AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

EPIDEMIOLOGY

Rapid and Specific Detection of the *Escherichia coli* Sequence Type 648 Complex within Phylogroup F

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ABSTRACT The *Escherichia coli* sequence type 648 complex (STc648) is an emerging lineage within phylogroup F—formerly included within phylogroup D—that is associated with multidrug resistance. Here, we designed and validated a novel multiplex PCR-based assay for STc648 that took advantage of (i) four distinctive single-nucleotide polymorphisms in *icd* allele 96 and *gyrB* allele 87, two of the multilocus sequence typing alleles that define ST648; and (ii) the typical absence within STc648 of *uidA*, an *E. coli*-specific gene encoding β-glucuronidase. Within a diverse 212-strain validation set that included 109 STs other than STc648, from phylogroups A, B1, B2, C, D, E, and F, the assay exhibited 100% sensitivity (95% confidence interval [CI], 82% to 100%) and specificity (95% CI, 98% to 100%). It functioned similarly well in two distant laboratories that used boiled lysates or DNAzol-purified DNA as the template DNA. Thus, this novel multiplex PCR-based assay should enable any laboratory equipped for diagnostic PCR to rapidly, accurately, and economically screen *E. coli* isolates for membership in STc648.

KEYWORDS *Escherichia coli*, antimicrobial resistance, diagnostics, molecular epidemiology, polymerase chain reaction, sequence type, strain typing

Received 20 September 2016 **Returned for modification** 16 October 2016 **Accepted** 13 January 2017

Accepted manuscript posted online 18 January 2017

Citation Johnson JR, Johnston BD, Gordon DM. 2017. Rapid and specific detection of the *Escherichia coli* sequence type 648 complex within phylogroup F. J Clin Microbiol 55:1116–1121. https://doi.org/10.1128/JCM.01949-16.

Editor Daniel J. Diekema, University of Iowa College of Medicine

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Escherichia coli, an important cause of extraintestinal infections in humans and animals (1), is highly clonal. Each of its seven recognized phylogenetic groups (phylogroups) comprises numerous individual sequence types (STs), as defined by multilocus sequence typing (MLST) (2). Among thousands of distinct *E. coli* STs, a dozen or so ST complexes (i.e., groups of closely related STs) account for most human extraintestinal *E. coli* infections, and so are regarded as extraintestinal pathogenic *E. coli* (ExPEC) (3, 4); a similarly small proportion account for most antimicrobial-resistant *E. coli* infections (4–7). Thus, an *E. coli* isolate's ST can be highly informative regarding its likely pathogenic and resistance capabilities (8, 9).

Phylogroup F is related closely to phylogroup B2, the origin of most human clinical *E. coli* isolates, and phylogroup D, the origin of most non-B2 ExPEC strains (2, 10). Prior to its recognition as a distinct phylogroup, its members were usually classified under group D, including by a PCR-based phylotyping assay that delineates only four major *E. coli* phylogroups (11). An updated version of that assay differentiates phylogroup F from phylogroup D (2).

Within phylogroup F, the sequence type 648 complex (STc648) is reported increasingly as an emerging resistance-associated lineage (4). In multiple studies of resistant *E. coli* from diverse sources and locales, STc648 has been the first, second, or third most prevalent STc, accounting for up to 28% of isolates (12–19). The reported resistance phenotypes for different STc648 strains include fluoroquinolones, extended-spectrum cephalosporins (CTX-M-type enzymes and CMY-2), carbapenems (OXA-48 and NDM and KPC variants), fosfomycin (*fosA3*), and colistin (*mcr-1*) (13, 14, 20–30). STc648 is distrib-

TABLE 1 Performance characteristics of the *Escherichia coli* sequence type 648 complex (STc648) PCR assay with 212 validation strains

Laboratory	No. of isolates			Assay performance characteristics[a]	
	STc648	Non-STc648	Total	Sensitivity, % PPV (95% CI)	Specificity, % NPV (95% CI)
1	10	50[b]	60	100 (69–100)	100 (93–100)
2	9	143[c]	152	100 (66–100)	100 (97.5–100)
Total	19	193	212	100 (82–100)	100 (98–100)

