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Novel Integron Gene Cassette Arrays Identified in a Global Collection of Multi-Drug Resistant Non-Typhoidal *Salmonella enterica*

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Abstract

Investigation of integron carriage in a global collection of multi-drug resistant *Salmonella enterica* identified 3 unique class 1 integron gene cassette arrays not previously reported in this species. The present study used PCR and DNA sequence analysis to characterize the structure of these gene cassette arrays. A ~4.0 kb integron containing the gene cassette array *arr2/cmlA5/bla*$_{OXA10}$/ *aadA*1 was found in isolates belonging to serovars Isangi and Typhimurium from South Africa. A ~6.0 kb integron containing the gene cassettes *aac(6′)IIc/ereA2/*IS*1247/aac/ arr/ereA2* was found in isolates belonging to serovar Heidelberg from the Philippines. In this gene cassette array, the insertion sequence, IS*1247*, and two putative resistance genes, disrupt the erythromycin resistance gene cassette. Finally, a ~6.0 kb integron containing the gene cassette *qacH/dfrA32/ereA1/aadA2/cmlA/ aadA1* was found in serovar Stanley isolates from Taiwan. This integron, which has not been previously reported in any bacterial species, contains a new dihydrofolate reductase gene cassette sequence designated *dfrA32*, with only 90% sequence similarity to previously reported *dfrA* cassettes. The *S. enterica* integrons described in the present study represent novel collections of resistance genes which confer multi-drug resistance and have the potential to be widely disseminated among *S. enterica* as well as other bacterial species.

Introduction

Salmonella enterica is a leading cause of bacterial gastro-enteritis in humans worldwide [33]. While most *S. enterica* infections are self-limiting, serious cases often require treatment with antibiotics. For several decades, an increase in antibiotic resistance has been noted in *S. enterica*, as in other gram-negative bacteria [6]. Class 1 integrons contribute significantly to antibiotic resistance in gram-negative organisms [28]. Integrons are genetic structures capable of capturing and integrating gene cassettes that typically encode antibiotic resistance determinants; they therefore have the ability to confer novel combinations of drug resistance to the bacteria in which they reside. Integrons are frequently associated with plasmids and are

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therefore easily transferable among and between different bacterial species [5]. Due to their mobility and ability to quickly acquire diverse resistance determinants, integrons are uniquely adapted to transfer and disseminate antibiotic resistance.

Commonly reported integrons in *S. enterica* are small, usually containing 1 to 3 antibiotic resistance cassettes [16,18,25]. However, integrons containing larger numbers of cassettes have been reported in other bacteria [9,19]. In a previous study, we found that the majority of integrons in a global collection of *S. enterica* contained 1 to 3 gene cassettes [13]. The present study describes the gene cassette arrays of 3 large, novel integrons identified from the same global collection of multi-drug resistant *S. enterica*.

Materials and Methods

Bacterial Isolates

Salmonella enterica isolates were obtained from laboratories in South Africa, Taiwan, and the Philippines between 2001 and 2002 as part of a large global investigation of multi-drug resistant *S. enterica* [13]. Other than country of origin, no epidemiological information is available for these isolates. Representative isolates from this collection that harbored integron gene cassette arrays ≥4 kb were selected for further characterization (Table 1).

Gene Cassette Identification

DNA preparation and PCR amplification of integron gene cassette arrays were accomplished as previously described [13]. Primers specific for integron 5′ and 3′ conserved segments (CS) [15] were used for initial sequencing followed by additional sequencing with previously described primers for each of the integrons: *arr2/cmlA5/bla*$_{OXA10}$*/aadA1*; arr2_R, cmlA5_F, cmlA_F, cmlA_R, oxa10_R, aadA1_R: *aac(6′) IIc/ereA2/*IS*1247/aac/arr2/ereA2;* aac(6′)IIc-F, ere_est_R, IS_F, TNP_F, arr_accA_R, arr_R2, ere_F2: *qacH/dfrA32/ ereA1/aadA2/cmlA/ aadA1*; qacH-F, dfr17_F, ere2-R, aadA2_R, cmlA_R_internal, cml_R2, aadA1_R_S [13]. Sequencing was performed using the BigDye terminator 3.1 kit (Applied Biosystems) according to the manufacturer’s instructions. Capillary sequence analysis of sequencing products was performed on a 3730 DNA sequence analyzer (Applied Biosystems). Sequence analysis utilized the Lasergene 7.0.0 software package (DNAStar, Madison, WI). BLAST analysis was performed on resulting sequences to determine gene cassette homologies (http://www.ncbi.nlm.nih.gov/BLAST) [1].

