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Antimicrobial susceptibility, integron carriage, genetic relationship and presence of some important virulence genes of the integron-carrier strains of Shigella sonnei (n=230) and Shigella flexneri (n=22) isolated from stool samples of patients in Hungary between 1998 and 2008 were investigated. Sixty-seven per cent (168/252) of the strains were resistant to sulfamethoxazole/trimethoprim (SxT) followed by streptomycin (S, 47 %), ampicillin (A, 32 %) and tetracycline (Tc, 28 %). Thirty-six per cent (90/252) exhibited multidrug resistance, mostly showing SSxTTc or ASSxTc, ASSxTc resistance patterns. An S. sonnei strain of imported origin was resistant to ceftaxime and harboured a blaCTX-M-95-type extended-spectrum β-lactamase gene. Altogether 33 % of the S. sonnei (n=75) and 14 % of the S. flexneri (n=3) strains had either class 1 or class 2 integrons or both. The variable regions encoded aadA1 or dfrA1-aadA1 genes for the class 1 and dfrA1-sat2-aadA1 or dfrA1-sat2 genes for the class 2 integrons. Pulsed-field gel electrophoresis analysis revealed that those strains that have different integron types represented different genetic clusters. The Shiga toxin (stx1) gene was identified in one S. sonnei strain and the cdtB gene was detected in an S. flexneri strain. The results reveal the high incidence of antibiotic resistance among Shigella isolates and the presence of the stx1 gene in S. sonnei and the cdtB gene in S. flexneri. The genetic diversity of Shigella spp. isolated recently in Hungary was also demonstrated.

INTRODUCTION

Shigellosis is one of the frequently diagnosed diarrhoeal diseases both in developing and industrialized countries. Antibiotic treatment is usually indicated for individuals with moderate or severe symptoms of shigellosis, because it can reduce the duration and severity of symptoms, excretion of organisms and prevent potentially lethal complications. However, increasing antimicrobial resistance and carriage of mobile genetic elements that encode antimicrobial resistance genes, such as class 1 and class 2 integrons of Shigella spp., has been reported worldwide (Oh et al., 2003; Peirano et al., 2005; Pan et al., 2006; Kuo et al., 2008). Essential events in the pathogenesis of Shigella infections include bacterial invasion of epithelial cells, escape from the phagosome, intracellular multiplication, extension into adjacent epithelial cells and induction of apoptosis in macrophages (Guichon et al., 2001). Several virulence factors have been associated with Shigella spp., the most common being the ability to colonize and invade the intestinal cells. Another virulence factor, related mainly to Shigella dysenteriae, is its capacity to produce an exotoxin called Shiga toxin (Stx), which is not excreted by the bacteria but is released only during cell lysis (Cantey, 1985). Despite its clear toxigenicity, the role of Stx in

Abbreviations: CDT, Cytotoxinh distending toxin; ESBL, extended-spectrum β-lactamase; MDR, multidrug resistance; PFGE, pulsed-field gel electrophoresis; RifR, rifampicin resistant; Stx, Shiga toxin; TC, transconjugant.
shigellosis is not clear, since it is known that Stx is not essential for invasion or cellular lysis. Cytotoxic distending toxin (CDT) is also a potent cytotoxin that initiates a eukaryotic cell cycle block at the G2 stage prior to mitosis. CDT is produced by a number of bacteria including S. dysenteriae. The presence of CDT in Shigella spp. is uncommon; however, few strains have been tested so far.

At the beginning of the 1990s, there were approximately 1500–2000 shigellosis cases reported in Hungary. Since then, the number of cases has decreased gradually, but 50–150 cases annually have still been reported in recent years (Epi Info of the National Center for Epidemiology; http://www.oek.hu). Because of the lack of data on antimicrobial resistance, genetic diversity and virulence traits of recent Hungarian Shigella isolates, this study was undertaken to investigate the above-mentioned characteristics of selected strains isolated between 1998 and 2008.

METHODS

Bacterial strains and serotyping. Shigella sonnei (n=230) and Shigella flexneri (n=22) strains isolated from stool samples of patients of outbreak and sporadic cases were identified and serotyped by standard biochemical and serological methods and sent to the Phage Typing and Molecular Epidemiology Department of the National Center for Epidemiology for further characterization. The strains were received and identified between 1998 and 2008 in the Institutes of the National Public Health and Medical Officer Service network which covers the whole country. The strains were stored in nutrient agar slants at room temperature and represented patients of both sexes and age groups from 3 to 48 years.

