MOLECULAR TAXONOMY AND EPIDEMIOLOGY

Restriction endonucleases in clinical isolates of *Shigella* spp.

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Thirteen restriction endonuclease-containing strains were isolated from a collection of 186 clinical isolates of *Shigella* spp. Among these, eight and five isolates carried isoschizomers of *EcoRII* and *NciI*, respectively. The former restriction-modification (R-M) system was homologous to that of *EcoRII* and was located on plasmids with sizes of 46.6 or 55.6 kb. Isolates producing *NciI* isoschizomers contained a 5.7-kb non-transferable plasmid. Together with antimicrobial susceptibility tests and plasmid profile studies, it is concluded that these two R-M systems are not widely spread but confined to strains with similar antibiotic resistance and plasmid profile.

Introduction

Restriction-modification (R-M) systems exist in many bacterial species [1]. Restriction endonucleases serve to degrade foreign invading DNA and methyltransferases protect the host DNA from its own restriction endonuclease [2]. Some R-M systems are coded for on naturally occurring plasmids and they may contribute to the maintenance of plasmid stability [3–5]. In Hong Kong, infection with *Shigella* spp. is endemic and constitutes *c.* 33% of the notified cases of diarrhoea [6]. Many strains of these species are resistant to multiple antibiotics and carry plasmids [7, 8]. To explore the diversity of restriction endonucleases in *Shigella* spp. and find if restriction endonuclease production is a useful character for epidemiological studies, this study correlated the occurrence of restriction endonucleases with serotype, antibiotic resistance and plasmid profile, in a local collection of clinical isolates of *Shigella* spp.

Materials and methods

Shigella strains

A total of 186 single isolates of *Shigella* spp. comprising 124 strains of *S. flexneri*, 61 of *S. sonnei* and one of *S. boydii*, collected from two local hospitals between 1985 and 1994, was examined.

Screening for restriction endonucleases

The isolates were grown at 37°C overnight in 5 ml of L broth: bacterial tryptone (Difco) 1%, bacterial yeast extract (Difco) 0.5%, NaCl 1% w/v. The cells from 1.5 ml of the broth culture were collected by centrifugation and resuspended in 0.4 ml of column buffer (10 mM Tris-HCl, pH 7.5, 5 mM β -mercaptoethanol, 1 mM EDTA, glycerol 5%, 1 mM phenylmethylsulphonyl fluoride). After disruption of the cells by sonication in an ice-water bath and centrifugation to remove the cell debris, 1 μ l of the supernate was incubated with 14 μ l of a reaction mixture containing 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol and a mixture of phage T7, adenovirus-2 and phage lambda DNA at 37°C for 15 min. The digested product was analysed by electrophoresis on an agarose 0.8% gel in Tris-borate-EDTA (TBE) buffer [9].

Isolates in which sonicates exhibited restriction endonuclease activity were grown overnight in 300 ml of L broth at 37°C with shaking. The cultures were centrifuged at 3840 *g* for 15 min. The cell pellet was resuspended in 15 ml of column buffer and the cells were lysed as described previously [10]. The lysate was loaded on to a heparin-agarose column (18 $\times$ 10 cm). Restriction endonuclease was eluted with a 0–1 M NaCl gradient in 300 ml of column buffer and 1-ml fractions were collected.

Antimicrobial susceptibility tests and serotype determination

The antibiotic susceptibility of the *Shigella* isolates was determined by the agar dilution method as described

previously [7]. The isolates were serotyped by slide agglutination with specific antisera (Wellcome Research Laboratories, Beckenham).

Southern hybridisation

Plasmid DNA was extracted from the isolates according to the method of Kado and Liu [11], electrophoresed on an agarose 0.7% gel in TBE buffer [9] and stained with ethidium bromide for UV visualisation. Plasmids separated on gels were then transferred on to Hybond-N+ nylon membranes (Amersham) by a vacuum blotting system (Vacugene, LKB, Bromma, Sweden). Membranes were hybridised with a 400-bp *Bam*HI-*Eco*RV fragment within the *Eco*RII restriction endonuclease-encoding gene. Pre-hybridisation was at 65°C for 1 h in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution [12], denatured salmon sperm DNA 100 mg/L and SDS 0.5%. Hybridisation was performed at 65°C for 12 h in a solution containing 6 × SSC, 5 × Denhardt's solution, denatured salmon sperm DNA 100 mg/L and labelled probe at a concentration of 1 ng/ml. The probe was labelled with [α -³²P] dCTP (Amersham) with the Megaprime™ DNA labelling kit (Amersham) according to the manufacturer's instructions. Unincorporated radioactive nucleotides were removed by passing through prepacked Bio-Spin® chromatography columns (BioRad Laboratories, CA, USA). Membranes were washed with 2 × SSC, SDS 0.1% for 5 and 10 min at room temperature with 0.5 × SSC, SDS 0.1% for 15 min at 65°C and finally with 0.1 × SSC, SDS 0.1% twice for 15 min at 65°C. Hybridised membranes were exposed to X-ray films (Kodak, Rochester, NY, USA) at -70°C for 1-3 days.

Results

Among the 186 isolates examined, 13 were found to contain restriction endonucleases. Table 1 summarises

their characteristics. The partially purified enzymes were used to digest DNA preparations of known sequence. The enzymes were shown to be either *Nci*I (Fig. 1a) or *Eco*RII (Fig. 1b) isoschizomers which recognise CCSGG or CCWGG, respectively. The isolates containing *Nci*I isoschizomers were resistant to only one or two antibiotics and all harboured a 5.7-kb plasmid (Fig. 2a and Table 1). This plasmid was not transferable to an *Escherichia coli* strain by either conjugation or transformation.

