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Research Article

Characterization of Multidrug Resistant ESBL-Producing *Escherichia coli* Isolates from Hospitals in Malaysia

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The emergence of *Escherichia coli* that produce extended spectrum β-lactamases (ESBLs) and are multidrug resistant (MDR) poses antibiotic management problems. Forty-seven *E. coli* isolates from various public hospitals in Malaysia were studied. All isolates were sensitive to imipenem whereas 36 were MDR (resistant to 2 or more classes of antibiotics). PCR detection using gene-specific primers showed that 87.5% of the ESBL-producing *E. coli* harbored the bla_{TEM} gene. Other ESBL-encoding genes detected were bla_{OXA}, bla_{SHV}, and $bla_{\mathrm{CTX\text{-}M}}$. Integron-encoded integrases were detected in 55.3% of isolates, with class 1 integron-encoded *intI1* integrase being the majority. Amplification and sequence analysis of the 5′CS region of the integrons showed known antibiotic resistance-encoding gene cassettes of various sizes that were inserted within the respective integrons. Conjugation and transformation experiments indicated that some of the antibiotic resistance genes were likely plasmid-encoded and transmissible. All 47 isolates were subtyped by PFGE and PCR-based fingerprinting using random amplified polymorphic DNA (RAPD), repetitive extragenic palindromes (REPs), and enterobacterial repetitive intergenic consensus (ERIC). These isolates were very diverse and heterogeneous. PFGE, ERIC, and REP-PCR methods were more discriminative than RAPD in subtyping the *E. coli* isolates.

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1. Introduction

Escherichia coli are one of the main bacterial pathogens responsible for nosocomial infections especially in immunocompromised patients [1]. Extended-spectrum β-lactamases (ESBLs) are enzymes produced by Gram-negative bacilli that mediate resistance to penicillin, cephalosporins, and monobactams and are commonly recognized in Enterobacteriaceae and *Pseudomonas aeruginosa* [2]. Although most ESBLs are mutants of TEM and SHV enzymes, the CTX-M type β-lactamases which have become important, originated from β-lactamases found in environmental species of the genus *Kluyvera,* and this enzyme hydrolyzes cefotaxime and cefriaxone but is weakly active against ceftazidime [3, 4]. These enzymes are present worldwide with more than 50 variants [4]. The emergence of ESBL-producers along with multiple resistant isolates poses a serious problem in the hospital setting. The widespread uses of antibiotics coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and gene cassettes in integrons are factors that contribute to the increase in antibiotic resistance in bacterial pathogens [1].

Rapid and discriminative subtyping methods are essential for determining the epidemiology of isolates in order to design rational control methods. Available subtyping methods for *E. coli* include PFGE, plasmid profiling, ribotyping and PCR-based typing methods such as arbitrary-primed PCR, repetitive extragenic palindromes (REPs), and enterobacterial repetitive intergenic consensus (ERIC) [5].

A study from Malaysia reported the presence of the *ampC* gene and SHV-5 ESBL in clinical isolates of *E. coli* [6]. However, the genotypic characterization of other resistant isolates has not been reported so far. The objectives of this study were to determine the antimicrobial resistance and ESBL profiles of *E. coli* isolated from 5 public Malaysian hospitals and to determine their genetic diversity using PCR-based fingerprinting techniques and PFGE. The presence of resistance genes and integrons was also determined via PCR, and their transferability was determined by conjugation and transformation.

2. Materials and Methods

2.1. Bacterial Strains. In this retrospective study, 47 nonrepeat *E. coli* isolates were collected in 2004 from 47 different patients selected randomly from intensive care units of 5 public hospitals located in different parts of Malaysia. The participating hospitals were Kota Bharu Hospital ($n = 10$), Sultanah Aminah Hospital ($n = 23$), Kuala Lumpur Hospital ($n = 10$), Ipoh Hospital ($n = 2$), and Queen Elizabeth Hospital ($n = 2$). The isolates were from tracheal aspirates ($n = 18$), urine ($n = 6$), body fluids ($n = 1$), blood ($n = 7$), pus ($n = 6$), bile ($n = 1$), catheter tips ($n = 6$), and unknown ($n = 2$). They were identified by standard laboratory methods in the respective hospitals. They were stored in cryovials containing Luria-Bertani broth with 50% glycerol (Invitrogen, USA) at −20°C and −85°C.

2.2. Genotyping by RAPD, REP, and ERIC. Crude DNA from the *E. coli* obtained by direct cell lysate was used for random amplified polymorphic DNA (RAPD) analysis using primers OPAB04 [7] and OPB17 [8] with cycling conditions as previously described (Table 1).

Enterobacterial repetitive intergenic consensus (ERIC) analysis was performed using primer ERIC-1R (Operon Biotechnologies GmbH, Germany) while repetitive extragenic palindrome (REP) analysis was carried out using REP oligonucleotides (Operon Biotechnologies GmbH, Germany) as primers previously reported [9] (Table 1).

Each PCR reaction was carried out in a 25 μL volume using 1.5 U of *Taq* DNA polymerase (Promega, Madison, Wis, USA) in the reaction buffer provided by the manufacturer containing 2.5 mM $MgCl_2$, 50 μM of each deoxynucleoside triphosphate, 0.3 μM of the selected primer and 5 μL of DNA template. Aliquots (10 μL) of each PCR product were subjected to electrophoresis on 1.5% agarose gel.