Antimicrobial susceptibility testing and detection of the extended-spectrum β-lactamase (ESBL) gene. Antibiotic susceptibility testing of all isolates was done by the disc diffusion method on Mueller–Hinton agar using antibiotic discs (Oxoid) as follows: ampicillin (10 μg), amoxicillin (10 μg), cefotaxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (30 μg), streptomycin (10 μg), sulnamide (300 μg), trimethoprim (5 μg), trimethoprim-sulphamethoxazole (1.5/23.75 μg) and tetracycline (30 μg). The zones of growth inhibition and the breakpoints were evaluated according to CLSI recommendations (CLSI, 2005). Escherichia coli ATCC 25922 was used as a reference strain. Phenotypic confirmation of ESBL production of the cefotaxime-resistant isolate was performed by double disc approximation test and Etests (Ab Biodisk). Multidrug resistance (MDR) was defined as resistance to three or more classes of antibiotics.

Conjugal transfer of the ESBL gene and its localization by Southern hybridization. For testing the transferability of the blaCTX-M-55 gene of the S. sonnei 2/08 isolate, the plasmid-free E. coli J53 rifampicin resistant (Rit+) strain was used as recipient. Transconjugants (TCs) were selected on Mueller–Hinton agar plates supplemented with 5 mg cefotaxime 1L and 300 mg rifampicin 1L and tested by API 20E (bioMérieux). The transfer of the plasmids and the presence of the transmitted antimicrobial resistance genes were tested by the methods described above. Plasmid DNA was purified from selected TCs, separated by electrophoresis, transferred to Hybond-N+ membrane (Roche Diagnostics) using a Vacuum Blotter (model 785; Bio-Rad Laboratories) and probed with the 32P-labelled blaCTX-M PCR amplicon obtained from the S. sonnei strain 2/08 as described previously (Nőgrády et al., 2006).

PCR assays and sequencing. The ESBL-producing isolate was tested for the presence of the blaTEM, blaCTX-M and blaSHV genes by PCR as described previously (Damjanova et al., 2008). The blaCTX-M amplicon obtained was purified and sequenced by using standard methods. All strains were tested for the presence of class 1 and class 2 integrons by PCR methods according to Lévesque et al. (1995) and White et al. (2001), respectively. The resistance genes carried by the detected integrons were determined by sequencing followed by BLAST analysis at http://www.ncbi.nlm.nih.gov. The PCR primers 5’CS and 3’CS and hep74 and hep51 were used for sequencing both ends of the different class 1 and class 2 amplicons, respectively. In addition, for the 1456, 1586 and 2158 bp amplicons, internal primers were also used to continue the sequencing until the resistance genes inserted in each amplicon were identified.

The presence of stx genes was tested with the Lin up/Lin down stx universal primers (Lin et al., 1993), and stx genes were typed by using stxl specific B54/B55 and stx2 specific B56/B57 PCR primers (China et al., 1996). Detection of cdtB gene was performed with cdtB universal PCR primers (Toth et al., 2003). The type of the cdtB genes was determined by direct sequencing of the amplicons using the primers used for PCR amplification.

Macro-restriction analysis by pulsed-field gel electrophoresis (PFGE). PFGE was carried out according to the standardized Shigella protocol of the CDC PulseNet (Ribot et al., 2006). The Salmonella Braenderup H9812 strain was used as a molecular standard. PFGE-generated DNA profiles were entered into the Fingerprinting II Software (Bio-Rad Laboratories) for analysis. Cluster analysis was performed by the unweighted pair-group method with arithmetic averages (UPGMA); DNA relatedness was calculated on the basis of the Dice coefficient. A 1.5 % position tolerance and 1.0 % optimization setting were applied. As bands smaller than 33.3 kb cannot be detected with certainty, they were not included in the analysis. Strains with 100 % identical macro-restriction patterns were considered to belong to the same pulsotype.

RESULTS

Antibiotic and multidrug resistance

From 1998 to 2008, a total of 3428 dysenteric shigellosis cases were reported in Hungary and our laboratory received 1024 Shigella isolates; 624 were S. sonnei and 400 S. flexneri. This collection can be considered as representative since our laboratory is the national reference laboratory for phage typing Shigella isolates; it receives Shigella isolates with epidemiological significance from public health laboratories and hospitals in every region of Hungary for phage typing.

