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Genetic analysis of antibiotic-resistance determinants in multidrug-resistant *Shigella* strains isolated from Chilean children

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SUMMARY

A total of 162 clinical isolates of *Shigella* collected from children in a semi-rural community of Chile were examined for the presence of genetic determinants of resistance to ampicillin, chloramphenicol, tetracycline, and trimethoprim. Ampicillin resistance was most frequently associated with the presence of bla_{OXA} in *S. flexneri* and with bla_{TEM} in *S. sonnei*. The bla_{OXA} gene but not bla_{TEM} was located in class 1 integrons. The *dhfr*Ia gene encoding for resistance to trimethoprim was associated to class 2 integrons and detected exclusively in *S. flexneri*, whereas *dhfr*IIIc was found in all *S. sonnei* strains and in 10% of the *S. flexneri* isolates. *Cat*, coding for choramphenicol resistance, and bla_{OXA} genes were located in the chromosome in all cases, whereas *tetA* gene, coding for tetracycline resistance, and bla_{TEM}, *dhfr*Ia and *dhfr*IIIc genes were found either in the chromosome or in conjugative plasmids. Our results show a heterogenous distribution of antibiotic-resistance determinants between *S. flexneri* and *S. sonnei*.

INTRODUCTION

Infections due to *Shigella* spp. are a major cause of diarrhoeal disease. Worldwide, more than 100 million diarrhoeal episodes occur per year among children from 0 to 4 years of age in developing countries [1]. The peak mean annual incidence of shigellosis, estimated to be 9·0–12·6 cases per 100 000, occurs among children aged 12–47 months, but the incidence among infants under 12 months and over 48 months is also high [1]. Although living standards are better in Chile than in other South American countries, *Shigella* remains an important enteric pathogen among children and is associated with 22% of cases of bloody diarrhoea and 5·4% of non-dysenteric diarrhoea. Two different species, *S. flexneri* and *S. sonnei*, account for almost all disease and make virtually equal contributions [2].

Antibiotic treatment of shigellosis is recommended because it reduces the duration of illness and the rate of the transmission of the disease by shortening the excretion period of the pathogen [3]. However, antibiotic therapy of *Shigella* spp. in Chile, as in other countries, has been compromised by the emergence of strains resistant to the antibiotics most widely used

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for treatment of the disease [4, 8]. Surveillance of enteric infections due to *Shigella* isolated during the period 1995–1997 from patients in a semi-rural community north of Santiago revealed that 73% of the 226 isolates were resistant to ampicillin, 37% to cotrimoxazol, 44% to chloramphenicol and 48% to tetracycline [9]. Similar levels of resistance to these antimicrobials have been found in studies conducted in two Chilean hospitals [10, 11].

Because of the high levels of antibiotic resistance found in *Shigella* strains, the goal of this study was to characterize the antibiotic-resistance determinants in *Shigella* strains isolated from children in Chile, in order to gain knowledge on how *Shigella* spp. are progressively acquiring multidrug-resistance genes.

MATERIALS AND METHODS

Bacterial strains

A total of 162 clinical isolates, 81 *S. flexneri* and 81 *S. sonnei* strains, were characterized in this work. These strains were isolated in a previous study during the summer seasons of 1995, 1996 and 1997 in Colina, Chile, from stool samples of children under 5 years suffering from diarrhoea [9]. All strains were identified by standard microbiological and biochemical methods and their susceptibilities to antibiotics were determined by the disk diffusion method. Sixty-five per cent (53/81) of the *S. flexneri* isolates were resistant to three or more antibiotics. Among the resistant strains, 55% were Ap^r Cm^r Tet^r $Trim^r$, 17% were Ap^r Cm^r Tet^r, 11% were Ap^r Tet^r $Trim^r$ and 9% were Ap^r $Trim^r$. Out of the *S. sonnei* isolates, 47% were Ap^r $Trim^r$ and 38% were Ap^r Cm^r Tet^r [9]. Serotyping of *S. flexneri* strains was done using commercially available monoclonal antibodies (Reagensia AB, Stockholm, Sweden).

Transfer of resistance determinants and conjugal plasmid analysis

Antibiotic-resistant *Shigella* donor strains were conjugated with the recipient strain *E. coli* DH5α (Nal^r) as described [12]. Briefly, equal volumes of bacteria from exponential phase cultures were mixed and incubated at 37 °C for 3 h. Transconjugants were selected on Luria agar (1·5%) plates containing nalidixic acid (Nal, 15 μg/ml) and one of the following: ampicillin (Ap, 50 μg/ml); chloramphenicol (Cm, 40 μg/ml); tetracycline (Tet, 10 μg/ml) or trimethoprim (Trim, 20 μg/ml). To confirm the presence of plasmids and to estimate their sizes, plasmids from clinical isolates and from the transconjugants were isolated by standard protocols and analysed by electrophoresis in 0·8% agarose gels stained with ethidium bromide [13]. The resistance genes in the transconjugants were identified by PCR amplification.

