New integron gene arrays from multiresistant clinical isolates of members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa* from hospitals in Malaysia

Sue-Bee Kor,¹ Quok-Cheong Choo² and Choy-Hoong Chew¹

¹Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Kampar, Perak, Malaysia
²Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Kampar, Perak, Malaysia

This study investigated 147 multidrug-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates from hospitalized patients in Malaysia. Class 1 integrons were the most dominant class identified (45.6%). Three isolates were shown to contain class 2 integrons (2.0%), whilst one isolate harboured both class 1 and 2 integrons. No class 3 integrons were detected in this study. In addition, the *sul1* gene was amplified in 35% of isolates and was significantly associated with the presence of integrase genes in an integron structure. RFLP and DNA sequencing analyses revealed the presence of 19 different cassette arrays among the detected integrons. The most common gene cassettes were those encoding resistance towards aminoglycosides (*aad*) and trimethoprim (*dfr*). As far as is known, this study is the first to identify integron-carrying cassette arrays such as *aadA2-linF*, *aacC3-cmlA5* and *aacA4-catB8-aadA1* in the Malaysian population. Patients’ age was demonstrated as a significant risk factor for the acquisition of integrons (*P*<0.028). Epidemiological typing using PFGE also demonstrated a clonal relationship among isolates carrying identical gene cassettes in *Klebsiella pneumoniae* and *P. aeruginosa* but not in *Escherichia coli* isolates.

**INTRODUCTION**

Dissemination of antibiotic resistance genes by horizontal gene transfer has led to the rapid emergence of multidrug resistance (MDR) among bacteria. Infections caused by MDR Gram-negative bacteria are now recognized as important causes of morbidity and mortality among hospitalized patients (Paterson & Bonomo, 2005; Walsh et al., 2005). The family *Enterobacteriaceae*, for example, contains genera that inhabit the intestinal tracts of humans, which are frequently exposed to various antibiotics, frequently causing higher potential for the dissemination of antibiotic resistance genes (Goldstein et al., 2001), whilst *Pseudomonas aeruginosa* is an also important pathogen for immunocompromised patients (Driscoll et al., 2007).

Integrons participate in site-specific recombination, playing a major role in disseminating antibiotic resistance. Many antibiotic resistance genes found in Gram-negative bacteria are part of a gene cassette integrated into integrons (Recchia & Hall, 1995). Class 1 integrons are the most prevalent and well-characterized integrons (Martínez-Freijoo et al., 1999). Integrons possess three essential components in the 5’-conserved segment (CS): an *intI* gene encoding an integrase, a specific recombination site (*attI*) and a promoter that directs transcription of the gene cassettes. Most class 1 integrons contain an additional resistance gene, *sul1*, in the 3’-CS, which confers sulfonamide resistance (Fluit & Schmitz, 1999). Class 2 and 3 integrons contain the integrase genes *intI2* and *intI3*, respectively, whose products are 40 and 61% identical to class 1 integrase (*intI1*), respectively (Recchia & Hall, 1995).

In Malaysia, Lim et al. (2009a, b) reported the presence of class 1 integrons in extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae*. However, no studies have been carried out to elucidate the presence of the three classes of integrons (classes 1, 2 and 3) among the MDR *Enterobacteriaceae* and *Pseudomonas* spp., which are commonly associated with hospital-acquired infections in Malaysia, as well as to characterize the gene cassettes present within the integron-positive isolates. Thus, this study reports the patterns of...
antibiotic susceptibility in association with the presence of integrons and gene cassettes, as well as the clonal relationship among integron-positive clinical isolates with similar gene cassettes among the integron-positive clinical isolates in Malaysia.

METHODS

Bacterial strains. A total of 147 MDR Enterobacteriaceae and Pseudomonas isolates were collected from in-patients in a government-funded hospital and several private hospitals in Malaysia from May 2009 to October 2010. The isolates were collected randomly from various sites of the in-patients, and identified by an API system (bioMérieux). In this study, bacterial isolates resistant to more than three antibiotics were termed MDR isolates. All bacteria isolates were preserved and suspended in 40% (v/v) glycerol and stored at −70 °C.