[a]PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval. PPV and NPV depend on the prevalence of STc648 in the population, so are not generalizable to other populations.
[b]STs represented ($n = 50$): 10, 12, 14, 23, 38, 43, 44, 46, 47, 48, 49, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 64, 66, 67, 68, 69, 70, 71, 73, 75, 84, 85, 86, 88, 127, 131, 141, 144, 167, 372, 393, 394, 405, 410, 550, 576, 598, 617, 618, and 3856.
[c]STs represented ($n = 68$): 38, 59, 62, 68, 104, 106, 117, 126, 131, 132, 135, 141, 144, 349, 354, 357, 362, 372, 394, 405, 457, 501, 549, 646, 648, 681, 714, 720, 967, 976, 1158, 1166, 1257, 1276, 1340, 1386, 1674, 1865, 1867, 1876, 1877, 1880, 1883, 1919, 1925, 1931, 2141, 2171, 3276, 3290, 3291, 3306, 3307, 3308, 3573, 3619, 3622, 3637, 3711, 4557, 5155, 5156, 5797, 5799, 5800, 6120, 6663, and 6664.

uted globally and occurs as a pathogen and commensal of humans and animals (whether food producing, companion, or wild) and in the environment (12–39).

Detection of STc648 is relevant now for molecular epidemiological studies, and could prove useful for clonal trend surveillance and patient management (7, 8). However, conventional MLST (e.g., http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) is expensive and labor-intensive, and whole-genome-based *in silico* MLST (7) currently has limited availability. Therefore, we sought to develop a multiplex PCR-based assay for STc648 to enable easy and inexpensive screening for STc648 among *E. coli* isolates.

RESULTS

***In silico* predictions.** A query of the Enterobase database (https://enterobase.warwick.ac.uk/species/index/ecoli) identified 328 entries (i.e., strains) corresponding with STc648. These represented 61 total STs, including ST648 proper plus 51 single-locus variants and 9 two-locus variants. ST648 proper, with *icd96* and *gyrB87*, accounted for 246 (75%) of the 328 entries. The complex's 60 non-ST648 STs were represented by a single entry each, except for seven multiple-entry STs (with 2, 2, 2, 3, 3, 7, and 10 entries each; 29 entries total). Of the 60 non-ST648 STs, 49 (82%) also contained *icd96* and *gyrB87*, accounting collectively for an additional 71 (22%) of the 328 entries. Thus, 50 STs within STc648 (i.e., ST648 and 49 others) contained *icd96* and *gyrB87*, accounting collectively for 317 (97%) of the total entries. The remaining 10 STs, which accounted collectively for only 11 entries (3.4% of 328), included 3 STs with *icd96* but not *gyrB87* (3 entries) and 7 with *gyrB87* but not *icd96* (8 entries). Thus, the combination of *icd96* plus *gyrB87* was highly sensitive (97%) for STc648, which was represented mainly by ST648 proper.

Assay performance. In both study laboratories, when the assay was tested against 212 validation set isolates (including 19 STc648 isolates and 193 non-STc648, from 109 different STs), it was 100% accurate in differentiating STc648 and non-STc648 isolates (Table 1). Therefore, its overall performance characteristics were estimated at 100% for sensitivity, specificity, and positive and negative predictive values, with associated 95% confidence intervals of 82% to 100% for sensitivity and positive predictive value, and 98% to 100% for specificity and negative predictive value (Table 1).

In laboratory 1, none of the 50 non-STc648 validation isolates yielded a product from either the *icd96* or *gyrB87* primers. In contrast, in laboratory 2, 14 (10%) of 143 non-STc648 validation isolates yielded products with the *icd96* ($n = 7$) or *gyrB87* ($n = 7$) primers. In 5 strains, this phenomenon corresponded with the presence of authentic *icd96* ($n = 3$) or *gyrB87* ($n = 2$), whereas in 9 strains, it corresponded with an alternate allele of *icd* (*icd52* or *icd6*) or *gyrB* (*gyrB97* or *gyrB180*). The presence of binding sites for the *gyrB87* primers in these *gyrB* alleles was confirmed, with the distinctive allele-

defining single-nucleotide polymorphisms (SNPs) occurring outside or toward the 5′ end of the primer-binding sites, which likely enabled amplification. In contrast, in the *icd* alleles, the binding sites for the forward *icd96* primer had a SNP at the 3′ end, which likely prevented amplification. Thus, although neither the *icd96* nor *gyrB87* primers were entirely specific for the targeted alleles, nearly all strains that amplified with a given primer pair contained the targeted allele, and amplification with both primer pairs was limited to STc648 strains.

