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Multilocus Sequence Typing versus Pulsed-Field Gel Electrophoresis for Characterization of Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolates

Lucia L. Nemoj, ^{1,3*} Mamuka Kotetishvili, ¹ Justine Tigno, ^{1,4} Ananda Keefer-Norris, ¹
Anthony D. Harris, ^{1,3} Eli N. Perencevich, ^{1,3} Judith A. Johnson, ^{2,3} Dave Torpey, ^{1,3}
Alexander Sulakvelidze, ¹ J. Glenn Morris, Jr., ¹ and O. Colin Stine ¹

Department of Epidemiology and Preventive Medicine¹ and Department of Pathology,² University of Maryland School of Medicine, and VA Maryland Health Care System,³ Baltimore, Maryland, and Department of Pathology, Case Western Reserve University, Cleveland, Ohio⁴

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Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* strains are emerging pathogens. Molecular typing of ESBL-producing *E. coli* is useful for surveillance purposes, to monitor outbreaks and track nosocomial spread. Although pulsed-field gel electrophoresis (PFGE) is the current “gold standard” for bacterial molecular typing, multilocus sequence typing (MLST) may offer advantages. Forty ESBL-producing *E. coli* isolates were selected at random from a cohort of intensive care unit patients who had active surveillance perirectal cultures done. PFGE identified 19 unique PFGE types (PT) among the 40 isolates; MLST identified 22 unique sequence types. MLST had greater discriminatory ability than PFGE for ESBL-producing *E. coli*. Simpson’s indices of diversity for PFGE and MLST were 0.895 and 0.956, respectively. There were five clonal complexes (CCs) (isolates with differences of no more than two loci) that each contained multiple PT, but each PT was found in only one CC, indicating genetic consistency within a CC. MLST has clear utility in studies of ESBL-producing *E. coli*, based on a greater discriminatory ability and reproducibility than PFGE and the ability to a priori define genetically related bacterial strains.

Pulsed-field gel electrophoresis (PFGE) is the most widely used tool for molecular typing of bacterial strains (1), but new DNA fingerprinting techniques, including multilocus sequence typing (MLST), are emerging as alternatives, particularly when information regarding evolutionary history is needed (30). MLST is a relatively new technique where multiple genes (loci) are sequenced to measure genetic relatedness and analyze sequence variation between alleles from many strains (20). MLST has already been used to characterize a number of pathogenic bacteria, including *Neisseria meningitidis*, *Enterococcus* spp., *Staphylococcus aureus*, and *Campylobacter* spp. (3, 4, 7, 21). In studies comparing MLST to PFGE among *Salmonella* spp., *Vibrio cholerae*, *Enterococcus faecalis*, and *S. aureus* isolates, MLST has been found to have similar or greater discriminatory ability than PFGE (17, 18, 21, 27). However, these results may not be generalizable to all species, since a study comparing MLST to PFGE in *Escherichia coli* O157:H7 found that PFGE had a greater discriminatory ability than MLST did (26).

MLST has not yet been used to characterize extended-spectrum beta-lactamase (ESBL)-producing *E. coli* isolates. ESBL production, which confers resistance to all penicillins and cephalosporins, is found in *E. coli* and other members of the family *Enterobacteriaceae*. In the United States, the prevalence of ESBL carriage ranges between 0 and 25%, with a national