Antimicrobial susceptibility determination. Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI, 2011) using a Kirby–Bauer disc diffusion assay on Mueller–Hinton agar (Oxoid). The susceptibility profile was determined using 18 antibiotics (Oxoid): amikacin (30 μg), aminoglycoside (3 μg), ampicillin (10 μg), cephalosporin (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), norfloxacin (10 μg), streptomycin (10 μg), sulamethoxazole (100 μg), tetracycline (30 μg), tigecycline (15 μg), trimethoprim (5 μg) and trimethoprim/sulamethoxazole (25 μg).

Template DNA preparation. Total DNA was extracted using a fast-boil method. The bacterial isolates were incubated in tryptic soy broth for 20 h at 37 °C with constant shaking. Subsequently, 1.5 ml inoculum was subjected to centrifugation at 11200 g for 5 min and the bacterial pellet was resuspended in 300 μl sterile deionized water. The cells were then lysed by boiling for 5 min and incubated immediately on ice for 2 min. The cell debris was again pelleted by centrifugation at 11200 g for 2 min. The supernatant was aspirated and stored at −20 °C until subsequent PCR analysis.

Integron analyses. To determine the presence of integron carriage, a degenerate primer pair (hep35 and hep36), complementary to a conserved region of the integron-encoded integrase genes intI1, intI2 and intI3, was used. The resultant PCR products were digested further using RsaI or Hinfl to determine the class of integron (White et al., 2000). Subsequently, characterization of the 3′ region was performed by detecting sulfonamide resistance genes (sul1) in all the bacterial isolates using primers sul1_F and sul1_R (Stokes & Hall, 1989).

Following the amplification of integron-encoded genes, class 1 and 2 integron cassette regions were amplified with primer pairs 5′-CS/3′-CS and hep74/heps1, respectively (Lévesque et al., 1995; White et al., 2000). The primer Int2f, which is specific to the 3′ region of the integrase gene by PCR

PFGE. PFGE was carried out using a contour-clamped homogeneous-field apparatus (CHEF Mapper XA System; Bio-Rad). The protocol used was based on the standardized PulseNet PFGE protocol (Centers for Disease Control and Prevention, GA, USA) with minor modifications for self-optimized conditions. Briefly, the genomic DNA was digested with XbaI and separated on a 1.0% pulse-field certified agarose gel (Bio-Rad) in 0.5× TBE buffer. The conditions for electrophoresis were 6 V cm⁻¹ for 20 h with increasing pulse time variation for different bacterial species.

Dendrograms of similarity were calculated based on the Dice coefficient and clustering by UPGMA, constructed using BioNumerics Fingerprint types and cluster analysis software (Applied Maths). PFGE patterns with a similarity of ≥80% were considered to be epidemiologically related.

Statistical analysis. Data were processed and analysed using spss software version 17 for Windows. A χ² test was used for analysis on categorical variables and a Mann–Whitney U test was used to analyse continuous variables. All P values were based on two-tailed tests of significance, with P<0.05 being considered statistically significant.

RESULTS

Antimicrobial susceptibility profile

For the purpose of analysis, both intermediate and resistant organisms were grouped together as resistant. In this study, the majority of the Enterobacteriaceae and P. aeruginosa showed resistance to ampicillin (98.0%), augmentin (94.6%), cefuroxime (92.5%) and ceftriaxone (89.1%). In contrast, the isolates were sensitive to imipenem, meropenem (both at 91.16%) and amikacin (85.0%), as illustrated in Table 1.

Overall, bacterial resistance was mostly towards the penicillin and cephalosporin groups, and to a lesser extent towards the carbapenem and aminoglycoside groups, except for streptomycin. Up to 66.0% of the isolates in this study were streptomycin resistant. High levels of resistance of >50% were also observed towards tetracycline (68.0%), the sulfonamide group (60%) and ciprofloxacin (52.4%). Alarmingly, 40.8% of the isolates were observed to be resistant towards the newly synthesized antibiotic tigecycline.

Detection of class 1, 2 and 3 integrases and the sul1 gene by PCR

PCR amplification of the integrase gene showed that 71 (48.3%) of the isolates were integron positive (Table 2). Restriction analysis using Hinfl and Rsal revealed that 94.4% of the isolates (67/71) contained class 1 integrons, 4.2% (3/71) contained a class 2 integron, and only one isolate harboured both class 1 and 2 integrons. No class 3 integrons were detected. Among all isolates tested, a high proportion of integrons was detected in Klebsiella sp. (33/40, 82.5%), followed by 61.7% of E. coli (21/34), 60.0% of P. aeruginosa (9/15) and Morganella sp. (3/5), 33.3% of Citrobacter sp. and Proteus sp. (1/3) and lastly 9.1% (3/33) of Enterobacter sp. isolates (Table 2). No integrons were detected in Providencia and Serratia isolates.