Patients were not treated with antibiotics before isolation procedures. Our study strains were selected randomly from the collection of strains and therefore the frequency of drug resistance should correspond with the actual antibiotic resistance rate in Hungary.

Antimicrobial resistance of 230 S. sonnei and 22 S. flexneri isolates was tested against 15 antibiotics as described in Methods. The incidence of antimicrobial resistance and the resistance patterns are shown in Fig. 1 and Table 1, respectively. Eighty-three per cent (191/230) of S. sonnei and 77 % (17/22) of S. flexneri strains were antibiotic resistant. Most of the S. sonnei strains were resistant to...
sulfamethoxazole/trimethoprim (67%), followed by streptomycin (45%), ampicillin (27%) and tetracycline (26%). Some strains were resistant to chloramphenicol (3%), kanamycin (3%), nalidixic acid (2%) or gentamicin (1%), either alone or in combination. Seventy-seven per cent (17/22) of *S. flexneri* isolates were resistant to ampicillin, 64% (14/22) to sulfamethoxazole/trimethoprim, streptomycin and chloramphenicol, and 50% (11/22) to tetracycline. None of the strains had ciprofloxacin or enrofloxacin resistance but one *S. sonnei* strain (2/08) of imported origin was resistant to cefotaxime. PCR assays and sequence analysis revealed that strain 2/08 harboured a *bla*<sub>CTX-M-55</sub>-type ESBL gene. Conjugational transfer experiments were conducted by mating this Ctx<sup>r</sup> *S. sonnei* with *E. coli* J53 Rif<sup>r</sup>. Investigation of Ctx<sup>r</sup>Rif<sup>r</sup> TCS revealed that only the Ctx resistance of the donor isolate was transferred. Southern hybridization of the donor strain and selected TCS revealed that the *bla*<sub>CTX-M-55</sub> gene was carried by a conjugative 90 kb plasmid (data not shown).

Thirty-three per cent (76/230) of the *S. sonnei* strains and 64% (14/22) of the *S. flexneri* strains were resistant to three or more antibiotics; details are shown in Table 1.

The number of the strains investigated yearly varied between 7 and 36. Of the 230 *S. sonnei* isolates, 76 proved to be multidrug resistant and the proportion of MDR *S. sonnei* isolates has been showing an increasing tendency. Considering 2- or 3-year rather than 1-year periods, the number of isolates tested was similar and therefore the proportions of MDR *S. sonnei* strains were more comparable (Fig. 2).

### Integron carriage, PFGE patterns and virulence genes

Thirty-three per cent (75/230) of the *S. sonnei* and 14% (3/22) of the *S. flexneri* strains had either class 1 or class 2 integrons or both (Fig. 3 and Table 2). Two types of class 1 integrons were identified, both were characteristic of *S. sonnei* only. An 855 bp class 1 integron encoding *aadA1* was detected in three *S. sonnei* strains. Thirteen *S. sonnei* strains carried a 1586 bp class 1 integron encoding *dfrA1-aadA1* genes. Two types of class 2 integrons were detected. A 2158 bp class 2 integron was the most frequent, found in 58 *S. sonnei* and three *S. flexneri* strains carrying the gene cassette *dfrA1-sat2-aadA1*. Six *S. sonnei* strains had a 1456 bp class 2 integron which carried the *dfrA1-sat2* gene.
cassette. The integrons are referred to by their rounded-off sizes (0.9, 1.5, 1.6 and 2.2 kb) later in the text and in Fig. 3.

