54:H21 (1), O57:H21 (1), O64:H21 (1), O91:H21 (2), O111:H21 (1), O113:H21 (1), O146:H21 (1)	9	O111:H ⁻ (1), O127:H ⁻ (1), OUT:H ⁻ (1)	3
Hg23	<i>flhC</i>	4	O8:H23 (1), O149:H23 (1), O158:H23 (1)	3	OUT:HUT (1)	1
Hg24	<i>flhC</i>	1	O23:H24 (1)	1		0
Hg25	<i>flhC</i>	18	O28:H25 (1), O30:H25 (1), O88:H25 (2), O103:H25 (3), O156:H25 (1), O165:H25 (1), O177:H25 (1)	10	O28ac:HUT (1), O153:HUT (1), O165:H ⁻ (5), OUT:HUT (1)	8
Hg26	<i>flhC</i>	7	O38:H26 (1), O41:H26 (1), O48:H26 (1), O80:H26 (1), O131:H26 (1), O157:H26 (1)	6	O143:HUT (1)	1
Hg27	<i>flhC</i>	19	O2:H27 (1), O58:H27 (1), O107:H27 (3), O119:H27 (1), O126:H27 (2), O174:H27 (1), OUT:H27 (1)	10	O86:H ⁻ (8), O111:H ⁻ (1) ^c	9
Hg28	<i>flhC</i>	16	O91:H28 (1), O132:H28 (1), O148:H28 (2), O152:H28 (1), O175:H28 (1), O185:H28 (1), O187:H28 (1)	8	O145:H ⁻ (8)	8
Hg29	<i>flhC</i>	3	O133:H29 (2), OUT:H29 (1)	3		0
Hg30	<i>flhC</i>	8	O11:H30 (1), O27:H30 (1), O41:H30 (1), O62:H30 (2), O86:H30 (1), O124:H30 (1), O153:H30 (1)	8		0
Hg31	<i>flhC</i>	8	O73:H31 (1), O83:H31 (1), O126:H31 (1), O175:H31 (5)	8		0
Hg32	<i>flhC</i>	7	O26:H32 (1), O114:H32 (1), O184:H32 (1), OUT:H32 (1)	4	O2:H ⁻ (1), O86:H ⁻ (1), O140:H ⁻ (1)	3
Hg33	<i>flhC</i>	11	O60:H33 (1), O92:H33 (2), O95:H33 (1), O99:H33 (1), O101:H33 (1), O106:H33 (1)	7	O83:H ⁻ (1), O92:H ⁻ (2), O101:H ⁻ (1)	4
Hg34	<i>flhC</i>	9	O6:H34 (2), O32:H34 (1), O132:H34 (1), O145:H34 (2), O160:H34 (1), O176:H34 (1), OUT:H34 (1)	9		0
Hg35	<i>flhA^a</i>	1	O128:H35[Hg2 ⁺] (1)	1		0
Hg36	<i>flhA^a</i>	2	O172:H36[Hg16 ⁺] (1), O26:H36[Hg27 ⁺] (1)	2		0
Hg37	<i>flhC</i>	2	O42:H37 (2)	2		0
Hg38	<i>flhC</i>	5	O75:H38 (1), O89:H38 (1), O134:H38 (1), O152:H38 (1), O157:H38 (1)	5		0
Hg39	<i>flhC</i>	2	O110:H39 (1), O157:H39 (1)	2		0
Hg40	<i>flhC</i>	7	O40:H40[Hg47] (1), ^b O41:H40 (1), O51:H40 (1), O58:H40 (1), O79:H40 (1), O102:H40 (1), O124:H40 (1)	7		0
Hg41	<i>flhC</i>	10	O44:H41 (1), O169:H41 (9)	10		0
Hg42	<i>flhC</i>	3	O25:H42 (3)	3		0
Hg43	<i>flhC</i>	3	O157:H43 (1), O159:H43 (2)	3		0
Hg44	<i>flhA^a</i>	1	O14:H44 (1)	1		0
Hg45	<i>flhC</i>	7	O1:H45 (1), O18:H45 (1), O153:H45 (2), O157:H45 (1)	5	O157:HUT (1), OUT:H ⁻ (1)	2
Hg46	<i>flhC</i>	0		0		0
Hg47	<i>flhA^a</i>	2	O128abc:H47[Hg2 ⁺] (1), O156:H47[Hg11 ⁺] (1)	2		0
Hg48	<i>flhC</i>	2	O138:H48 (2)	2		0
Hg49	<i>flhC</i>	7	O8:H49 (1), O28ab:H49 (1), O39:H49 (1), O51:H49 (2), O181:H49 (2)	7		0
Hg51	<i>flhC</i>	1	O86:H51 (1)	1		0
Hg52	<i>flhC</i>	1	O8:H52 (1)	1		0
Hg53	<i>flhA</i>	0		0		0
Hg54	<i>flhA^a</i>	1	O161:H54[Hg21 ⁺] (1)	1		0
Hg55	<i>flhA^a</i>	1	O75:H55[Hg38 ⁺] (1)	1		0
Hg56	<i>flhC</i>	1		0	O103:H ⁻ (1)	1