In terms of characterization of the 3′-CS of integrons, only 34.7% (n=51) of the isolates carried the sulfonamide
of the 51 sul1-positive isolates, 80.4% (n = 41) of them were found to carry integrase genes as well. There were 66 isolates that did not harbour either of these integron markers.

### Characterization of integron-borne gene cassettes

Among the 71 isolates that tested positive for the integrase gene, intI1 and intI2 gene cassette regions were amplified...
successfully in 42 of them (59.2%). The size of the inserted gene cassettes in this study ranged from 0.7 to 2.6 kb. Characterization by PCR-RFLP using *Hinfl* separated all the cassette amplicons into 19 distinct profiles containing a combination of one to three gene cassettes in tandem (Table 3). Furthermore, the 150 bp product generated in combination of one to three gene cassettes was obtained in 33.8 % of the isolates typed here, five with identical *aadA6-orfD* and *aacC3-cmlA5* cassettes were grouped into one cluster. Two of the isolates containing *aadA6-orfD* gene cassettes (K18 and K31) formed another cluster with 80 % similarity. However, no distinct PFGE pattern was found within the *E. coli* isolates studied.

**DISCUSSION**

This study gives a representative picture of the prevalence of integrons within the hospital environment in Malaysia. The existence of integrons was confirmed in 48.3 % (71/147) of collected multiple antibiotic-resistant *Enterobacteriaceae* and *Pseudomonas* species. Class 1 integrons were the dominant class, with 94.4 % (67/71) of these integron-positive isolates having *intI1*. The proportion of class 1 integron carriage in these antibiotic-resistant isolates was comparable to that in other studies reported worldwide (Martínez-Freijo et al., 1998; Chang et al., 2000).

Table 2 showed that class 1 and 2 integrons are widespread in different species of *Enterobacteriaceae*. Interestingly, class 1 integrons were detected in 80 % of the *Klebsiella* species collected. A higher percentage of class 1 integrons was observed in this study than in a study by Lim et al. (2009a), in which class 1 integrase was found in only 41.2 % (21/51) of the ESBL *K. pneumoniae* strains. This observation suggests either an increase in antibiotic resistance within this species or possible variation in criteria during sample collection. Surprisingly, one of the *K. pneumoniae* isolates in our study was shown to harbour both *intI1* and *intI2*, which, to our knowledge, has never been reported in any other studies in Malaysia. Class 1 integrons were also found in 60 % of *P. aeruginosa*. The high occurrence of integrons in *P. aeruginosa* is in contrast to the study of Lim et al. (2009), in which only 19 % of the isolates were shown to carry class 1 integrons. This difference can be explained because only 69 % of the collected isolates were MDR in the study by Lim et al. (2009), compared with this study, in which a significant increase in MDR was observed in this bacterial species (100 %). However, Khosravi et al. (2011) demonstrated a high prevalence of class 1 integrons (54/90, 60.0 %) in clinical imipenem-resistant *P. aeruginosa* in Malaysia.

Gene cassettes in 43 of the isolates (59.2 %) were amplified successfully using 5′-CS/3′-CS and hep74/hep51 primer pairs. Five of these isolates generated amplicons of only 150 bp and were later shown to be an integron structure without any inserted gene cassette. In addition, no amplification product was obtained in 33.8 % of the
Table 3. The 14 RFLP groups of gene cassettes with their corresponding resistant phenotype and bacterial species identified