2.3. Genotyping by PFGE. PFGE was performed according to previously described protocols [20] with minor variations. Equal volumes of 1% Seakem Gold agarose (Cambrex Bio Science, Rockland, USA) and standardized cell suspension ($OD_{610} = 1.4$; approximately 1×10^8 cfu/mL) were mixed to form agarose plugs, and the bacteria lysed within the plugs with cell lysis buffer (50 mM Tris; 50 mM EDTA (pH 8.0), 1% Sacrosine, 1 mg/mL proteinase K) and incubated at 54°C for 3 hours. Plugs were then washed with sterile deionised water (twice) and TE buffer (4 times), then digested with 12 U of *Xba*I (Promega, Madison, Wis, USA), and incubated overnight at 37°C. The *Xba*I-digested DNA was separated on a CHEF-DRIII (BioRad, Hercules, CA, USA) with pulse times of 2.2–54.2 seconds at 200 V for 24 hours, and gels were photographed under UV light after staining with 0.5 μg/mL ethidium bromide.

2.4. Fingerprint Pattern Analysis. The banding patterns generated by RAPD, ERIC-PCR, REP-PCR, and PFGE were analyzed using GelCompar II, version 2.5 (Applied Maths, Kortrijk, Belgium). PCR fingerprints and PFGE profiles were assigned arbitrary designation and analyzed by defining a similarity (Dice) coefficient F [21]. Cluster analysis based on the unweighted pair group method with arithmetic averages (UPGMA) with a position tolerance of 0.15 was done using the GelCompar II software.

2.5. Antimicrobial Susceptibility Testing and Screening for ESBL. Eighteen antimicrobial agents were tested by the disk diffusion method in accordance with CLSI guidelines [22]: ampicillin 10 μg, piperacillin 100 μg, amoxicillin-clavulanic acid 20 μg/10 μg, cefriaxone 30 μg, ceftazidime 30 μg, cefepime 30 μg, cefoperazone 75 μg, aztreonam 30 μg, imipenem 10 μg, amikacin 30 μg, streptomycin 10 μg, gentamicin 30 μg, kanamycin 30 μg, tetracycline 30 μg, chloramphenicol 30 μg, ciprofloxacin 5 μg, trimethoprim-sulfamethoxazole 75 μg, and nalidixic acid 30 μg (Oxoid Ltd., Basingtoke, Hampshire, England). Isolates were screened for ESBL production by the CLSI disk diffusion method using cefriaxone 30 μg, ceftazidime 30 μg, and aztreonam 30 μg [22]. The double-disk synergy test was performed according to established protocols and results interpreted as described previously [23].

Phenotypic confirmatory test was performed with 30 μg ceftazidime, 30/10 μg ceftazidime-clavulanic acid (Becton, Diskson & Company, Maryland, USA), 30 μg cefotaxime and 30/10 μg cefotaxime-clavulanic acid (Becton, Diskson & Company, Maryland, USA) disks on Mueller-Hinton agar. The results were interpreted as described [22]. *E. coli* isolates ATCC 25922 and ATCC 35218, *Klebsiella pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853 were used as controls.

2.6. Detection of ESBL Genes. β-lactamase genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{VEB}, bla_{DHA}) were detected by PCR using reverse and forward primer pairs listed in Table 1. Boiled suspension of bacterial cells was used as DNA template, and cycling parameters were as previously described [10–13] with minor modifications (Table 1). Primers 1R, 1, 8F, 8R, 2F, 2R, 9F, and 9R [15] were used for further subgrouping of the $bla_{CTX\text{-}M}$ gene into CTX-M groups 1, 2, 8/25, and 9. All amplified products obtained were sequenced to validate their identities.

Two ESBL-producing isolates (EC19 and EC31) were further tested by using primers specific for bla_{ACT}, bla_{GES}, bla_{VIM}, bla_{PER}, and bla_{FOX} genes using conditions as listed in Table 1.

Table 1: Primer sequences and PCR conditions.