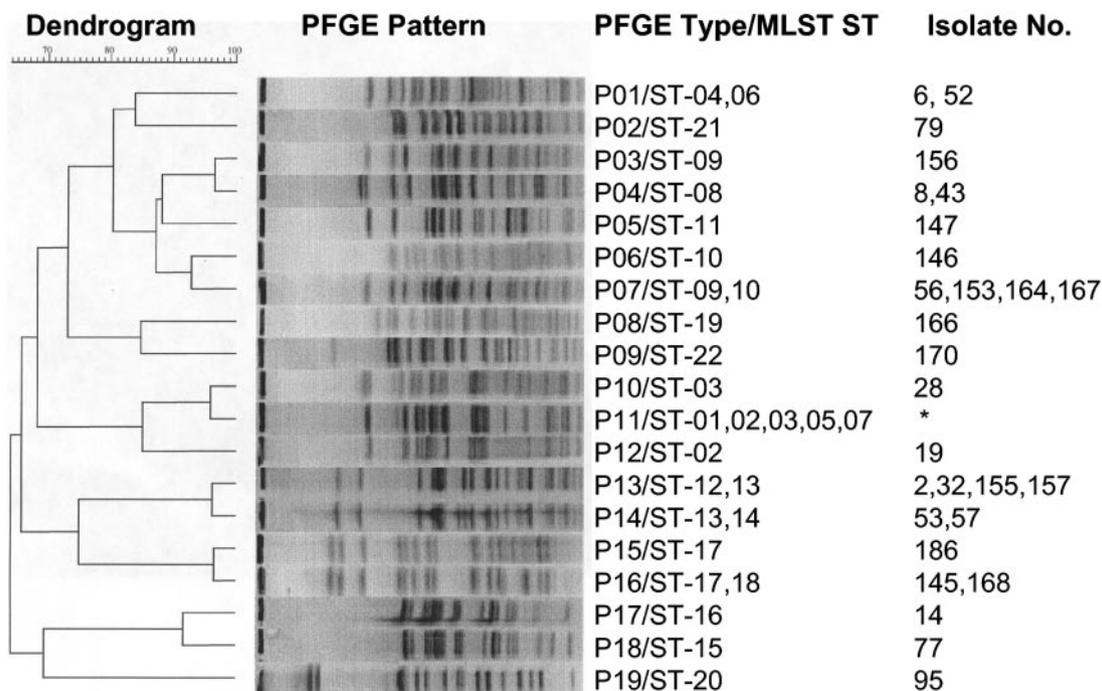
average of approximately 6%, and these numbers are increasing (2, 24). ESBL-producing *E. coli* bacteria cause nosocomial infections, including bloodstream infections, urinary tract infections, and pneumonias, and are associated with increased patient morbidity and mortality (29). Molecular typing of ESBL-producing *E. coli* is useful as a framework for hospital epidemiologists, microbiologists, and clinicians to conduct surveillance studies, monitor outbreak situations, and track the nosocomial spread of emerging pathogens. The aim of this study was to compare the discriminatory ability and potential utility of PFGE and MLST for the genetic characterization of ESBL-producing *E. coli* isolates.

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MATERIALS AND METHODS

Bacterial isolates. Forty ESBL-producing *E. coli* isolates were chosen at random from a cohort of intensive care unit (ICU) patients who had perirectal surveillance cultures taken upon admission, every week, and upon discharge from the ICU from October 2001 through September 2002. Perirectal swabs were plated directly onto MacConkey agar with 1 µg/ml of ceftazidime (Lilly) to screen for resistant isolates. Single lactose-fermenting colonies were streaked for isolation on Trypticase soy agar with 5% sheep blood. Plates were incubated for 18 to 24 h at 35 to 37°C before identification and susceptibility testing. Isolates were identified to the species level with API 20E (BioMerieux). *E. coli* isolates were tested for ESBL production by disk diffusion according to NCCLS guidelines (22, 23). Strains were stored at –80°C in Trypticase soy broth with 20% glycerol. For DNA isolation, single colonies were inoculated into Trypticase soy broth and incubated for 18 to 24 h at 37°C. Genomic DNA was isolated using Prepman Ultra (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer’s guidelines.

* Corresponding author. Mailing address: Department of Epidemiology and Preventive Medicine, University of Maryland School of Medicine, MSTF Building, Room 9-34, 10 South Pine Street, Baltimore, MD 21201. Phone: (410) 706-0082. Fax: (410) 706-4581. E-mail: lnemoj@epi.umaryland.edu.



*P11 isolates 4,16,20,27,44,69,85,91,158,162,163,165

FIG. 1. PFGE dendrogram with the corresponding MLST sequence types of the ESBL-producing *E. coli* isolates. Each row represents a unique PFGE type with its unique PFGE pattern. The corresponding MLST sequence type (ST) is similarly shown with the isolate number(s). For example, the first row depicts PFGE type 1 (P01) which corresponded to MLST ST-04 and ST-06 from isolates 6 and 52.

