Distribution and Content of Class 1 Integrons in Different Vibrio cholerae O-Serotype Strains Isolated in Thailand

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In this study, 176 clinical and environmental Vibrio cholerae strains of different O serotypes isolated in Thailand from 1982 to 1995 were selected and studied for the presence of class 1 integrons, a new group of genetic elements which carry antibiotic resistance genes. Using PCR and DNA sequencing, we found that 44 isolates contained class 1 integrons harboring the aadB, aadA2, blaP1, dfrA1, and dfrA15 gene cassettes, which encode resistance to gentamicin, kanamycin, and tobramycin; streptomycin and spectinomycin; β-lactams; and trimethoprim, respectively. Each cassette array contained only a single antibiotic resistance gene. Although resistance genes in class 1 integrons were found in strains from the same epidemic, as well as in unrelated non-O1, non-O139 strains isolated from children with diarrhea, they were found to encode only some of the antibiotic resistance expressed by the strains. Serotype O139 strains did not contain class 1 integrons. However, the appearance and disappearance of the O139 serotype in the coastal city Samutsakorn in 1992 and 1993 were associated with the emergence of a distinct V. cholerae O1 strain which contained the aadA2 resistance gene cassette. A 150-kb self-transmissible plasmid found in three O1 strains isolated in 1982 contained the aadB gene cassette. Surprisingly, several strains harbored two integrons containing different cassettes. Thus, class 1 integrons containing various resistance gene cassettes are distributed among different V. cholerae O serotypes of mainly clinical origin in Thailand.

In the past, the prevalence of antibiotic resistance of Vibrio cholerae was low and routine susceptibility testing was not recommended (29, 35). However, reports of V. cholerae strains resistant to commonly used antibiotics are appearing with increasing frequency and susceptibility testing is now recommended for monitoring of resistance among toxigenic V. cholerae O1 and O139, the two serotypes which cause cholera (4, 20, 23, 29, 54).

The importance of non-O1, non-O139 serotypes of V. cholerae as causes of diarrhea are increasingly being recognized (13, 14, 18, 33, 41). Although high prevalences of multiple-antibiotic-resistant non-O1, non-O139 strains have been reported recently, little is known about the mechanisms of resistance (14, 33, 41). Studies of non-O1, non-O139 strains from Thailand showed that strains frequently contained small-size plasmids. However, the plasmids did not appear to encode antibiotic resistance (14, 18).

Antibiotic resistance genes may be acquired and transmitted through several mechanisms, of which the acquisition of genes through mobile genetic elements and dissemination through horizontal transfer are of special interest. In addition to plasmids and conjugative transposons, integrons have also been described as vehicles for the acquisition of resistance genes (24, 44; for reviews, refer to references 25, 38, and 46). Among the three classes of integrons that have been identified, class 1 integrons are prevalent among clinical isolates. Class 1 integrons were originally defined as being composed of two conserved segments, the 5′ conserved segment (5′-CS) and the 3′ conserved segment (3′-CS), and an internal variable region which contains gene cassettes encoding antibiotic resistance determinants (24, 44). The 5′-CS contains the intI1 gene, which encodes the type 1 integrase. This integrase is responsible for site-specific insertion and excision of gene cassettes (11). Also, the 5′-CS contains the attI1 site, which is responsible for recombination. The 3′-CS contains the quaEΔ1 and sul1 genes, which encode resistance to quaternary ammonium compounds and to sulfonamides, respectively. However, class 1 integrons do not always contain the entire 3′-CS (38, 46).

Some information is available about the distribution and importance of class 1 integrons in encoding antibiotic resistance in bacterial enteropathogens, and recent studies indicate that class 1 integrons may be widespread in multiple-drug-resistant clinical isolates (26, 27, 30), e.g., Salmonella enterica Typhimurium (40, 52). Dalsgaard et al. (15) characterized V. cholerae O1 isolated in Vietnam from 1979 to 1996 and found that strains isolated after 1990 were resistant to sulfonamides and streptomycin and harbored class 1 integrons containing an aadA2 gene cassette. A comparison of phenotypic and genotypic characteristics of the Vietnamese O1 strains isolated after 1990 with a distinct O1 strain recently found in Samutsakorn, Thailand, suggests that they belong to the same clone (15, 17).

