Multilocus Sequence Typing (MLST) for Characterization of Enterobacter cloacae

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Introduction

Enterobacter cloacae is an important emerging pathogen, which sometime causes respiratory infection, surgical site infection, urinary infection, sepsis, and outbreaks at neonatal units [1–4]. This organism is an important emerging pathogen, which sometime causes respiratory infection, surgical site infection, urinary infection, sepsis, and outbreaks at neonatal units [1–4]. Extended-spectrum β-lactamases (ESBLs) and carbapenemases have been reported to be widespread in E. cloacae [5]. The factors dominantly contributing to drug resistance of E. cloacae are the plasmid-encoded CTX-M family of ESBLs, the KPC family of serine carbapenemases, and the VIM, IMP, and NDM-1 metallo-β-lactamases [5,6]. Several molecular epidemiological methods, including pulsed-field gel electrophoresis, restriction fragment length polymorphism, and ribotyping, are routinely applied for typing of bacteria. In addition to those methods, multilocus sequence typing (MLST) is becoming a gold standard method with advances in sequencing technology. MLST can also be used to analyze the genetic relations between isolates. Therefore, MLST would be useful for analysis of the epidemiology of E. cloacae. Although molecular typing methods have been applied to characterize clinical isolates of E. cloacae [7,8], previous studies focused mostly on discrimination of drug resistance genes. Recently, methods for discriminating E. cloacae complex comprised of Enterobacter asburiae, E. cloacae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigi, and Enterobacter rhusiopathiae based on hsp60 and tpsB genotyping, multilocus sequence analysis, and comparative genomic hybridization have been evaluated [9]. MLST for E. cloacae has not been reported previously. Here, we designed an MLST scheme for E. cloacae based on seven housekeeping genes and evaluated its performance for discriminating clinical isolates.

Materials and Methods

Bacterial strains

Five E. cloacae strains the complete genome sequences of which have been determined (ATCC 13047, NCTC 9394, ENHKU 01, SCF1, and EeWSU 1; hereafter, genome strains) were used to design PCR primers. One hundred one clinical isolates collected at National Center for Global Health and Medicine Hospital and a commercial clinical laboratory (BML Inc, Saitama, Japan) during 2007–2013 were used to evaluate the performance of the MLST scheme developed in the present study (Table 1).

Bacterial growth and biochemical identification

All strains were stored at –80°C, plated on sheep blood agar (Nissui Plate Sheep Blood Agar; Nissui, Tokyo, Japan) and cultured at 37°C overnight. Biochemical characterization was performed by Microscan Walkaway96SI (Siemens Healthcare Diagnostic, Inc., West Sacramento, CA) and VITEK 2 (SYSMEX bioMérieux Co., Ltd., Lyon, France) in a hospital laboratory and at a clinical testing company.

DNA preparation

Bacteria were grown on sheep blood agar at 37°C overnight. A single colony was suspended in molecular biology grade water, and the suspension was heated at 95°C for 5 min. After centrifugation, the supernatant was used as the PCR template.

Primers for MLST

The MLST scheme was developed according to the general guidelines described previously [10]. Primers to amplify internal fragments of candidate genes were designed based on the five...
Table 1. E. cloacae strains/clinical isolates used in this study and accession numbers of target sequences.