PFGE. PFGE was performed according to the PulseNet protocol of the Centers for Disease Control and Prevention (2a). The agarose-embedded bacterial genomic DNA was digested with restriction enzyme XbaI at 37°C for 4 hours. Electrophoresis was performed on 1% agarose gel with 0.5× Tris-borate-EDTA buffer. The electrophoretic conditions were optimized for separation of the 24- to 600-kb XbaI-digested macrorestriction fragments. The following PFGE parameters were applied: voltage of 5.6 V/cm, initial switch time of 1.71 seconds, final switch time of 54.17 seconds, and run time of 20 h. Electrophoresis was conducted by using a CHEF-Mapper (Bio-Rad Laboratories, Hercules, CA). A dendrogram (Fig. 1) was constructed with Fingerprinting DST Molecular Analyst software (Bio-Rad Laboratories). The patterns were compared by means of the Jaccard coefficient of band-based similarity using the unweighted pair group method. A tolerance of 1.75% in band position was applied. Isolates with identical PFGE banding patterns were considered identical PFGE types.

MLST. Standard DNA amplification and sequencing with 27F and 519R primers (11) of 16S rRNA was performed to confirm that all isolates were *E. coli* isolates. Internal fragments from eight housekeeping genes (loci) and two antibiotic resistance genes were selected for amplification and sequencing. Housekeeping genes that are conserved in *E. coli*, involved with intermediary metabolism, and previously shown to work in *E. coli* and closely related species were chosen: *gnd*, *glnA*, *aroE*, *arcA*, *mdh*, *adh*, *gyrB*, and *purA*. The antibiotic resistance genes were *tem* and *shv*. Four of these genes (*gnd*, *aroE*, *arcA*, and *mdh*) have previously been used in *E. coli* (26), and *glnA* has been used in *Salmonella* spp. (18). The *adh*, *gyrB*, and *purA* genes have recently been used as part of an MLST protocol for *E. coli*; the primers used are shown in Table 1 and are available at http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli/documents/primersColi_html. The same primer was used for amplification and sequencing. Amplification conditions for *adh*, *gyrB*, and *purA* were as follows: an initial denaturation at 95°C (2 min), followed by 35 cycles, with 1 cycle consisting of denaturation at 94°C (1 min), annealing at 56°C (1 min), and elongation at 72°C (1 min). PCR amplification of approximately 700-bp fragments of *tem* and *shv* were performed as outlined previously (28). All negative reactions were repeated twice. Bidirectional sequencing of amplified products was performed with a Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Inc., Foster City, CA) and the ABI 3700 automatic sequencer. The raw trace files were read, and the sequences from both directions assembled into a single contig using Phred (5, 6), Phrap

(10), and Consed (9). Low-quality sequence was removed from the ends, and the sequences were aligned with ClustalX (13).

MLST data analysis. Each unique nucleotide sequence was assigned a unique allele number. The allelic profile for each isolate was determined and consisted of a line listing the allele number for each gene in turn. Isolates were then assigned a sequence type (ST) according to their allelic profiles. Isolates were considered genetically identical and hence the same ST if they were identical at all 10 loci. An MLST dendrogram was constructed from the data matrix of allelic mismatches with START, applying unweighted pair group method with averages. Isolates were grouped into clonal complexes (CC) by BURST implemented in the program START, if they differed at no more than two loci (14).

Simpson's index of diversity (*D*) is an index of discrimination for bacterial typing methods and was calculated for both PFGE and MLST using the following formula (12):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j - 1)$$

In the above equation, *N* is the total number of strains in the sample population, *S* is the total number of types described, and *n_j* is the number of strains belonging to the *j*th type.

Nucleotide sequence accession numbers. The housekeeping genes *gnd*, *glnA*, *aroE*, *arcA*, *mdh*, *adh*, *gyrB*, and *purA* and two antibiotic resistance genes, *tem* and *shv*, were sequenced for each isolate and deposited in GenBank under accession numbers AY832931 to AY833007.