However, it is unknown if the Thai V. cholerae O1 strain also contains class 1 integrons (17). Recently, Falbo et al. (21) found chromosomally located class 1 integrons containing the aadA1 gene cassette among V. cholerae O1 strains isolated during a cholera outbreak in Albania and Italy in 1994.

In this background, the objective of the present study was to determine the distribution and importance of class 1 integrons in encoding antibiotic resistance among a large collection of different V. cholerae O serotypes isolated from clinical and environmental sources in Thailand. As the vast majority of strains containing class 1 integrons are resistant to sulfonamides, we selected strains with different patterns of suscepti-
specific class 1 primer set, qacE\(\Delta\)I and sulI, directed at the 3'-CS of class 1 integrons (37, 38). However, as a few class 1 integrons do not contain the qacE\(\Delta\)I and sulI genes, these would not be detected using the selected primers. PCR was carried out by suspension of one bacterial colony in 100 \(\mu\)l of sterile distilled H\(_2\)O, which was then boiled for 15 min at 95°C. After lysis, the suspension was centrifuged at 13,000 rpm (Biofuge 13; Heraeus Sepatech) for 2 min and stored at −20°C. For each PCR, 5 \(\mu\)l of the lysis suspension was used. The PCR amplification was performed by the method of Aarestrup et al. (1) and optimized to detect amplicons in the range of 300 to 1,500 bp. The annealing temperature was set at 58°C. Strains yielding a PCR product with the class 1 primers were further amplified with the integron primers in-F and in-B (F is forward, and B is backward), which amplify the region between the 5'-CS and the 3'-CS, yielding products of various sizes, depending on the number and length of the inserted gene cassettes (Fig. 1 and Table 1). The in-B primer anneals at the 3'-CS. Primers in-F and addA-B were used to determine if the integron contained a gene cassette encoding resistance to streptomycin and spectinomycin (Table 1) (31). Primers intI1-F and blap1-B were used to determine if the integron contained the β-lactam resistance gene cassette using an annealing temperature of 52°C (55). All of the PCR primers used are listed in Table 1. S. enterica serotype Typhimurium strain 9720921 and Acinetobacter sp. strain R4-96 were included as positive and negative controls, respectively (40). A 100-bp molecular mass standard (GIBCO BRL, Gaithersburg, Md.) was used as a size marker during electrophoresis of PCR products.

Amplified DNA was purified before sequencing using Microspin S-400HR columns purchased from Pharmacia Biotech, and the nucleotide sequence was determined by cycle sequencing using the AmpliTaqFS dye terminator kit and a 373A automatic sequencer (Applied Biosystems/Perkin-Elmer, Foster City, Calif.) (6) or by the Pharmacia Biotech ALF automated DNA sequencing apparatus. To analyze the identity of determined sequences, a comparison was made with gene banks using the BLAST software (3). The DNAsis software (Hitachi Software Engineering Co., Ltd.) was used to identify restriction enzymes for restriction fragment length polymorphism (RFLP) analysis of amplicons showing similar sizes.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the ada\(\Delta\)2, dflA1 and orf, dfrA15, adaB, and blap1 gene cassettes have been assigned GenBank accession nos. AF221903, AF221901, AF221900, AF221902, and AF221899, respectively.

**RESULTS**

An approximately 800-bp 3'-CS PCR product was obtained from 44 of the 176 V. cholerae strains studied using the primers qacE\(\Delta\)I-F and sulI-B. However, the majority of epidemiologically unrelated clinical strains which showed resistance to sulfonamides did not, based on lack of PCR amplification, contain the sulI gene. Only a single environmental strain amplified this region. The phenotypic characteristics and resistance gene cassettes found in strains containing class 1 integrons are further described below (see also Tables 2 and 3).