| Strain/Isolate | Target gene | Accession # or isolation year |
|----------------|-------------|------------------------------|
|                | ST          | dnaA | fusA | gyrB | leuS | pyrG | rplB | rpoB |                  |
| ATCC13047      | 1           | 1    | 1    | 1    | 1    | 1    | 1    | 1    | NC_014121.1      |
| EcWSU1         | 2           | 2    | 2    | 2    | 2    | 2    | 2    | 2    | NC_016514.1      |
| ENHKU01        | 3           | 3    | 3    | 3    | 3    | 3    | 3    | 3    | NC_018405.1      |
| NCTC9394       | 4           | 4    | 4    | 4    | 4    | 4    | 4    | 4    | FP929040.1       |
| SCF1           | 5           | 5    | 5    | 2    | 5    | 5    | 5    | 5    | NC_014618.1      |
| NCGM1          | 6           | 6    | 6    | 4    | 6    | 6    | 4    | 6    | 2007             |
| NCGM2          | 7           | 7    | 7    | 5    | 7    | 7    | 6    | 7    | 2007             |
| NCGM3          | 69          | 7    | 8    | 5    | 7    | 8    | 6    | 7    | 2007             |
| NCGM4          | 77          | 8    | 9    | 6    | 8    | 9    | 6    | 8    | 2011             |
| NCGM5          | 74          | 8    | 33   | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM6          | 78          | 8    | 9    | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM7          | 75          | 8    | 33   | 7    | 9    | 9    | 6    | 8    | 2012             |
| NCGM8          | 83          | 9    | 6    | 8    | 6    | 10   | 4    | 6    | 2012             |
| NCGM9          | 82          | 9    | 6    | 14   | 10   | 11   | 4    | 6    | 2012             |
| NCGM10         | 78          | 8    | 9    | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM11         | 73          | 8    | 33   | 6    | 12   | 6    | 8    | 2012             |
| NCGM12         | 71          | 8    | 33   | 6    | 11   | 9    | 6    | 8    | 2012             |
| NCGM13         | 74          | 8    | 33   | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM14         | 8           | 10   | 10   | 9    | 12   | 13   | 4    | 33   | 2012             |
| NCGM15         | 9           | 11   | 4    | 4    | 13   | 14   | 4    | 9    | 2012             |
| NCGM16         | 74          | 8    | 33   | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM17         | 78          | 8    | 9    | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM18         | 76          | 8    | 9    | 10   | 9    | 9    | 6    | 8    | 2012             |
| NCGM19         | 70          | 8    | 33   | 11   | 9    | 9    | 6    | 8    | 2012             |
| NCGM20         | 78          | 8    | 9    | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM21         | 78          | 8    | 9    | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM22         | 72          | 8    | 33   | 6    | 14   | 9    | 6    | 8    | 2012             |
| NCGM23         | 74          | 8    | 33   | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM24         | 74          | 8    | 33   | 6    | 9    | 9    | 6    | 8    | 2012             |
| NCGM25         | 55          | 42   | 11   | 52   | 37   | 23   | 16   | 3    | 2012             |
| NCGM26         | 36          | 32   | 12   | 22   | 31   | 31   | 8    | 28   | 2012             |
| NCGM27         | 58          | 44   | 32   | 12   | 9    | 35   | 6    | 6    | 2012             |
| NCGM28         | 50          | 4    | 4    | 4    | 6    | 37   | 4    | 25   | 2012             |
| NCGM29         | 39          | 35   | 25   | 35   | 47   | 48   | 12   | 20   | 2012             |
| NCGM30         | 66          | 52   | 21   | 20   | 44   | 45   | 4    | 6    | 2012             |
| NCGM31         | 64          | 50   | 20   | 17   | 44   | 45   | 12   | 32   | 2012             |
| NCGM32         | 59          | 45   | 27   | 31   | 56   | 25   | 11   | 27   | 2012             |
| NCGM33         | 62          | 48   | 4    | 15   | 42   | 39   | 4    | 9    | 2012             |
| NCGM34         | 32          | 3    | 24   | 3    | 35   | 3    | 16   | 17   | 2012             |
| NCGM35         | 27          | 26   | 16   | 25   | 53   | 22   | 9    | 15   | 2012             |
| NCGM36         | 26          | 25   | 31   | 24   | 52   | 21   | 9    | 15   | 2012             |
| NCGM37         | 30          | 29   | 18   | 32   | 33   | 29   | 8    | 30   | 2012             |
| NCGM38         | 54          | 41   | 3    | 54   | 37   | 3    | 15   | 17   | 2012             |
| NCGM39         | 20          | 19   | 2    | 46   | 26   | 51   | 2    | 13   | 2012             |
| NCGM40         | 79          | 9    | 22   | 14   | 6    | 39   | 4    | 9    | 2012             |
| NCGM41         | 67          | 7    | 34   | 5    | 7    | 15   | 6    | 7    | 2012             |
| NCGM42         | 46          | 4    | 4    | 4    | 13   | 39   | 4    | 6    | 2012             |
| NCGM43         | 12          | 13   | 2    | 45   | 24   | 52   | 2    | 14   | 2012             |
Table 1. Cont.