In contrast, the isolates containing *Eco*RII isoschizomers harboured multiple plasmids with similar, although non-identical, profiles (Fig. 2a). Hybridisation of the plasmid DNA with a probe containing part of the *Eco*RII gene showed that there was high homology between the *Eco*RII gene and a 46.6- or 55.6-kb plasmid (Fig. 2b). These plasmids were transferable to *E. coli* by conjugation.

The plasmid profiles of 113 isolates that did not contain restriction endonucleases were determined. Ten isolates with multiple plasmids also contained a 5.7-kb plasmid and of these, one was resistant to streptomycin only and one to streptomycin and tetracycline. On the other hand, three isolates resistant to multiple antibiotics contained a 46.6-kb or a 55.6-kb plasmid, together with other plasmids of different sizes.

Discussion

Restriction endonucleases have been found in many different bacteria. Usually, isoschizomers from different species have unrelated sequences [13]. Nevertheless, there are only a few reports on the use of restriction enzymes in epidemiological studies. Miyahara *et al.* found isoschizomers of restriction endonuclease *Pst*I to occur at a high frequency in *Yersinia enterocolitica* serotype O8 strains [14]. Subsequently, this group has also correlated the occurrence of three

Table 1. Characteristics of *Shigella* isolates with restriction endonuclease activity

<i>Shigella</i> strain	Serotype	Antibiotic resistance	Restriction endonuclease	Isoschizomer of
11573	F2	ST	<i>Sfi</i> HK11573I	<i>Nci</i> I
11087	F2	S	<i>Sfi</i> HK11087I	<i>Nci</i> I
11086	F2	S	<i>Sfi</i> HK11086I	<i>Nci</i> I
7462	So	ASTCKSuTmSxtG	<i>Sso</i> HK7462I	<i>Eco</i> RII
11572	F3	S	<i>Sfi</i> HK11572I	<i>Nci</i> I
10695	So	ST	<i>Sso</i> HK10695I	<i>Nci</i> I
7234	So	ASTCSuTmSxtG	<i>Sso</i> HK7234I	<i>Eco</i> RII
6873	So	ASTCKSuTmSxtG	<i>Sso</i> HK6873I	<i>Eco</i> RII
2374	F1	ASTCSu	<i>Sfi</i> HK2374I	<i>Eco</i> RII
2731	F1	ASTCSuTmSxtGCthMaCp	<i>Sfi</i> HK2731I	<i>Eco</i> RII
1794	So	ASTCSuTmSxtG	<i>Sso</i> HK1794I	<i>Eco</i> RII
8401	F1	ASTCKSuTmSxt	<i>Sfi</i> HK8401I	<i>Eco</i> RII
10790	So	ASTCKSuTmSxtG	<i>Sso</i> HK10790I	<i>Eco</i> RII

F1, *flexneri* serotype 1; F2, *flexneri* serotype 2; F3, *flexneri* serotype 3; So, *sonnei*; A, ampicillin; S, streptomycin; T, tetracycline; C, chloramphenicol; K, kanamycin; Su, sulphamethoxazole; Tm, trimethoprim; Sxt, cotrimoxazole; G, gentamicin; Cth, cephalothin; Ma, cephamandole; Cp, cefoperazone.

Fig. 1. (a) Digestion of (1) non-methylated pUC19 and (2) M13mp18DNA by (a) 4, (b) 2, (c) 1 and (d) 0.5 μ l of *Shigella* 10695 enzyme. (b) Digestion of (1) non-methylated pBR322 and (2) non-methylated pACYC184 DNA by (a) 4, (b) 2, (c) 1 and (d) 0.5 μ l of *Shigella* 10790 enzyme. M: λ hindIII marker.

Fig. 2. (a) Profile and (b) Southern hybridisation of plasmid DNA from *Shigella* isolates containing *Eco*RII or *Nci*I isoschizomers. Lanes 1–4, *Shigella* isolates 11573, 11087, 11086 and 7462; 5, supercoiled DNA ladder (BRL cat no. S622SA); 6, marker 28R823 of 221 kb; 7, markers 40R448, RT641, 48R626 of 119, 91 and 62 kb, respectively; 8, markers 40R268, 40R646 of 98 and 58 kb, respectively; 9–17 *Shigella* isolates 11572, 10695, 7234, 6873, 2374, 2731, 1794, 8401 and 10790.

restriction endonucleases to three serotypes of *Salmonella* [15].

In the present study, 13 of the 186 *Shigella* isolates were found to contain *Nci*I or *Eco*RII isoschizomers.

There appeared to be no relationship between serotype and the occurrence of these enzymes in *Shigella*, indicating that the two characteristics had not co-evolved. On the other hand, there was a correlation between the two restriction endonucleases, plasmid

profile and antibiotic resistance. The *NciI* isoschizomer was found only in isolates resistant to one or two antibiotics and having a single 5.7-kb plasmid. The plasmid profiles of isolates containing the *EcoRII* isoschizomers were similar; the strains were resistant to multiple antibiotics (Table 1) and only a very few strains containing a 46.6- or 55.6-kb plasmid had escaped the detection of *EcoRII* isoschizomer. Therefore, these two R-M systems may be used in addition to plasmid profile and antibiotic resistance for tracing *Shigella* strains. As the DNA encoding *EcoRII* isoschizomers in *Shigella* were able to hybridise to an *EcoRII* gene probe, and the latter gene is carried on a large R plasmid with conjugal transfer ability [3, 16], it is likely that both the *EcoRII* R-M system and its *Shigella* counterpart have the same origin. The identity of the *Shigella* plasmid which carries the *EcoRII* isoschizomer gene is being sought, in order to reveal if the R plasmid in *E. coli* has spread to these *Shigella* strains.

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