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Identification and characterization of *Shigella boydii* 20 serovar nov., a new and emerging *Shigella* serotype

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Analysis of 163 putative *Shigella* isolates from Canada and the USA showed biochemical reactions consistent with *Shigella* species, although none of the isolates reacted with antiserum raised against any of the well-established or provisional *Shigella* serotypes. All these isolates, provisionally designated serotype SH108, were positive for the *ipaH* gene and the invasion-associated locus. All fermented mannitol, were serologically indistinguishable from each other and showed no reaction in antisera prepared against *Escherichia coli* serotypes O1 to O181. PCR-RFLP analysis of the genes involved in O-antigen synthesis revealed a common pattern among these isolates that was distinct from recognized *Shigella* serotypes and *E. coli*. Between 1999 and 2003, isolates from across Canada were submitted to the National Laboratory for Enteric Pathogens for antibiotic susceptibility testing, phage typing and PFGE. These assays revealed heterogeneity among the members of this serotype. Antimicrobial susceptibility testing with seven antibiotics identified six profiles, with 90 % (45/50) of the isolates resistant to four or more antibiotics and 72 % (36/50) resistant to five or more. All isolates were typable using a panel of 16 phages, with 11 different phage types (PTs) represented. The most common PTs found were PT 3 (64 %), PT 6 (10 %) and PT 16 (6 %). Analysis of *Xba*I-restricted genomic DNA revealed 16 highly related patterns that were not readily distinguishable from those obtained for some other *Shigella* serotypes. The World Health Organization Collaborating Center for *Shigella* has added serotype SH108 to the *Shigella* scheme as *S. boydii* serotype 20 (serovar nov.). Strain SH108 (isolate 99-4528) is the reference strain for this serotype.

INTRODUCTION

Infections with *Shigella* species cause approximately 600 000 deaths worldwide annually. Two-thirds of all cases and most of the deaths occur among children under 10 years of age (Chin, 2000). Although more prevalent in developing countries, shigellosis is a worldwide problem. Transmission of the disease is primarily by person-to-person contact through contaminated hands (Butler, 2000). Outbreaks typically occur under conditions involving close physical contact, such as those encountered in day-care centres, nursing homes, custodial institutions, cruise ships, aboriginal reservations and crowded refugee camps, with poor hygiene practices and contaminated food or water serving as the vehicle for infection (Brian *et al.*, 1993; Chin, 2000; Edwards, 1999; Janda & Abbott, 1998). The infectious dose of *Shigella* species is low, with 10–100 bacteria sufficient to cause disease (International Commission on Microbiological

Abbreviation: PT, phage type.

Specifications for Foods, 1996; Trevejo *et al.*, 1999; WHO Scientific Working Group, 1980).

Shigellosis is an acute infectious bacterial disease causing inflammatory enteritis in humans. Clinical manifestations of classical bacillary dysentery include fever, vomiting, abdominal pain, tenesmus (painful straining to pass stools) and stools containing blood and mucus resulting from invasion of the intestinal mucosa by the pathogen (Edwards, 1999; Janda & Abbott, 1998; Keusch & Bennish, 1998; Keusch, 1998). Despite this, however, many cases present with only a watery diarrhoea (Chin, 2000; Wathen-Grady *et al.*, 1985). Shigellae are host-adapted to the human intestine (Janda & Abbott, 1998). Illness is usually self-limited and lasts an average of 4–7 days, while the severity of illness and the case-fatality rate are functions of the age and pre-existing nutritional state of the host, as well as of the serotype of bacteria causing disease (Chin, 2000).

The genus *Shigella* is divided into four subgroups that for medical purposes continue to be treated as species: subgroup A (*Shigella dysenteriae*), subgroup B (*Shigella flexneri*), subgroup C (*Shigella boydii*), and subgroup D (*Shigella sonnei*) (Bopp *et al.*, 2003; Chin, 2000). *S. flexneri*, *S. boydii* and *S. dysenteriae* account for most cases of human disease in developing countries, whereas *S. sonnei* is more commonly isolated in developed countries (Janda & Abbott, 1998). *S. boydii* is relatively rare in developed countries and is typically associated with individuals who have travelled to endemic areas.