| Strain/Isolate | ST  | Target gene | Accession # or isolation year |
|----------------|-----|-------------|------------------------------|
| NCGM44         | 78  | 8 9 6 9 6 6 | 2012                         |
| NCGM45         | 28  | 27 14 26 54 26 10 16 | 2012 |
| NCGM46         | 25  | 24 14 43 52 27 18 21 | 2012 |
| NCGM47         | 38  | 34 18 33 32 30 8 31 | 2012 |
| NCGM48         | 41  | 37 25 49 30 49 21 20 | 2012 |
| NCGM49         | 17  | 16 2 45 25 55 7 14 | 2012 |
| NCGM50         | 40  | 36 26 36 49 50 12 20 | 2012 |
| NCGM51         | 20  | 19 2 46 26 51 2 13 | 2012 |
| NCGM52         | 34  | 30 18 38 29 34 8 22 | 2012 |
| NCGM53         | 43  | 39 27 50 48 49 12 26 | 2012 |
| NCGM54         | 20  | 19 2 46 26 51 2 13 | 2012 |
| NCGM57         | 45  | 4 4 14 6 39 4 6 | 2012 |
| NCGM57         | 78  | 8 9 6 9 9 6 8 | 2012 |
| NCGM58         | 29  | 28 14 27 55 20 10 15 | 2012 |
| NCGM59         | 57  | 43 3 51 36 18 16 19 | 2012 |
| NCGM60         | 33  | 3 3 53 37 19 16 19 | 2012 |
| NCGM61         | 63  | 49 20 19 45 45 4 32 | 2012 |
| NCGM62         | 78  | 8 9 6 9 9 6 8 | 2012 |
| NCGM63         | 65  | 51 4 21 41 42 4 6 | 2012 |
| NCGM64         | 51  | 4 4 4 6 37 4 6 | 2012 |
| NCGM65         | 18  | 17 13 44 19 2 2 14 | 2012 |
| NCGM66         | 50  | 4 4 4 6 37 4 25 | 2012 |
| NCGM67         | 10  | 11 4 4 40 39 4 6 | 2012 |
| NCGM68         | 53  | 40 17 39 15 46 11 10 | 2012 |
| NCGM69         | 11  | 12 2 48 18 54 13 14 | 2012 |
| NCGM70         | 52  | 4 8 18 43 40 4 25 | 2012 |
| NCGM71         | 23  | 22 15 39 17 47 11 10 | 2012 |
| NCGM72         | 81  | 9 4 15 13 43 4 24 | 2012 |
| NCGM73         | 78  | 8 9 6 9 9 6 8 | 2012 |
| NCGM74         | 31  | 3 24 3 35 17 16 17 | 2012 |
| NCGM76         | 19  | 18 2 41 22 51 2 13 | 2012 |
| NCGM77         | 68  | 7 8 5 7 36 6 7 | 2012 |
| NCGM78         | 21  | 20 30 28 50 16 20 12 | 2012 |
| NCGM80         | 48  | 4 4 4 39 41 4 25 | 2012 |
| NCGM81         | 15  | 14 2 30 20 51 2 14 | 2012 |
| NCGM82         | 14  | 13 2 47 23 53 2 14 | 2012 |
| NCGM83         | 47  | 4 4 4 39 39 19 25 | 2012 |
| NCGM84         | 80  | 9 4 14 6 11 4 9 | 2012 |
| NCGM85         | 49  | 4 4 4 40 38 4 23 | 2012 |
| NCGM86         | 50  | 4 4 4 6 37 4 25 | 2012 |
| NCGM87         | 78  | 8 9 6 9 9 6 8 | 2012 |
| NCGM88         | 78  | 8 9 6 9 9 6 8 | 2012 |
| NCGM89         | 62  | 48 4 15 42 39 4 9 | 2012 |
| NCGM90         | 16  | 15 2 40 21 52 2 14 | 2012 |
| NCGM91         | 50  | 4 4 4 6 37 4 25 | 2012 |
| NCGM92         | 24  | 23 15 23 16 28 11 11 | 2012 |
| NCGM94         | 56  | 42 3 52 37 23 16 3 | 2012 |
genome strains (Table 2). Sequences of the target genes in the five strains were aligned to choose suitable region for the primers using Genetyx (Genetyx Corporation, Tokyo, Japan). Candidate genes were selected based on previously published genotyping schemes for members of the *E. cloacae* complex [9] and *dnaA* was added to increase the resolution. The primers targeted seven housekeeping genes (*dnaA, fusA, gyrB, leuS, pyrG, rplB*, and *rpoB*) (Table 2).

### PCR conditions and amplicon sequencing

The amplification reactions were performed in 20 μL using 1 μL of DNA extract as the template. The temperature program was as follows: 2 min of initial denaturation at 95°C followed by 25 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 10 s, and primer extension at 72°C for 60 s. After confirmation of amplification by electrophoresis, the PCR amplicons were treated with ExoSAP-IT (USB, Cleveland, OH) to remove the excess primers according with the manufacturer’s instructions, and sequenced using the primers listed in Table 2 by the dideoxy chain termination method on an ABI 3130XL Genetic analyzer or an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

### Sequence alignment and phylogenetic analysis

Genetyx (Genetyx Corporation, Tokyo, Japan) was utilized to align and edit the sequences of five *E. cloacae* genome strains as well as those obtained from the clinical isolates by Sanger sequencing.

### Table 1. Cont.

| Strain/Isolate | ST | Target gene | Accession # or isolation year |
|----------------|----|-------------|------------------------------|
| NCGM95         | 37 | dnaA 19     | 2012                         |
| NCGM96         | 35 | fusA 28     | 2013                         |
| NCGM97         | 44 | gyrB 37     | 2013                         |
| NCGM98         | 42 | leuS 46     | 2013                         |
| NCGM99         | 78 | pyrG 14     | 2013                         |
| NCGM100        | 24 | rplB 6      | 2013                         |
| NCGM101        | 22 | rpoB 16     | 2013                         |
| NCGM102        | 60 | NCGM103     | 2013                         |
| NCGM104        | 61 | NCGM104     | 2013                         |

NCGM75, NCGM78 and NCGM93 were unused in this study. All isolates named with NCGM were collected during 2007-2013 at laboratories located in Japan.