PFGE was performed on the 75 integron-positive S. sonnei strains. By conducting PFGE with the integron-harbouring Shigella sonnei strains, we aimed to focus and explore potential relationships between the genotypes of these strains and the different integron types. These experiments revealed that S. sonnei strains harbouring different integrons belong to different clusters as well. PFGE patterns, resistotype and approximate size of the integron(s) of the integron-carrier S. sonnei strains are shown in Fig. 3. PFGE analysis of the S. sonnei strains revealed that the 75 integron-carrier strain represented 65 different pulsotypes. The organisms clustered into two large clusters: A and B with 65.93 % similarity. Interestingly, with one exception (strain 30/98), all strains carrying either the 1.6 or 0.9 kb class 1 integron grouped in cluster A. Strains 58/01 and 35/01 carrying both the 1.6 kb class 1 and the 2.2 kb class 2 integron, grouped in cluster B. With only two exceptions (strains 10/05 and 4/05, which have a 2.2 kb class 2 integron and belonged to cluster A), those strains that had either the 2.2 or 1.5 kb class 2 integron grouped to cluster B. No correlation with either geographical origin or isolation time, or dominance of any pulsotype was detected. Both large clusters contained sporadic and outbreak strains. Within the two large clusters, several smaller groups were differentiated, but no clonal selection of any pulsotype was observed. Interestingly, the S. sonnei 4/00 strain was found to be susceptible to the antibiotics tested but had a class 2 integron of 2.2 kb (Fig. 3).

One MDR S. sonnei strain (75/02) carried the stx1 gene. S. sonnei 75/02 had a class 1 integron of 1.6 kb and was resistant to ampicillin, streptomycin, sulfamethoxazole/trimethoprim and tetracycline (Fig. 2). None of the S. flexneri had the stx gene, but strain 6/05 carried the cdtB gene. Sequencing revealed that the cdtB gene was identical to cdtB III/V genes of E. coli strains available in GenBank.

**DISCUSSION**

Our results indicate that the antibiotic resistance of Hungarian Shigella isolates to older drugs, such as sulfamethoxazole/trimethoprim, streptomycin, ampicillin and tetracycline is frequent. We could not find any region-specific characteristic antibiotic resistance pattern, but the Sm, SxT-resistant S. sonnei strains could represent a widely spread clone in the country. It is worth mentioning that ampicillin, cotrimoxazole (sulfamethoxazole/trimethoprim) and chloramphenicol are the most commonly used antibiotics for Shigella infections. Our results also revealed an increasing proportion of MDR S. sonnei over time (Fig. 2). Twice as many MDR S. sonnei strains were identified in the urban than than rural regions (data not shown). To our knowledge, there are no published data available about the antibiotic resistance of Shigella isolates during this period either from Hungary or from the region of Europe.

Of the quinolones, only nalidixic acid resistance was detected in 2 % of S. sonnei isolates and no fluoroquinolone resistance was found. One S. sonnei strain of imported origin was resistant to cefotaxime and harboured a bla<sub>CTM-M-55</sub>-type ESBL gene. Resistance to the third-generation cephalosporins mediated by ESBL enzymes is a rare phenomenon, although some studies have suggested that this may be an emerging problem in Shigella spp. Isolation of ESBL-producing shigellae has been reported since the late 1990s. In recent years, retrospective studies have described the results of searches for ESBL producers among large collections of S. sonnei isolates (Xiong et al., 2007; Acikgoz et al., 2008). Most of these cases were detected in Asia, where bacillary dysentery is still a frequent disease diagnosed without microbiological examination and treated by antibiotics empirically. Although there are reports on bla<sub>CTX-M</sub> production by Klebsiella spp. (Damjanova et al., 2008) and Salmonella spp. (Toth et al., 2007) in Hungary, there has been no report on the antimicrobial susceptibility of Hungarian shigellae isolated in recent years, and this is the first report on the isolation of an ESBL-producing S. sonnei strain in Hungary.

The prevalence of class 1 integrons reported from S. sonnei worldwide varies widely from 6 % in Japan (Ahmed et al., 2006) to 46 % in Vietnam (Iversen et al., 2003). Among the Hungarian S. sonnei strains, 7 % contained a class 1 integron, which carried either the dfrA1-aadA1 (conferring