**V. cholerae non-O1, non-O139 strains isolated at the Children Hospital, Bangkok.** Sixty-nine V. cholerae non-O1, non-O139 strains recovered from children as part of a cholera surveillance conducted from August 1993 to July 1995 at the Children Hospital in Bangkok were studied (14). These strains represented 37 different O serotypes, of which none contained the cholera toxin gene. The strains were originally tested for

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**TABLE 1.** PCR primers used for identification and characterization of integrons and gene cassettes in V. cholerae O serotypes isolated in Thailand

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Accession no.</th>
<th>Position of primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>in-F(^6) (5'-CS)</td>
<td>GCC ATC CAA GCA GCA AGC</td>
<td>U12338</td>
<td>1416→1433</td>
<td>12</td>
</tr>
<tr>
<td>in-B(^6) (3'-CS)</td>
<td>AAG CAG ACT TGA CTC CAT</td>
<td>U12338</td>
<td>4831→4814</td>
<td>12</td>
</tr>
<tr>
<td>attI1-F</td>
<td>CGG GCA TTC AAG CAG CAC GGC C</td>
<td>U12338</td>
<td>1414→1435</td>
<td>12</td>
</tr>
<tr>
<td>qacE(\Delta)I-F</td>
<td>ATC GCA ATA GTT GGC GAA GT</td>
<td>X15370</td>
<td>211→230</td>
<td>44</td>
</tr>
<tr>
<td>sulI-B</td>
<td>GCA AGG CCG AAA CCC GGC CC</td>
<td>X12869</td>
<td>1360→1341</td>
<td>47</td>
</tr>
<tr>
<td>blap1-F(^a)</td>
<td>CGC TTC CCG TTA ACA AGT AC</td>
<td>Z18955</td>
<td>348→367</td>
<td>55</td>
</tr>
<tr>
<td>blap1-B(^b)</td>
<td>CTG GTT CAT TCC AGA TAG CG</td>
<td>Z18955</td>
<td>767→748</td>
<td>55</td>
</tr>
<tr>
<td>adaA-B</td>
<td>ATT GCC CAG TCG GCA GGC</td>
<td>D43625</td>
<td>1933→1916</td>
<td>31</td>
</tr>
</tbody>
</table>

\(^a\) Accession numbers are from the published sequences in the GenBank database.

\(^b\) F, forward nucleotide sequence (5'→3'); B, backward nucleotide sequence (3'→5').

\(^c\) ada\(\Delta\)2 is identical to the pse1 gene (55).
susceptibility to 12 antibiotics by disk diffusion including ampicillin, chloramphenicol, ciprofloxacin, colistin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfonamides, TMP, trimethoprim-sulfamethoxazole; TET, tetracycline; CHL, intermediate chloramphenicol resistance. DNA sequencing showed that the 739-bp amplicon contained the dfrA15 gene cassette, as indicated multiply resistant (14). Of 40 strains that were resistant to colistin, streptomycin, sulfisoxazole, 28 showed further resistance and were designated multiply resistant (14).

Nine of the V. cholerae non-O1, non-O139 strains, all resistant to sulfonamides, contained class 1 integrons (Table 2). A PCR product of approximately 739 bp was obtained from each of five strains using the in-F and in-B primers (Fig. 2 and Table 2). DNA sequencing showed that the 739-bp amplicon of strain VO-556 contained a trimethoprim gene cassette, as well as 5′-CS and 3′-CS sequences (GenBank accession no. AF221890). The gene cassette showed 100% identity to the dfrA15 trimethoprim resistance gene cassette and part of the conserved segments (GenBank accession no. AF221900) (2). DNA sequencing of the 739 bp showed 100% identity to the dfrA15 trimethoprim resistance gene cassette and an open reading frame (GenBank accession no. AF221901) (48).

Strain VO-258 contained one integron, and PCR with the in-F and in-B primers (Fig. 2 and Table 2). DNA sequencing showed that the 739-bp amplicon was obtained. As shown below, the integron containing the blaP1 gene yielded an 1,197-bp amplicon when the in-F and in-B primers were used (Table 2). We cannot explain why only one amplicon was obtained.