### Table 2. Primers for *E. cloacae* MLST scheme.

| Name      | Sequence (5’–>3’) | Position in the target gene |
|-----------|-------------------|----------------------------|
| Amplification primers | | |
| *dnaA-f2* | AYAACCCGCCTGGTTCTTGTATGCGGCTGCAC | 500–527* |
| *dnaA-r* | KGCCAGGCCATGCGCTGTTGCAGCGG | 1222–1248* |
| *fusA-f2* | TCGGGTTCGTTAACAAAATGGACCGTAT | 413–440* |
| *fusA-r2* | TCGCCAGACGCGCCCAGAGCCAGACCACAT | 1291–1318 |
| *gyrB-f* | TCGACGAAGCGCTCGCGGGTCACTGTAA | 143–170 |
| *gyrB-r* | GCAGAACCGCCCGCCTCCCTTCA | 1268–1295 |
| *leuS-f2* | GATCARCTSCCGGTKATCCTGCCGGAAG | 1342–1369* |
| *leuS-r* | ATAGCCGCAATTGCGGTATTGAAGGTCT | 2159–2186* |
| *pyrG-f* | AYCCBGAYGTBATTGCRCAYMAGGCGAT | 56–83* |
| *pyrG-r* | GCRICGRATYTYCVCCTTCTHTGTCCCCACC | 563–590* |
| *rplB-f* | GTAAACCGACATCTCCGCGTCGCCGCCGCC | 17–44* |
| *rplB-r* | ACCCTTGGTCTGAAACGCCACGGAGT | 735–762* |
| *rpoB-f* | CCAGAACCGGCTGGGAACATCGGGCTG | 252–280* |
| *rpoB-r* | CCACGAGATCGAAGGCTCACGCTCCTTCTG | 973–1000* |
| Sequencing primers | | |
| *gyrB-r3-seq* | GCAGAACCGCCGCCGGAGTCCCTTCCT | 1269–1295 |
| *gyrB-f3-seq* | AAAACCGGACTATGTTGCGGCCCTT | 484–510* |
| *fusA-r2-seq* | ATCTCTTACGGTGTATGCGGCTACATC | 1094–1121* |

*These primers were used for sequencing of respective amplicons.

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Phylogenetic analysis using concatenated MLST loci created by the STRAT2 software [11] was performed using CLUSTAL W hosted by DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp). The dataset used contained only one isolate/ST to prevent bias toward a clonal population for strains with the same epidemiological history. The tree was drawn using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Circles indicate each clade. The START2 software was used to generate the concatenated loci sequence and calculate the number of nucleotide differences and ratio of nonsynonymous to synonymous substitutions (dN/dS) [11]. Tajima’s D statistic [12], Fu’s F and D statistic [13] and Ramos-Onsins & Rozas’ R2 [14] were analyzed using DnaSP 5.10.1 [15].

Figure 1. Unrooted UPGMA tree of concatenated sequences from combinations of seven MLST loci. Phylogenetic analysis using concatenated MLST loci created by the STRAT2 software was performed using CLUSTAL W hosted by DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp). The dataset used contained only one isolate/ST to prevent bias toward a clonal population for strains with the same epidemiological history. The tree was drawn using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Circles indicate each clade.

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Table 3. Characteristics of E. cloacae MLST loci.

| Locus  | dnaA  | fusA  | gyrB  | leuS  | pyrG  | rplB  | rpoB  |
|--------|-------|-------|-------|-------|-------|-------|-------|
| Amplicon size (bp) | 1151  | 906   | 1153  | 845   | 535   | 746   | 944   |
| Sequence target size (bp) | 442   | 646   | 434   | 578   | 259   | 607   | 545   |
| dN/dS ratio* | 0.0019 | 0.1682 | 0.0274 | 0.023 | 0.0576 | 0.0166 | 0.028 |
| Number of variable sites* | 71    | 59    | 60    | 104   | 106   | 17    | 77    |
| Percentage of variable sites | 16.1  | 9.1   | 13.8  | 18.0  | 40.9  | 2.8   | 14.1  |

*Based on the sequences of the genome strains.

# Nonsynonymous synonymous to synonymous substitution ratio.

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To examine linkage disequilibrium among the seven genes analyzed in this study, the index of association (IA) values were calculated in START2 by the classical (Maynard Smith) and standardized (Haubold) methods [11].

Accession numbers of sequences determined in this study

DNA sequences of the alleles determined in this study was deposited in DNA databank of Japan under the accession number following. The accession numbers are listed in Table 6.

Results and Discussion

Development of a MLST scheme for E. cloacae

The PCR primers designed for the E. cloacae MLST scheme are listed in Table 2. Candidate genes were selected based on previously published genotyping schemes for members of the E. cloacae complex [9] and dnaA was added to increase the resolution. Because hsp60 was also included in the genotyping scheme in the previous study, we designed several combinations of primer sets and attempted to obtain amplicons. However, none of the clinical isolates tested yielded the amplicon. Thus, hsp60 was omitted from the MLST scheme. The target amplicon sizes of dnaA and gyrB were larger than 1 kb (Table 3) to locate the primers in the conserved sequence. The percentage of variable sites at each locus ranged from 2.8 (rplB) to 40.9 (pyrG) (Table 3). The discriminatory

Index of association

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Table 4. Allele frequencies of the MLST scheme for E. cloacae.

