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Evaluation of a Method Using Three Genomic Guided *Escherichia coli* Markers for Phylogenetic Typing of *E. coli* Isolates of Various Genetic Backgrounds

Kouta Hamamoto,[a] Shuhei Ueda,[a,b] Yoshimasa Yamamoto,[b,c] Itaru Hirai[a,b]

Laboratory of Microbiology, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan[a]; Japan Science and Technology Agency/Japan International Cooperation Agency, Science and Technology Research Partnership for Sustainable Development (JST/JICA, SATREPS),[b] and Osaka Prefectural Institute of Public Health, Higashinari-ku,[c] Osaka, Japan

Genotyping and characterization of bacterial isolates are essential steps in the identification and control of antibiotic-resistant bacterial infections. Recently, one novel genotyping method using three genomic guided *Escherichia coli* markers (GIG-EM), *dinG*, *tonB*, and dipeptide permease (DPP), was reported. Because GIG-EM has not been fully evaluated using clinical isolates, we assessed this typing method with 72 *E. coli* collection of reference (ECOR) environmental *E. coli* reference strains and 63 *E. coli* isolates of various genetic backgrounds. In this study, we designated 768 bp of *dinG*, 745 bp of *tonB*, and 655 bp of DPP target sequences for use in the typing method. Concatenations of the processed marker sequences were used to draw GIG-EM phylogenetic trees. *E. coli* isolates with identical sequence types as identified by the conventional multilocus sequence typing (MLST) method were localized to the same branch of the GIG-EM phylogenetic tree. Sixteen clinical *E. coli* isolates were utilized as test isolates without prior characterization by conventional MLST and phylogenetic grouping before GIG-EM typing. Of these, 14 clinical isolates were assigned to a branch including only isolates of a pandemic clone, *E. coli* B2-ST131-O25b, and these results were confirmed by conventional typing methods. Our results suggested that the GIG-EM typing method and its application to phylogenetic trees might be useful tools for the molecular characterization and determination of the genetic relationships among *E. coli* isolates.

Several species of antibiotic-resistant bacteria have been found to be causative agents in frequent nosocomial infections. To understand and contain nosocomial infections, the genetic relationships between the causative bacterial isolates need to be identified. To this end, many analytical methods have been developed, including pulsed-field gel electrophoresis (PFGE) (1), ribotyping (2), arbitrarily primed PCRs, such as enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and repetitive element sequence-based PCR (rep-PCR) (3), and several multilocus sequence typing (MLST) schemes (4–7).

PFGE and ribotyping are principally based on restriction fragment length polymorphisms (RFLP). PFGE in particular has been utilized as one of the gold standard methods, because standard protocols for certain pathogens, such as *Escherichia coli* serotype O157, have been established and are available online at the PulseNet website (Centers for Disease Control and Prevention, USA) (8). It is relatively simple to obtain reproducible results, i.e., PFGE banding patterns, and perform comparisons of the consequent PFGE banding patterns among test isolates. However, it may be difficult to detect intrachromosomal rearrangements and recombination. PFGE is applicable for an assessment of clonality among test isolates but may not be suitable for considerations of genetic ancestor-descendant relationships.

Both ERIC-PCR and rep-PCR target repetitive DNA sequence elements, which are sporadically located throughout the bacterial genome. These PCR methods are convenient and appropriate for testing isolates using larger sample sizes. However, the number, size, and thickness of the amplified DNA fragments tend to vary among test isolates. Unclear results from ERIC-PCR or rep-PCR may make an assessment of the genetic relationships among test isolates difficult.

Generally, the results obtained by analytical methods based on PCR can be affected by factors such as the purity and complexity of template DNA and type of thermal cycler. In contrast, MLST is a nucleotide sequence-based method and is therefore regarded as one of the most reliable analytical methods for the comparison of genetic backgrounds among bacterial isolates (9). Sequences of seven or eight well-conserved housekeeping genes, selected as genetic markers, are utilized to determine the sequence type (ST) of each isolate. MLST is able to correctly classify bacterial isolates. Because of the reliability of the method, MLST has been well utilized in the analyses of bacterial isolates causing nosocomial infections, despite the labor and cost involved. The obtained sequences are applicable to phylogenetic analysis for the determination of the genetic relationships among test isolates and for drawing phylogenetic trees using several phylogenetic methods.