Strain VO-258 contained one integron, and PCR with the strain(s) was performed (lanes a to i), in-F and blaP1-B (lanes l and m), and qacE

**TABLE 2. Phenotypic characteristics and resistance gene cassettes found by PCR in V. cholerae strains containing class 1 integrons**

<table>
<thead>
<tr>
<th>V. cholerae strain(s)</th>
<th>Date of isolation (day/mo/yr or mo/yr)</th>
<th>O serotype*</th>
<th>Antibiotic$^b$</th>
<th>Size(s) (bp) of PCR product(s) obtained with in-F and in-B</th>
<th>Aminoglycoside resistance gene $aadA^{2c}$</th>
<th>β-Lactam resistance gene $blaP1^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children Hospital strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO-258</td>
<td>27/08/93</td>
<td>O8</td>
<td>COL; STR; SUL; TET</td>
<td>1,009 $^{a}$</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>VO-556</td>
<td>17/09/93</td>
<td>O151</td>
<td>AMP; STR; SUL; TMP</td>
<td>739 $^{e}$</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>VO-2339</td>
<td>30/01/94</td>
<td>O62</td>
<td>AMP; SUL; TMP</td>
<td>739 $^{e}$</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>VO-3198</td>
<td>28/03/94</td>
<td>O78</td>
<td>AMP; CHL; STR; SUL; TMP; TET</td>
<td>1,009, 1,197 $^{f}$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VO-3417</td>
<td>22/04/94</td>
<td>O183</td>
<td>AMP; STR; SUL; TMP; TET</td>
<td>739 $^{e}$</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>VO-3892</td>
<td>12/12/94</td>
<td>O110</td>
<td>AMP; COL; KAN; NAL; NEO; STR; SUL; TMP</td>
<td>1,009, 1,197 $^{f}$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VO-5398</td>
<td>25/01/95</td>
<td>O13</td>
<td>AMP; COL; NAL; STR; SUL; TMP</td>
<td>1,009, 1,197 $^{f}$</td>
<td>+</td>
<td>+</td>
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<tr>
<td>VO-6059A</td>
<td>22/03/95</td>
<td>O152</td>
<td>AMP; STR; SUL; TMP</td>
<td>739 $^{e}$</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Strains from patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 strains</td>
<td>05/09–06/90</td>
<td></td>
<td>COL; CHL; NEO; STR; SUL; TMP; TET</td>
<td>1,009, 1,237 $^{f}$</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>292/90</td>
<td>05/09–06/90</td>
<td></td>
<td>COL; NEO; STR; SUL; TMP; TET</td>
<td>1,237 $^{f}$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Samutsakorn strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1075/25</td>
<td>15/09/82</td>
<td>O1</td>
<td>AMP; COL; CHL; NEO; GEN; KAN; STR; SUL; TMP; TET</td>
<td>744 $^{f}$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1076/25</td>
<td>15/09/82</td>
<td>O1</td>
<td>AMP; COL; CHL; NEO; GEN; KAN; STR; SUL; TMP; TET</td>
<td>744 $^{f}$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>64/26</td>
<td>11/10/82</td>
<td>O1</td>
<td>AMP; COL; NEO; GEN; KAN; STR; SUL; TMP; TET</td>
<td>744 $^{f}$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20 strains</td>
<td>13/11/92–09/05/96</td>
<td>O1</td>
<td>AMP; COL; STR; SUL</td>
<td>1,009 $^{f}$</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Seafood strain SKF-09</td>
<td>02/02/94</td>
<td></td>
<td>AMP; COL; CHL; STR; SUL; TMP</td>
<td>1,009, 1,197 $^{f}$</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* O serotype according to the scheme of Shimada et al. (43).

$^b$ AMP, ampicillin; CHL, chloramphenicol; COL, colistin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfonamides; TMP, trimethoprim-sulfamethoxazole; TET, tetracycline; CHL, intermediate chloramphenicol resistance.