| Allele | dnaA | fusA | gyrB | leuS | pyrG | rplB | rpoB |
|--------|------|------|------|------|------|------|------|
| 1      | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| 2      | 1    | 12   | 2    | 1    | 2    | 11   | 1    |
| 3      | 5    | 5    | 4    | 1    | 4    | 1    | 3    |
| 4      | 13   | 18   | 13   | 1    | 1    | 26   | 1    |
| 5      | 1    | 1    | 4    | 1    | 1    | 1    | 1    |
| 6      | 1    | 3    | 21   | 10   | 1    | 30   | 12   |
| 7      | 4    | 1    | 1    | 4    | 1    | 1    | 5    |
| 8      | 24   | 4    | 1    | 1    | 1    | 5    | 24   |
| 9      | 5    | 14   | 1    | 22   | 23   | 2    | 5    |
| 10     | 1    | 1    | 1    | 1    | 1    | 2    | 2    |
| 11     | 2    | 1    | 1    | 1    | 1    | 2    | 6    |
| 12     | 1    | 1    | 1    | 1    | 1    | 5    | 1    |
| 13     | 3    | 1    | 3    | 1    | 1    | 1    | 4    |
| 14     | 1    | 3    | 4    | 1    | 1    | 1    | 8    |
| 15     | 1    | 3    | 3    | 1    | 1    | 1    | 3    |
| 16     | 1    | 1    | 1    | 2    | 1    | 7    | 1    |
| 17     | 1    | 1    | 1    | 1    | 1    | 1    | 4    |
| 18     | 1    | 3    | 1    | 1    | 1    | 1    | 1    |
| 19     | 3    | 2    | 2    | 1    | 1    | 1    | 2    |
| 20     | 1    | 3    | 1    | 1    | 1    | 1    | 4    |
| 21     | 1    | 1    | 1    | 1    | 1    | 1    |
| 22     | 1    | 1    | 1    | 1    | 1    |
| 23     | 2    | 1    | 2    | 1    | 2    |
| 24     | 1    | 3    | 1    | 3    |
| 25     | 1    | 1    | 2    | 1    | 1    | 1    |
| 26     | 1    | 1    | 1    | 1    | 1    |
| 27     | 1    | 2    | 1    | 1    | 1    |
| 28     | 1    | 1    | 1    | 1    | 1    | 1    | 2    |
| 29     | 1    | 1    | 1    | 1    | 1    |
| 30     | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| 31     | 1    | 1    | 1    | 1    | 1    | 1    |
| 32     | 1    | 1    | 1    | 1    | 1    | 1    | 2    |
| 33     | 1    | 1    | 1    | 1    | 1    | 1    |
| 34     | 1    | 1    | 1    | 1    | 1    | 1    |
| 35     | 1    | 1    | 1    | 1    |
| 36     | 1    | 1    | 1    | 1    |
| 37     | 1    | 1    | 1    | 1    |
| 38     | 1    | 1    | 1    | 1    |
| 39     | 1    | -    | 2    | 2    | 2    |
| 40     | 1    | -    | 1    | 2    | 1    | -    |
| 41     | 1    | -    | 1    |
| 42     | 2    | -    | 1    | 2    |
| 43     | 1    | -    | 1    | 1    |
| 44     | 1    | -    |
| 45     | 1    | -    | 1    |
| 46     | 1    | -    | 1    |
| 47     | 1    | -    | 1    | 1    |
| 48     | 2    | -    | 1    | 1    | -    |
| 49     | 1    | -    | 1    | 1    |

Table 4. Cont.

| Allele | dnaA | fusA | gyrB | leuS | pyrG | rplB | rpoB |
|--------|------|------|------|------|------|------|------|
| 50     | 1    | -    | 1    | 1    | 1    | -    | -    |
| 51     | 1    | -    | 1    | 1    |
| 52     | 1    | -    | 2    | 2    | 2    |
| 53     | -    | -    | 1    | 1    | 1    | -    |
| 54     | -    | -    | 1    | 1    | 1    |
| 55     | -    | -    | 1    | 1    | -    |
| 56     | -    | -    | 1    | 1    | -    |

Unique 52 34 54 56 56 21 33

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Table 5. Analysis of neutrality tests of genes used to develop the MLST scheme.

| Gene | Tajima’s D | Fu and Li’s D* | Fu and Li’s F* | R2 |
|------|------------|----------------|---------------|----|
| dnaA | -0.51656ns | -1.10953ns    | -1.05928ns    | 0.10537ns |
| fusA | -2.56811*  | -4.52388*     | -4.56688*     | 0.11307ns |
| gyrB | -0.75309ns | -1.08782ns    | -1.14955ns    | 0.10381ns |
| leuS | -0.75309ns | -1.08782ns    | -1.14955ns    | 0.10381ns |
| pyrG | 1.55553ns  | 4.00283*      | 3.65452*      | 0.10252ns |
| rplB | 2.60808*   | 4.22457*      | 4.36815*      | 0.12713ns |
| rpoB | 1.35637ns  | 2.48230ns     | 2.48825ns     | 0.11489ns |

Tajima’s D statistic [12], Fu’s D and F statistic [13] and Ramos-Onsins & Rozas’ R2 [14] were analyzed using DnaSP 5.10.1 [15].

*Statistically significant (P < 0.05).
ns: Non significant.