S. boydii isolates can be difficult to distinguish from *S. flexneri* and enteroinvasive *Escherichia coli*. Isolates of this organism differ mainly in that they are typically agglutinated by *S. boydii* polyvalent group C antiserum and the corresponding monovalent factor antisera specific for serotypes 1–19. They are also differentiated by a recently described *rfb* RFLP technique (Coimbra *et al.*, 1999, 2001) that has proved to be an effective molecular method for rapid characterization of known *Shigella* O serotypes and for the detection of new *Shigella* O serotypes.

In 1999, the National Laboratory for Enteric Pathogens (NLEP) in Winnipeg, Canada, received eight lactose-non-fermenting, D-mannitol-fermenting *Shigella* isolates biochemically resembling *S. flexneri*. These isolates did not agglutinate in antisera specific for the group or type antigens of *S. flexneri* nor in antisera reactive with other recognized and provisional serotypes of *Shigella*. They also lacked the PCR-RFLP patterns characteristic of the molecular serotypes of the recognized *Shigella* serotypes (Coimbra *et al.*, 1999). We have presented here laboratory and epidemiological findings on a collection of clinical isolates received in Canada and the USA that represent a newly emerging serotype of *S. boydii* designated *S. boydii* serotype 20 by the Centers for Disease Control and Prevention (CDC) in Atlanta.

METHODS

Strains. The *S. boydii* isolates that were characterized in this study were submitted, along with any associated information about the cases from which they were derived, by provincial Public Health Laboratories across Canada and state public health laboratories across the USA for confirmation of identification and serotyping results (Table 1; Demczuk *et al.*, 2001; Kalluri *et al.*, 2004). Isolates were routinely grown on nutrient agar and stored at −80 °C in either double-strength skimmed milk or trypticase soy broth containing 10 % glycerol. Isolate 99-4528, designated the *S. boydii* serotype 20 reference strain by the CDC, was used as a control for all tests. Strain 99-4528 has been submitted to the American Type Culture Collection (ATCC) and will be made available to others in the scientific community.

Biochemical tests and serotyping. *S. boydii* is typically non-motile, oxidase negative and catalase positive. Indole is variable, the methyl red test is positive, Voges–Proskauer and Simmons' citrate reactions are negative, and lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase tests are negative. H_2S is not produced, urea is not hydrolysed and there is no growth in KCN broth. Carbohydrates are usually fermented and these include glucose (in the absence of gas production), D-mannitol, arabinose, trehalose and mannose (Holt *et al.*, 1994). The biochemical reactions of the isolates were determined using the methods of Edwards and Ewing (Bopp *et al.*, 2003; Ewing, 1986). Serological identification was performed by slide agglutination with polyvalent, somatic (O) antigen grouping sera, followed by testing

Table 1. Distribution of isolates of *S. boydii* serotype 20 in Canadian provinces (1999–2003)

BC, British Columbia; AB, Alberta; MB, Manitoba; ON, Ontario; QC, Quebec; NB, New Brunswick; NS, Nova Scotia. ND, None detected.

Year	No. of isolates							
	BC	AB	MB	ON	QC	NB	NS	Total
1999	3	1	ND	2	2	ND	ND	8
2000	3	ND	ND	3	5	ND	ND	11
2001	9	5	2	6	2	1	ND	25
2002	3	1	ND	1	ND	ND	1	6
2003	4	1	ND	ND	ND	ND	ND	5
Total	22	8	2	12	9	1	1	55

with monovalent antisera for specific serotype identification (Bopp *et al.*, 2003; Ewing, 1986).