Primers	Oligonucleotide sequence (5′ to 3′)	PCR conditions	Reference	Expected size (bp)
TEM-F TEM-R	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1 min at 58°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	[10]	867
SHV-F SHV-R	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1 min at 60°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	[10]	867
OXA-F OXA-R	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1 min at 60°C, 2 min at 72°C; 1 cycle of 10 min at 72°C	[10]	885
CTX-MU1 CTX-MU2	ATGTGCAGYACCAGTAARGT TGGGTRAARTARGTSACCAGA	1 cycle of 7 min at 94°C; 35 cycles of 50 sec at 94°C, 40 sec at 50°C, 1 min at 72°C; 1 cycle of 5 min at 72°C	[11]	593
DHA-1U DHA-1L	CACACGGAAGGTTAATTCTGA CGGTTATACGGCTGAACCTG	1 cycle of 5 min at 94°C; 35 cycles of 30 sec at 94°C, 45 sec at 50°C, 1 min at 72°C; 1 cycle of 8 min at 72°C	[12]	970
VEB-1A VEB-1B	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	1 cycle of 5 min at 96°C; 30 cycles of 1 min at 96°C, 1 min at 55°C, 2 min at 72°C; 1 cycle of 10 min at 72°C	[13]	1014
IntI1-F IntI1-R	GGTCAAGGATCTGGATTTGG ACATGCGTGTAAATCATCGTC	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C; 1 cycle of 10 min at 72°C	[14]	500
5′CS 3′CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	1 cycle of 10 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 54°C, 2 min at 72°C; 1 cycle of 8 min at 72°C	[14]	—
IntI2-F IntI2-R	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	Same as for *int*1	[14]	740
attI2-F orfX-R	GACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 59.5°C, 3.5 min at 72°C; 1 cycle of 10 min at 72°C	[14]	2000
IntI3-F IntI3-R	AGTGGGTGGCGAATGAGTG TGT TCT TGT ATC GGC AGG TG	1 cycle of 12 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C; 1 cycle of 8 min at 72°C	[14]	600
1, 8F 1R 2F 2R 8R 1,8F 9F 9R	GCSATGTGCAGCACCAGTAA ACAAACCGTYGGTGACGATT CTCAATASCGCCATTCCAGG CCGTGGGTTACGATTTTCGC GTCGTACCATAAYCRCCGCT GCSATGTGCAGCACCAGTAA ARTGCAACGGATGATGTYCG GAT GAT TCT CGC CGC TGA AG	1 cycle of 5 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 63°C, and 1 min at 72°C; 10 min at 72°C.	[15]	666 355 529 846
GES-A GES-B	CTTCATTCACGCACTATTAC TAACTTGACCGACAGAGG	1 cycle of 5 min at 95°C; 30 cycles of 1 min at 95°C, 45 sec at 55°C, 1 min 30 sec at 72°C; 1 cycle of 8 min at 72°C	[16]	827
VIM-F VIM-R	AGTGGTGAGTATCCGACAG ATGAAAGTGCGTGGAGAC	Same as GES-A	[17]	225
FOXUP1F FOXUP1R	CACCACGAGAATAACC GCCTTGAACTCGACCG	Same as GES-A	[18]	1184
NHAmpCF NHAmpR	ATTCGTATGCTGGATCTCGCCACC CATGACCCAGTTCGCCATATCCTG	Same as GES-A	[18]	396
PER-A PER-B	GGGACARTCSKATGAATGTCA GGGYSGCTTAGATAGTGCTGAT	Same as GES-A		—

Table 1: Continued.

Primers	Oligonucleotide sequence (5′ to 3′)	PCR conditions	Reference	Expected size (bp)
OPAB04	GC ACG CGT T	1 cycle of 2 min 30 sec at 94°C; 35 cycles of 30 sec at 94°C, 1 min at 47°C, and 1 min at 72°C; 4 min at 72°C.	[7]	—
OPB17	AGGGAACGAG	Same as OPAB04	[8]	—
REP	GCG CCG ICA TGC GGC ATT	1 cycle of 7 min at 94°C; 30 cycles of 30 sec at 94°C, 1 min at 44°C, 8 min at 72°C for 30 cycles; 16 min at 65°C.	[9]	—
ERIC-1R	ATGTAACGTCCTGGGGATTCAC	1 cycle of 2 min and 30 sec at 94°C; 35 cycles of 30 sec at 94°C, 1 min at 47°C, 1 min at 72°C; 1 cycle of 4 min at 72°C	[19]	—

Clinical isolates of *E. coli* were used as the positive controls for bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{OXA} genes. No positive controls were available for detection of bla_{VEB}, bla_{ACT}, bla_{GES}, bla_{VIM}, bla_{PER}, bla_{FOX}, and bla_{DHA} genes and subgrouping of CTX-M group amplified 2, 8/25, and 9.

2.7. Detection of Class 1, 2, and 3 Integrons. Class 1, 2, and 3 integrons were detected by PCR using established primers and conditions as listed in Table 1. Selected amplified products were sequenced to corroborate their identities.

2.8. Transfer of Antibiotic Resistance Determinants. Transfer of resistance genes was attempted in broth using nalidixic resistant recipient *E. coli* JM109 (*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rk^-, m_k^+), *rel*A1, *sup*E44, λ-, Δ (*lac-pro*AB), (F′, *tra*D36, *pro*AB, *lacI*^{q}ZΔZM15). Transconjugants were selected on Luria-Bertani agar supplemented with ampicillin (100 μg/mL) plus nalidixic acid (100 μg/mL) (Sigma Aldrich, USA).

Transformation experiments were carried out for isolates in which conjugation failed to produce positive results. Plasmid DNA was extracted from ESBL-producing *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and was transformed by electroporation into electrocompetent *E. coli* DH10B (F^-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G *ton*A). Transformants were selected on Luria-Bertani agar plates containing 100 μg/mL ampicillin (Sigma Aldrich, USA).

Size determination of the plasmids from transconjugants and transformants was carried out by digestion with *Eco*RI or *Sph*I (Promega, Madison, Wis, USA), and the products separated in 0.8% agarose gels at 70 V for 4 hours.

3. Results

3.1. PCR-Based Fingerprinting. Three PCR-based DNA fingerprinting methods were used to subtype the 47 *E. coli* isolates. ERIC-PCR analysis differentiated the 47 isolates into 45 unique profiles (F = 0.54–1.0) whereas RAPD using the OPAB04 and OPB17 primers generated 44 and 43 profiles, respectively, (F = 0.41–1.0 for the OPAB04 primer and F = 0.36–1.0 for the OPB17 primer, see Figures 1(a) and 1(b)). REP-PCR differentiated the 47 isolates into 45 distinct profiles (F = 0.53–1.0, see Figure 1(c)). All three PCR-based methods were reproducible as identical profiles were obtained in separate experiments using the same set of isolates.