With the rapid advancement of next-generation sequencing

Received 27 January 2015 **Returned for modification** 15 February 2015
Accepted 19 March 2015

Accepted manuscript posted online 25 March 2015

Citation Hamamoto K, Ueda S, Yamamoto Y, Hirai I. 2015. Evaluation of a method using three genomic guided *Escherichia coli* markers for phylogenetic typing of *E. coli* isolates of various genetic backgrounds. J Clin Microbiol 53:1848–1853. doi:10.1128/JCM.00227-15.

Editor: E. Munson

Address correspondence to Itaru Hirai, hiraii@med.u-ryukyu.ac.jp.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.00227-15.

doi:10.1128/JCM.00227-15

TABLE 1 Test isolates used in this study

ID[a]	ST	Ph.G[b]	Specimen type	Patient type	O25 gene
N0021	131	B2	Urine	Inpatient	+
N0055	131	B2	Urine	Outpatient[c]	+
N0057	131	B2	Urine	Inpatient	+
N0058	131	B2	Urine	Outpatient[c]	+
N0127	131	B2	Urine	Outpatient[c]	+
N0211	131	B2	Vaginal discharge	Inpatient	+
N0214	131	B2	Sputum	Outpatient[c]	+
N0222	131	B2	Urine	Outpatient[c]	+
N0223	131	B2	Urine	Outpatient[c]	+
N0226	405	D	Feces	Outpatient[c]	ND[d]
N0269	131	B2	Urine	Inpatient	+
N0327	131	B2	Urine	Outpatient[c]	+
N0349	131	B2	Urine	Inpatient	+
N0995	131	B2	Urine	Inpatient	+
N1011	95	B2	Urine	Outpatient	+
N1032	131	B2	Urine	Outpatient[c]	+

[a] ID, identification.
[b] Ph.G, phylogenetic group.
[c] Outpatient with past hospitalization history.
[d] ND, not detected.

technology and its application to microbiology research, whole-genome sequence information from various bacterial species has become widely accessible. Consequently, phylogenetic analysis using whole-genome sequence information can identify genetic ancestor-descendant relationships among isolates. Sahl, Matalka, and Rasko (10) used *E. coli* as a model to perform phylogenetic analysis using whole-genome sequences and designated three potential genetic markers (genomic guided *E. coli* markers [GIG-EM]) to classify the test isolates. They reported that the classification by the phylogenetic tree with whole-genome sequences was consistent with the phylogenetic grouping of *E. coli*, which mainly consists of A, B1, B2, and D groups (11, 12). However, classification by the phylogenetic tree drawn with concatenated sequences obtained using current MLST schemes was not completely concordant with the phylogenetic grouping of the test isolates (10). Sahl, Matalka, and Rasko (10) also reported that the phylogenetic group classifications of the reference isolates were matched with those of the reference *E. coli* strains by the phylogenetic tree drawn with concatenated sequences of the three GIG-EM. However, further evaluation of the usefulness of applying the GIG-EM method to analyses of clinical *E. coli* isolates from patients with nosocomial infections is important. Therefore, we evaluated the GIG-EM classification method using various *E. coli* isolates, including clinical isolates and isolates from asymptomatic healthy individuals.

MATERIALS AND METHODS

Isolates. A total of 63 *E. coli* isolates possessing bla_{CTX-M} were examined in this study. Thirty-two KC series *E. coli* isolates were isolated from fecal specimens from asymptomatic healthy Thai individuals in 2008 (see Table S1 in the supplemental material) (13, 14). Fifteen JO series *E. coli* isolates were obtained from fecal specimens from nursing home residents in the Kinki region of Japan in 2010 (15). All the JO isolates were *E. coli* B2-ST131-O25b, except for strain JO120, which was *E. coli* B2-ST131 without the O25b gene. The 16 N isolates were randomly selected from 97 *E. coli* isolates producing extended-spectrum β-lactamase (ESBL), which were obtained from the Okinawa Prefectural Nambu Medical Center between June 2013 and July 2014 (Table 1). The bacterial species of the N isolates were determined by the Vitek 2 system (bioMérieux, Marcy l'Etoile, France). ESBL production of the N isolates was confirmed according to CLSI guideline M100-S23 (16) and PCR with *Ex Taq* (TaKaRa Bio, Inc., Ōtsu, Japan) with specific primers targeting pan-bla_{CTX-M} (13).

Phylogenetic analyses. The STs of the isolates were determined by the MLST method described by Wirth et al. (4). Phylogenetic grouping of *E. coli* isolates using three genetic markers, such as *chuA*, *yjaA*, and TspE4C2, and O25b PCR confirmation of the *E. coli*-ST131 isolates were performed according to previously described protocols (12, 17).