Phage typing. Bacteriophages were isolated from municipal raw sewage samples. Phage isolation, propagation, purification and typing were conducted using standard methods (Adams, 1959; Ahmed *et al.*, 1987; Anderson & Williams, 1956). A phage typing scheme was developed for *S. boydii* using 16 phages that were selected on the basis of distinct lytic reactions.

R-typing. Antimicrobial resistance patterns (R-types) were determined using BBL Sensi-Disc antimicrobial susceptibility discs (Becton Dickinson) according to procedures previously outlined by Bauer *et al.* (1966) and the NCCLS (2001). Antibiotics used in this study included ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), streptomycin (10 μg), sulfadiazine (0·25 μg), tetracycline (30 μg) and trimethoprim/sulfamethoxazole (1·25/23·75 μg).

PCR tests. Isolates were tested for the presence of the *ipaH* gene and for the invasion-associated locus diagnostic for *Shigella* and enteroinvasive *E. coli* as described by Sethabutr *et al.* (1993). PCR was used to demonstrate the presence or absence of *stx* genes and was performed according to the methods described by Paton & Paton (1997).

PFGE. PFGE was done according to previously described protocols (CDC, 2000; Gautom, 1997). The *S. sonnei* F2353 standard strain was used as the size marker. Briefly, bacteria were grown overnight in brain–heart infusion broth, washed in cell suspension buffer (100 mM Tris/HCl pH 8·0, 100 mM EDTA) and suspended in the same buffer to give a density reading of approximately 0·50 in a Dade turbidity meter (Baxter Diagnostics). Proteinase K (Roche Molecular Biochemicals) was added to washed cells to a concentration of 1 mg ml^{-1}. Bacteria were then directly embedded in an equal volume of 1·2 % SeaKem Gold agarose (Mandel Scientific) and prepared using TE buffer (10 mM Tris/HCl pH 8·0, 1 mM EDTA) containing 1 % SDS. Solidified plugs were transferred to 1·5 ml cell lysis buffer (50 mM Tris/HCl pH 8·0, 50 mM EDTA, 1 % Sarkosyl, 0·1 mg proteinase K ml^{-1}) and incubated at 54 °C in a water bath with shaking at 200 r.p.m. for 2 h. Plugs were washed twice at 50 °C with distilled water (18 MΩ quality) and three times for 15 min with TE. All washes were in water baths with shaking as outlined above. Plug slices were equilibrated in 100 μl buffer H (Roche Molecular Biochemicals) at 37 °C for 15 min and digested at 37 °C for 2 h with 50 U *Xba*I in 200 μl fresh buffer H. After digestion, plugs were equilibrated in 0·5× TBE (0·89 M Tris/HCl, 0·89 M boric acid, 0·02 M disodium EDTA, pH 8·4; Roche Molecular Biochemicals) in 1 % SeaKem Gold agarose at 14 °C. Initial and final switch times were 2·2 and 54·2 s, respectively, and the total run time was 22 h. Following electrophoresis, gels were stained with ethidium bromide (0·5 μg ml^{-1}) and imaged using the GelDoc 1000 system (Bio-Rad).

