Identification of a group of shigella-like isolates as *Shigella boydii* 20

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Infections by *Shigella* species are an important cause of diarrhoeal disease worldwide. Of 4198 *Shigella* isolates received by the French National Reference Centre for *Escherichia coli* and *Shigella*, 180 from patients with diarrhoea and dysentery in 2000–2004 did not react with any available polyclonal rabbit antisera used to identify the established *Shigella* serogroups. This study describes the molecular and phenotypic characteristics of these isolates in seroagglutination tests, molecular serotyping (*rfb*-RFLP and *fliC*-RFLP), ribotyping, detection of invasivity and enterotoxins genes, and antibiotic sensitivity. All isolates gave biochemical reactions typical of *Shigella boydii*, were mannitol-positive and indole-negative. They all carried invasion-associated genes, enterotoxin 2 [ShET-2] and an IS630 sequence. They had a unique ribotype that was distinct from all other *Shigella* and *E. coli* patterns. Further characterization by *rfb*-RFLP clearly distinguished this serogroup from all other *Shigella* or *E. coli* O-groups. The *fliC*-RFLP pattern corresponded to P4, an F-pattern which is associated with 10 different serogroups of *S. boydii*. A new antiserum prepared against strain 00-977 agglutinated all 180 isolates and cross-agglutination and absorption studies with anti-00-977 serum and anti-CDC 99-4528 (reference for the newly described *S. boydii* serogroup 20) serum showed identical antigenic structure. Furthermore, strains 00-977 and CDC 99-4528 had the same molecular serotype, ribotype and virulence genes.

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INTRODUCTION

Shigellae cause diseases ranging from diarrhoea to bacillary dysentery, an invasive infection of the human colon affecting humans in developing countries, and are associated with poor hygiene conditions. In industrialized or developed countries, common-source outbreaks occur sporadically and several outbreaks have been reported (Butler, 2000; Legros, 2004). The diarrhoeal disease is highly contagious due to its low infectious dose.

The genus *Shigella* comprises four subgroups that historically have been treated as species (*Enterobacteriaceae* Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies, 1958): subgroup A is referred to as *Shigella dysenteriae*, subgroup B as *Shigella flexneri*, subgroup C as *Shigella boydii* and subgroup D as *Shigella sonnei*. In fact, these four so-called species constitute with *Escherichia coli* a single genomic species (Brenner *et al.*, 1972, 1973), with the exception of *S. boydii* 13, which is synonymous with *Escherichia alberti* (Brenner *et al.*, 1982; Huys *et al.*, 2003). Phylogenetically, the shigellae (except *S. boydii* 13) are distributed in

seven clusters within the *E. coli* branch (Lan & Reeves, 2002).

Shigella species cannot be easily identified by biochemical properties alone, and definitive identification requires serotyping (Ewing *et al.*, 1958; Ewing & Lindberg, 1984). Based on somatic antigen (O-antigen) properties, *S. dysenteriae* is subdivided into 15 serogroups (Bopp *et al.*, 2003) and 2 provisional serogroups (Ansaruzzaman *et al.*, 1995; Coimbra *et al.*, 2001b; Melito *et al.*, 2005). *S. flexneri* is subdivided into six serogroups and *S. sonnei* has a single serogroup which is divided into biotypes a, d, e, f and g (Janda & Abbott, 1988). Previously *S. boydii* consisted of 19 serogroups. In 1986, Ewing (1986) acknowledged 17 serogroups, including two provisional serogroups represented by reference strain 2710-54 as *S. boydii* 16 and 3615-53 as *S. boydii* 17. Gross *et al.* (1980) reported a new provisional serogroup with strain E10163, isolated in Bangladesh. Brenner and the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Enterobacteriaceae* (Brenner, 1984) recommended that the strain be added to the scheme as *S. boydii* 18. More biochemical and serological studies were presented by

Wathen-Grady *et al.* (1985), and subsequently the designation *S. boydii* 19 for the provisional serogroup E16553 was published by Gross *et al.* (1982), Bopp *et al.* (2003) and Ansaruzzaman *et al.* (2005).