DISCUSSION

This novel multiplex PCR-based assay for *E. coli* STc648, which targets four STc648-associated SNPS in *icd* and *gyrB* and capitalizes on the *uidA*-negative status of STc648 strains, proved to be 100% sensitive and specific in distinguishing STc648 from 108 diverse non-STc648 *E. coli* STs, including many within phylogroup F, the origin of STc648. Only 7% of the 212 non-STc648 isolates provided an amplicon for either *icd* or *gyrB*; none provided amplicons for both loci. The assay performed well in two geographically separate laboratories, one using boiled lysates and the other using DNAzol-purified DNA as PCR template. Thus, this novel assay should enable ready and reliable detection of *E. coli* STc648 in any laboratory equipped for conventional PCR.

The new *E. coli* STc648-specific assay can be used in conjunction with published assays that detect ST131 and its subsets (40–42), eight other major clonal subsets within phylogroup B2 (43), and three important phylogroup D-derived clonal subsets (ST69, the O15:K52:H1–ST31/ST393 complex, and ST405) (44–46), for an extensive clonal analysis of extraintestinal *E. coli* isolates. Because several of these lineages are typically multidrug-resistant (4), such clonal typing may help to identify reservoirs of resistance, track epidemiological trends, and guide empirical antimicrobial therapy selection (8).

The typical absence of *uidA* (β-glucuronidase) in STc648 isolates may spuriously reduce the prevalence of STc648 among *E. coli* isolates, if isolates are identified as *E. coli* based in part on their production of β-glucuronidase. We show that this distinctive characteristic of STc648 can be advantageous for distinguishing STc648 from other *E. coli* lineages, all or nearly all of which are *uidA* positive.

The limitations of this study include that the validation set was not exhaustive with respect to non-ST648 STs (which is inevitable, given the enormous number of *E. coli* STs), that the observed predictive values are not generalizable to populations with a different prevalence of STc648, and that minor genetic variation will inevitably lead to some false-positive and false-negative assay results. The strengths of this study include the extensively diverse validation set, the participation of two geographically distant laboratories, and the use of boiled lysates and purified DNA as PCR templates.

Comment. For many bacteria, whole-genome sequence analysis will likely supersede PCR-based typing in the not-too-distant future (7). However, at present, this approach remains unavailable for routine use. Thus, in the near term, PCR-based assays, such as this novel SNP-based multiplex assay for *E. coli* STc648, will likely remain useful for efforts to understand, prevent, diagnose, and treat extraintestinal *E. coli* infections, including those caused by emerging multidrug-resistant lineages, such as STc648.

MATERIALS AND METHODS

Assay development. STc648 was defined operationally as including ST648 and its single- and double-locus variants. To develop the STc648-specific assay, we used an approach similar to that we used for developing other STc-specific PCR-based assays (40, 41, 43–45). First, we assessed the STc648-defining alleles at each of the seven Achtman MLST loci for specificity (or quasi-specificity) to STc648. Using the most promising loci, we next identified STc648-specific (or quasi-specific) SNPs by aligning the published alleles using MEGA6 and scrutinizing the alignments. We then assessed the candidate SNPs for their suitability as primer targets on the basis of the characteristics of the immediate flanking sequences and, within a given gene, the distances of different SNPs from one another. The most promising SNP pairs were used to design forward and reverse primer pairs in different genes, with the 3′ end of each primer being one of the selected SNPs. Primer pairs were designed for use in combination, to add specificity and to enable multiplexing.

TABLE 2 Primers used in the *Escherichia coli* sequence type 648 complex (STc648) multiplex PCR assay

Primer[a]	Primer sequence	Length (nt)	T_m (°C)[c]	% GC	Amplicon size (bp)	ST648 SNP (nucleotide position in gene)
icd96_F18	ACCACTCCGGTTGGTGGt[b]	18	61	61	297	T (181)
icd96_R22	AGAACACGGCTTAATACCGATg[b]	22	60	45.5		C (439)
gyrB87_F18	ATGGTGCGTTTCTGGCCc[b]	18	64	61	143	C (174)
gyrB87_R18	TCTTTGCCGTCGCGCTTa[b]	18	64	56		T (282)
*uidA_For	GCGTCTGTTGACTGGCAGGTGGTGG	25	70	64	510	Not applicable
*uidA_Rev	GTTGCCCGCTTCGAAACCAATGCCT	25	69	56		Not applicable

[a]Primers for *icd* and *gyrB* are novel to this study. Primers for *uidA* are from Walk et al. (47).
[b]Nucleotides specific for the targeted SNP are lowercase.
[c]Based on Primer3 web-based software (http://primer3plus.com).