Molecular serotyping. Molecular serotyping was done according to previously published protocols with minor changes (Coimbra *et al.*, 1999, 2001). Bacteria were grown at 37 °C for 14–18 h on nutrient agar containing 1·5 % NaCl. Bacteria were embedded in low-melting-point agarose, lysed and washed according to the CDC PulseNet protocol for this organism (CDC, 2000) as outlined for PFGE (above). Each agarose plug was melted at 67 °C for 15 min in 500 μl TE buffer and 5 μl volumes were used for long PCR. The PCR primers 482 and 412, complementary to JUMPstart and *gnd*, were used with cycling conditions as previously described (Coimbra *et al.*, 1999). Amplicons were separated by gel electrophoresis in 0·75 % agarose gels using DNA Molecular Weight Marker III (Roche Diagnostics) to aid product size estimation. Between 8 and 20 μl of product was digested for 2 h at 37 °C with 10 U *Mbo*II restriction enzyme (New England Biolabs), followed by denaturation of the enzyme for 10 min at 72 °C. Digested samples were fractionated for 5 h at 140 V (constant voltage) in 2 % agarose gels made with 50 % typing-grade agarose and 50 % Agarose 1000 (Invitrogen Life Technologies) using a 1 kb PlusDNA Ladder (Invitrogen Life Technologies) to allow gel normalization. RFLP patterns were normalized and analysed as outlined below using Bionumerics 2.1 software (Applied Maths). These patterns were compared with a library created in-house using all *E. coli* and *Shigella* serotype reference strains, as well as any additional isolates serotyped at the National Microbiology Laboratory (NML), Canada, using reference and molecular serotyping techniques.

Sequencing. Sequencing of the 16S rRNA gene was performed as described by Edwards *et al.* (1989), using an ABI 3100 gene analyser (Applied Biosystems).

Analysis of data. Interpretation of the PFGE patterns was aided by the Bionumerics version 2.1 software. All associations obtained using this software package were checked visually by at least two laboratory staff familiar with PFGE interpretation. For the isolates analysed in this study, each unique pattern was given a separate designation. Similarity coefficients were obtained within Bionumerics by calculating Dice coefficients. Automated cluster analysis was performed within Bionumerics using the unweighted pair group method with arithmetic means (UPGMA) as a way of organizing banding patterns for visual comparison. Band position tolerances and optimization values of 1 % were used throughout. All pattern matches were confirmed visually.

RESULTS

From 1999 to 2003, a total of 55 laboratory-confirmed cases of *S. boydii* serotype 20 infection were reported in seven Canadian provinces (Table 1). No seasonality was observed in the isolation or in the incidence of disease caused by these organisms. Infection with *S. boydii* serotype 20 was associated with recent travel to Cuba, Ethiopia, India, Guatemala and Mexico. *S. boydii* infections in Canada showed a steady increase between 1999 and 2000 (Table 1). Surveillance data indicated that in 1999 *S. boydii* serotype 20 represented 18 % of laboratory-confirmed cases of *S. boydii* infection in Canada. By the year 2000, serotype 20 was the most frequent causative agent (26 %) of *S. boydii* infections in Canada.

Phenotypic characterization of *S. boydii* serotype 20

S. boydii reference strain 99-4528 appeared microscopically as Gram-negative straight rods. The biochemical reactions of serotype 20 strains are typical of those observed for other *S. boydii* serotypes (Table 2). Laboratory findings from reciprocal agglutination studies showed that the O antigen of strain 99-4528 was unique. This strain showed no agglutination in antisera prepared against the established and provisional serotypes of *Shigella* or in antisera specific for *E. coli* antigens O1 to O181. Of the various recognized *Shigella* serotypes and provisional serotypes, only the reference strain for *S. boydii* serotype 6 agglutinated in antiserum prepared against the O antigen of reference strain 99-4528 of *S. boydii* serotype 20. Adsorption of this antiserum with the reference strain to *S. boydii* serotype 6 was required to ensure the specificity of the *S. boydii* serotype 20 antiserum.

The *S. boydii* phage typing scheme was capable of subdividing isolates from well-characterized serotypes. There was a wide distribution of phage types (PTs) in reference strains

Table 2. Biochemical reactions of *S. boydii* serotype 20 reference strain SH108 (99-4528)

TSI, Triple-sugar iron; V, variable.