Two isolates, EC14 and EC34, from the same hospital but from different wards, yielded identical profiles by all the 3 methods. Two other blood isolates, EC12 and EC24, from 2 different patients in the same ward, were also indistinguishable by their ERIC, REP, and RAPD profiles. ESBL-producing isolates EC4, EC9, and EC20 were clonally related by both RAPD and REP-PCR. Isolates EC4 and EC9 were indistinguishable by RAPD using the OPAB04 primer whereas isolates EC4 and EC20 were indistinguishable by RAPD using the OPB17 primer. However, isolates EC4 and EC9 were in the same cluster (92% similarity) based on their ERIC-PCR profiles.

3.2. PFGE with XbaI-Digested Genomic DNA. *Xba*I-digested genomic DNA of the 47 *E. coli* isolates resulted in 44 distinct pulsed-field profiles (PFPs) comprising 12–26 restriction fragments. The 2 *E. coli* strains that had identical ERIC-PCR, REP-PCR and RAPD profiles (i.e., EC12 and EC24) were similarly indistinguishable by their PFPs with both sharing all 14 restriction fragments.

Two other isolates, EC1 and EC4 which were indistinguishable by PFGE but were distinguishable in their ERIC, RAPD and REP-PCR profiles, had 39%–68% similarities. Similarly, ERIC, RAPD, and REP-PCR differentiated isolates EC37 and EC39 that displayed identical PFPs. Both EC37 and EC39 were isolated from the same hospital. On the other hand, isolates EC14 and EC34 that displayed identical ERIC, RAPD, and REP-PCR profiles could be differentiated by PFGE (F = 0.81).

3.3. Combined Analysis. A dendrogram based on the combined fingerprints generated by ERIC-PCR, RAPD, REP-PCR, and PFGE was constructed (Figure 2). All the 47 isolates were differentiated into 46 combined subtypes (Table 2). Two isolates, EC12 and EC24, with the combined profile E12R12A11B12X11 were identical in their ERIC, RAPD, REP, and PFGE profiles. Three other isolates (EC4, EC9, and EC20) were grouped within the same cluster and