<table>
<thead>
<tr>
<th>RFLP group</th>
<th>Approximate amplicon size (kb)</th>
<th>Gene cassette(s)*</th>
<th>Bacterial species (n)†</th>
<th>Resistant phenotype‡</th>
<th>No. isolates resistant to antibiotic§§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AK</td>
</tr>
<tr>
<td>1a</td>
<td>0.7</td>
<td>dfrA5</td>
<td>EC (1)</td>
<td>TMP</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>0.7</td>
<td>aadB</td>
<td>KO (1), KP (1)</td>
<td>GN</td>
<td>0</td>
</tr>
<tr>
<td>1c</td>
<td>0.7</td>
<td>dfrA7</td>
<td>KP (4)</td>
<td>TMP</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>aadA1</td>
<td>EC (1)</td>
<td>S</td>
<td>0</td>
</tr>
<tr>
<td>3a</td>
<td>1.2</td>
<td>blaP1</td>
<td>KP (1)</td>
<td>AM/AmC</td>
<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>1.2</td>
<td>dfrA1-orfC</td>
<td>MM (1)</td>
<td>TMP</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>aadA6-orfD</td>
<td>PA (3), KO (1)</td>
<td>S</td>
<td>4</td>
</tr>
<tr>
<td>5a</td>
<td>1.6</td>
<td>dfrA15-aadA1</td>
<td>KP (1)</td>
<td>TMP/S</td>
<td>0</td>
</tr>
<tr>
<td>5b</td>
<td>1.6</td>
<td>dfrA16-aadA2</td>
<td>KP (1)</td>
<td>TMP/S</td>
<td>0</td>
</tr>
<tr>
<td>5c</td>
<td>1.6</td>
<td>dfrA17-aadA5</td>
<td>EC (1), KP (1), MM (1)</td>
<td>TMP/S</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1.9</td>
<td>dfrA12-orfF-aadA2</td>
<td>EA (1), EC (1), MM (1),</td>
<td>TMP/S</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>2.4</td>
<td>aacA4-catB8-aadA1</td>
<td>KP (4), PA (1)</td>
<td>AK/C/S</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>2.6</td>
<td>aacA4-catB3-dfrA1</td>
<td>EC (2)</td>
<td>AK/C/TMP</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>1.0 + 1.6</td>
<td>aadA1 + dfrA1-aadA1</td>
<td>EC (1)</td>
<td>S and TMP/S</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1.0 + 2.3</td>
<td>aadA2 + dfrA1-aadA5</td>
<td>ECL (1)</td>
<td>S and TMP/S</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>1.3 + 2.3</td>
<td>aadA6-orfD + aacC3-cmlA5</td>
<td>PA (5)</td>
<td>S and GN/C</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>1.6 + 1.9</td>
<td>dfrA17-aadA5 + aadA2-LinF</td>
<td>EC (1)</td>
<td>TMP/S and S, DA</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>1.9 + 2.2</td>
<td>dfrA12-orfF-aadA2 + dfrA1-sat1-aadA1</td>
<td>KP (1)</td>
<td>TMP/S and TMP/S</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>2.2</td>
<td>dfrA1-sat1-aadA1</td>
<td>EC (2), ECL (1)</td>
<td>TMP/S</td>
<td>1</td>
</tr>
</tbody>
</table>

| Total      |                               |                  |                        |                      | 42 | 14 | 29 | 38 | 41 | 40 | 41 | 25 | 39 | 38 |

*Class 2 cassettes are underlined.
†Bacterial species: EA, Enterobacter aerogenes; EC, Escherichia coli; ECL, Enterobacter cloacae; MM, Morganella morgani; KO, Klebsiella oxytoca; KP, K. pneumoniae; PA, P. aeruginosa; PM, Proteus mirabilis. Bold indicates bacterial isolates carrying similar gene cassettes that were further subjected to PFGE for analysis of clonal relatedness.
‡AK, Amikacin; GN, gentamicin; S, streptomycin; AM, ampicillin; AmC, augmentin; TMP, trimethoprim; C, chloramphenicol; SMZ, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; DA, clindamycin.
§Antibiotic relevance to the resistant phenotype of integron-associated resistance genes is shown in bold. Isolates that are not correlated with antibiotic-resistant phenotypes are underlined.
Integrons in Enterobacteriaceae and P. aeruginosa

...integrase-positive isolates. This was probably due to the lack of a 5’- or 3’-CS, as stated by other investigators (White et al., 2000; Moura et al., 2007). Indeed, rearrangements by IS26 in the integrons may have mediated changes to the class 1 integron structure that included loss of parts of the 3’-CS (Labbate et al., 2008; Doublet et al., 2009; Juan et al., 2010) and 5’-CS (Miriagou et al., 2005). Furthermore, class 1 integrons with a complete tni module and no 3’-CS are being found with increasing frequency, demonstrating the structural diversity of class 1 integrons (Betteridge et al., 2011).