During a period of 5 years (January 2000–December 2004), the French National Reference Center for *Escherichia coli* and *Shigella* (FNRCECS) received a total of 4198 isolates of *Shigella* isolated from diverse laboratories in France in different geographical locations (France and abroad) from patients suffering from diarrhoea. *S. sonnei*, the most predominant species, accounted for 50 %, followed by *S. flexneri* (35 %), *S. boydii* (5 %) and *S. dysenteriae* (4 %). One hundred and eighty isolates (6 %) were serologically not typable.

A new provisional serogroup represented by strain 99-4528 isolated in Canada was reported in an epidemiological investigation. This strain was designated by the World Health Organization (WHO) Collaborating Centre for *Shigella* the reference strain for *S. boydii* 20 by Kalluri *et al.* (2004) but no microbiological or molecular features were reported. Very recently, Woodward *et al.* (2005) reported a study on the same new serogroup with laboratory and epidemiological information on a collection of clinical isolates in Canada and the USA.

In the work presented here, we have studied the 180 non-typable *Shigella* isolates above. We describe their characteristics with respect to biochemical test results, presence of pathogenicity genes, ribotype pattern (Grimont & Grimont, 1986; Coimbra *et al.*, 2001c; Machado *et al.*, 1998), molecular serotyping with RFLP of the amplified O-antigen gene cluster (*rfb* genes) (Coimbra *et al.*, 1999, 2000) and RFLP of the amplified cryptic flagellin gene (*fliC*) (Coimbra *et al.*, 2001a; Machado *et al.*, 2000), and agglutination by a serum raised against representative strain 00-977. The reference strain of *S. boydii* 20 (CDC 99-4528), received recently, was agglutinated by anti-00-977 serum, and was studied using the same methods, and compared with the other strains.

METHODS

Bacterial isolates. A total of 180 isolates of *Shigella* from diverse laboratories from patients in different geographical locations in France (145) and abroad (35) that did not agglutinate with available *Shigella* antisera were studied. Strain CDC 99-4528, the reference strain for *S. boydii* 20, was received from Dr N. Strockbine, CDC, Atlanta, GA, USA.

Biochemical tests. Cultures were grown at 37 °C on Drigalski agar for identification. All isolates (180) were subjected to the following biochemical tests: motility, glucose (acid and gas), H₂S, indole production, lysin decarboxylase, ornithine decarboxylase, dulcitol, glycerol, lactose, D-mannitol, mucate, rhamnose and D-xylose. Twenty-seven randomly chosen clinical isolates were subjected to the 36 biochemical tests listed in Table 1 by conventional methods as described by Ewing (1986).

Seroagglutination. Slide agglutination was performed with appropriate antisera raised against all recognized *Shigella* antigens

Table 1. Biochemical reactions of serogroup 00-977 (*S. boydii* 20)

+, Positive (1–2 days); –, negative.

Biochemical reaction	Reaction
Motility	–
Glucose (acid)	+
Glucose (gas)	–
H ₂ S	–
Urease	–
Indole production	–
Lysine decarboxylase	–
Arginine dihydrolase	–
Ornithine decarboxylase	–
Tryptophan deaminase	–
β-Galactosidase (ONPG)	–
Christensen's citrate	–
Simmons' citrate	–
Gelatin	–
Voges-Proskauer (37 °C)	–
NO ₂ from NO ₃	+
Oxidase	–
Adonitol	–
L-Arabinose	+
Dulcitol	–
Glycerol	–
myo-Inositol	–
Lactose	–
Malonate	–
Maltose	–
D-Mannitol	+
D-Mannose	+
Melibiose	–
Mucate	–
Raffinose	–
Rhamnose	–
Salicin	–
Sorbitol	–
Sucrose	–
Trehalose	+
D-Xylose	–

(Eurobio). In addition, two antisera against strains 00-977 and CDC 99-4528 were raised by injecting New Zealand rabbits with heat-killed bacteria (Ewing & Lindberg, 1984). Cross-absorptions were performed with both antisera and both reference strains.