To investigate the divergence of the dihydrofolate reductase encoded by the newly identified *dfrA32* gene cassette, predicted amino acid sequences of 21 *dfrA* genes (Table 2) were obtained from GenBank (www.ncbi.nlm.nih.gov) [3] and were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) [17]. This alignment was used to create a rooted neighbor-joining tree by the method of Saitou and Nei [27], also using ClustalW2. The neighbor-joining tree was displayed using PhyloDraw (http://pearl.cs.pusan.ac.kr/phylodraw) [8]. ClustalW2 was also used to create an alignment of the predicted amino acid sequences of *dfrA32* and its closest homolog, *dfrA17* (GenBank Accession no. FJ895301), and to perform an alignment of the *dfrA32* and *dfrA7 attC* sites.

Plasmid Analysis

Plasmids were isolated from *S. enterica* isolates using the Qiagen Midi Kit (Qiagen, Maryland) according to the manufacturer’s directions. Electroporation of plasmids into electrocompetent *Escherichia coli* Top 10 cells (Invitrogen, Carlsbad, CA) was performed using a MicroPulser (Bio-Rad, Hercules, CA). *E. coli* transformants were selected on Luria–Bertani agar supplemented with the appropriate antibiotic (150 μg/ml erythromycin, 50 μg/ml chloramphenicol, or 50 μg/ml rifampin). Antibiotic resistance of *S. enterica* isolates and *E.*

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coli transformants was determined using the disc diffusion method (Becton, Dickinson and Co, Sparks, MD) on Mueller–Hinton agar according to the guidelines of the Clinical Laboratory Standards Institute [20]. Antibiotics tested were: ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), streptomycin (10 μg), ampicillin/sulbactam (10 μg/10 μg), ceftazidime (30 μg), nalidixic acid (30 μg), gentamicin (10 μg), aztreonam (30 μg), trimethoprim-sulfamethoxazole (1.25 μg/ 23.75 μg), rifampin (5 μg), and erythromycin (15 μg). *Escherichia coli* ATCC25922 was included as a control in all antibiotic susceptibility testing. The plasmid preparations from *E. coli* transformants were PCR amplified with 5′ and 3′ CS primers to identify gene cassette arrays.

For isolates which did not generate transformants, plasmid carriage was further investigated by performing restriction endonuclease digestion on the purified plasmid preparations with 20 units of *Hind*III in a 10 μl volume containing 1× NEB2 buffer (New England Biolabs, Ipswich, MA) and 0.1 μg BSA. The digestion mixture was incubated at 37°C for 2 h and subjected to electrophoresis on a 0.7% gel and stained with ethidium bromide to visualize plasmid bands.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of integrons *arr2/cmlA5/* bla_{OXA10}*/aadA1, aac(6′)IIc/ereA2/* IS*1247/aac3/arr/ereA2*, and *qacH/dfrA32/ereA1/aadA2/cmlA/aadA1* have been deposited in the GenBank/EMBL/DDBJ under accession nos. GU067640, GU067641, and GU067642, respectively.

Results

Antibiotic Resistance

All *S. enterica* isolates harboring integron cassette arrays ≥4.0 kb exhibited resistance to at least 7 antimicrobials, including ampicillin, ampicillin/sulbactam, tetracycline, chloramphenicol, streptomycin, and sulfamethoxazole. Isolates of identical serovar displayed similar resistance patterns (Table 1).