Genomic guided *E. coli* marker phylogenetic typing. The three selected markers *dinG*, *tonB*, and DPP were amplified with PrimeSTAR Max DNA polymerase (TaKaRa Bio, Inc.) using the primers described by Sahl, Matalka, and Rasko (10). The amplified DNA fragments were purified with the QIAquick gel extraction kit (Qiagen K.K., Tokyo, Japan), according to the manufacturer's product manual. The purified DNA fragments were subjected to sequence analysis using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies Japan, Tokyo, Japan). The obtained sequences of the three genetic markers of the 63 *E. coli* isolates (*dinG*, GenBank accession no. LC032292 to LC032354; *tonB*, GenBank accession no. LC032229 to LC032291; and DPP, GenBank accession no. LC032166 to LC032228) were processed and concatenated using the MEGA software version 6.06 (18). The reference sequences of *dinG* (GenBank accession no. JQ283606 to JQ283677), *tonB* (GenBank accession no. JQ283534 to JQ283605), and DPP (GenBank accession no. JQ283462 to JQ283533) of the *E. coli* collection of reference (ECOR) environmental *E. coli* reference strains (19), which were previously submitted by Sahl, Matalka, and Rasko (10), were verified using GenBank (20). The concatenated sequences were utilized to construct phylogenetic trees by the neighbor-joining method (21) using the MEGA software.

TABLE 2 Length and location of the GIG-EM genetic markers

Marker	Length (bp)	Location[a]
dinG	768	2861569–2862336
tonB	745	2853210–2853954
DPP	655	2955610–2956264

[a] Regions of the GIG-EM genetic markers are indicated corresponding to the genome sequence of *E. coli* strain K-12 substrain MG1655 (GenBank accession no. CP009685).

RESULTS

After several preliminary considerations, the 72 retrieved reference sequences of the ECOR strains were processed using the MEGA software. Consequently, 768 bp of *dinG*, 745 bp of *tonB*, and 655 bp of DPP were chosen (Table 2). Using the concatenated sequences, phylogenetic trees were drawn using the MEGA software (Fig. 1A). Bootstrap confidence levels of >95 were observed at some branches, particularly those including whole phylogenetic group B2 isolates. This demonstrated that the B2 branches in the phylogenetic trees were well supported. As shown in Fig. 1A, the reference ECOR isolates were assigned to their respective phylogenetic groups by following the original GIG-EM phylogenetic tree (10).

Forty-seven *E. coli* isolates (32 KC and 15 JO) were utilized to compare the distributions of the examined *E. coli* isolates on the phylogenetic trees based on sequences obtained using the GIG-EM typing method and conventional MLST (Fig. 2). The KC group consisted of isolates having 27 STs and belonging to four phylogenetic groups. In contrast, all JO isolates were identified as *E. coli* B2-ST131. The KC isolates were sporadically distributed, and the JO isolates localized to one branch on both the GIG-EM and MLST trees. With the exception of phylogenetic group B2 isolates, no clear relationship was evident between phylogenetic group and classification by GIG-EM and MLST.