Antimicrobial susceptibility testing. Susceptibility to antimicrobial agents was determined on Mueller–Hinton (MH) agar by the disc diffusion method following the guidelines of the French National Antibigram Committee (communiqué 2003, www.sfm.asso.fr/nouv/general.php?pa=2). The following antimicrobial discs (Bio-Rad) were used: amoxicillin, ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin–tazobactam, cephalothin, cefamandole, cefoperazone, cefoxitin, ceftriaxone, ceftazidime, moxalactam, aztreonam, imipenem, streptomycin, spectinomycin, kanamycin, tobramycin, netilmicin, gentamicin, amikacin, nalidixic acid, ofloxacin, ciprofloxacin, sulfonamides, trimethoprim, chloramphenicol and tetracycline.

Detection of pathogenicity genes by PCR. DNA was prepared (Grimont & Grimont, 1995) from 27 isolates and examined by PCR for the presence of the invasion-associated locus (*ial*) and the invasion plasmid antigen H (*ipaH*) of *Shigella* as described by Frankel *et al.* (1990) and Sethabutr *et al.* (1993). PCR assays to demonstrate the presence of Shiga toxin (*stx*), *Shigella* enterotoxin 1 (ShET-1; gene *set1*), *Shigella* enterotoxin 2 (ShET-2; gene *sen*) and IS630 were performed as described by Lin *et al.* (1993), Vargas *et al.* (1999) and Hounig *et al.* (1997) in a DNA thermal cycler (GenAmp 9700; Applied Biosystems). Amplification products were subjected to gel electrophoresis in 2% agarose and detected by staining with ethidium bromide.

Molecular serotyping

***rfb*-RFLP.** Analysis of the *rfb* gene cluster by amplification and restriction of the amplified product with endonuclease *Mbo*II was carried out as described by Coimbra *et al.* (1999). Patterns were analysed and compared against the established *Shigella* and *E. coli* database (Coimbra *et al.*, 1999, 2000) using programs of the Taxotron package (Grimont, 2000). Molecular masses of the fragments were estimated by the Spline algorithm (Grimont, 2000). In pattern comparisons, the maximum fragment size variation accepted ranged from 7% for 500 bp to 3.5% for 4000 bp.

***fliC*-RFLP.** Analysis of the cryptic flagellin gene (*fliC*) by amplification and restriction of the amplified product with endonuclease *Hha*I was done following the method of Machado *et al.* (1998). Patterns were analysed and compared against the established *Shigella* and *E. coli* database (Machado *et al.*, 2000; Coimbra *et al.*, 2001a) using the Taxotron package. Molecular masses of the fragments were estimated by the Schaffer and Sederoff algorithm (Grimont, 2000). In pattern comparisons, the percentage of tolerated fragment size variation was set to 3.5%.

Ribotyping. Fifty isolates were subjected to automated ribotyping using the RiboPrinter Microbial Characterization System (Qualicon) according to the manufacturer's instructions. Restriction endonuclease digestion by *Mlu*I, gel separation, transfer and hybridization with a chemiluminescent-labelled DNA probe containing the rRNA operon from *E. coli* were done automatically in 8 h. Ribotype numbering and identification were generated by this system. The gel images were also analysed with programs of the Taxotron package, and ribopatterns were compared to the *Shigella* and *E. coli* *Mlu*I ribotype databases. A maximum fragment size variation of 5% was accepted.

RESULTS

Biochemical tests

All of the 180 isolates were non-motile rods, fermented D-mannitol and failed to produce indole. The biochemical reactions of strain 00-977 are presented in Table 1.

Serological tests

Except for five rough isolates (2.8%), all isolates (180) and the reference strain CDC 99-4528 (*S. boydii* 20) agglutinated solely with the antiserum raised against strain 00-977. Subsequently, an anti-CDC 99-4528 serum was prepared. Strains 00-977 and CDC 99-4528 agglutinated with the two antisera raised against these two strains (both

at 1/500). After cross-absorptions of the two antisera with the two strains, no agglutinations were observed with the strains: antiserum against strain CDC 99-4528 absorbed with strain 00-977 did not agglutinate strain CDC 99-4528, and conversely antiserum against strain 00-977 absorbed with strain CDC 99-4528 did not agglutinate strain 00-977.