PCR amplifications

All reactions were carried out in a DNA Thermal Cycler Mastercycler® (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) using boiled bacterial suspensions as a source of template DNA and the primer pairs listed in Table 1. Amplifications consisted of 30 cycles of denaturation for 1 min at 92 °C, annealing for 30 s at different temperatures as indicated in Table 1, and extension for 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. PCR products were analysed by electrophoresis in 1% agarose gels stained with ethidium bromide. Positive control templates for amplification of the various resistance determinants included plasmid pUC19 for the bla_{TEM} gene; plasmid pACYC184 for the *cat* gene; and strain SC2, a derivative of *S. typhi* strain carrying transposon Tn*10* for the *tetA* gene [14]. The *dhfr*Ia and *dhfr*IIIc genes were amplified from the *Shigella* strains used in this study, and the amplicons were sequenced by the Laboratory of Ecology at the Pontificia Universidad Católica de Chile.

Southern hybridization analyses

Chromosomal DNA was isolated from liquid cultures of *Shigella* strains by the cetyltrimethylammonium bromide method [15], digested with *Bgl*I (Gibco-BRL, Gaithersburg, MD, USA) overnight at 37 °C and separated by gel electrophoresis. Gels were depurinated, denatured, and neutralized, and DNA was transferred to a positively charged nylon membrane (Biodyne, Gibco-BRL). Overnight hybridization and subsequent washings were performed under high-stringency conditions at 65 °C, using probes labelled with dCTP-biotin using the random primer BioPrime® DNA labelling system (Gibco-BRL). Hybridization was detected by using the non-radioactive HRP Southern Blotting (KPL, Gaithersburg, MD, USA) detection system and X-OMAT™ Kodak films (Rochester, NY, USA).

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Table 1. *Oligonucleotide primers used in this study*

Gene	Accession no. or reference	PCR product size (bp)	Annealing temp.	Nucleotide sequence (5′ to 3′)
bla_{TEM}	SFU48775	559	52 °C	GTTGGGTGCACGAGTGGGTTACATC
				AAGGGCCGAGCGCAGAAGTGTGC
bla_{OXA}	AJ238349	713	52 °C	ACTGTCGCATCTCCATTATTTGA
				ATCGCATTTTTCTTGGCTTTTAT
tetA	AF162223	751	52 °C	CTGGATTACTTATTGCTGGCTTTTT
				CACCTTGCTGATGACTCTTTGTTTG
*dhfr*Ia	X00926	367	50 °C	GGAGTGCCAAAGGTGAACAGC
				GAGGCGAAGTCTTGGGTAAAAAC
*dhfr*IIIc	U09273	247	43 °C	GAGCTTCCGGGTGTTCGTGAC
				CTTCCATGCCATTCTGCTCGTAGT
*dhfr*VII	AF139109	204	55 °C	CATATAATCAGTGGCTCCTTGTTG
				TGTAGATTTGACCGCCACCAGA
cat	U81140	1003	52 °C	AACGACCCTGCCCTGAAACC
				TTGCGCCGAATAAATACCTG
int1	[16]	280	50 °C	CCTCCCGCACGATGAGGC
				TCCACGCATCGTCAGGC
int2	[16]	233	50 °C	TTATTGCTGGGATTAGGC
				ACGGCTACCCTCTGTTATC

RESULTS

Prevalence of antibiotic-resistance genes in *Shigella* spp.

In this study, 81 *S. flexneri* and 81 *S. sonnei* isolates were investigated. Serotyping of *S. flexneri* strains revealed that serotypes 2a and 2b were the most commonly found (70%). No consistent correlation between serotype and antimicrobial drug-resistance pattern was found. Serotype 2a was most frequently associated to $Ap^r\ Cm^r\ Cot^r\ Tet^r\ Trim^r$ and $Ap^r\ Cm^r\ Tet^r$ phenotypes (11% and 12% respectively), whereas serotype 2b was found predominantly in $Ap^r\ Cm^r\ Cot^r\ Tet^r\ Trim^r$ and $Ap^r\ Cm^r\ Tet^r\ Trim^r$ strains (11% for each phenotype).