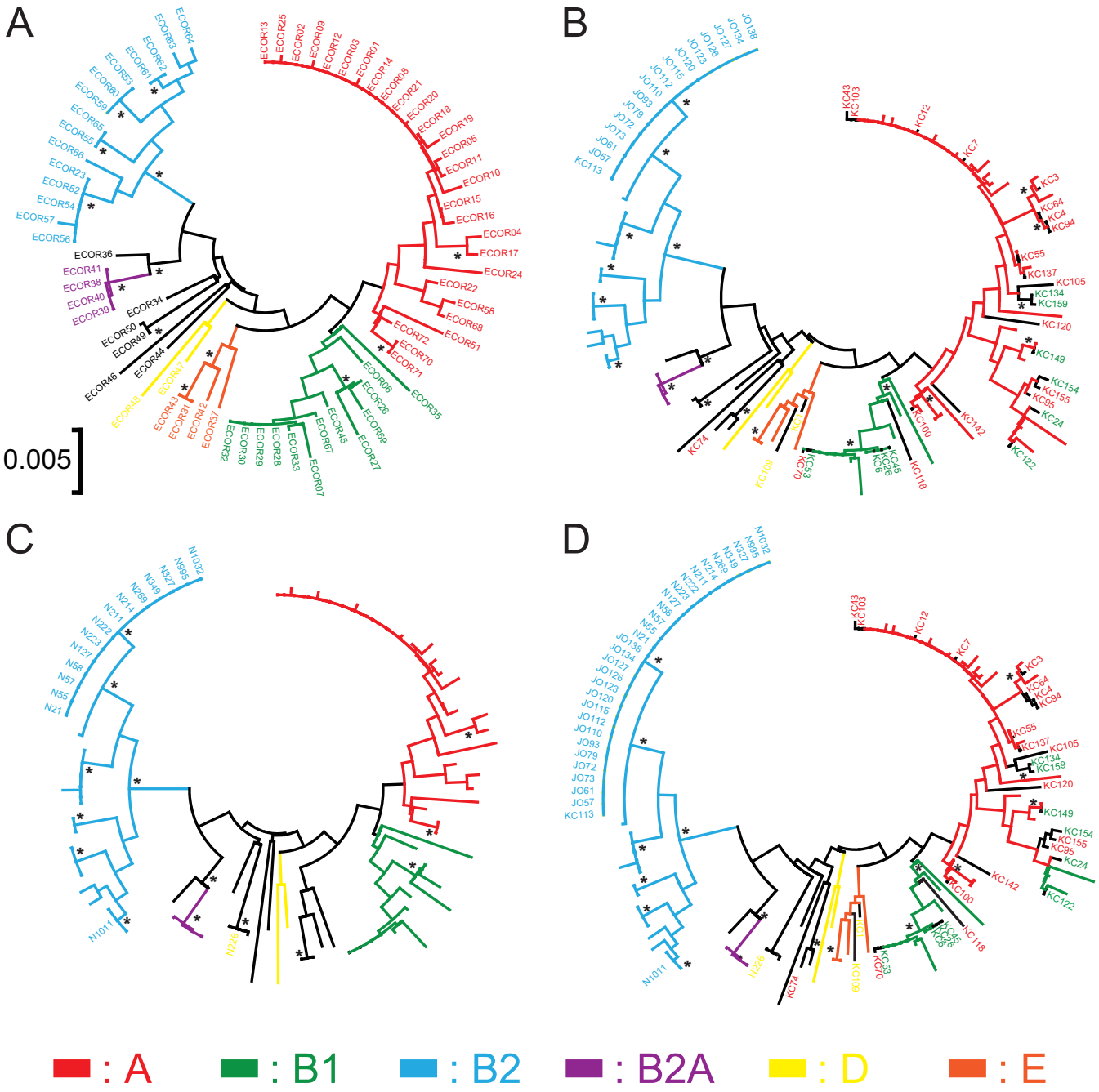

FIG 1 GIG-EM phylogenetic trees. Phylogenetic trees are drawn based on concatenations of the genetic markers *dinG*, *tonB*, and DPP of the ECOR *E. coli* reference strains (A), of the ECOR reference strains with KC series and JO series isolates (B), of the ECOR reference strains with N series isolates, which were utilized as test isolates (C), and of the ECOR reference strains with all *E. coli* isolates (D). *, branches with bootstrap confidence levels of >95.

Generally, the shapes of the phylogenetic trees and the bootstrap confidence levels of individual branches were easily altered by the number and character of input subjects. Therefore, the 47 *E. coli* isolates were subjected to the already established GIG-EM phylogenetic trees drawn using the ECOR reference strains (Fig. 1B). A similar trend was observed in the GIG-EM phylogenetic tree compared with the phylogenetic tree including the 72 ECOR reference strains. Isolates with the same ST, such as KC4 and KC94, were properly assigned to the same branch. However, isolates from phylogenetic groups A and B1 were not effectively separated and were localized together on the tree. Interestingly, *E. coli* B2-ST131 isolates were still located within a clade with a higher bootstrap confidence level.