Antimicrobial susceptibility

All 27 isolates tested were susceptible to piperacillin and piperacillin-tazobactam, cephalothin, cefamandole, cefoperazone, cefoxitin, ceftriaxone, ceftazidime, moxalactam, aztreonam, imipenem, kanamycin, tobramycin, netilmicin, gentamicin, amikacin, nalidixic acid, ofloxacin, ciprofloxacin and chloramphenicol. They were all resistant to amoxicillin, ticarcillin, ticarcillin-clavulanic acid, streptomycin, spectinomycin and trimethoprim; 17 (63.0%) isolates were also resistant to sulfonamide and tetracycline, 6 (22.2%) isolates to sulfonamide and 2 (7.4%) to tetracycline.

Detection of pathogenicity genes by PCR

All isolates tested and the reference strain CDC 99-4528 were positive for *ial* and *ipaH* invasion-associated genes, *sen* and IS630 sequences. One strain isolated in 2000 was rough and carried *ipaH* but not *ial*. The PCR tests for *stx* and *set1* genes were negative for all strains tested.

Fig. 1. *Mbo*II-RFLP patterns (and schematic representation) of the amplified *rfb* gene cluster. Lanes: 1 and 4, molecular mass marker (mixture of lambda-*Hind*III and AmpliSize Molecular Ruler); 2, 00-977; 3, CDC 99-4528; same RN1 type.

***rfb*-RFLP**

A single restriction pattern was shown by all 27 isolates and by the reference strain CDC 99-4528 (Fig. 1). This unique pattern was clearly different from any other pattern in our database for *Shigella* and *E. coli* O groups, including the unique *rfb* pattern of *S. flexneri* serogroups 1–5.

***fliC*-RFLP**

All the 27 isolates and the reference strain CDC 99-4528 gave a single restriction pattern (Fig. 2). This pattern was identical to pattern P4, which is associated with *S. boydii* serogroups 1, 2, 3, 4, 6, 8, 10, 11, 14 and 18; *S. flexneri* 6; and *S. dysenteriae* serogroups 4, 5, 6, 7, 9, 11 and 12.

Ribotyping

Fifty isolates tested and the reference strain CDC 99-4528 showed the same ribotype, RQ1 (Fig. 3), which was unique. This ribotype was compared with those in the *Shigella* and *E. coli* *Mlu*I ribotype databases and was clearly different from any other profile (Fig. 4). The closest profile in the database was C1b/3/8/10b/18 of *S. boydii* (Coimbra *et al.*, 2001c), with seven common bands and two different bands.

DISCUSSION

The FNRCECS received 180 atypical strains of *Shigella* isolated from patients from January 2000 to December

Fig. 2. *Hha*I-RFLP patterns (and schematic representation) of the *Shigella* amplified *fliC* gene. Lanes: 1, 6 and 11, molecular mass marker (AmpliSize Molecular Ruler); 2, 00-977; 3, 01-6104; 4, 01-6419; 5, 02-110; 7, 04-059; 8, 00-3176; 9, 02-4425; 10, CDC 99-4528; same P4 type.

Fig. 3. Ribotypes of four *Shigella boydii* 20 strains. Lanes: 1, 4 and 7, molecular mass marker; 2, 00-977; 3, 01-6104; 5, 04-059; 6, CDC 99-4528; same ribotype RQ1.

Fig. 4. Ribotypes of 20 serogroups of *Shigella boydii*.

2004 representing a mean of 4.3 % of the total *Shigella* strains. The incidence of antigenically atypical strains which did not agglutinate with any available serum of *Shigella* was high in 2000 and accounted for 12.8 % of *Shigella* strains. Since 2001, the incidence has been stable and they account for 1.5–3.1 % of *Shigella* strains. For 91 patients, there was a history of recent travel to Africa (Algeria, Burkina Fasso, Egypt, Ivory Coast, Mali, Morocco, Mauritania, Togo, Tunisia), Asia (Bangladesh, India, Kazakhstan, Yemen), America (Brazil, French Guyana, Cuba, St Domingue, Peru) and also Bosnia.