^aAll strains which are positive by non-*flhC*-targeting primers were also positive by any type of *flhC*-targeting primers.^bHg40 (*flhC*)-positive O40:H40 isolate was also positive with Hg47 (non-*flhC*).^cO111:H⁻ isolate was positive with Hg27 (*flhC*) and Hg33 (*flhC*).

type is conformed to non-*fliC* types, not *fliC* (4). Our validation resulted in the identification of new combinations of flagellar genes *fliC*-H16 and *flkA*-H36 as well as *fliC*-H40 and *flkA*-H47 and finally the presence of two *fliC* genes in the same strain, *fliC*-H27 and *fliC*-H33. In addition to H type-identified strains, we used nonmotile isolates to evaluate the *E. coli* H-genotyping PCR. As the reasons for losing motility may not be involved in mutations on *fliC*, all 76 nonmotile strains used in this study could be subtyped into any of several Hg types by using our method of targeting *fliC*. This point is one of the great advantages of DNA-based H typing, allowing broader subtyping than the serological H-typing platform.

Although *fliA*_H17 was found in the H17 reference strain P12b as the gene encoding H17 antigen (36), two previous studies reported that *fliA*_H17 was not detected in the strain P12b (9, 37). Additionally, Joensen et al. (9) showed that the sequence of *fliA*_H17 was not detected in the genomes of all three H17 isolates tested. To resolve this complex issue, more H17 strains need to be examined. Although we designed an H17-targeting primer based on *fliA*_H17 sequence, a PCR product was not obtained from the H17 reference strain P12b. Because the validity of the H17 primer was not confirmed by using our H type reference strain set, we did not incorporate it into the multiplex PCR kits.

In conclusion, we present a basic set of PCR primers for the identification of all known *E. coli* H types and confirmed that the complete and practical platform for molecular H typing, *E. coli* H-genotyping PCR, could accurately and specifically identify the H types of all reference strains and most wild-type strains. The system provides usable information to evaluate the routes, sources, and prevalence of agents. An emergence of novel serotypes in investigations and surveillance of pathogenic *E. coli* was also noticed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.00190-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Yuiko Kato for technical assistance.

This research was partially supported by the Research Program on Emerging and Reemerging Infectious Diseases from the Japan Agency for Medical Research and Development (AMED) (JP15fk0108008).

Members of the Pathogenic *E. coli* Working Group in Japan include the following: Aichi Prefectural Institute of Public Health (Masakado Matsumoto), Ibaraki Prefectural Institute of Public Health (Yuri Unno), Oita Prefectural Institute of Health and Environment, Okazaki City Public Health Center, Okayama Prefectural Institute for Environmental Science and Public Health (Hiroshi Nakajima and Hideaki Kariya), Kagoshima Prefectural Institute for Environmental Research and Public Health (Nodoka Hozumi), Kitakyusyu City Institute of Environmental Sciences, Gifu Prefectural Research Institute for Health and Environmental Sciences (Yoshihiko Kameyama, Makiko Noda, and Yukiko Kadokura), Kumamoto Prefectural Institute of Public Health and Environmental Science (Atsumi Obara and Seiya Harada), Kobe City Institute for Environment and Public Health (Natsuki Hama and Ryohei Nomoto), Saitama Prefectural Institute of Public Health (Takayuki Kurazono), Saga Prefectural Center of Public Health and Sanitary, Shiga Prefectural Institute of Public Health, Shizuoka Institute of Environment and Hygiene, Shimane Prefectural Institute of Public Health and Environmental Science (Yoshie Tsunomori), Sendai City Institute of Public Health (Toshinobu Hoshi), Chiba City Research Institute for Environmental Sciences and Public Health (Tomoko Kitahashi), Tokushima Pharmaceutical and Environmental Sciences Center, Toyama Institute of Health (Keiko Kimata and Junko Isobe), Nagasaki Prefectural Institute for Environmental Research and Public Health (Hiroko Ojima and Yumika Takaki), Niigata Prefectural