**Table 2. Integrons of Shigella isolates**

<table>
<thead>
<tr>
<th>Integron</th>
<th>Gene(s)</th>
<th>Size (bp)</th>
<th>S. sonnei (n=230)</th>
<th>S. flexneri (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td>aadA1</td>
<td>885</td>
<td>3 (1.3 %)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>dfrA1-aadA1</td>
<td>1856</td>
<td>13 (5.7 %)</td>
<td>0</td>
</tr>
<tr>
<td>Class 2</td>
<td>dfrA1-sat2</td>
<td>1456</td>
<td>6 (2.6 %)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>dfrA1-sat2-aadA1</td>
<td>2158</td>
<td>56 (24.3 %)</td>
<td>3 (13.6 %)</td>
</tr>
</tbody>
</table>
resistance to trimethoprim and spectinomycin/streptomycin, respectively) or the aadA1 gene cassette. These cassette arrays have also been found in human Salmonella isolates from 2002 to 2003 in Hungary (Nőgrády et al., 2005), indicating potential interspecies spread of class 1 integrons. Consistent with the literature, among the examined S. sonnei we observed the predominance (24 %) of a class 2 integron of 2.2 kb having the dfrA1-sat2-aadA1 cassette array conferring resistance to trimethoprim, streptothricin and spectinomycin/streptomycin, respectively. This Tn7-borne class 2 integron is common in S. sonnei (Peirano et al., 2005; Pan et al., 2006). Three of the 22 S. flexneri had the same 2.2 kb class 2 integron that was detected in S. sonnei as well, but no other integron was found in any S. flexneri strains. Beside Shigella spp., this class 1 integron with the dfrA1-aadA1 gene cassette and this class 2 integron with dfrA1-sat2-aadA1 gene cassette appear to be common also in E. coli strains isolated from livestock, water or human clinical samples in various parts of the world (e.g. the USA, Portugal, Kenya, China) (Solberg et al., 2006; Moura et al., 2007). Furthermore, Moura et al. (2007) described both integrons not only in E. coli but also in Aeromonas sp. and Morganella morganii isolated from a slaughterhouse wastewater treatment plant in Portugal.

PFGE profiling is a common method for assessment of the possible relatedness of individual clinical isolates of a particular bacterial species for epidemiological studies. In our study, PFGE typing could distinguish 65 different pulsortypes among the 75 integron-carrier S. sonnei isolates. The similarities among the isolates ranged from 66 to 100 %. Two large clusters were identified in the dendrogram generated, which, with few exceptions, separated the class 2- and class 1-harbouring isolates. Within these large and relatively distantly related lineages, there are several smaller, more closely related groups, but the dissemination of any clone was not detected.

Production of Shiga toxin was recognized first in S. dysenteriae type 1. It is most likely that a transducing Shigella bacteriophage has mobilized the stx gene of S. dysenteriae type 1, which has spread to different species where, during its adaptation to the host, it became defective or kept its lytic character. It was demonstrated in more than 200 serotypes of E. coli (Shiga toxigenic E. coli) strains but it is rare in other Shigella spp. (Nataro & Kaper, 1998). In E. coli, the stx1 and stx2 genes are located in the genome of frequently inducible phages while in S. dysenteriae type 1 the stx genes are present in the chromosome on a defective prophage (McDonough & Butterton, 1999). Here we report the existence of stx1 gene in an S. sonnei strain and as a novelty the presence of cdtB III/V gene in an S. flexneri. Earlier, Strauch et al. (2001) reported for the first time the existence of a self-transferable stx1-harbouring bacteriophage in an S. sonnei strain.

Johnson & Lior (1987) screened a set of Shigella isolates for Shiga toxin and CDT. Shiga toxin was produced by only S. dysenteriae type 1 strains (5/12) and CDT was produced by four strains of S. dysenteriae type 2 and one of the 19 isolates of S. boydii type 7. None of the S. flexneri (n=23) or S. sonnei (n=2) isolates tested produced Stx or CDT. Okuda et al. (1995) screened for cdtA gene among Shigella isolates by hybridization. The cdtA gene frequently (8/35) characterized the S. dysenteriae strains, and one of 100 S. sonnei strains also was cdt-positive, but none of the 133 S. flexneri strains had a cdt gene.

In summary, we identified the most frequent antimicrobial resistance patterns as well as class 1 and 2 integrons carried, and provided data about some virulence characteristics and the genetic diversity of the integron-carrier S. sonnei and S. flexneri isolated in recent years in Hungary. For the first time, we report ESBL resistance in Shigella encoded by the blaCTX-M-55 ESBL gene, located on a 90 kb conjugative plasmid from Hungary. Furthermore, this is also the first report on the cdtB gene in S. flexneri and one of the rare reports about stx-harbouring S. sonnei. Knowledge of the antimicrobial resistance pattern and PFGE pulsotype of Shigella strains circulating in the country may be helpful in choosing appropriate antimicrobial therapy and also in the epidemiological characterization of shigellosis in Hungary.

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REFERENCES


Characterization of Hungarian shigellae