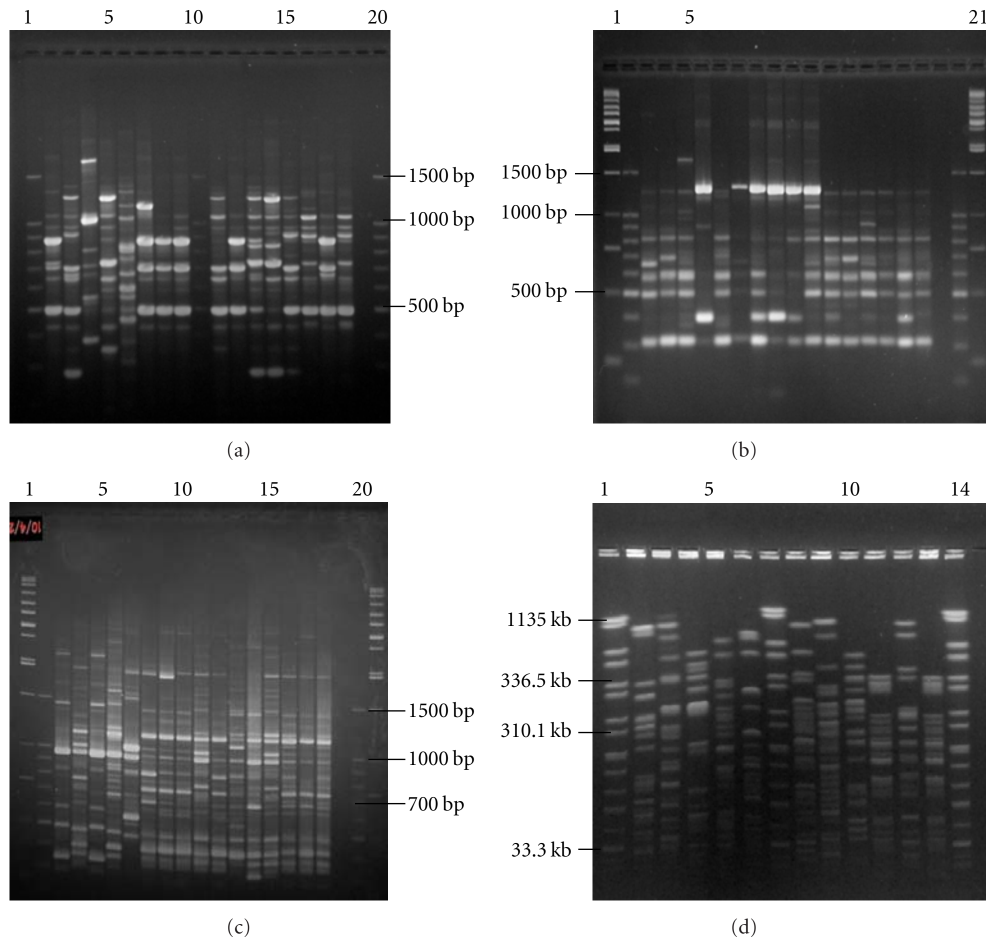

Figure 1: (a) Representative ERIC-PCR profiles for *E. coli* isolates. Lanes 1, 10, 20: 100 bp DNA ladder, lanes 2–4: EC1-EC3; lanes 5–9: EC17, EC19, EC18, EC30, EC34, lanes 11–18: EC32, EC14, EC41, EC39, EC42, EC22, EC45, EC46; lane 19: negative control. (b) Representative RAPD profiles for *E. coli* isolates. Lanes 1, 21: 1 kb DNA ladder; lane 2, 20: 100 bp DNA ladder; lanes 3–18: EC5, EC8, EC13, EC15, EC7, EC44, EC12, EC24, EC25, EC29, EC35, EC40, EC26, EC43, EC46, EC47; lane 19: negative control. (c) Representative REP-PCR profiles for *E. coli* isolates. Lane 1, 21: 1 kb DNA ladder; lane 2, 20: 100 bp DNA ladder; lanes 3–18: EC5, EC8, EC13, EC15, EC7, EC44, EC12, EC24, EC25, EC29, EC35, EC26, EC40, EC43, EC46, EC47; lane 19: negative control. (d) Representative PFGE profiles for *E. coli* isolates. Lanes 1, 7, 14: *Salmonella* Braenderup H9812 Standard DNA marker; lanes 2–6: EC4, EC9, EC38, EC36, EC18; lanes 8–13: EC28, EC31, EC40, EC22, EC45, EC46.

were clonally related (more than 85% similarity). Isolates EC14 and EC34 were also grouped together within the same cluster and were clonally related (more than 94% similarity).

3.4. Antimicrobial Susceptibility. The antibiotic resistant rates for the *E. coli* isolates were as follows: ampicillin 77%, piperacillin 64%, tetracycline 53%, trimethoprim-sulfamethoxazole 43%, cefoperazone and kanamycin 30% each, nalidixic acid 28%, chloramphenicol 26%, ciprofloxacin 23%, gentamicin 21%, amoxicillin-clavulanic acid 17%, cefriaxone ceftazidime and aztreonam 11% each, and amikacin 2%. All 47 isolates were sensitive to imipenem. Majority of the isolates were sensitive to cefepime except EC28, EC34, and EC37 that showed intermediate susceptibility. Among them, 36 isolates (76.5%) were multidrug-resistant. The cephalosporin resistant isolates were also resistant to ampicillin and 71% of them resistant to tetracycline. A large number of isolates that were resistant to aminoglycosides (72%) were also resistant to tetracycline.

Thirty-six (76.5%) isolates were presumptive ESBL producers based on initial screening. Using the double-disk synergy test, only 3 ESBL producing isolates were detected. However, based on the phenotypic confirmatory test, 10 isolates were found to be ESBL producers. All isolates that tested positive for ESBL were also multidrug-resistant.

3.5. Detection of Genes Encoding ESBLs. Established primers were used on the genomic and plasmid DNA of the 47 *E. coli* isolates for the following ESBL-encoding genes: bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{DHA}, and bla_{VEB}. The $bla_{\mathrm{TEM\text{-}1}}$ gene was detected in 35 (74.5%) whereas bla_{SHV}-, $bla_{\mathrm{CTX\text{-}M}}$-, and bla_{OXA}-specific amplicons were detected in only 3, 8, and 2 isolates, respectively. Of the 35 bla_{TEM} isolates, 7 also harbored $bla_{\mathrm{CTX\text{-}M}}$ and 1 had bla_{SHV}. Three ESBL genes were