Integron Characterization

The Isangi isolates and the Typhimurium isolate from South Africa contained a gene cassette array comprised of the cassettes *arr2/cmlA5/*bla_{OXA10}*/aadA1*. The *arr2* gene cassette, which was originally described in *Pseudomonas aeruginosa*, encodes an ADP-ribosyl transferase and confers resistance to rifampin [29]. The *cmlA5* cassette encodes a chloramphenicol efflux protein and contains its own promoter [19]. The bla_{OXA10} gene cassette encodes resistance to the *β*-lactam class of antibiotics. In this cassette array, the bla_{OXA10} cassette was contiguous with the *aadA*1 cassette due to the lack of a majority of the *attC* site, which usually terminates an integron gene cassette [19]. Truncations of the *attC* site have been reported [23]. The *aadA*1 gene cassette confers resistance to aminoglycoside antibiotics. All of the isolates carrying the *arr2/cmlA5/* bla_{OXA10}*/aadA1* gene cassette array were resistant to rifampin, chloramphenicol, ampicillin, and streptomycin, as expected if all cassettes in this integron were expressed, and were additionally resistant to tetracycline, ceftazidime, gentamicin, aztreonam, and trimethoprim-sulfamethoxazole. Serovar Isangi isolates were also resistant to nalidixic acid.

Plasmids were prepared from the serovar Isangi isolate Sal01259 and the serovar Typhimurium isolate Sal02433 and these plasmids were transferred by electroporation to *E. coli*. The *E. coli* transformants exhibited resistance patterns similar to those of the *S. enterica* isolates (Table 1). PCR amplification of plasmids and of plasmid transformed *E. coli* cell lysates with 5′ and 3′ CS primers generated a product identical in size to that obtained from the original isolates.

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Plasmids isolated from Isangi Sal01261 and Sal01274 gave similar transfer of resistance and PCR amplification of gene cassette arrays using the 5′ and 3′ CS primers (Table 1).

These data suggest that the rifampin, sulfamethoxazole, chloramphenicol, and ampicillin resistances were transferred to *E. coli* on a plasmid bearing the integron gene cassette array *arr2/cmlA5/bla*$_{OXA10}$*/aadA1*. Other plasmid located resistance determinants not part of the integron may be responsible for the transfer of additional resistances. Nalidixic acid resistance was not transferred from serovar Isangi isolates to *E. coli*; this resistance is most likely due to mutation in the chromosomally encoded topoisomerase [26].

The Heidelberg isolates contained the ~6 kb integron gene cassette array *aac(6′)IIc/ereA2/*IS*1247/aac3/arr/ ereA2*. The *aac(6′)IIc* gene cassette encodes a 6′-*N*-aminoglycoside acetyltransferase [10]. The *ereA2* gene cassette, which normally confers resistance to erythromycin [4,22], is interrupted at nucleotide position 188 by IS*1247* [30] and the putative gene cassettes *aac3* and *arr*. The *aac3* and *arr* genes are not technically integron gene cassettes, as they both lack an *attC* site. The *aac3* gene shows 79.8% sequence similarity to the *N*-acetyltransferase, *aac3-Vb* from *Serratia marcescen*s [24,31]. The *arr* gene displays 74% sequence identity to a rifampin ADP-ribosyl transferase from a *Rhodopseudomonas palustris* gene [14,31]. The 3′ end of the *ereA2* cassette, beginning at nucleotide 189, resides downstream of the *arr* gene.

Serovar Heidelberg isolates harboring the *aac(6′)IIc/ereA2/*IS*1247/aac3/arr/ereA2* integron gene cassette array were resistant to rifampin, nalidixic acid, gentamicin, aztreonam, and trimethoprim-sulfamethoxazole as well as ampicillin, ampicillin/sulbactam, tetracycline, chloramphenicol, and streptomycin.