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| Allele | Accession # | Allele | Accession # | Allele | Accession # | Allele | Accession # |
|--------|-------------|--------|-------------|--------|-------------|--------|-------------|
| dnaA_allele1 | AB774293 | fusA_allele1 | AB774304 | gyrB_allele1 | AB774314 | leuS_allele1 | AB774325 |
| dnaA_allele2 | AB774294 | fusA_allele2 | AB774305 | gyrB_allele2 | AB774315 | leuS_allele2 | AB774326 |
| dnaA_allele3 | AB774295 | fusA_allele3 | AB774306 | gyrB_allele3 | AB774316 | leuS_allele3 | AB774327 |
| dnaA_allele4 | AB774296 | fusA_allele4 | AB774307 | gyrB_allele4 | AB774317 | leuS_allele4 | AB774328 |
| dnaA_allele5 | AB774297 | fusA_allele5 | AB774308 | gyrB_allele5 | AB774318 | leuS_allele5 | AB774329 |
| dnaA_allele6 | AB774298 | fusA_allele6 | AB774309 | gyrB_allele6 | AB774319 | leuS_allele6 | AB774330 |
| dnaA_allele7 | AB774299 | fusA_allele7 | AB774310 | gyrB_allele7 | AB774320 | leuS_allele7 | AB774331 |
| dnaA_allele8 | AB774300 | fusA_allele8 | AB774311 | gyrB_allele8 | AB774321 | leuS_allele8 | AB774332 |
| dnaA_allele9 | AB774301 | fusA_allele9 | AB774312 | gyrB_allele9 | AB774322 | leuS_allele9 | AB774333 |
| dnaA_allele10 | AB774302 | fusA_allele10 | AB774313 | gyrB_allele10 | AB774323 | leuS_allele10 | AB774334 |
| dnaA_allele11 | AB774303 | fusA_allele11 | AB809745 | gyrB_allele11 | AB774324 | leuS_allele11 | AB774335 |
| dnaA_allele12 | AB809704 | fusA_allele12 | AB809746 | gyrB_allele12 | AB809769 | leuS_allele12 | AB774336 |
| dnaA_allele13 | AB809705 | fusA_allele13 | AB809747 | gyrB_allele13 | AB809770 | leuS_allele13 | AB774337 |
| dnaA_allele14 | AB809706 | fusA_allele14 | AB809748 | gyrB_allele14 | AB809771 | leuS_allele14 | AB774338 |
| dnaA_allele15 | AB809707 | fusA_allele15 | AB809749 | gyrB_allele15 | AB809772 | leuS_allele15 | AB809812 |
| dnaA_allele16 | AB809708 | fusA_allele16 | AB809750 | gyrB_allele16 | AB809773 | leuS_allele16 | AB809813 |
| dnaA_allele17 | AB809709 | fusA_allele17 | AB809751 | gyrB_allele17 | AB809774 | leuS_allele17 | AB809814 |
| dnaA_allele18 | AB809710 | fusA_allele18 | AB809752 | gyrB_allele18 | AB809775 | leuS_allele18 | AB809815 |
| dnaA_allele19 | AB809711 | fusA_allele19 | AB809753 | gyrB_allele19 | AB809776 | leuS_allele19 | AB809816 |
| dnaA_allele20 | AB809712 | fusA_allele20 | AB809754 | gyrB_allele20 | AB809777 | leuS_allele20 | AB809817 |
| dnaA_allele21 | AB809713 | fusA_allele21 | AB809755 | gyrB_allele21 | AB809778 | leuS_allele21 | AB809818 |
| dnaA_allele22 | AB809714 | fusA_allele22 | AB809756 | gyrB_allele22 | AB809779 | leuS_allele22 | AB809819 |
| dnaA_allele23 | AB809715 | fusA_allele23 | AB809757 | gyrB_allele23 | AB809780 | leuS_allele23 | AB809820 |
| dnaA_allele24 | AB809716 | fusA_allele24 | AB809758 | gyrB_allele24 | AB809781 | leuS_allele24 | AB809821 |
| dnaA_allele25 | AB809717 | fusA_allele25 | AB809759 | gyrB_allele25 | AB809782 | leuS_allele25 | AB809822 |
| dnaA_allele26 | AB809718 | fusA_allele26 | AB809760 | gyrB_allele26 | AB809783 | leuS_allele26 | AB809823 |
| dnaA_allele27 | AB809719 | fusA_allele27 | AB809761 | gyrB_allele27 | AB809784 | leuS_allele27 | AB809824 |
| dnaA_allele28 | AB809720 | fusA_allele28 | AB809762 | gyrB_allele28 | AB809785 | leuS_allele28 | AB809825 |
| dnaA_allele29 | AB809721 | fusA_allele29 | AB809763 | gyrB_allele29 | AB809786 | leuS_allele29 | AB809826 |
| dnaA_allele30 | AB809722 | fusA_allele30 | AB809764 | gyrB_allele30 | AB809787 | leuS_allele30 | AB809827 |
| dnaA_allele31 | AB809723 | fusA_allele31 | AB809765 | gyrB_allele31 | AB809788 | leuS_allele31 | AB809828 |
| dnaA_allele32 | AB809724 | fusA_allele32 | AB809766 | gyrB_allele32 | AB809789 | leuS_allele32 | AB809829 |
| dnaA_allele33 | AB809725 | fusA_allele33 | AB809767 | gyrB_allele33 | AB809790 | leuS_allele33 | AB809830 |
| dnaA_allele34 | AB809726 | fusA_allele34 | AB809768 | gyrB_allele34 | AB809791 | leuS_allele34 | AB809831 |
| dnaA_allele35 | AB809727 | gyrB | AB809792 | leuS_allele35 | AB809832 |
| dnaA_allele36 | AB809728 | gyrB | AB809793 | leuS_allele36 | AB809833 |
| dnaA_allele37 | AB809729 | gyrB | AB809794 | leuS_allele37 | AB809834 |
| dnaA_allele38 | AB809730 | gyrB | AB809795 | leuS_allele38 | AB809835 |
| dnaA_allele39 | AB809731 | gyrB | AB809796 | leuS_allele39 | AB809836 |
| dnaA_allele40 | AB809732 | gyrB | AB809797 | leuS_allele40 | AB809837 |
| dnaA_allele41 | AB809733 | gyrB | AB809798 | leuS_allele41 | AB809838 |
| dnaA_allele42 | AB809734 | gyrB | AB809799 | leuS_allele42 | AB809839 |