Test	Reaction
H_2S (TSI)	−
Urease	−
Indole	V
Methyl red (37 °C)	+
Voges–Proskauer (37 °C)	−
Simmons' citrate	−
Christensen's citrate	−
Sodium acetate	−
Sodium malonate	−
Mucate	−
KCN	−
Motility	−
Lysine decarboxylase	−
Arginine dihydrolase	−
Ornithine decarboxylase	−
Phenylalanine deaminase	−
β-Galactosidase (ONPG)	−
Catalase	+
Oxidase	−
Glucose (acid)	+
Glucose (gas)	−
Lactose	−
Sucrose	−
Mannitol	+
Dulcitol	−
Salicin	−
Adonitol	−
Inositol	−
Sorbitol	+
Arabinose	+
Raffinose	−
Rhamnose	−
Maltose	−
Xylose	+
Trehalose	+
Cellobiose	−
Mannose	+
Glycerol	+
Esculin	−

belonging to *S. boydii* serotypes 1–19. The 50 *S. boydii* serotype 20 isolates were subdivided into 11 different PTs and none of the isolates tested was untypable. The most common PT, representing 64 % of S. *boydii* 20 isolates, was PT 3 (Table 3).

Of the 50 *S. boydii* serotype 20 isolates investigated using antimicrobial susceptibility testing, 45 (90 %) were resistant to more than four antibiotics (Table 4). Two isolates were resistant to six of the antibiotics tested and belonged to PT 5. The association between antibiotic R-type, PT, PFGE pattern and the country of travel for isolates from individuals with travel-associated disease is outlined in Table 5.

Table 3. PT distribution of *S. boydii* serotype 20 isolates in Canada (1999–2002)

PT	No. of isolates	Percentage
1	1	2
2	2	4
3	32	64
5	2	4
6	5	10
8	1	2
10	1	2
11	1	2
14	1	2
16	3	6
17	1	2
Total	50	100

Genotyping and subtyping

All the isolates tested carried the *ipaH* gene and invasion-associated locus, but did not carry any detectable *stx* genes. Sequencing of the 16S rRNA gene locus indicated that the *S. boydii* serotype 20 type strain was closely related to *S. boydii* ATCC 9207 (data not shown). Molecular serotyping through RFLP analysis of the locus responsible for O-antigen biosynthesis indicated that serotype 20 could be distinguished from serotypes 1–19 (Fig. 1). PFGE analysis using *Xba*I was performed on all *S. boydii* serotype 20 isolates and individual reference isolates of *S. boydii* serotypes 1–19. The resulting PFGE patterns were compared (Fig. 2) with *Xba*I PFGE patterns of a small number of *S. sonnei* and *S. flexneri* randomly selected from the PulseNet Canada *Shigella* PFGE database to determine whether PFGE would be a valuable tool for: (i) differentiating among serotypes; and (ii) differentiating among strains within serotype 20.

The collection of 50 serotype 20 isolates was divided into 16 distinct *Xba*I patterns that differed by only a few minor bands against a relatively conserved band arrangement. All serotype 20 isolates showed greater than 90 % pattern similarity to each other, although there appeared to be sufficient pattern variability within even this small population to make PFGE using *Xba*I useful for confirmation of traceback investigations, disease surveillance and detection of case clusters that may be outbreaks. With the exception of serotypes 1, 18 and 19, serotype 20 isolates were less than 85 % similar to the other *S. boydii* serotypes. PFGE patterns of the *S. boydii* serotype 20 isolates clustered more closely with PFGE fingerprints from some *S. sonnei* isolates than with isolates from other *S. boydii* serotypes, indicating that the clustering of PFGE patterns in this population does not reflect phylogenetic relationships. *S. flexneri* PFGE fingerprints also clustered among the *S. boydii* and *S. sonnei* patterns; the isolate showing the greatest PFGE pattern difference was

Table 4. Antibiotic resistance profiles of *S. boydii* serotype 20 isolates with corresponding PTs

A, ampicillin; C, chloramphenicol; Ci, ciprofloxacin; S, streptomycin; Su, sulfadiazine; T, tetracycline; Tm, trimethoprim/sulfamethoxazole.