Institute of Public Health and Environmental Sciences (Junko Aoki), Niigata City Institute of Public Health and Environment (Kazunari Yamamoto and Yukihiro Taoka), Higashi-Osaka City Research Center of Environment and Public Health, Fukui Prefectural Institute of Public Health and Environmental Science (Akihiro Nagata and Satomi Iwasaki), Fukuoka Institute of Health and Environmental Sciences, Fukuoka City Institute for Hygiene and the Environment, Miyagi Prefectural Institute of Public Health and Environment, Miyazaki Prefectural Institute for Public Health and Environment (Nami Tsuru and Shuji Yoshino), Yamagata Prefectural Institute of Public Health, Yamaguchi Prefectural Institute of Public Health and Environment (Hitoshi Ohtsuka, Mitsuhiro Kameyama, and Noriko Obane), and Yokohama City Institute of Health (Atsuko Ogawa and Yuko Matsumoto).

REFERENCES

- Orskov F, Orskov I. 1992. *Escherichia coli* serotyping and disease in man and animals. *Can J Microbiol* 38:699–704. <https://doi.org/10.1139/m92-115>.
- Samatey FA, Imada K, Nagashima S, Vonderviszt F, Kumasaka T, Yamamoto M, Namba K. 2001. Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. *Nature* 410:331–337. <https://doi.org/10.1038/35066504>.
- Orskov I, Orskov F, Bettelheim KA, Chandler ME. 1975. Two new *Escherichia coli* O antigens, O162 and O163, and one new H antigen, H56, withdrawal of H antigen H50. *Acta Pathol Microbiol Scand B* 83:121–124.
- Feng L, Liu B, Liu Y, Ratiner YA, Hu B, Li D, Zong X, Xiong W, Wang L. 2008. A genomic islet mediates flagellar phase variation in *Escherichia coli* strains carrying the flagellin-specifying locus *flk*. *J Bacteriol* 190:4470–4477. <https://doi.org/10.1128/JB.01937-07>.
- Ratiner YA. 1998. New flagellin-specifying genes in some *Escherichia coli* strains. *J Bacteriol* 180:979–984.
- Ratiner YA, Sihvonen LM, Liu Y, Wang L, Siitonen A. 2010. Alteration of flagellar phenotype of *Escherichia coli* strain P12b, the standard type strain for flagellar antigen H17, possessing a new non-*flhC* flagellin gene *flnA*, and possible loss of original flagellar phenotype and genotype in the course of subculturing through semisolid media. *Arch Microbiol* 192:267–278. <https://doi.org/10.1007/s00203-010-0556-x>.
- Tominaga A. 2004. Characterization of six flagellin genes in the H3, H53 and H54 standard strains of *Escherichia coli*. *Genes Genet Syst* 79:1–8. <https://doi.org/10.1266/ggs.79.1>.
- Wang L, Rothmund D, Curd H, Reeves PR. 2003. Species-wide variation in the *Escherichia coli* flagellin (H-antigen) gene. *J Bacteriol* 185:2936–2943. <https://doi.org/10.1128/JB.185.9.2936-2943.2003>.
- Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM, Scheutz F. 2015. Rapid and easy *in silico* serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *J Clin Microbiol* 53:2410–2426. <https://doi.org/10.1128/JCM.00008-15>.
- Ramos Moreno AC, Cabilio Guth BE, Baquerizo Martinez M. 2006. Can the *flhC* PCR-restriction fragment length polymorphism technique replace classic serotyping methods for characterizing the H antigen of enterotoxigenic *Escherichia coli* strains? *J Clin Microbiol* 44:1453–1458. <https://doi.org/10.1128/JCM.44.4.1453-1458.2006>.
- Fields PI, Blom K, Hughes HJ, Helsel LO, Feng P, Swaminathan B. 1997. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length polymorphism test for identification of *E. coli* O157:H7 and O157:NM. *J Clin Microbiol* 35:1066–1070.