| dnaA_allele43 | AB809735 | gyrB | AB809800 | leuS_allele43 | AB809840 |
| dnaA_allele44 | AB809736 | gyrB | AB809801 | leuS_allele44 | AB809841 |
| dnaA_allele45 | AB809737 | gyrB | AB809802 | leuS_allele45 | AB809842 |
| dnaA_allele46 | AB809738 | gyrB | AB809803 | leuS_allele46 | AB809843 |
| dnaA_allele47 | AB809739 | gyrB | AB809804 | leuS_allele47 | AB809844 |
| dnaA_allele48 | AB809740 | gyrB | AB809805 | leuS_allele48 | AB809845 |
| Allele | Accession | Allele | Accession | Allele | Accession | Allele | Accession |
|--------|-----------|--------|-----------|--------|-----------|--------|-----------|
| dnaA_allele49 | AB809741 | gyrB_allele49 | AB809806 | leuS_allele49 | AB809846 |
| dnaA_allele50 | AB809742 | gyrB_allele50 | AB809807 | leuS_allele50 | AB809847 |
| dnaA_allele51 | AB809743 | gyrB_allele51 | AB809808 | leuS_allele51 | AB809848 |
| dnaA_allele52 | AB809744 | gyrB_allele52 | AB809809 | leuS_allele52 | AB809849 |
|           |          | gyrB_allele53 | AB809810 | leuS_allele53 | AB809850 |
|           |          | gyrB_allele54 | AB809811 | leuS_allele54 | AB809851 |
|           |          | leuS_allele55 | AB809852 |          |          |
|           |          | leuS_allele56 | AB809853 |          |          |
| pyrG_allele1 | AB774339 | rplB_allele1 | AB774353 | rpoB_allele1 | AB774361 |
| pyrG_allele2 | AB774340 | rplB_allele2 | AB774354 | rpoB_allele2 | AB774362 |
| pyrG_allele3 | AB774341 | rplB_allele3 | AB774355 | rpoB_allele3 | AB774363 |
| pyrG_allele4 | AB774342 | rplB_allele4 | AB774356 | rpoB_allele4 | AB774364 |
| pyrG_allele5 | AB774343 | rplB_allele5 | AB774357 | rpoB_allele5 | AB774365 |
| pyrG_allele6 | AB774344 | rplB_allele6 | AB809896 | rpoB_allele6 | AB774366 |
| pyrG_allele7 | AB774345 | rplB_allele7 | AB809897 | rpoB_allele7 | AB809912 |
| pyrG_allele8 | AB774346 | rplB_allele8 | AB809898 | rpoB_allele8 | AB809913 |
| pyrG_allele9 | AB774347 | rplB_allele9 | AB809899 | rpoB_allele9 | AB809914 |
| pyrG_allele10 | AB774348 | rplB_allele10 | AB809900 | rpoB_allele10 | AB809915 |
| pyrG_allele11 | AB774349 | rplB_allele11 | AB809901 | rpoB_allele11 | AB809916 |
| pyrG_allele12 | AB774350 | rplB_allele12 | AB809902 | rpoB_allele12 | AB809917 |
| pyrG_allele13 | AB774351 | rplB_allele13 | AB809903 | rpoB_allele13 | AB809918 |
| pyrG_allele14 | AB774352 | rplB_allele14 | AB809904 | rpoB_allele14 | AB809919 |
| pyrG_allele15 | AB809854 | rplB_allele15 | AB809905 | rpoB_allele15 | AB809920 |
| pyrG_allele16 | AB809855 | rplB_allele16 | AB809906 | rpoB_allele16 | AB809921 |
| pyrG_allele17 | AB809856 | rplB_allele17 | AB809907 | rpoB_allele17 | AB809922 |
| pyrG_allele18 | AB809857 | rplB_allele18 | AB809908 | rpoB_allele18 | AB809923 |
| pyrG_allele19 | AB809858 | rplB_allele19 | AB809909 | rpoB_allele19 | AB809924 |
| pyrG_allele20 | AB809859 | rplB_allele20 | AB809910 | rpoB_allele20 | AB809925 |
| pyrG_allele21 | AB809860 | rplB_allele21 | AB809911 | rpoB_allele21 | AB809926 |
| pyrG_allele22 | AB809861 | rpoB_allele22 | AB809922 |          |          |
| pyrG_allele23 | AB809862 | rpoB_allele23 | AB809928 |          |          |
| pyrG_allele24 | AB809863 | rpoB_allele24 | AB809929 |          |          |
| pyrG_allele25 | AB809864 | rpoB_allele25 | AB809930 |          |          |
| pyrG_allele26 | AB809865 | rpoB_allele26 | AB809931 |          |          |
| pyrG_allele27 | AB809866 | rpoB_allele27 | AB809932 |          |          |
| pyrG_allele28 | AB809867 | rpoB_allele28 | AB809933 |          |          |
| pyrG_allele29 | AB809868 | rpoB_allele29 | AB809934 |          |          |
| pyrG_allele30 | AB809869 | rpoB_allele30 | AB809935 |          |          |
| pyrG_allele31 | AB809870 | rpoB_allele31 | AB809936 |          |          |
| pyrG_allele32 | AB809871 | rpoB_allele32 | AB809937 |          |          |
| pyrG_allele33 | AB809872 | rpoB_allele33 | AB809938 |          |          |
| pyrG_allele34 | AB809873 |          |          |          |          |
| pyrG_allele35 | AB809874 |          |          |          |          |
| pyrG_allele36 | AB809875 |          |          |          |          |
| pyrG_allele37 | AB809876 |          |          |          |          |
| pyrG_allele38 | AB809877 |          |          |          |          |
ability of the different loci, measured as number of alleles, varied from 21 (rplB) to 56 (leuS and pyrG) (Table 4). The average number of alleles at each locus was 43.9, providing the potential to distinguish approximately $2.1 \times 10^{11}$ different sequence types (STs).