Antibiogram	No. of isolates	PTs represented
ACSSuTTm	2	PT 5 (2)
ASSuTTm	34	PT 1 (1), PT 2 (1), PT 3 (22), PT 6 (4), PT 8 (1), PT 11 (1), PT 14 (1), PT 16 (2), PT 17(1)
SsuTTm	9	PT 3 (9)
SsuTm	3	PT 2 (1), PT 3 (1), PT 10 (1)
ACST	1	PT 6 (1)
CT	1	PT 16 (1)

Table 5. Antibiotic resistance profiles, PTs and PGFE profiles of *S. boydii* serotype 20 isolates associated with travel

Year	No. of cases	Province*	Country of travel	R-type†	PT	PFGE pattern‡
2002	1	BC	Guatemala	Sensitive	3	SBXAI.0037
2002	1	BC	Guatemala	Sensitive	3	SBXAI.0038
2001	1	AB	India	ST	4	SBXAI.0016
2001	1	AB	India	SsuTTm	4	SBXAI.0009
2001	1	MB	Ethiopia	ASSuTTm	4	SBXAI.0011
2001	1	QB	Mexico	SSuTTm	4	SBXAI.0017
2002	1	NS	Cuba	ASSuTTm	3	SBXAI.0038

*For definitions, see Table 1.

†For definitions, see Table 4.

‡SBXAI, *S. boydii* restricted with *Xba*I.

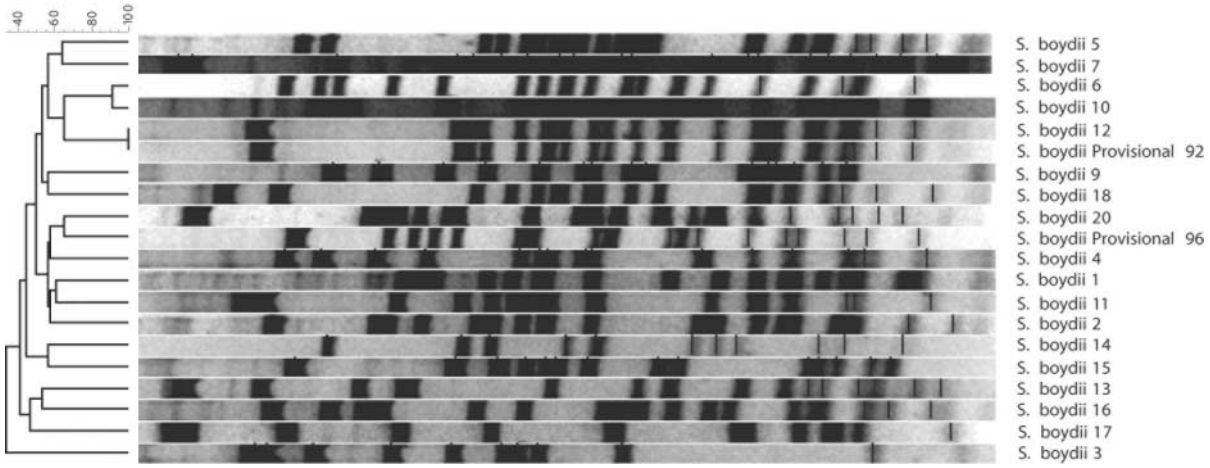

Fig. 1. O-antigen molecular serotyping patterns produced by PCR-RFLP.

S. boydii serotype 13 (Fig. 2). Because *S. boydii* isolates of serotypes other than 20 have either been rarely associated with human disease in Canada or have not been referred to our reference laboratory for more complete characterization, it was not possible to determine whether the PFGE patterns of the isolates used in this study were characteristic of each serotype as a whole.