Repeated attempts to purify plasmids from Heidelberg isolates Sal02519, Sal02520, and Sal02521 failed to yield *E. coli* transformants on appropriate selection. Plasmids were not evident in these isolates when assessed by restriction endonuclease digestion with *Hind*III.

The 3 representative serovar Stanley isolates contained a gene cassette array that has not been previously described. This novel ~6 kb array contains the gene cassettes *qacH/dfrA32/ereA1/aadA2/cmlA/aadA1*. The Stanley isolates containing this gene cassette array were resistant to erythromycin, streptomycin, chloramphenicol, and trimethoprim/sulfamethoxazole, consistent with the resistance cassettes carried on the integron. The *qacH* cassette encodes an efflux pump that confers resistance to quaternary ammonium compounds and ethidium bromide [19]. Resistance for these compounds was not tested in this study.

The second gene cassette in this array exhibited only 90% sequence identity to its nearest match, *dfrA17*, a dihydrofolate reductase integron gene cassette, found in *Shigella flexneri* (Genbank accession number FJ895301, unpublished). A number of dihydrofolate reductase gene cassette variants that confer trimethoprim resistance have been described [32]. The *dfrA* gene cassette identified in the Stanley gene cassette array, encodes a predicted 157 amino acid protein variant which differs from *dfrA17* FJ895301 at 11 amino acid residues (Fig. 1). This cassette has been named *dfrA32* following the suggested naming guidelines [21]. Since the majority of amino acid changes are conservative, the trimethoprim resistance observed in all of the Stanley isolates is predicted to be due to expression of the *dfrA32* cassette. A neighbor-joining tree of the predicted amino acid sequences of 21 dihydrofolate reductase genes present in GenBank, along with *dfrA32*, indicates that the DHFR protein product of *dfrA32* is most similar to those of *dfrA17* and *dfrA7* (Fig. 2). The *attC* site of *dfrA32* is most similar to that of *dfrA7* (Fig. 3).

The *ereA1* gene cassette encodes a type 1 erythromycin esterase, which has been reported in *E. coli* [4]. The gene cassette series *aadA2/cmlA/aadA1* has been reported in *E. coli* (GenBank

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Accession no EF113389) and *S. enterica* serovar Typhimurium [2]. Both *aadA2* and *aadA1* confer resistance to aminoglycosides while *cmlA* confers chloramphenicol resistance.

While a plasmid band was detected in the serovar Stanley isolates by restriction endonuclease digestion, plasmid preparations from these isolates did not amplify a 6 kb gene cassette array product nor were repeated attempts at plasmid transformations of *E. coli* successful, suggesting that the integron may not be located on a plasmid.

Discussion

The present study describes several *S. enterica* integron gene cassette arrays not previously identified in that bacterium. These cassette arrays are interesting not only for their size and content but also for their possible origins and their implications for spread of multiple antibiotic resistance.

The integron gene cassette array *arr2/cmlA5/bla*$_{\mathrm{OXA10}}$*/ aadA1* identified in Isangi and Typhimurium *S. enterica* isolates contains a series of gene cassettes identical to those in an integron found on a plasmid in *P. aeruginosa* in China (unpublished, GenBank accession number EU886979) and as a segment of a previously reported larger, complex class 1 integron in *Acinetobacter baumanii* strain AYE from France [9]. This sequence of gene cassettes has not been reported in *S. enterica.* As was previously reported [13], the serovar Isangi and Typhimurium isolates differ in genetic lineage as defined by MLST as well as by serovar. The successful transfer of this integron gene cassette array to *E. coli* by electroporation provides evidence that the array is mobile and therefore may have been horizontally transferred between the 2 different genetic lineages of *S. enterica* or transferred to the Isangi and Typhimurium serovars from another common source.