To determine the suitability of using the GIG-EM typing method for analyzing clinical isolates, we examined 16 randomly selected N series *E. coli* isolates producing CTX-M-type ESBL obtained from a Japanese prefectural hospital. Except for strain N0226, 15 of the 16 *E. coli* isolates localized to the phylogenetic B2

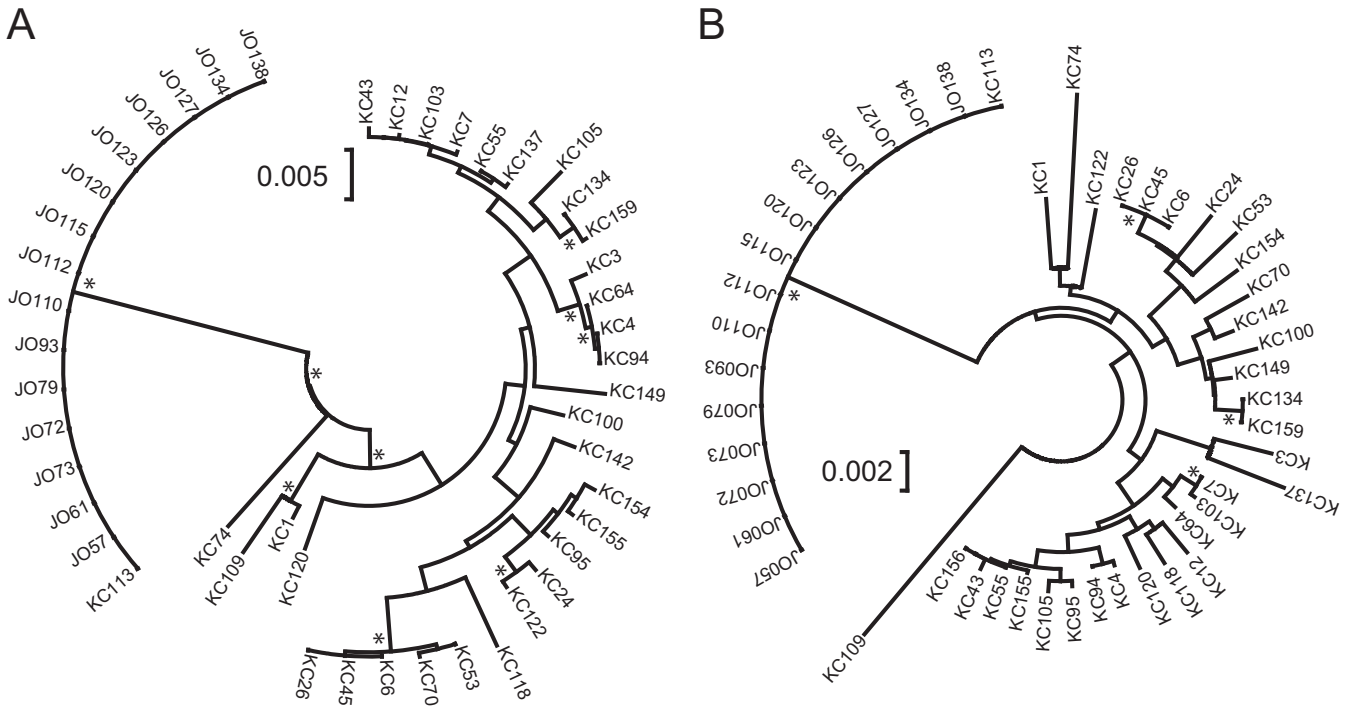

FIG 2 Comparison of GIG-EM and MLST phylogenetic trees. Phylogenetic trees are drawn based on concatenations of the genetic markers of the GIG-EM typing method (A) and conventional MLST typing method (B) of KC series and JO series isolates. *, branches with bootstrap confidence levels of >95.

branch. Among them, 14 isolates were distributed in the same clade as the *E. coli* B2-ST131 isolates (Fig. 1B to D). To confirm the classification obtained from the GIG-EM phylogenetic tree, the phylogenetic group and ST of each N isolate were determined. In addition, PCR was used to detect the gene encoding O25b serotypes of the N series isolates, because an association between O25b serotype and *E. coli* B2-ST131 strains was previously reported (22, 23). The 14 *E. coli* isolates were subsequently confirmed to be *E. coli* B2-ST131-O25b. In addition, a single group B2 isolate was identified as *E. coli* B2-ST95, and one non-B2 group isolate was identified as *E. coli* D-ST405. Taken together, our results indicate that the GIG-EM typing method consistently classified the test *E. coli* clinical isolates according to their STs. In particular, the discrimination of phylogenetic group B2 isolates in the GIG-EM phylogenetic tree was well supported by a high bootstrap confidence level.