- Madic J, Peytavin de Garam C, Vingadassalon N, Oswald E, Fach P, Jamet E, Auvray F. 2010. Simplex and multiplex real-time PCR assays for the detection of flagellar (H-antigen) *flhC* alleles and intimin (*eae*) variants associated with enterohaemorrhagic *Escherichia coli* (EHEC) serotypes O26:H11, O103:H2, O111:H8, O145:H28 and O157:H7. *J Appl Microbiol* 109:1696–1705.
- Wang L, Rothmund D, Curd H, Reeves PR. 2000. Sequence diversity of the *Escherichia coli* H7 *flhC* genes: implication for a DNA-based typing scheme for *E. coli* O157:H7. *J Clin Microbiol* 38:1786–1790.
- Iguchi A, Iyoda S, Ohnishi M. 2012. Molecular characterization reveals three distinct clonal groups among clinical Shiga toxin-producing *Escherichia coli* strains of serogroup O103. *J Clin Microbiol* 50:2894–2900. <https://doi.org/10.1128/JCM.00789-12>.
- Beutin L, Delannoy S, Fach P. 2015. Genetic diversity of the *flhC* genes encoding the flagellar antigen H19 of *Escherichia coli* and application to the specific identification of enterohemorrhagic *E. coli* O121:H19. *Appl Environ Microbiol* 81:4224–4230. <https://doi.org/10.1128/AEM.00591-15>.
- Beutin L, Delannoy S, Fach P. 2015. Sequence variations in the flagellar antigen genes *flhC*25 and *flhC*28 of *Escherichia coli* and their use in identification and characterization of enterohemorrhagic *E. coli* (EHEC) O145:H25 and O145:H28. *PLoS One* 10:e0126749. <https://doi.org/10.1371/journal.pone.0126749>.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680. <https://doi.org/10.1093/nar/22.22.4673>.
- Iguchi A, Iyoda S, Seto K, Morita-Ishihara T, Scheutz F, Ohnishi M. 2015. *Escherichia coli* O-genotyping PCR: a comprehensive and practical platform for molecular O serogrouping. *J Clin Microbiol* 53:2427–2432. <https://doi.org/10.1128/JCM.00321-15>.
- Schmidt H, Geitz C, Tarr PI, Frosch M, Karch H. 1999. Non-O157:H7 pathogenic Shiga toxin-producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. *J Infect Dis* 179:115–123. <https://doi.org/10.1086/314537>.
- Terajima J, Izumiya H, Wada A, Tamura K, Watanabe H. 1999. Shiga toxin-producing *Escherichia coli* O157:H7 in Japan. *Emerg Infect Dis* 5:301–302. <https://doi.org/10.3201/eid0502.990222>.
- Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA. 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J Infect Dis* 192:1422–1429. <https://doi.org/10.1086/466536>.
- Beutin L, Krause G, Zimmermann S, Kaulfuss S, Gleier K. 2004. Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. *J Clin Microbiol* 42:1099–1108. <https://doi.org/10.1128/JCM.42.3.1099-1108.2004>.
- Eklund M, Scheutz F, Siitonen A. 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. *J Clin Microbiol* 39:2829–2834. <https://doi.org/10.1128/JCM.39.8.2829-2834.2001>.
- Kappeli U, Hachler H, Giezendanner N, Beutin L, Stephan R. 2011. Human infections with non-O157 Shiga toxin-producing *Escherichia coli*, Switzerland, 2000–2009. *Emerg Infect Dis* 17:180–185. <https://doi.org/10.3201/eid1702.100909>.
- Pradel N, Livrelli V, De Champs C, Palcoux JB, Reynaud A, Scheutz F, Sirot J, Joly B, Forestier C. 2000. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. *J Clin Microbiol* 38:1023–1031.
- Toma C, Martinez Espinosa E, Song T, Miliwebsky E, Chinen I, Iyoda S, Iwanaga M, Rivas M. 2004. Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing *Escherichia coli*. *J Clin Microbiol* 42:4937–4946. <https://doi.org/10.1128/JCM.42.11.4937-4946.2004>.
- Urdahl AM, Beutin L, Skjerve E, Wasteson Y. 2002. Serotypes and virulence factors of Shiga toxin-producing *Escherichia coli* isolated from healthy Norwegian sheep. *J Appl Microbiol* 93:1026–1033. <https://doi.org/10.1046/j.1365-2672.2002.01787.x>.
- Ju W, Rump L, Toro M, Shen J, Cao G, Zhao S, Meng J. 2014. Pathoge-