RESULTS

PFGE types. Nineteen PFGE types (P1 to P19) were identified among the 40 ESBL *E. coli* isolates (Fig. 1). Isolates were included within the same PFGE type if they had identical PFGE patterns. There was one dominant PFGE type, P11, which included 12 of the 40 isolates. P07 and P13 each con-

TABLE 1. Amplification and sequencing primers for *adk*, *gyrB*, and *purA*^a

Gene	Primer sequence (5'→3')
<i>adk</i>	ATTCTGCTTGCGCTCCGGG
.....	CCGTCAACTTTCGCGTATTT
<i>gyrB</i>	TCGGCGACACGGATGACGGC
.....	ATCAGGCCTTCACGCGCATC
<i>purA</i>	CGCGCTGATGAAAGAGATGA
.....	CATACGGTAAGCCACGCAGA

^a The primers for the remaining seven genes (*gnd*, *glnA*, *aroE*, *arcA*, *mdh*, *tem*, and *shv*) have previously been published as cited in the text.

tained four isolates. P01, P04, P14, and P16 each encompassed two isolates, and the remaining 12 ESBL-producing *E. coli* isolates were singletons and represented unique PFGE types.

MLST sequence types. Eight housekeeping genes (loci), *gnd*, *glnA*, *aroE*, *arcA*, *mdh*, *adk*, *gyrB*, and *purA*, and two antibiotic resistance genes, *tem* and *shv*, were amplified and sequenced for each isolate. Six isolates did not amplify *tem*, and 10 isolates did not amplify *shv*, after multiple attempts. These were assigned an allele number of 100 in Table 2. There were 10, 7, 7, 7, 8, 6, 7, 9, 5, and 11 unique alleles identified at each locus, respectively. Twenty-two unique MLST sequence types (ST) were identified among the 40 ESBL *E. coli* isolates and are shown in Table 2. An MLST dendrogram of these isolates was constructed with START software and is shown in Fig. 2.

Discriminatory ability. Simpson's index of diversity (*D*) was used as an index of discrimination for bacterial typing to compare MLST to PFGE. For the 19 PFGE types, *D* was 0.895. For the 22 sequence types identified by MLST, *D* was 0.956. These indices indicate that if two isolates were sampled randomly from this population, then on 89.5% and 95.6% of occasions,

they would fall into different types; an index of greater than 0.90 is desirable (12).

Correlation between PFGE and MLST. A one-to-one correlation between PFGE types and ST did not exist (Fig. 1 and Table 2). Some isolates with the same PFGE type had multiple STs, and conversely, isolates with the same ST had multiple PFGE types. For example, there were 12 isolates with P11 that had ST 1, 2, 3, 5, and 7. All these isolates were in CC1 and were differentiated by changes only at the *shv* locus. Conversely, isolates with ST2 had P11 and P12 (Table 2). However, isolates with an identical ST had distinct, but related PFGE patterns, differing by one or two bands, suggesting that isolates considered closely related by the criteria of Tenover et al. correspond to our CCs (32). However, there was one notable exception: CC1 included P01, P10, P11, and P12. P10, P11, and P12 were clustered in the PFGE dendrogram, but P01 was unrelated to P10, P11, and P12.

Clonal groups and degree of deviation from ancestral alleles. MLST sequence types were grouped using START software (14) into CCs consisting of all isolates with an identical ST or a ST with a single-locus difference from another ST within the CC (Fig. 2). There were five CCs. CC1 included ST-1, -2, -3, -4, -5, -6, and -7 and P01, P10, P11, and P12. CC2 included ST-8, -9, and -10 and P03, P04, P06, and P07. CC3 included ST-12, -13, and -14 and P13 and P14. CC4 included ST-15 and -16 and P17 and P18. CC5 included ST-17 and -18 and P15 and P16. The remaining five isolates were singletons (ST-11, -19, -20, -21, and -22) and did not cluster into any CC. One of these isolates (isolate number 147) with ST-11 differed from its ancestral allele at three loci, *gnd*, *tem*, and *shv* by 27, 3, and 2 nucleotides, respectively. The ancestral allele is the most common allele at each locus (20). Two singleton isolates