DISCUSSION

E. coli isolates producing ESBL have frequently been detected in various clinical specimens, regardless of whether the infection was nosocomial or community acquired. These ESBL-producing *E. coli* isolates have been detected even in asymptomatic healthy individuals, especially in Asian countries (13, 14, 24). Along with epidemiological analysis to determine the factors contributing to the widespread distribution of ESBL-producing bacteria, effective phylogenetic typing methods are essential to understanding the distribution of ESBL-producing bacteria. In this study, we evaluated the currently proposed GIG-EM typing method with 63 ESBL-producing *E. coli* isolates of different origins and varied genetic backgrounds.

First, we considered the sequence length of each genetic marker included in the GIG-EM typing method using ECOR reference isolates as a model. The optimal sequence length was determined to be long enough to include maximum variation among the reference sequences, yet short enough to be analyzed by a single sequence reading. While a shorter sequence length of the genetic markers might be advantageous in sequencing, it may be disadvantageous in discriminatory power. In our preliminary consideration of the sequence length, shorter sequences distorted phylogenetic trees in comparison with the phylogenetic tree drawn using the whole-genome sequences reported by Sahl, Matalka, and Rasko (10).

In molecular epidemiology, the ST and phylogenetic group were important indices for indicating genetic relationships among the target bacterial isolates. As shown in Fig. 1A, the phylogenetic group classification of the ECOR reference isolates, i.e., A, B1, B2, B2A, D, and E, was relatively consistent with the topology of the GIG-EM phylogenetic tree (10). However, in our results, the phylogenetic groups of the test isolates were not always associated with locations in the GIG-EM phylogenetic tree (Fig. 1B). Because the *chuA* gene is absent from strains in groups B1 and A, phylogenetic grouping was based on the presence (B1) or absence (A) of an anonymous DNA region, TSPE4.C2 (17). It is possible that mutation in primer-targeting sequences of the TSPE4.C2 region could affect assignment to the correct phylogenetic group of certain *E. coli* isolates. This discrepancy between STs and phylogenetic groups in our test isolates was observed in both the GIG-EM and MLST trees (Fig. 2). Considering genetic distances and the number of branches with a bootstrap confidence level of >95, the classification of the isolates by the GIG-EM typing method was more accurate than that with the MLST tree. In contrast to phylogenetic groups B1 and A, the classification of phylogenetic

group B2 requires triple-positive detection of *chuA*, *yjaA*, and TSPE4.C2 (17). Therefore, the assignment of an isolate to phylogenetic group B2 can be done with relative certainty. Both GIG-EM- and MLST-based phylogenetic trees effectively separated B2 isolates in accordance with their classification as phylogenetic group B2, supported by higher bootstrap confidence levels (Fig. 2).

We then evaluated the GIG-EM typing method, using clinical isolates, before performing the genetic characterization of these isolates. *E. coli* B2-ST131-O25b has been recognized as one of the pandemic ESBL-producing *E. coli* clones (25, 26). Therefore, the detection and confirmation of *E. coli* B2-ST131-O25b are important, especially in nosocomial infection. In addition to the ST131 strain, an *E. coli* D-ST405 isolate and a B2-ST95 isolate were also identified in this study. *E. coli* D-ST405 has frequently been reported as one of the ESBL-producing *E. coli* clones in many countries, particularly in Japan (27–35). Another *E. coli* isolate, B2-ST95, was reported as an extraintestinal pathogenic *E. coli* strain exhibiting high virulence, which potentially originated from poultry (36). These isolates were located separately from *E. coli* B2-ST131 isolates in the phylogenetic tree (Fig. 1C). In our results, all strains of *E. coli* isolates with the same ST, such as ST131, were classified in one branch in the GIG-EM phylogenetic tree. This meant that those isolates could be typed in the GIG-EM phylogenetic tree and confirmed with conventional phylogenetic grouping and O-antigen determination by PCR or serological methods (Table 1 and Fig. 1C and D).

The GIG-EM typing method, which utilizes only three genetic marker sequences, is a less laborious analytical method than conventional MLST schemes. Therefore, using GIG-EM to acquire information about *E. coli* isolates, including pandemic nosocomial clones, may facilitate the identification of STs of clinical isolates from patients with nosocomial and community-acquired infections. Taken together, our results indicate that typing and classification of *E coli* isolates with the GIG-EM method can be a useful tool to determine molecular and genetic relationships among *E. coli* isolates and could replace conventional MLST methods.

ACKNOWLEDGMENTS

We thank A. Shingaki, R. Nakama, and Y. Tohma for their technical assistance.

This work was supported in part by Technology Agency/Japan International Cooperation Agency, Science, and Technology Research Partnership for Sustainable Development (JST/JICA, SATREPS).

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