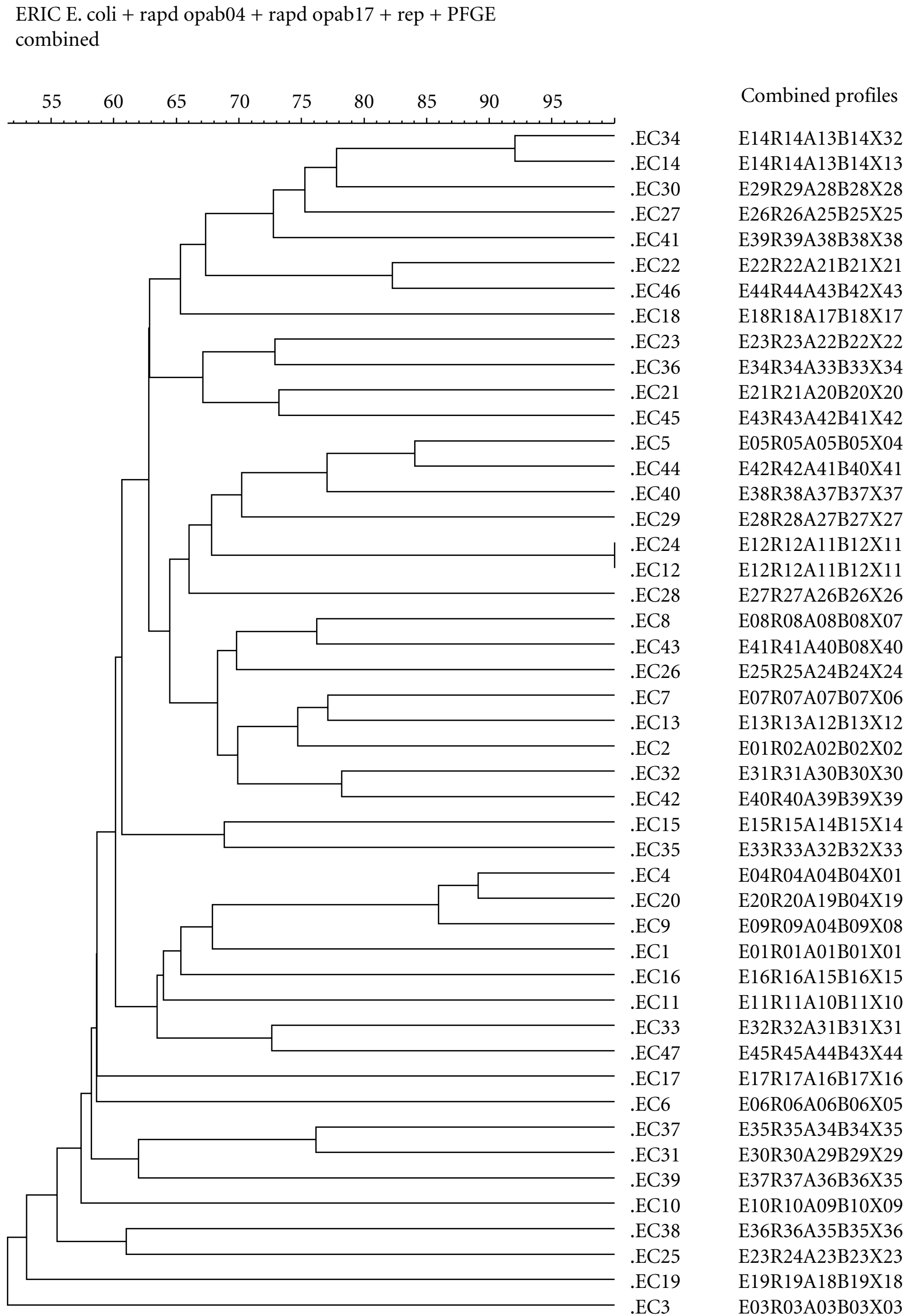

Figure 2: Dendrogram generated using UPGMA based on Dice coefficients of similarity for the clustering of the *E. coli* combined profile. The dotted blue vertical line indicates 80% similarity level.

detected in *E. coli* isolate EC7: bla_{SHV}, bla_{OXA}, and $bla_{\text{CTX-M}}$. bla_{DHA}, and bla_{VEB} were not detected in any of the isolates. All bla_{TEM}, bla_{SHV}, and bla_{OXA} genes were carried on plasmids whereas 6 of the 8 $bla_{\text{CTX-M}}$ genes were plasmid-borne. No ESBL-encoding gene was detected in 2 presumptive ESBL-producers, EC19 and EC31. Sequencing of the amplified products indicated complete identity with the respective gene sequences (i.e., bla_{TEM}, bla_{SHV}, bla_{OXA}, and $bla_{\text{CTX-M}}$) in the NCBI database. Further analysis of the bla_{TEM} sequences indicated that the *E. coli* isolates harbored the TEM-1 subgroup whereas for bla_{OXA}, the subgroup found in the isolates was OXA-1. In the case of the $bla_{\text{CTX-M}}$ gene, DNA sequences analysis indicated that 3 of the 8 isolates detected belonged either to subgroup CTX-M-15 or to subgroup CTX-M-28. However, it was not possible to further subgroup the remaining 5 $bla_{\text{CTX-M}}$ genes, as the primers used did not amplify the entire $bla_{\text{CTX-M}}$ reading frame. The subsequent use of primers 1R, 1, 8F, 2F, 2R, 8R, 9F, and 9R [15] to further subgroup the 5 $bla_{\text{CTX-M}}$ genes could only indicate that they did not belong to subgroups 1, 2, 8/25, and 9. Similarly, we

Table 2: Antimicrobial resistance, size of plasmids and ESBL genes detected in the selected donor *E. coli* isolates, and their respective transconjugants and transformants.

No.	Resistance profile	ESBL-encoding gene detected by PCR	Plasmid donor (kb)	Plasmid transformant/transconjugant (kb)	Resistance profile of transformant/ transconjugant	ESBL-encoding gene transferred	Mode of transfer
EC7	AMP, PIP, TET, CRO, FEP, NAL, SXT, CHL, CFP, CAZ, KAN, ATM	bla_{OXA}, bla_{SHV}, bla_{CTX-M}	135	55	AMP, PIP, TET, CRO, FEP, NAL, SXT, CFP, KAN, ATM	bla_{OXA}, bla_{SHV},	Transformation
EC10	AMP, PIP, TET, NAL, SXT, CHL, CAZ, KAN, CIP, STR, GEN	bla_{TEM}	310	310	AMP, PIP, TET, NAL, SXT, CHL, CIP, STR, GEN	bla_{TEM}	Transformation
EC12	AMP, PIP, TET, NAL, SXT, CHL, KAN, STR	bla_{TEM}	50	50	AMP, PIP, TET, SXT, KAN, STR	bla_{TEM}	Transformation
EC18	AMP, PIP, TET, SXT, STR, CRO	bla_{TEM}	190	190	AMP	bla_{TEM}	Conjugation
EC24	AMP, PIP, TET, NAL, SXT, CHL, KAN, CIP, GEN	bla_{TEM}, bla_{CTX-M}	60	60	AMP, PIP, TET, NAL, SXT, CHL, CIP, GEN	bla_{TEM}, bla_{CTX-M}	Transformation
EC31	AMP, PIP, TET, SXT, CHL, CFP, KAN, CRO, STR	ND	90	40	AMP, PIP, SXT, CFP	ND	Conjugation
EC36	AMP, PIP, TET, SXT, CFP, CIP, STR, KAN, GEN, NAL, CHL, SXT	bla_{TEM}	300	300	AMP, PIP, TET, SXT, CIP, STR, GEN, CHL, NAL	bla_{TEM}	Transformation
EC46	AMP, PIP, STR	bla_{TEM}	50	50	AMP, PIP, STR	bla_{TEM}	Conjugation

were unable to subgroup the bla_{SHV} genes as the primers used only amplified a portion of the gene and not the entire reading frame.