$^c$ PCR product of 752 bp obtained using primers in-F and aadA-B (GenBank accession no. AF221903).

$^d$ PCR product of 762 bp obtained using primers in-F and blaP1-B (GenBank accession no. AF221899).

$^e$ DNA sequencing showed the 739 bp showed 100% identity to the dfrA15 trimethoprim resistance gene cassette (GenBank accession no. AF221890) (2).

$^{f}$ DNA sequencing of the 1,237 bp showed 100% identity to the dfrA15 trimethoprim resistance gene cassette and an open reading frame (GenBank accession no. AF221901) (48).

$^{g}$ Non-O1, non-O139 strains isolated at Children Hospital in Bangkok, Thailand (14).

$^{h}$ Non-O1, non-O139 strains isolated from patients in Thailand (18).

$^{i}$ O1 strains isolated from patients in Samutsakorn, Thailand (17).

$^{j}$ Non-O1, non-O139 strain isolated from seafood in Samutsakorn, Thailand (18).

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FIG. 2. Examples of PCR products of V. cholerae O1 strains isolated in Thailand by using the primers in-F and in-B (lanes b to i), in-F and aadA-B (lanes j and k), in-F and blaP1-B (lanes l and m), and qacE. The 739-bp amplicon of strain VO-556 (lanes n and o). Lanes: a, 100-bp molecular mass standard; b, strain VO-3417; c, strain 1075/25; d, strain 292/90; e, strain VO-258; f, strain 30/90; g, strain 1076/25; h, S. enterica serotype Typhimurium strain 9720921 (positive control); i, Acinetobacter sp. strain R4-96 (negative control); j, strain 1076/25; k, strain 9720921 (positive control); l, strain SKF-09; m, strain 9720921 (positive control); n, strain VO-557; o, strain 9720921 (positive control); p, PCR mixture, (negative control); q, 100-bp molecular mass standard.
TABLE 3. RFLP analyses of resistance gene cassettes found in class 1 integrons in V. cholerae

<table>
<thead>
<tr>
<th>Gene cassette</th>
<th>Size (bp)</th>
<th>Restriction enzyme used</th>
<th>Restriction fragment sizes (bp)</th>
<th>GenBank no. of identical sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aadA2</td>
<td>1,009</td>
<td>HindIII</td>
<td>216, 275, 517</td>
<td>D43625</td>
<td>31</td>
</tr>
<tr>
<td>dfrA1</td>
<td>1,237</td>
<td>ClaI</td>
<td>436, 801</td>
<td>X17477</td>
<td>48</td>
</tr>
<tr>
<td>dfrA15</td>
<td>739</td>
<td>HindIII, SphI</td>
<td>315, 424</td>
<td>Z85311, Z85310</td>
<td>2</td>
</tr>
<tr>
<td>aadB</td>
<td>744</td>
<td>SphI</td>
<td>175, 569</td>
<td>L06418</td>
<td>10</td>
</tr>
<tr>
<td>blap1</td>
<td>1,197</td>
<td>MfeI</td>
<td>258, 939</td>
<td>AF071555</td>
<td>9</td>
</tr>
</tbody>
</table>

a The amplicon contained both the dfrA1 gene cassette and an open reading frame. See also Fig. 1.

b The sequence with this GenBank no. included some of the aadA2 gene sequence located downstream of the dfrA15 gene cassette.

in-F and aadA-B primers confirmed the presence of an aadA2 aminoglycoside resistance gene cassette (GenBank accession no. D43625) which confers resistance to streptomycin and spectinomycin (Fig. 2; Tables 1 and 2) (8, 31).