The 162 strains were investigated to elucidate the mechanisms of resistance to ampicillin, chloramphenicol, tetracycline and trimethoprim. To identify the resistance determinant(s) present in each isolate, PCR amplifications were performed with primer pairs specific for the bla_{OXA}, bla_{TEM}, *cat*, *tetA*, *dhfr*Ia, *dhfr*IIIc and *dhfr*VII genes (Table 1). In both species, resistance to chloramphenicol was associated with the presence of the *cat* gene whereas resistance to tetracycline was coded by the *tetA* gene. Ampicillin resistance of *S. flexneri* was explained in most cases (68%) by the presence of the bla_{OXA} gene and in 22% of the cases by the bla_{TEM} gene. In contrast, the bla_{TEM} gene was the most prevalent ampicillin determinant in *S. sonnei* (59%). Approximately 10% of isolates of *S. flexneri* and 3% of *S. sonnei* carried both the bla_{OXA} and the bla_{TEM} genes (Fig. 1*a*). Resistance to trimethoprim was associated with the presence of the *dhfr*Ia or *dhfr*IIIc genes. As shown in Figure 1*b*, the *dhfr*Ia gene was found in most *S. flexneri* strains (86%) whereas all *S. sonnei* isolates carried the *dhfr*IIIc gene. Only one *S. flexneri* out of 162 *Shigella* isolates harboured both genes simultaneously. No *dhfr*VII gene determinant was detected in the $Trim^r$ isolates.

We examined for the presence of integrons to determine their contribution to the observed resistance patterns. PCR using specific primers for class 1 and class 2 integrase genes [16] showed that 67% of *S. flexneri* strains contained either one or both integrons. In contrast, only *int1* was detected in *S. sonnei* strains (30% of the isolates, Fig. 1*c*). Class 1 integron was associated with the *oxa* gene, and class 2 integron with the *dhfr*Ia gene. These results indicate that the genes coding for ampicillin and trimethoprim resistance are distributed differently in *S. flexneri* and *S. sonnei*. In addition, the prevalence of class 1 and class 2 integrons is also different in both species.

Transfer of antibiotic-resistance genes and plasmid analysis

Gram-negative human pathogens that progressively acquire multidrug-resistance generally carry conjugative plasmids encoding antibiotic-resistance

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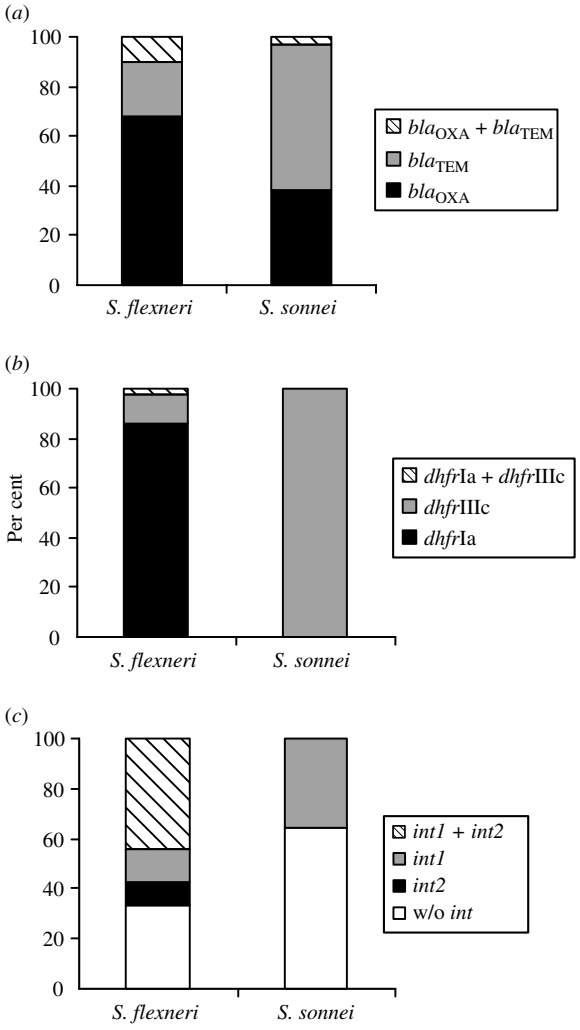

Fig. 1. Distribution of ampicillin and trimethoprim resistance determinants and integrase genes in *Shigella* strains. All the gene determinants were detected by PCR using genomic DNA from *S. flexneri* and *S. sonnei* isolates. (*a*) A total of 60 *S. flexneri* and 71 *S. sonnei* ampicillin-resistant strains were analysed. (*b*) A total of 50 *S. flexneri* and 42 *S. sonnei* trimethoprim-resistant strains were analysed. *dhfr*VII gene marker was not detected in the Trim^r isolates. (*c*) All the 81 *S. flexneri* and 81 *S. sonnei* strains were analysed.