TABLE 2. MLST sequence types and PFGE types of ESBL-producing *E. coli* isolates

Isolate(s)	ST ^a	PFGE type	CC ^b
16, 27, 44, 85, 91, 158	ST-01 (2-2-1-1-1-12-1-1-1-4)	P11	CC1
19, 20	ST-02 (2-2-1-1-1-12-1-1-1-6)	P11, P12	CC1
4, 28	ST-03 (2-2-1-1-1-12-1-1-1-8)	P10, P11	CC1
6	ST-04 (2-2-1-1-1-12-1-1-1-2)	P01	CC1
69	ST-05 (2-2-1-1-1-12-1-1-1-9)	P11	CC1
52	ST-06 (2-2-1-1-1-12-1-1-1-10)	P01	CC1
162, 163, 165	ST-07 (2-2-1-1-1-12-1-1-1-100)	P11	CC1
8, 43	ST-08 (3-3-2-3-2-2-13-16-1-1)	P04	CC2
156, 167	ST-09 (3-3-2-3-2-2-13-16-100-4)	P03, P07	CC2
56, 146, 153, 164	ST-10 (3-3-2-3-2-2-13-16-1-100)	P06, P07	CC2
147	ST-11 (10-3-2-3-2-2-13-16-9-26)	P05	Singleton
32, 155, 157	ST-12 (6-3-2-2-3-2-15-14-4-2)	P13	CC3
2, 57	ST-13 (6-3-2-2-3-2-15-14-100-2)	P13, P14	CC3
53	ST-14 (6-3-2-2-3-2-15-14-100-10)	P14	CC3
77	ST-15 (8-3-2-2-2-2-13-13-10-4)	P18	CC4
14	ST-16 (8-3-2-2-2-2-13-13-5-23)	P17	CC4
145, 186	ST-17 (18-21-6-3-8-3-9-11-5-24)	P15, P16	CC5
168	ST-18 (18-21-6-3-8-3-9-11-5-25)	P16	CC5
166	ST-19 (21-22-8-9-9-11-3-8-50-2)	P08	Singleton
95	ST-20 (15-12-5-6-6-7-4-7-1-100)	P19	Singleton
79	ST-21 (14-10-4-5-5-11-2-2-1-100)	P02	Singleton
170	ST-22 (22-23-7-8-10-10-2-3-100-100)	P09	Singleton
Total no.	22	19	5

^a The ST consists of the allelic profile or allele at each locus in turn: *gnd*, *glnA*, *aroE*, *arcA*, *mdh*, *adk*, *gyrB*, *purA*, *tem*, and *shv*

^b A CC is defined as isolates with the identical ST or STs that differ at no more than two loci. Singletons are unique STs that do not cluster into a CC.