PCR with the in-F and in-B primers yielded two amplicons of 1,009 and 1,197 bp in strains VO-3198, VO-4967, and VO-5398. PCR with the in-F and aadA-B primers and DNA sequencing of the 1,009-bp amplicon from strain VO-3198 confirmed the presence of the aadA2 aminoglycoside resistance gene cassette (GenBank accession no. AF221903) (8, 31) (Fig. 2; Tables 1 and 2). RFLP analysis of the 1,009-bp amplicon using HindIII, which has two restriction sites in the 1,009-bp product but none in the 1,197-bp amplicon, suggested that strains VO-3198, VO-4967, VO-5398, and VO-258 also contained the aadA2 gene cassette (Table 3). PCR with the in-F and blap1-B primers yielded a 762-bp product corresponding to the conserved regions of the integron, together with the blaP1 gene cassette, that encode the β-lactamase PSE-1 (Fig. 2; Tables 1 and 2) (55). DNA sequencing of the 1,009-bp product from strain VO-3198 confirmed these findings, showing 100% identity to the blaP1 gene and the 3′CS and 3′-CS sequences (GenBank accession no. Z10955 and AF071555) (9, 55). Digestion with MfeI, which had a single restriction site in the 1,197-bp fragment, but none in the 1,009-bp amplicon, demonstrated identical fragments for all three of the strains, suggesting that these strains contained the same 764-bp β-lactamase-encoding blap1 cassette (Table 3) (40).

V. cholerae non-O1, non-O139 strains recovered from patients in Thailand. Nineteen V. cholerae non-O1, non-O139 strains were selected from 41 strains recovered from stool samples from patients with diarrhea (18), including 13 strains isolated in 1990 during an epidemic of a cholera-like disease among Khmer refugees in a camp in Aranyaprathet, Thailand (5).

Ten sulfonamide-resistant non-O1, non-O139 strains isolated from the Khmer refugees contained class 1 integrons each yielding PCR amplicons of 1,009 and 1,237 bp using the in-F and in-B primers, whereas strain 292/90 yielded a single product of 1,237 bp (Fig. 2 and Table 2). PCR with the in-F and aadA-B primers confirmed the presence of an aadA2 gene cassette (Fig. 2; Tables 1 and 2). DNA sequencing showed that the 1,237-bp amplicon of strain 292/90 showed 100% identity to the dfrA1 (dfrP1) gene encoding resistance to trimethoprim (GenBank accession no. X00926) (22) and an open reading frame downstream of the dfrA1 gene (GenBank accession no. AF221901). This open reading frame was previously noted by Sundström and Sköld (48). Digestion with ClaI, which had a single restriction site on the 1,237-bp amplicon, suggested that each strain contained the dfrA1 gene cassette (Table 3). The remaining strains were intermediately resistant or sensitive to sulfonamides (18).

V. cholerae O1 strains recovered from patients in Samutsakorn, Thailand. Thirty-one clinical V. cholerae O1 strains were selected from 70 strains isolated from 1982 to 1996 in Samutsakorn, a port city 30 km southwest of Bangkok (17). We showed in a previous study that the disappearance of V. cholerae O139 from Samutsakorn was associated with distinct phenotypic and genotypic changes in O1 strains isolated during and after the O139 epidemic, including the development of resistance to streptomycin and sulfisoxazole (17).

Twenty V. cholerae O1 strains isolated during and after the O139 epidemic, but also within a 10-months period just before the appearance of the O139 serotype, showed resistance to sulfonamides and streptomycin and contained class 1 integrons (Fig. 2; Table 2). PCR and RFLP analysis as described above confirmed the presence of the aadA2 aminoglycoside resistance gene cassette (Table 2). One strain isolated in October 1991 showed resistance to sulfonamides and streptomycin but did not contain class 1 integrons.

Strains 1075/25, 1076/25, and 64/26 were isolated by Tabtieng et al. (49) during a cholera outbreak among children in 1982 and found to carry a conjugative 150-kb plasmid conferring multiple-drug resistance. Each strain contained class 1 integrons, and the use of the in-F and in-B primers yielded a PCR product of 744 bp (GenBank accession no. AF221902) (Table 2). DNA sequencing and RFLP analysis with SphI revealed that the 744-bp amplicon of each of the strains contained the aadA2 gene cassette, as well as 5′-CS and 3′-CS sequences. This gene cassette showed 100% identity to the aadA2 gene, which encodes resistance to gentamicin, kanamycin, and tobramycin (GenBank accession no. L06418) (Table 3) (10). Conjugation of the strains with E. coli K-12 strain J53-1 yielded one type of exconjugant which showed identical antibiograms and contained the 150-kb plasmid, suggesting that the entire antibiotic resistance pattern was plasmid encoded (Table 2). Further, PCR of the exconjugants yielded the 744-bp amplicon, showing that the aadA2 gene cassette was located and transferred on the 150-kb plasmid.