3.6. Class 1, 2, and 3 Integrons. Forty-seven *E. coli* isolates were screened for the presence of integrases encoded on class 1, 2, and 3 integrons. The class 1 integron-encoded *intI1* integrase gene was detected in 25 isolates while 4 isolates tested positive for class 2-encoded *intI2* integrase. One isolate, EC24, was found to harbor both *intI1* and *intI2*. No class 3 integron was detected. Majority of the integrons were found to be plasmid-encoded (16 of the 25 *intI1* and 3 of the 4 *intI2* detected).

Isolates that were positive for class 1 and 2 integrons were further analyzed for the presence of inserted gene cassettes within the variable region by using the primer pair 5′_CS/3′_CS for class 1 integrons and primer pair orfx/attI2 for class 2 integrons. Amplified products of different sizes were obtained from 17 of the 25 *intI1*-positive isolates and sequencing indicated the presence of 5 different types of known gene cassettes: *aadA5-dfrA17, dfrA7, aadA1-aadB-cmlA6, dfrA12-aadA2-orfF* and *aadA1*. Using the attI2/orfX primer pair for *intI2*-positive isolates resulted in a 2 kb

amplified product which, when sequenced, contained the *dfrA1-aadA1-sat2* gene cassette. The *aadA2, aadA5,* and *aadB* genes encode resistance to aminoglycosides whereas *sat2* encode resistance to strepthoricin. Both *dfrA12* and *dfrA17* encode resistance to trimethoprim and *cmlA6* to chloramphenicol. Although trimethoprim and strepthoricin were not used in our study, the presence of gene cassettes encoding resistance to aminoglycosides and chloramphenicol coincided with the resistance profiles of the respective isolates. The majority of the integron-positive isolates (24 of 27) were multidrug-resistant.

3.7. Transfer of Resistance Determinants. Conjugation experiments were carried out for 7 randomly-selected ESBL-producers and transfer of this phenotype to the recipient nalidixic acid-resistant *E. coli* JM109 was successful in only 3 of the 7 isolates (38%). However, it should be noted that only broth matings were carried out in this study and not filter matings and thus the transmissibility potential for the other 4 isolates could not be fully ascertained. All transconjugants were resistant to ampicillin and piperacillin except for the EC18 which remained susceptible to piperacillin. Streptomycin resistance was cotransferred in the EC46 transconjugant whereas for the EC31 transconjugant, trimethoprim-sulfomethaxazole and cefoperazone resistances were also transferred (Table 2).

Identical *Eco*RI and *Sph*I restriction profiles were obtained from plasmids extracted from the donor *E. coli* and their respective transconjugants except for EC31. In this case, based on the restriction profiles obtained, the plasmid extracted from the transconjugant was smaller than the plasmid obtained from the donor EC31 strain (approximately 40 kb from the transconjugant as compared to ~90 kb from the donor) although they shared a number of common restriction bands (Table 2). Plasmids extracted from the transconjugants were used to transform electrocompetent *E. coli* DH10B cells. Plasmids extracted from the resulting ampicillin resistant DH10B transformants showed identical *Eco*RI restriction profiles with those from their respective transconjugants. The DH10B transformants also displayed identical antibiotic resistance profiles as their respective transconjugants, strongly implying that these antibiotic resistance determinants were plasmid-borne.

Both bla_{TEM} and *intI*1 were detected on the plasmids extracted from the EC18 and EC46 donor, transconjugants as well as their subsequent transformants indicating that these 2 genes were likely present on the plasmid that was transferred. None of the common ESBL-encoding genes were detected in EC31 although this isolate harbored class 1 integron-encoded *intI* that was detected from the plasmid in the EC31 transconjugant and transformant suggesting that the transferred plasmid harbored a class 1 integron.

Transformation was carried out for the 4 isolates in which conjugation was not successful and another ESBL-positive randomly chose isolate. Plasmids were extracted from these 5 isolates and electroporated into recipient *E. coli* DH10B cells. All transformants obtained were resistant to ampicillin, piperacillin, tetracycline, and trimethoprim-sulfamethoxazole. Plasmids extracted from the transformants showed identical *Eco*RI restriction profiles when compared to their respective donor plasmids except for isolate EC7 in which the plasmid isolated from the transformant was smaller (~55 kb) when compared to the hospital isolate (~135 kb) (Table 2). Both bla_{TEM} and *intI1* genes were also detected from the plasmids isolated from the donor isolates as well as the transformants except for isolate EC7 and its transformant.

4. Discussion

Genotyping by PCR-based methods and PFGE showed the 47 *E. coli* clinical isolates to be genetically diverse and heterogeneous. This is expected as the isolates were randomly selected from different hospitals and sources. Similar observations were reported by Mugnaioli et al. [24] and Woodford et al. [16]. We found that ERIC and REP-PCR yielded practically identical results and able to differentiate strains which were indistinguishable by RAPD. Although PCR-based fingerprinting is rapid, it is more susceptible to technical variations than PFGE especially reproducibility. Thus PFGE is considered the better method for subtyping of *E. coli,* even though it is relatively laborious and time-consuming compared to PCR-based methods.