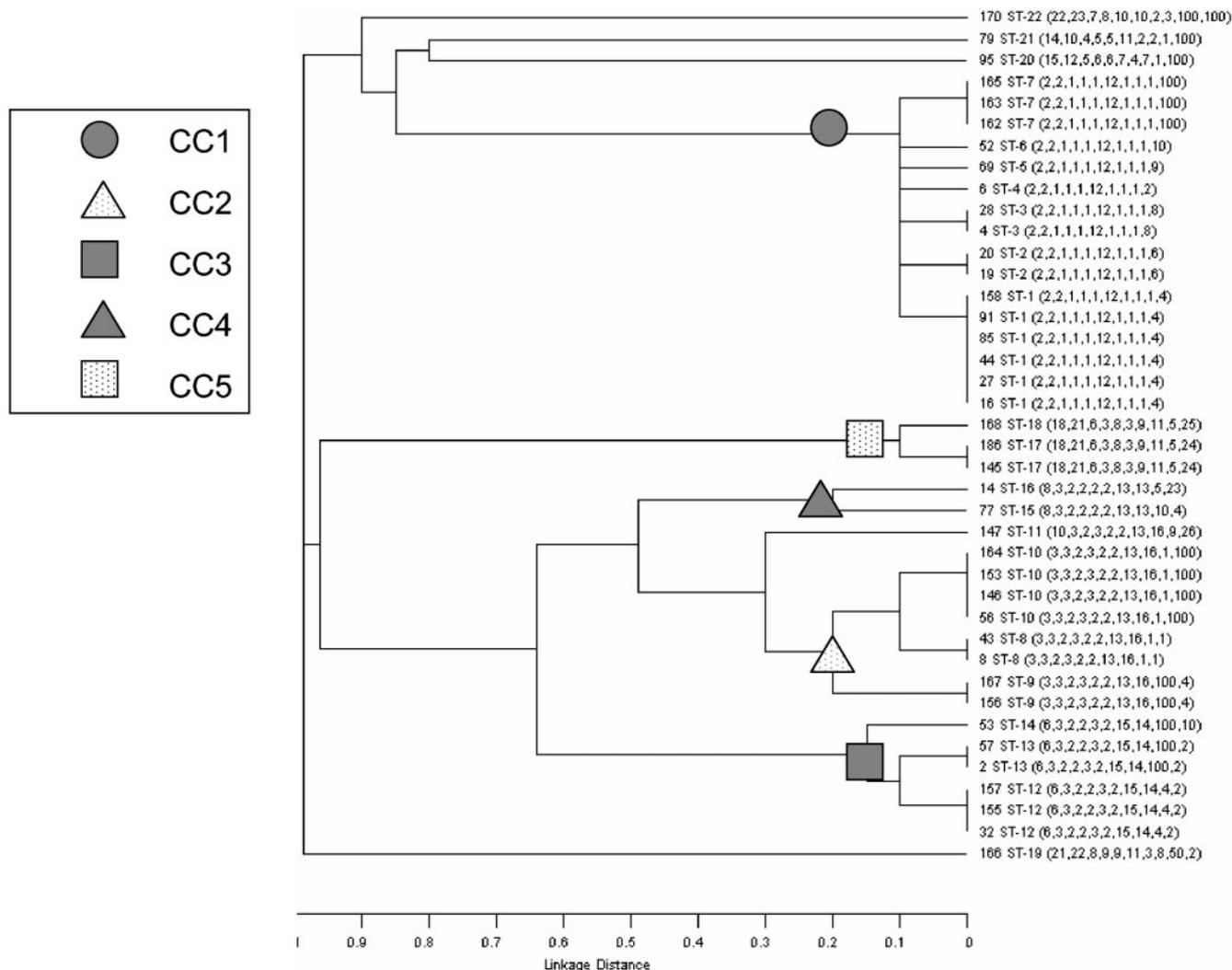


FIG. 2. MLST dendrogram of ESBL-producing *E. coli* isolates. The 40 ESBL *E. coli* isolates are shown and labeled as isolate number, sequence type (ST), and allelic profile in parentheses. Isolates with identical STs are shown on the same vertical branch. There are five CCs defined as isolates with the identical ST or a ST that differ at no more than two loci.

(95 and 79) had both distinct and unrelated MLST sequence types and distinct and unrelated PFGE types by criteria of Tenover et al. (32). An additional two isolates (166 and 170) had unrelated ST (ST-19 and -22) but related PFGE types (P08 and P09), with a genetic similarity of 0.84.

DISCUSSION

In our study of ESBL-producing *E. coli* isolates, MLST had a higher index of discrimination (*D*) than PFGE and achieved a *D* greater than 0.90, as would be desirable for a bacterial typing method (12); *D* for PFGE was 0.895. However, the high level of discrimination using MLST was achieved only with the addition of antibiotic resistance genes. MLST is most commonly done using housekeeping genes alone. Excluding *tem* and *shv* would have resulted in 10 MLST sequence types with a discrimination index of 0.786, or less discriminatory ability than PFGE.

These observed differences in *D* may be due to the spectrum

of changes detected by PFGE and MLST. PFGE examines both nucleotide changes that are in specific restriction sites and changes that involve large insertions or deletions of DNA, while MLST detects nucleotide changes within the amplified gene fragment. These changes in PFGE type are interpreted as changes in an XbaI restriction site or the addition or deletion of a large block(s) of DNA; the latter has been observed in *E. coli* O157:H7 (19). Selection of nonhousekeeping genes, hypervariable regions for MLST, PCR of variable-length tandem repeat loci, octamer-based genome scanning, and PCR/sequence-based methods have been used for subpopulations of *E. coli* O157:H7 and yielded high diversity indexes (15, 16, 25). The addition of two antibiotic resistance genes, in particular the *shv* gene, where the most variability was observed, increased the discriminatory index to 0.956. The increased discrimination may reflect horizontal transfer of plasmid-borne resistance genes within a conserved genetic background or more rapid change within *tem* and *shv* due to selective pressure. Further, the mere presence or absence of the two anti-

biotic resistance genes tested may on their own be useful to discriminate among ESBL-producing *E. coli* isolates.