The *aac(6′)IIc/ereA2/*IS*1247/aac/arr/ereA2* integron contains an insertion sequence interrupting the gene cassette *ereA2,* which normally confers erythromycin resistance. A potential ancestor of this integron may be the integron *aac(6′)IIc/er*eA2, found on a plasmid in the newly emerged *S. enterica* serovar Keurmassar from Senegal in 2000 [11]. The *aac(6′) IIc/ereA2/*IS*1247/aac/arr/ereA2* integron has previously been reported in a *Klebsiella oxytoca* isolate from a single patient in Paris [31] and on an *Enterobacter cloacae* plasmid [7]. Although plasmid localization was not demonstrated for the *K. oxytoca* integron, the existence of this identical and unusual integron in these diverse bacteria isolated in different geographic locations suggests that this unique structure can be mobilized across bacterial species.

To our knowledge, the *qacH/dfrA32/ereA1/aadA2/cmlA/ aadA1* integron found in serovar Stanley isolates has not been reported in any bacterial species. The 3′ end of this *S. enterica* integron resembles a plasmid-borne 3.2 kb *aadA2/cmlA/aadA1* integron reported in *E. coli* [19]. Since integrons are believed to preferentially add cassettes at the first position [12], this *E. coli* integron could be a progenitor of the *S. enterica* serovar Stanley integron described here. The *dfrA32* cassette in this integron is novel in that it demonstrates only 90% sequence similarity to any reported dihydrofolate reductase genes. While the exact origin of the *dfrA32* cassette is unknown, its similarity to *dfrA17* and *dfrA7*, which have been found in *E. coli*, *Shigella*, and *Salmonella* suggest that this unique cassette may have evolved from these potential early progenitors. Since *E. coli*, *S. flexneri*, and *S. enterica* share environmental niches, it is conceivable that *dfrA32* arose through lateral gene transfer and mutation.

The integron gene cassette arrays described in this report have not been previously observed in *Salmonella*. These findings are unique and have important implications for development of multidrug resistance in *S. enterica*. The integron's capacity for creating and disseminating complex collections of resistance elements and the importance of horizontal gene transfer in

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the expansion of antibiotic resistance are reinforced by these data. Integrons and the gene cassettes they contain exist as a common pool which has the capacity to be exchanged by many bacterial species. Multiple drug resistance determinants in one genetic structure exacerbate the problem of antibiotic resistance as selection for any gene cassette within an integron selects for all of the cassettes. The present study further emphasizes the vital role integrons play in the spread of antibiotic resistance throughout the microbial community.

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FJ895301      MKISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMGVLPNRKYA 60
dfrA29        MKISLISAVSENGVIGSGPDIPWSAKGEQLIFKALTYNQWLLVGRKTFDSMGVLPNRKYA 60
                                      *     *

FJ895301      VVSKNGISSSNENVLVFPSIENALKELSKVTDHVYVSGGGQIYNSLIEKADIIHLSTVHV 120
dfrA29        VVSKNGISGSNENVLVFPSIENALQELSKITDHVYISGGGQIYESLIEKADIIHLSTIHV 120
                      *               *    *     *       *             *