- nicity islands in Shiga toxin-producing *Escherichia coli* O26, O103, and O111 isolates from humans and animals. Foodborne Pathog Dis 11: 342–345. <https://doi.org/10.1089/fpd.2013.1696>.
29. Dallman T, Smith GP, O'Brien B, Chattaway MA, Finlay D, Grant KA, Jenkins C. 2012. Characterization of a verocytotoxin-producing enteroaggregative *Escherichia coli* serogroup O111:H21 strain associated with a household outbreak in Northern Ireland. J Clin Microbiol 50:4116–4119. <https://doi.org/10.1128/JCM.02047-12>.
30. Murakami K, Etoh Y, Ichihara S, Maeda E, Takenaka S, Horikawa K, Narimatsu H, Kawano K, Kawamura Y, Ito K. 2014. Isolation and characteristics of Shiga toxin 2f-producing *Escherichia coli* among pigeons in Kyushu, Japan. PLoS One 9:e86076. <https://doi.org/10.1371/journal.pone.0086076>.
31. Bielaszewska M, Prager R, Vandivinit L, Musken A, Mellmann A, Holt NJ, Tarr PI, Karch H, Zhang W. 2009. Detection and characterization of the fimbrial *sfp* cluster in enterohemorrhagic *Escherichia coli* O165:H25/NM isolates from humans and cattle. Appl Environ Microbiol 75:64–71. <https://doi.org/10.1128/AEM.01815-08>.
32. Iguchi A, Shirai H, Seto K, Ooka T, Ogura Y, Hayashi T, Osawa K, Osawa R. 2011. Wide distribution of O157-antigen biosynthesis gene clusters in *Escherichia coli*. PLoS One 6:e23250. <https://doi.org/10.1371/journal.pone.0023250>.
33. Feng PC, Keys C, Lacher D, Monday SR, Shelton D, Rozand C, Rivas M, Whittam T. 2010. Prevalence, characterization and clonal analysis of *Escherichia coli* O157: non-H7 serotypes that carry *eae* alleles. FEMS Microbiol Lett 308:62–67. <https://doi.org/10.1111/j.1574-6968.2010.01990.x>.
34. Schimmer B, Nygard K, Eriksen HM, Lassen J, Lindstedt BA, Brandal LT, Kapperud G, Aavitsland P. 2008. Outbreak of haemolytic uraemic syndrome in Norway caused by *stx2*-positive *Escherichia coli* O103:H25 traced to cured mutton sausages. BMC Infect Dis 8:41. <https://doi.org/10.1186/1471-2334-8-41>.
35. Sonntag AK, Prager R, Bielaszewska M, Zhang W, Fruth A, Tschape H, Karch H. 2004. Phenotypic and genotypic analyses of enterohemorrhagic *Escherichia coli* O145 strains from patients in Germany. J Clin Microbiol 42:954–962. <https://doi.org/10.1128/JCM.42.3.954-962.2004>.
36. Liu B, Hu B, Zhou Z, Guo D, Guo X, Ding P, Feng L, Wang L. 2012. A novel non-homologous recombination-mediated mechanism for *Escherichia coli* unilateral flagellar phase variation. Nucleic Acids Res 40:4530–4538. <https://doi.org/10.1093/nar/gks040>.
37. Beutin L, Strauch E, Zimmermann S, Kaulfuss S, Schaudinn C, Mannel A, Gelderblom HR. 2005. Genetical and functional investigation of *fliC* genes encoding flagellar serotype H4 in wildtype strains of *Escherichia coli* and in a laboratory *E. coli* K-12 strain expressing flagellar antigen type H48. BMC Microbiol 5:4. <https://doi.org/10.1186/1471-2180-5-4>.