determinants. Therefore, we carried out conjugation experiments to distinguish whether the antibiotic-resistance genes in *Shigella* isolates were present in the bacterial chromosome or in conjugative plasmids. Each *Shigella* strain was mated with the recipient *E. coli* strain DH5α. Transconjugants were selected and tested for their antibiotic-resistance phenotypes by the disk diffusion method. The identities of the antibiotic-resistance genes were confirmed by PCR and Southern blot analyses performed in plasmid preparations from transconjugants (results not shown).

Table 2. *Antibiotic-resistance genes in conjugative plasmids in* Shigella *isolates*

Conjugative plasmid	Resistance profile of transconjugants	Resistance genes in conjugative plasmid
I	Tet	*tetA*
II	Trim	*dhfr*Ia
III	Trim–Tet	*dhfr*Ia–*tetA*
IV	Trim–Tet–Ap	*dhfr*Ia–*tetA*–*bla*$_{TEM}$
V	Trim–Ap	*dhfr*IIIc–*bla*$_{TEM}$

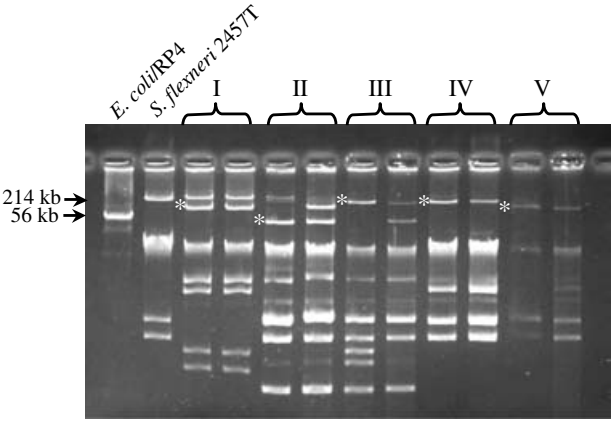

Fig. 2. Plasmid profiles of *Shigella* strains in 0·8 % agarose gel. * Indicates conjugative plasmids I–V obtained from two different strains.

The mating experiments revealed the presence of five different conjugative plasmids (named I–V) of molecular sizes ranging from approximately 50–120 kb (Fig. 2 and Table 2). Three plasmids (II–IV) were transferred only from *S. flexneri* donor strains and all had the *dhfr*Ia gene. Plasmid I containing the *tetA* gene was transferred only from *S. sonnei*. Plasmid V, carrying the *dhfr*IIIc and *bla*$_{TEM}$ genes, was the only conjugative plasmid harboured by both species. The *bla*$_{OXA}$ and *cat* genes were not transferable by conjugation, suggesting that they are chromosomally located. Based on the results from PCR amplifications, conjugation experiments and Southern blot analyses performed in 61 out of 162 strains, we assigned each resistance determinant to the *Shigella* chromosome or to a conjugal plasmid. The results (Table 3) demonstrate the high variability of genetic arrangements for a multidrug-resistance phenotype in *Shigella*. Indeed, we were able to distinguish five different arrangements for the Ap^r Cm^r Tet^r Trim^r phenotype (Table 3). Taken together, our results indicate that it is not

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Table 3. *Genetic localization of resistance genes in* Shigella *isolates*

Resistance phenotype	Chromosomal genes	Genes in conjugal plasmids	*S. flexneri* (*n*)	*S. sonnei* (*n*)
Ap, Cm, Tet, Trim	bla_{TEM}–*cat*–*tetA*	*dhfr*IIIc–bla_{TEM}	1	2
	bla_{OXA}–*cat*	*dhfr*Ia–*tetA*	4	0
	bla_{TEM}–bla_{OXA}–*cat*	*dhfr*Ia–*tetA*	1	0
	bla_{OXA}–*cat*–*tetA*	*dhfr*Ia	5	0
	bla_{OXA}–*dhf*Ia–*cat*–*tetA*	—	2	0
Ap, Cm, Tet	bla_{OXA}–*cat*–*tetA*	—	9	6
	bla_{OXA}–*cat*	*tetA*	0	2
	cat–*tetA*	*dhfr*IIIc–bla_{TEM}	0	1
Ap, Tet, Trim	—	*dhfr*Ia–bla_{TEM}–*tetA*	2	0
Ap, Trim	*dhfr*IIIc–bla_{TEM}	—	0	13
	—	*dhfr*IIIc–bla_{TEM}	1	0
Ap	bla_{TEM}	—	0	1
Sensitive	—	—	6	6

n, Number of isolates.

possible to establish *a priori* the genetic basis for the antibiotic-resistant phenotype of the strains.