These strains were characterized as *Shigella* spp. by the presence of the invasion-associated locus (*ial*) and the invasion plasmid antigen H (*ipaH*) gene. All fermented D-mannitol and might therefore be classified as either *S. boydii* or *S. flexneri*. Since *S. boydii* is characteristically D-mannitol-positive (98 %), arabinose-positive (94 %) and invariably raffinose-negative (Ewing, 1986), strains of this group have properties compatible with *S. boydii*.

Resistance of *Shigella* spp. to various antibiotics is extremely common and is increasing (Sack *et al.*, 1997). In this study, all the 27 strains tested were resistant to six antimicrobial agents, and 63.0 % were resistant to eight. Woodward *et al.* (2005) found resistance to five antimicrobial agents in 92 % of cases. If we consider the same antimicrobial agents (A-S-Su-T-Tm) as Woodward *et al.* (2005), we found a lower rate (63.0 %) due to a higher susceptibility to sulfonamides and tetracycline. We did not find any resistance to chloramphenicol in our strains, in contrast to Woodward *et al.* (2005), who found resistance in four strains. No correlation was observed between resistance and geographical origin.

Two novel toxins, ShET-1 encoded by the chromosomal gene *set1* and ShET-2 encoded by *sen*, associated with invasiveness of many *Shigella* spp., have been described in *S. flexneri* (Fasano *et al.*, 1995). It is now known that *sen* is located on the invasion plasmid and is present in most strains of *Shigella* (83 %) and in 75 % of EIEC (enteroinvasive *E. coli*) (Nataro *et al.*, 1995). ShET-2 has been reported in different species of *Shigella* causing travellers' diarrhoea by Vargas *et al.* (1999), but the prevalence of both enterotoxins in *S. boydii* serogroups had not been hitherto reported. We have studied the occurrence of these two genes in 27 isolates of the new serogroup of *Shigella* and in a collection of 424 diverse *Shigella* strains which comprised: 214 *S. flexneri*, 164 *S. sonnei*, *S. dysenteriae* and 38 *S. boydii* (unpublished data). We found that *sen* is present in all *S. boydii* and *S. dysenteriae* strains, in 61 % of *S. sonnei* strains and in 68 % of *S. flexneri* strains. These results agree with those of Vargas *et al.* (1999), who found *sen* in all *S. dysenteriae* strains and in 58 % of *S. sonnei* strains, with a lower rate for *S. flexneri* (14 %). In contrast, *set1* was absent from *S. boydii*, *S. dysenteriae*, *S. flexneri* and *S. sonnei*, but Vargas *et al.* (1999) found *set1* in 9 % of *S. flexneri* strains. Talukder *et al.* (2002) reported that *sen* was present in all *S. flexneri* 4, but that *set* was absent.

All isolates of our studied group of *Shigella* had *sen* but not *set*.

Houng *et al.* (1997) showed that all species of *Shigella* and all serogroups of *S. boydii* contain the IS630 sequence. We confirmed the presence of the sequence in strains studied here. Therefore, the IS630 sequence, as well as *ipaH* and *ial* gene sequences (Frankel *et al.*, 1990; Sethabutr *et al.*, 1993), can be amplified by PCR to allow the detection of *Shigella* or EIEC.

Though *Shigella* species are non-motile and lack flagella, a cryptic *fliC* gene is almost always present. Coimbra *et al.* (2001a) studied cryptic *fliC* alleles in the genome of *Shigella* serogroups by restriction analysis and found 17 restriction patterns. All patterns of our strains belong to *fliC* pattern P4 of Coimbra *et al.* (2001a), which is shown by 10 serogroups of *S. boydii* and 7 serogroups of *S. dysenteriae*.

The combination of biochemical reactions and *rfb*-RFLP (RN1) and *fliC*-RFLP (P4) data suggest that these isolates constitute a distinct group of *S. boydii*. They were assigned to serogroup *S. boydii* 20 by their reactions with an antiserum that reacted with the reference strain for this serogroup. The reciprocal cross-agglutinations and cross-absorption with both antisera confirmed the antigenic identity of the two reference strains. The diverse geographical origins of the strains and the long time span over which they were isolated raise questions about the epidemiology of this serogroup and its potentially widespread public health significance.

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