Isolates EC12 and EC24 were indistinguishable by both PFGE and PCR fingerprints. These two blood isolates were from different patients in the same ward, strongly suggesting a possible nosocomial spread. Interestingly, both isolates harbored plasmids of different sizes with EC12 harboring a plasmid of ~50 kb whereas EC24 contained a plasmid of ~60 kb. On the other hand, there were isolates that were identical in their PCR fingerprints but were differentiated by their PFGE profiles (e.g., isolates EC14 and EC34) and conversely, isolates that were indistinguishable by PFGE were differentiated by PCR (isolates EC1 and EC4, and EC37 and EC39). This shows that these methods are complementary and a combined analysis would give a finer perspective bearing in mind the drawbacks of each of these methods.

All 47 *E. coli* isolates analyzed in this study were susceptible to imipenem. This finding is similar to previous reports [16, 25]. Cefepime was equally sensitive at 98% which is a rate higher than the 80% sensitivity reported in Colombia [25] and 70% in China [26].

The resistance rates of chloramphenicol, trimethoprim-sulfomethaxazole, tetracycline, gentamicin, kanamycin, ciprofloxacin and nalidixic acid were 7% to 69% lower compared to a report by Alhaj et al. [27].

The resistance rates of *E. coli* isolates to ceftazidime (11%) and amoxillin-clavulanic acid (17%) were lower when compared to that from China—28% for ceftazidime and 84% for amoxillin-clavulanic acid [26]. *E. coli* resistance to amikacin in Malaysia (2% in this study) was still relatively low compared to 27% in Colombia [43] or 22.4% in Israel [28].

Forty *E. coli* isolates were classified as ESBL producers based on phenotypic or genotypic detection of ESBL: 38

isolates were classified as ESBL producers based on the genotypic detection of ESBL-encoding genes, and 2 others were categorized as ESBL producers based on the double-disk synergy and phenotypic confirmatory tests. However, these two isolates, EC7 and EC31, did not harbor any of the tested ESBL-encoding genes and may instead harbor other genes such as bla_{TLA}, bla_{IMP}, and bla_{CMY} [1, 29] that were not included in our test. Although the double-disk synergy test had been reported to be reliable and easy-to-use, its major disadvantage is that the distance of disk placement for optimal sensitivity has not been standardized [20, 29].

Analysis of the ESBL-encoding genes indicated that the majority of the ESBL-positive isolates harbored TEM-1 (88%) followed by CTX-M (20%), SHV (8%), and finally, OXA (5%). TEM-1 has been reported to be responsible for 90% of ampicillin resistance in *E. coli* [30]. The $bla_{\mathrm{CTX\text{-}M}}$ gene is considered the most prevalent ESBL-encoding gene worldwide and is replacing TEM and SHV types as the predominant ESBL in many European countries [31]. The presence of the $bla_{\mathrm{SHV\text{-}5}}$ gene in 11 ceftazidime resistant *E. coli* isolates from one Malaysian teaching hospital has been reported [6]. The specific SHV subtypes could not be confirmed in this study as the primers used only amplified a portion of the bla_{SHV} reading frame.

Analysis of integron-encoded integrases indicated that class 1 integron was the principal integron class in the Malaysian strains. Class 2 integron was in the minority, and no class 3 integron-encoded integrases were detected, a trend that has previously been reported [14, 32]. Four different gene cassettes, namely, the *aadA1, dfrA17-aadA5, dfrA12-orfF-aadA2,* and *aadA1-aadB-cmlA6* were found in the class 1 integron-positive isolates, and these have been previously described in *E. coli* as well as in other *Enterobacteriaceae* [15]. All 4 isolates positive for class 2 integron-encoded *intI2* harbored the *dfrA1-sat2-aadA1* gene cassette which has been reported [1, 33]. Conjugation and transformation experiments indicated that the majority of the integrons and some of the ESBL-encoding genes (in particular bla_{TEM}) were plasmid-encoded and transmissible. Plasmids that were isolated from the *E. coli* hospital isolates were estimated to be larger than 50 kb in agreement with the sizes reported previously [34, 35]. In 2 of the 3 successful conjugation experiments, plasmids with identical restriction profiles were isolated from the donor and the transconjugants. In the remaining case, based on the restriction profiles obtained, the plasmid that was isolated from the transconjugant was about 50 kb smaller than the donor EC31 strain (~90 kb), indicating either that the donor strain harbored more than a single type of plasmid and that only the plasmid of about 40 kb was transferred, or that only a ~40 kb portion of the original plasmid was successfully transferred by conjugation. The latter appeared to be a stronger possibility as separation of undigested plasmid DNA extracted from the parental EC31 strain seemed to indicate the presence of a single plasmid. Similar observations were also noted for the transformation experiments. Further characterization of these plasmids is clearly needed and is the subject of our on-going investigations. Nevertheless, our results indicate that most of the ESBL-encoding genes especially bla_{TEM} are carried on plasmids which are transmissible suggesting that the spread of ESBL and other antibiotic resistance determinants is likely to be plasmid-mediated in agreement with the conclusions made by other reports [36, 37] that plasmids are one of the main vehicles for spread of antibiotic resistance genes. This may have led to the high prevalence of ESBL-producers and multidrug resistance among *E. coli* hospital isolates in Malaysia.

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