Clinical V. cholerae O139 strains and environmental non-O1, non-O139 strains isolated from shrimp aquaculture and seafood. Class 1 integrons were not found among 11 representative V. cholerae O139 strains tested which showed resistance to furazolidone, streptomycin, trimethoprim, and sulfonamides and included strains isolated from patients in Bangladesh, India, and Thailand from 1992 to 1993 (19). Thus, the antibiotic resistances shown by the O139 strains were not associated with this type of integron.

Of 34 V. cholerae non-O1, non-O139 strains selected among 93 strains isolated from water, sediment, and shrimp in a shrimp production area in Thailand (16), none contained class 1 integrons. The strains tested included 23, 6, and 5 strains showing resistance (inhibition zone diameters of <19 mm), intermediate resistance (inhibition zone diameters between 20 and 22 mm), and susceptibility (inhibition zone diameters of ≥23 mm) to sulfonamides, respectively, when tested in disk diffusion assays by the Bauer-Kirby method (7, 18).

Twelve V. cholerae non-O1, non-O139 strains were selected among 23 strains isolated from seafood samples in Samutsakorn, Thailand (18). Ten and two strains were intermediate and fully resistant to sulfonamides, respectively (18). One sulfonamide-resistant strain contained two class 1 integrons containing amplicons of 1,009 or 1,197 bp (Table 2). PCR and RFLP analysis suggested that the amplicons found in the seafood isolate were identical to amplicons found in strains VO-3198, VO-4967, and VO-5398 from the Children Hospital, Bangkok.
bles 2 and 3). Thus, each of these strains seems to contain the aadA2 and blapI gene cassettes (Fig. 2; Tables 2 and 3).

**DISCUSSION**

In the present study, we showed that class 1 integrons containing antibiotic resistance gene cassettes were found mainly in epidemiologically related *V. cholerae* O serotypes isolated from outbreaks of diarrhea in Thailand. However, several epidemiologically unrelated *V. cholerae* non-O1, non-O139 strains isolated mainly from children with diarrhea also contained class 1 integrons. Obtained by PCR and DNA sequencing, the findings of aadB, aadA2, blapI, dfrA1, and dfrA15 resistance gene cassettes encoding resistances to gentamicin, kanamycin, and tobramycin; streptomycin and spectinomycin; β-lactams; and trimethoprim, respectively, represent the first report of the distribution of class 1 integrons among different *V. cholerae* O serotypes. The gene cassettes reported here corroborate previous findings of the content of class 1 integrons in *Enterobacteriaceae* and *Pseudomonas* spp., in which antibiotic resistance gene cassettes often encode resistances to aminoglycosides (32, 39, 42), β-lactams (40, 45), and trimethoprim (2, 48).

It should be noted that the majority of resistance genes, including determinants of resistance to ampicillin, chloramphenicol, neomycin, streptomycin, trimethoprim, and tetracycline, were not contained within class 1 integrons. Thus, although widely distributed, resistance genes on class 1 integrons seem to encode only a part of the antibiotic resistance reported in *V. cholerae*. As our PCR analysis was designed to detect relatively smaller-size amplicons, larger arrays of gene cassettes may have been missed. However, it should be noted that all of the strains yielding an amplicon with the primers qacEΔ1-F and sul1-B also showed a product when the in-F and in-B primers were used. Thus, it seems unlikely that larger arrays of gene cassettes were present.