The fusA locus had the highest dN/dS nonsynonymous (change of amino acid) to synonymous (no change of amino acid) substitution ratio. In contrast, the dN/dS ratio of dnaA was close to zero, suggesting that dnaA is under strong selection pressure. The rplB gene was omitted from the genotyping scheme in the previous study [9] because of a possibility that the gene is under positive selection pressure based on the two neutrality tests: Tajima’s D statistic [12] and Fu’s F statistic [13]. To validate departure of neutrality of each gene, we performed additional neutrality test: Ramos-Onsins & Rozas’ R2 test, which is more powerful at detecting population growth [14]. The R2 test did not detect any deviation from random evolution among any of the populations (Table 5), suggesting that it can not be excluded that rplB is also under neutral evolution. Thus, rplB was also included in the MLST scheme designed in this study. Among the 106 E. cloacae strains/isolates included in this study, 83 different STs were identified. Seventy-six of these STs were represented by only one strain. The data will be registered at pubmlst.org [16] to provide public analysis to MLST for E. cloacae.

To analyze the clonality of the strains/isolates, phylogenetic analysis using the concatenated sequence consisting of the loci was performed. The dataset used contain only one isolate/ST to prevent bias toward a clonal population for strains with the same epidemiological history. These strains clustered into three clades (Figure 1). To measure the extent of linkage equilibrium within a population by quantifying the amount of recombination among a set of sequences and detecting associations between alleles at different loci, I$\Lambda$ values [17] were calculated for each clade. I$\Lambda$ values of each clade indicated significant linkage disequilibrium between alleles (clade 1: $I\Lambda = 0.1593$, $P < 0.001$; clade 2: $I\Lambda = 0.1857$, $P < 0.001$; clade 3: $I\Lambda = 0.3184$, $P < 0.001$), and thus, a clonal structure of the population studied.

In conclusion, a robust and portable typing scheme for E. cloacae was established. This method, based on seven housekeeping genes, separated the species into three distinct lineages. The MLST scheme developed in this study could be used for further analysis of the epidemiology of E. cloacae. Thus, if homologous recombination does exist, it rarely contributes to the evolution of E. cloacae. Sequence data analysis revealed that large number of synonymous substitutions were detected in genes dnaA, gyrB, leuS, rplB and rpoB, suggesting that most nonsilent mutations are eliminated through purifying selection.

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**Author Contributions**

Conceived and designed the experiments: TMA KH NO TK. Performed the experiments: TMA. Analyzed the data: TMA KH. Contributed reagents/materials/analysis tools: MS TK. Wrote the paper: TMA TK.

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**Table 6.** Cont.

| pyrG     | rplB     | rpoB     |
|----------|----------|----------|
| Allele   | Accession| Allele   | Accession| Allele   | Accession |
| pyrG_allele39 | AB809878 | pyrG_allele40 | AB809879 | pyrG_allele41 | AB809880 |
| pyrG_allele42 | AB809881 | pyrG_allele43 | AB809882 | pyrG_allele44 | AB809883 |
| pyrG_allele45 | AB809884 | pyrG_allele46 | AB809885 | pyrG_allele47 | AB809886 |
| pyrG_allele48 | AB809887 | pyrG_allele49 | AB809888 | pyrG_allele50 | AB809889 |
| pyrG_allele51 | AB809890 | pyrG_allele52 | AB809891 | pyrG_allele53 | AB809892 |
| pyrG_allele54 | AB809893 | pyrG_allele55 | AB809894 | pyrG_allele56 | AB809895 |

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