After empirically optimizing PCR conditions and screening several candidate primer pairs against control strains, the best-performing primer pair combination (Table 2) and PCR conditions (described below) were used in validation experiments. For this, the selected *icd96* primers (267-bp product) and *gyrB87* primers (143-bp product) were combined with published primers for *uidA*, the *E. coli*-specific β-glucuronidase gene (510-bp product) (47), in a single-tube multiplex PCR (Table 2).

For a 15-μl reaction, the amplification mix included: 0.75 U GoTaq hot start polymerase (Promega), 1× GoTaq Flexi Buffer (Promega), 2.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates (dNTPs), 9 pmol ST648 primers, 0.6 pmol *uidA* control primers, 1.2 μl sample DNA, and H_2O to 15 μl. The cycling conditions were denaturation at 95°C for 2 min, 30 amplification cycles of 94°C for 20 s and 67°C for 45 s, extension at 72°C for 5 min, and then holding at 4°C.

PCR products were visualized in agarose gels. The presence or absence of the predicted amplicons for each of the primer pairs was inferred from the band size. A positive STc648 result was the presence of both the *icd96* and *gyrB87* amplicons and the absence of the *uidA* amplicon, which is uniformly absent within STc648, according to all 244 available STc648 genomes, representing 12 different ST within STc648 (unpublished data) (Table 3). A negative STc648 result was the absence of either the *icd96* or the *gyrB87* amplicon, or the absence of both amplicons and presence of the *uidA* amplicon. Other band combinations were considered indeterminate. As the assay yielded categorically positive or negative results when performed with freshly extracted DNA, blinding was not used.

Assay validation. The assay was validated in two different laboratories, one in the United States and one in Australia, using 212 total reference strains that represented, collectively, 108 STs, as determined by full or partial MLST. For partial MLST, allele combinations involving <7 loci that map to only one ST or STc were used to define an isolate's ST or STc. The phylogroups were inferred from the STc or were determined by multiplex PCR (2).

Laboratory 1 used the assay to screen, in duplicates, 60 total isolates, including 10 STc648 isolates and 50 non-STc648 isolates, which represented 50 different STs. The non-STc648 isolates and corresponding STs were from phylogroups (number per phylogroup) A (10), B1 (10), B2 (10), C (3), D (10), E (3), and F (4).

Laboratory 2 used the assay to screen 152 total isolates from phylogroups B2, D, and F, including 9 STc648 isolates and 143 non-STc648 isolates, which represented 68 different STs. The non-STc648 isolates and corresponding STs were distributed by phylogroup as follows: B2 (27 isolates, 27 STs), D (46 isolates, 22 STs), and F (70 isolates, 19 STs).

The ST overlap between the two laboratories included STc648 and nine other STs. PCR templates were boiled lysates in laboratory 1 and DNAzol-purified DNA (Thermo Fisher) in laboratory 2.

TABLE 3 Interpretation algorithm for the *Escherichia coli* sequence type 648 complex (STc648) multiplex PCR assay

Interpretation	Target gene/allele (band size)		
	gyrB87 (143 bp)	*icd96* (297 bp)	*uidA* (508 bp)
STc648	+	+	−
Non-STc648 *E. coli* with *gyrB87* (or related allele), not *icd96*	+	−	+/−[a]
Non-STc648 *E. coli* with *icd96* (or related allele), not *gyrB87*	−	+	+/−[a]
Non-STc648 *E. coli* without *gyrB87* or *icd96*	−	−	+
Indeterminate[b]	+	+	+
Indeterminate[c]	−	−	−

[a]Absence of *uidA* band implies *uidA*-negative *E. coli* (non-STc648).
[b]Expect *uidA* to be absent for STc648 (this result was not encountered among the validation set strains).
[c]Possibly represents *uidA*-negative *E. coli*, non-*E. coli*, no template DNA, or PCR failure (this result was not encountered among the validation set strains).

Statistical analysis. We calculated assay sensitivity, specificity, and positive and negative predictive values, along with 95% confidence intervals (CIs) (Table 1).

ACKNOWLEDGMENTS

The Minneapolis VA Medical Center clinical microbiology laboratory provided some of the isolates used in the study.

The opinions expressed here are strictly those of the authors and do not necessarily reflect those of the author's respective institutions or the Department of Veterans Affairs.

J.R.J. has received research grants or consultancies from Actavis/Forest, Crucell/Janssen, Merck, and Tetraphase, and has patent applications for tests to identify *E. coli* strains. The other authors report no conflicts of interest.

This material is based in part on work supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, grant no. 1 I01 CX000920-01 (to J.R.J.).

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