DISCUSSION

A surveillance of *Shigella* infections in a semi-rural community of Chile, from 1995 to 1997, revealed a high degree of resistance to the most commonly used antibiotics in the area and a high frequency (49%) of strains with multiple resistances [9]. The most frequent multidrug-resistance phenotypes found were Ap^r Cm^r Tet^r $Trim^r$ and Ap^r Cm^r Tet^r in isolates of *S. flexneri* and Ap^r Cm^r Tet^r and Ap^r $Trim^r$ in isolates of *S. sonnei*. These levels of resistance are representative of the current situation in our community. A surveillance of enteric infections conducted during the period 1998–2001 revealed that 51% of the *Shigella* isolates were resistant to three or more antibiotics. Among these isolates, 82% were resistant to ampicillin, 65% to cotrimoxazol, 53% to tetracycline and 49% to chloramphenicol (V. Prado, unpublished results). The *Shigella* isolates studied during the period 1998–2001, as well as all *Shigella* strains that have been studied in this work are sensitive to both nalidixic acid and fluoroquinolones.

For a better understanding of the molecular determinants of antimicrobial resistance, 162 *Shigella* strains from the surveillance spanning the period 1995–1997 were analysed in depth in this study. Molecular analysis of resistance genes revealed important differences between the two commonest *Shigella* spp. in Chile. Most *S. flexneri* isolates carry the *dhfr*Ia gene associated with the class 2 integron while only 10% contain *dhfr*IIIc. In contrast, in all *S. sonnei* isolates trimethoprim resistance is mediated by *dhfr*IIIc. Other authors have reported the presence of the *dhfr*Ia but not the *dhfr*IIIc gene in *S. flexneri* and *S. sonnei* strains isolated in Tanzania [8]. The *dhfr*VII gene, which has been described in other *Shigella* isolates [8] was not found, suggesting that the acquisition of this $Trim^r$ determinant by *Shigella* spp. is due to a more recent gene transfer event.

Ampicillin resistance is due to the presence of the bla_{TEM} gene in most *S. sonnei* strains whereas the bla_{OXA} gene is present more frequently in *S. flexneri*. Almost 10% of the Ap^r strains carry both the bla_{TEM} and bla_{OXA} genes. Similar results were obtained in studies conducted in Greece [17] and Denmark [18] but no *Shigella* strains expressing both the bla_{TEM} and bla_{OXA} genes were detected in Tanzania [8]. Interestingly, resistance to ampicillin among our *Shigella* isolates remains high even though ampicillin has not been used for the treatment of shigellosis in Chile since the 1980s. The chromosomal location of the bla_{OXA} gene associated with the class 1 integron and the presence of the bla_{TEM} gene in conjugative plasmids could explained the maintenance of the ampicillin-resistance phenotype in Chilean isolates.

We did not detect either class 2 integrons or the *dhfr*Ia gene in the *S. sonnei* isolates. Our results differ from studies conducted in Australia and Korea in which most *S. sonnei* strains carried class 2 integrons [19, 20].

Almost 50% of the *Shigella* isolates in our study are Cm^r. This resistance was explained in every case by the presence of a chromosomal *cat* gene.

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Chloramphenicol has been the antibiotic of choice in the treatment of shigellosis in Chile during the past years, resulting in a strong selective pressure for chloramphenicol resistance.

It is worth noting that there are pronounced genetic differences between *S. flexneri* and *S. sonnei* not only in the antibiotic-resistance genes that they harbour but also in their location in the chromosome or in conjugative plasmids. Five conjugative plasmids displaying different antibiotic-resistance patterns were found among our 162 isolates. Strains carrying these plasmids also contain different resistance genes in the chromosome (Tables 2 and 3). These results suggest that there may be a more restrictive barrier for the horizontal transfer of conjugal plasmids between the two *Shigella* spp. than between other Gram-negative donors and each of the potential *Shigella* recipients.

In conclusion, this work shows that *Shigella* strains present a heterogenous genetic background for antimicrobial-resistance phenotype and strengthen the need to conduct periodic surveillance for antibiotic resistance as well as to determine the antibiotic-resistance genes in *Shigella* strains in order to direct the clinical evaluation of antibiotic treatments for shigellosis.

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