There was a clear difference in the distribution and frequency of class 1 integrons among clinical and environmental *V. cholerae* non-O1, non-O139 strains, as only a single environmental strain recovered from seafood in Samutsakorn contained a class 1 integron. The non-O1, non-O139 strains from the shrimp production area in Thailand were isolated from ponds and coastal areas (16) and showed less antibiotic resistance than the clinical non-O1, non-O139 strains (unpublished results). It is likely that the higher frequency of class 1 integrons in clinical isolates is because such strains became dominant through the selective pressure caused by the therapeutic use of antibiotics.

Bagchi et al. (5) reported that during the cholera-like epidemic among the Khmers in 1982, children and pregnant women were treated with trimethoprim-sulfamethoxazole. Further, Dalsgaard et al. (18) showed that a high percentage (92%) of the non-O1, non-O139 Khmer outbreak strains showed resistance to trimethoprim-sulfamethoxazole. Thus, it is likely that the strains that acquired the dfrA1 gene cassette became predominant through selective pressure. The finding that non-O1, non-O139 strains showing resistance to several antibiotics also contained resistance gene cassettes located on integrons is of concern. The finding of trimethoprim resistance gene cassettes, including the recently described dfrA15 gene cassette (2), is of special concern, as trimethoprim is often used to treat diarrhea among children and pregnant women.

The appearance of the O139 serotype in Samutsakorn in 1993 and its sudden disappearance in 1994 were associated with the emergence of a sulfonamide-resistant *V. cholerae* O1 strain carrying a class 1 integron which contained the aadA2 resistance gene cassette. We reported earlier that this distinct O1 strain showed unique genotypes, as demonstrated by ribotyping, PFGE typing, and cholera toxin typing (17). Interestingly, Dalsgaard et al. (15) found that O1 strains isolated in Vietnam after 1990 also contained class 1 integrons which harbored the aadA2 gene cassette and showed a ribotype R1 identical to the ribotype seen among O1 strains isolated in Samutsakorn (17). Thus, it is likely that this distinct O1 strain was transferred between Thailand and Vietnam and became established as the main strain causing cholera. It remains to be determined how this strain acquired the class 1 integron and the aadA2 gene cassette and/or if the strain was introduced from a third country.

Although the class 1 integrons were first described by Stokes and Hall in 1989 (44), our findings of the aadA2 resistance gene among the three O1 strains isolated in 1982 from the cholera outbreak at the pediatric ward in Samutsakorn show that class 1 integrons have been present in *V. cholerae* in Thailand for a number of years (49). The demonstration of the class 1 integron’s being located on a 150-kb self-transmissible plasmid is, to our knowledge, the first report of a plasmid containing integrons in *V. cholerae*. Tabtieng et al. (49) reported that the 150-kb plasmid in each of the three strains belonged to incompatibility group C and contained genes coding for type II dihydrofolate reductase. The genes encoding the type II dihydrofolate reductase were not found as a class 1 gene cassette.

Interestingly, class 1 integrons were not found among any *V. cholerae* O139 strains. Thus, the 62-kb self-transmissible transposon-like SXT element described by Waldor et al. (53) among O139 strains, some of which were included in the present study, does not seem to carry class 1 integrons. The *V. cholerae* O1 El Tor strain which re-emerged in India and Bangladesh after the O139 epidemic were resistant to sulfamethoxazole, trimethoprim, and streptomycin (4, 34). Waldor et al. (53) showed that these O1 strains also contained the SXT element. It remains to be determined if the O1 strains from India and Bangladesh contain class 1 integrons.

Our findings show that class 1 integrons containing several different antibiotic resistance gene cassettes were distributed among different clinical *V. cholerae* O1 and non-O1, non-O139 serotypes in Thailand. Thus, PCR mapping of integrons and DNA sequencing of their genetic contents may be a useful epidemiological tool with which to study the evolution of multiresistance plasmids and dissemination of antibiotic resistance genes within *V. cholerae*. Although the resistance genes within the integrons encoded only a minor part of the antibiotic resistance detected, further studies in Thailand and elsewhere are needed to determine the importance of class 1 integrons in the horizontal acquisition and dissemination of antibiotic resistance genes.

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