DISCUSSION

Serological identification is a crucial step in the diagnosis of *Shigella* infections (Coimbra *et al.*, 2001). In 1985, *S. boydii* consisted of a total of 18 serotypes after the addition of three new serotypes, *S. boydii* serotypes 16, 17 and 18 (Gross *et al.*, 1980; Wathen-Grady *et al.*, 1985). Serotype 19, represented

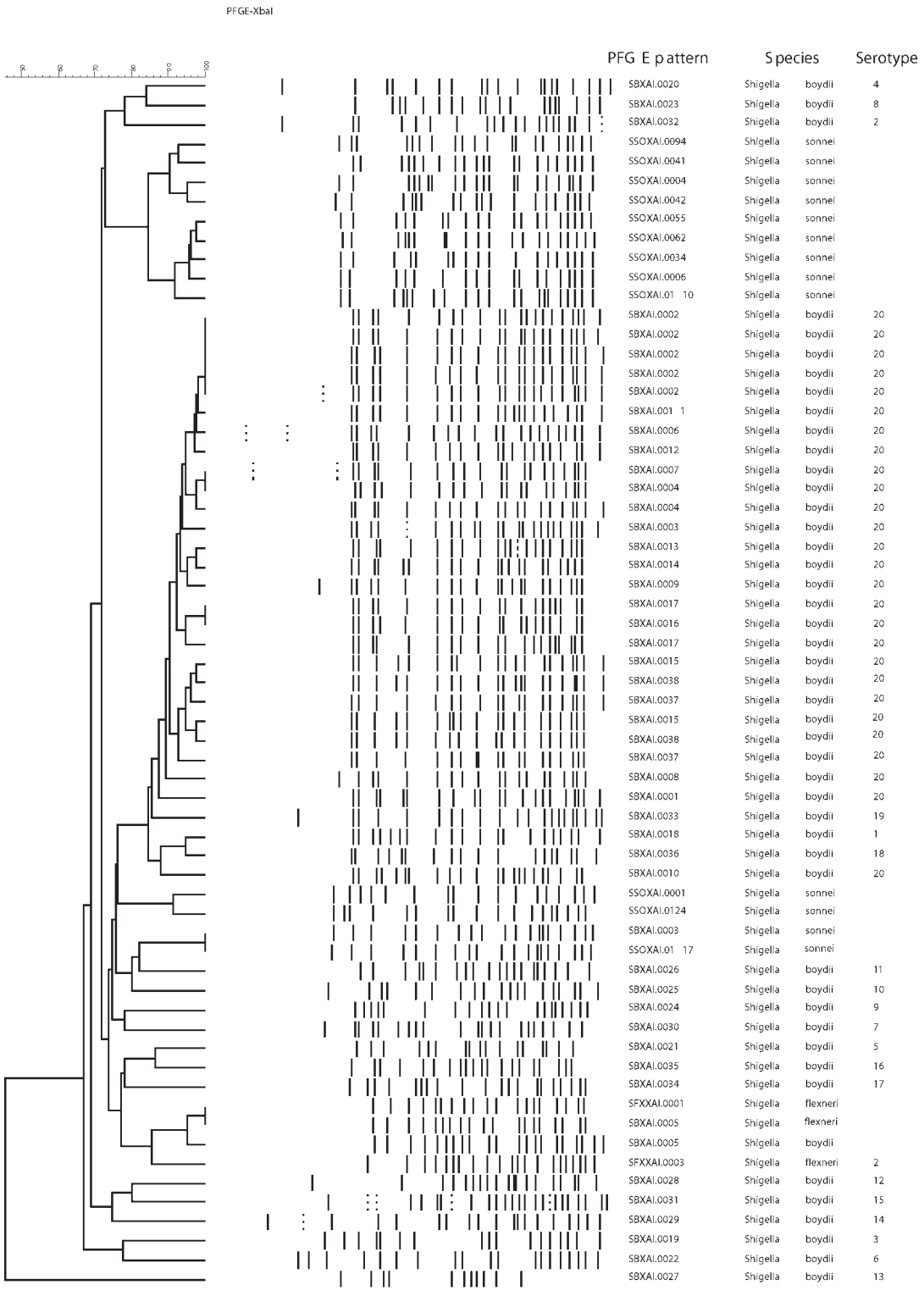

Fig. 2. Dendrogram showing the PFGE patterns of *S. boydii* serotype 20 isolates compared with other *Shigella* serotypes. The dendrogram was constructed with Bionumerics 2.1 software using the unweighted pair group method with arithmetic means (UPGMA) method. Thin lines were added by the program and show the location (peak of densitometry curve) of less-intense bands included in the analysis.

by strain E16553 and first described in 1982, was recently added to the scheme (Gross *et al.*, 1982).