FJ895301      EVEGDIKFPIMPENFNLVFEQFFMSNINYTYQIWKKG 157
dfrA29        EVEGDIKFPILPEGFNLVFEQFFVSNINYTYQIWKKG 157
                        *  *         *
```

* amino acid substitution

Fig. 1.

Comparison of predicted amino acid sequence of *dfrA32* gene cassette obtained from *S. enterica* serovar Stanley gene cassette array *qacH/dfrA32/ereA1/aadA2/cmlA/aadA1* to predicted amino acid sequence of closest match, *dfrA1*7 (GenBank accession number AF169041). *Asterisks* indicate amino acid substitutions

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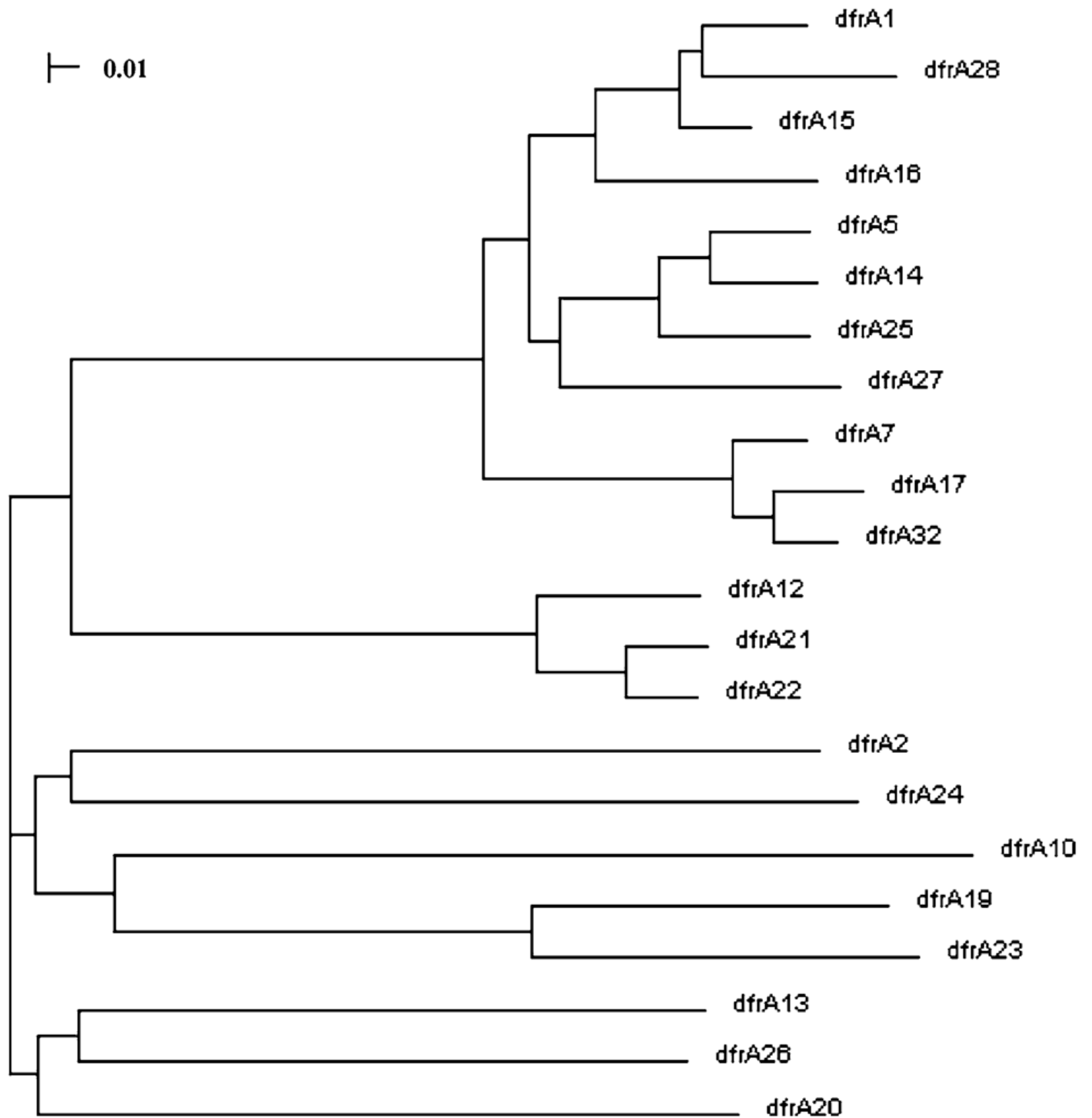

Fig. 2.

Neighbor-joining tree of aligned predicted amino acid sequences of 22 *dfrA* genes, showing relationship of predicted *S. enterica* serovar Stanley gene cassette *dfrA32* product to previously described DfrA proteins

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FJ854362 attC    CAAGTCGTTCCAGCACCAGTCGCTGCGCTCCTTGGACAGTTTTTAAGTCGCGGTTTTATG 60
dfrA32 attC      CAAGTCGTTGCAGCACCAGTCGCTCCGCTCCTTGGACAGTTTTTAAGTTGTGGTTTTATG 60
                          *              *                       * *

FJ854362 attC    GTTTTGCTGCGCAAAAGTATTCCATAAAACCACAACTTAAAAACTGCCGCTGAACTCGGC 120
dfrA32 attC      GTTTTGCTGCGCAAAAATATTCCATAAAACCACAACTTAAAAACTGCCGCTGAACTCGGC 120
                                 *
FJ854362 attC    GTTA 124
dfrA32 attC      GTTA 124
```