To our knowledge, this is the first report of cassette arrays such as dfrA5, aacA4-catB3-dfrA1 and dfrA1-aadA1 in E. coli isolated from clinical samples in Malaysia. These genes have, nevertheless, been seen in other countries, including Asian countries such as Indonesia and China (Waturangi et al., 2003; Wang et al., 2008). The array aacA4-catB3-dfrA1, which has been reported in Japan (Kumai et al., 2005) and China (Li et al., 2006), was also found in this study. This seems to indicate that these resistance cassettes are widespread in Asian countries. Interestingly, the rare combination of aadA2-linF was found in one of the E. coli isolates. The aadA2 and linF cassettes are individually frequently detected among Enterobacteriaceae isolates. However, sequences deposited in GenBank (accession nos FJ594765.1 and AY522431.4) showed that aadA2-linF was detected by two different studies in Salmonella enterica (J.-J. Yan, J.-J. Wu, W.-C. Ko, C.-S. Chiou and L.-R. Wang, 2009, unpublished data) and E. coli (González-Zorn et al., 2005). The current study is the first report, to our knowledge, of the presence of aadA2-linF in clinically isolated E. coli in Malaysia.

Lim et al. (2009a) amplified gene cassettes aadB and dfrA12-orfF-aadA2 from 57% (12/21) of the intI1-positive ESBL K. pneumoniae isolates in the local population. These similar cassettes were also observed in K. pneumoniae in this study. Interestingly, aacA4-catB8-aadA1, which was first reported in clinical K. pneumoniae from China (Gu et al., 2008) but never before in Malaysia, was also found to be widespread in four K. pneumoniae isolates in this study. This implicates the fast and wide dissemination of class 1 integrons and their gene cassettes to different parts of the world.

In P. aeruginosa, the class 1 integrin variable regions of all nine isolates were amplified successfully. The common gene cassettes detected were aadA6-orfD alone or in combination with aacC3-cmlA5. In particularly, the cassette aadA6-orfD was found in 89% of the isolates. This array seems to be widespread in other countries (Garza-Ramos et al., 2010; Shahcheraghi et al., 2010; Borgiai et al., 2011). Unlike aadA6-orfD and aacC3-cmlA5, the cassette aacA4-catB8-aadA1 detected in one of the isolates is usually reported in Acinetobacter baumannii (Turton et al., 2005), although this gene has been observed previously in P. aeruginosa (Jeong et al., 2009). All the gene cassettes identified in P. aeruginosa in the present study are deemed to be the first reported incidence in Malaysia, as not much research has been done in characterizing integrons from P. aeruginosa.

Despite the different combinations of gene cassettes obtained in this study, some gene cassettes have been observed to be frequently conserved over time. For example, in E. coli, gene cassettes such as aadA1, dfrA17-aadA5, dfrA1-aadA1 and dfrA12-orfF-aadA2 were first observed in the 1990s (Yu et al., 2003). These gene cassettes have also been reported in urinary E. coli isolates from Korea, Taiwan, Turkey, Finland and Australia (Heikkilä et al., 1993; Chang et al., 2000; White et al., 2000; Yu et al., 2003).

In this study, dissemination of integrons carrying identical gene cassettes was observed in K. pneumoniae and P. aeruginosa but not in E. coli. Eight K. pneumoniae isolates, however, generated a distinct cluster based on 80% similarity comprising three isolates carrying the dfrA7 gene cassette. This cluster of three strains with a similar XbaI
profile came from different types of specimens from one particular individual (bone, swab and tissue), suggesting the probability of the spread of an integron clone within the same host (patient). In *P. aeruginosa*, it is important to note that all five of the aadA6-orfD- and aacC3-cmlA5-carrying isolates were 80% similar. Interestingly, three of the isolates were isolated from different sites (tracheal aspirate, tissue and endotracheal tube secretion) of the same patient. The other two isolates carrying aadA6-orfD were both collected from tracheal aspirate isolates from two different patients from the same ward of the hospital but on different dates, suggesting the nosocomial spread of this clone in the hospital. This observation is supported by the studies of Nijssen *et al.* (2005) and Daikos *et al.* (2007), where cross-transmission of integron-carrying clones disseminating bacteria from patient to patient was observed more often in a nosocomial environment.

In conclusion, this study revealed the predominance of class 1 integrons and new integron-associated gene cassettes not reported previously in *P. aeruginosa* and *Enterobacteriaceae* such as *E. coli* and *K. pneumoniae* isolates from clinical settings in Malaysia. Both bacterial
classes are highly prevalent in hospital-acquired infections in Malaysia. This is believed to be the first description of the aadA2-lnif, aacC3-cmlA5 and aacA4-catB8-aadA1 gene cassettes in the Malaysian population.

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