In addition to its discriminatory ability, MLST defines genetically coherent groups derived from a common ancestor, i.e., CCs. While PFGE may also be able to do this, the point at which genetic relatedness is determined on a PFGE dendrogram, i.e., linkage distance, is arbitrary. This can be drawn at 0.60 or 0.80 or 1.00. Further complicating the analysis, bands of the same size are assumed to be identical in PFGE. However, unrelated fragments that are indistinguishable by size can occur by chance, especially as the genetic distance between strains increases. In contrast, MLST a priori defines CCs as those bacterial isolates that share a minimum of 8 of the 10 alleles with other members of the group. The ancestral allele in MLST is defined as the most common allele at each locus (20). In our data set, there were five CCs that each contained multiple PFGE types, but each PFGE type was found in only one CC. This indicates consistency within a CC. In addition, the observation of multiple PFGE types within a ST suggests that insertions and deletions of large blocks of DNA are more common in ESBL-producing *E. coli* isolates than either point mutations or homologous recombination.

MLST can detect lateral gene transfer events involving recombination between homologous loci. When most alleles show no change, an allele that differs from the ancestral allele by 1- or 2-nucleotide changes may result from spontaneous mutation. However, the *gnd-10* allele differed from the ancestral *gnd-3* allele by 27 bases and is unlikely to result from spontaneous mutation. The probability of these 27 nucleotide changes occurring by spontaneous mutation is in the order of 3.0×10^{-54} , following a probability calculation modeled after the model of Garg et al. (8). Instead these changes may represent a lateral gene transfer or recombination event. The *gnd* gene is the structural gene for 6-phosphogluconate dehydrogenase and is located adjacent to the *rfb* (O-antigen) locus. Studies with *E. coli* O55 and O157 and also *Salmonella enterica* suggest that recombination at the *gnd* allele is likely occurring and may be partially responsible for the emergence of pathogenic strains of *E. coli* O55 and O157 (31, 33).

In this study, 40 ESBL-producing *E. coli* isolates, out of a total of 62 isolates during the same 1-year period, were chosen at random for the purpose of typing by both MLST and PFGE. Consequently, it is difficult to state with certainty direct epidemiologic relationships, although this work is ongoing. However, MLST was able to separate the predominant PFGE type (P11) into five unique subtypes, which occurred over a 9-month period in two different ICUs. This suggests that MLST may be especially useful in certain situations, including transmission tracking over prolonged periods of time.

In general terms, MLST can be standardized for uniform application in distant laboratories and subsequent data are more easily disseminated and unambiguously compared. In contrast, despite strenuous efforts at standardization, PFGE data generated in various laboratories may be difficult to compare and can involve shipping bacterial isolates to a central laboratory for placement on a single agarose gel for direct comparison. MLST does not have these constraints. Primer sequences and amplification conditions can be shared readily via e-mail and subsequent data uploaded to a central database, as evidenced by the website www.mlst.net developed by David

Aanenesen. MLST may require different or additional loci for different bacteria or epidemiological situations.

ESBL-producing *E. coli* is an emerging pathogen in the hospital setting. The development of a standardized molecular typing technique would be useful to hospital epidemiologists, microbiologists, and clinicians as an effective tool for both surveillance and outbreak situations to track the spread and emergence of pathogens within and between hospitals. MLST had greater discriminatory ability than PFGE when antibiotic resistance genes were included in the schema. The additional utility of MLST includes the ability to a priori define genetically related bacterial strains, the ability to detect lateral gene transfer events, where recombination is likely to be occurring, and the unambiguous comparability of nucleotide sequence data among distant laboratories and hospitals. MLST is a useful modality to employ when studying the epidemiology of ESBL-producing *E. coli*.

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