*Nucleotide differences

Fig. 3.

Alignment of *attC* sites of *dfrA7* (GenBank accession number FJ854362) and *dfrA32* gene cassette (obtained from *S. enterica* serovar Stanley isolates from Taiwan). *Asterisks* indicate nucleotide substitutions

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Table 1

Origin, novel integron sequences, and resistance patterns of tested *S. enterica* isolates and associated plasmids

Isolate	Serovar	Geographic origin	Integron	Resistance pattern
Strains				
SAL01259	Isangi	South Africa	*arr2/cmlA5/bla*$_{OXA10}$/*aadA1*	AmpChlStrTetSamCazNalGenAtmSxtRif
SAL01261				
SAL01274				
SAL02433	Typhimurium	South Africa	*arr2/cmlA5/bla*$_{OXA10}$/*aadA1*	AmpChlStrTetSamCazGenAtmSxtRif
SAL02519	Heidelberg	Philippines	*aac(6')IIc/ereA2/IS1247/aac/arr2/ereA2*	AmpChlStrTetSamNalGenAtmSxtRif
SAL02520				
SAL02521				
SAL02530	Stanley	Taiwan	*qacH/dfrA32/ereA1/aadA2/cmlA/aadA1*	AmpChlStrTetSamSxtEry
SAL02533				
SAL02536				
Plasmids				
pSAL01259			*arr2/cmlA5/bla*$_{OXA10}$/*aadA1*	AmpChlStrTetSamCazGenAtmSxtRif
pSAL01261			*arr2/cmlA5/bla*$_{OXA10}$/*aadA1*	AmpChlStrTetSamCazGenAtmSxtRif
pSAL01274			*arr2/cmlA5/bla*$_{OXA10}$/*aadA1*	AmpChlStrTetSamCazGenAtmSxtRif
pSAL02433			*arr2/cmlA5/bla*$_{OXA10}$/*aadA1*	AmpChlStrTetSamCazGenAtmSxtRif

Amp ampicillin, *Chl* chloramphenicol, *Str* streptomycin, *Tet* tetracycline, *Sam* ampicillin-sulbactam; *Caz* ceftazidime, *Nal* nalidixic acid, *Gen* gentamicin, *Atm* aztreonam, *Sxt* trimethoprim-sulfamethoxazole, *Rif* rifampin, *Ery* erythromycin

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Table 2

GenBank accession numbers of *dfrA* genes used for alignment with *dfrA32*, obtained from *S. enterica* serovar Stanley integron gene cassette array

Cassette	Accession number	Cassette	Accession number	Cassette	Accession number
dfrA1	AY963803	*dfrA14*	NC_010886	*dfrA22*	FM957884
dfrA2	NZ_ACKQ01000046	*dfrA15*	FJ183470	*dfrA23*	AJ968952
dfrA5	FJ591050	*dfrA16*	AF174129	*dfrA24*	AJ972619
dfrA7	FJ854362	*dfrA17*	AF169041	*dfrA25*	FN252408
dfrA10	AY123253	*dfrA19*	NC_012556	*dfrA26*	AM403715
dfrA12	FJ950723	*dfrA20*	AJ605332	*dfrA27*	FJ976724
dfrA13	NZ_ACKX01000031	*dfrA21*	AJ870926	*dfrA28*	FN263373