S. boydii represents less than 1–2 % of the total *Shigella* isolated, except in the Indian subcontinent (Keusch & Bennish, 1998). In 1999, the NLEP in Canada identified what appeared to be a new emerging serotype of *S. boydii*. Epidemiological data identified seven cases that have been directly associated with travel to Cuba, Ethiopia, Guatemala, India and Mexico. While the other cases were not directly linked to travel outside Canada, the low prevalence of *S. boydii* endemic to this country suggests that they, too, might be travel associated. Cases of shigellosis associated with travel to Mexico and products exported from Mexico have been well documented (Crowe *et al.*, 1999). Outbreaks of *S. sonnei* infection in the USA and Canada in 1998 were associated with parsley imported from Mexico (Crowe *et al.*, 1999).

Between 2000 and 2003, the CDC received 108 isolates of *S. boydii* serotype 20 from 23 different states across the USA. An outbreak in California in 2001 was associated with 45 cases. At least 12 of the 45 *S. boydii* serotype 20 cases associated with this outbreak were associated with travel to Mexico (Kalluri *et al.*, 2004). As in Canada, the prevalence of cases associated with this serotype has increased dramatically in the USA. The epidemiology of this organism in the USA has recently been described by Kalluri *et al.* (2004).

The *S. boydii* phage typing scheme developed at the NML provided additional discrimination among *S. boydii* strains. PTs appeared to vary independently of serotype. Thus, while PT 3 was characteristic of a majority (64 %) of serotype 20 isolates, it was also found in the reference serotype 19 strain. Both PT and serotype could be used to obtain greater discrimination among strains.

Resistance to antimicrobial agents commonly used in the treatment of shigellosis is a continuing major problem in both developed and developing countries (Keusch & Bennish, 1998). *Shigella* resistant to multiple antibiotics has appeared in all areas of the world, a phenomenon related to the widespread use of antimicrobial reagents (Chin, 2000). In this study high levels of resistance were observed, with 45 (90 %) of the 50 isolates of *S. boydii* serotype 20 tested being resistant to four or more of the seven antibiotics used (Table 4). It has been estimated that 93·5 % of *Shigella* isolates associated with travel to developing nations are resistant to at least one antibiotic (Janda & Abbott, 1998), suggesting that most strains expressing multi-drug resistance may be associated with travel.

It is difficult to assess whether the overall PFGE pattern similarity of the *S. boydii* serotype 20 isolates is characteristic of the serotype as a whole or whether it results from isolates representative of a widely circulating dominant clonal type present in Canada at the time. *Xba*I PFGE fingerprints of serotypes 1, 18 and 19 were similar to those of serotype 20 strains. PFGE patterns from most serotypes exhibited similarities of 85 % or less, suggesting that the larger population of *S. boydii* strains is less homogeneous than the serotype 20 strains studied to date. However, only a few strains have so far been included in the *S. boydii* database and further study is required to validate these observations. Little is known about the population structure of *S. boydii* as a whole or how organisms vary with their place of origin. However, it is clear that *Xba*I PFGE pattern diversity within *S. boydii* is adequate for effective subtyping of strains.

The *Shigella* isolates described here, represented by the reference strain 99-4528, have been characterized using various phenotypic and genotypic epidemiological markers. Based on the identification procedures used, these strains have been designated *S. boydii* 20.

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