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Prevalence of Shiga toxin-producing *Shigella* species isolated from French travellers returning from the Caribbean: an emerging pathogen with international implications

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Abstract

Shiga toxins (Stxs) are potent cytotoxins that inhibit host cell protein synthesis, leading to cell death. Classically, these toxins are associated with intestinal infections due to Stx-producing *Escherichia coli* or *Shigella dysenteriae* serotype 1, and infections with these strains can lead to haemolytic–uraemic syndrome. Over the past decade, there has been increasing recognition that Stx is produced by additional *Shigella* species. We recently reported the presence and expression of *stx* genes in *Shigella flexneri* 2a clinical isolates. The toxin genes were carried by a new *stx*-encoding bacteriophage, and infection with these strains correlated with recent travel to Haiti or the Dominican Republic. In this study, we further explored the epidemiological link to this region by utilizing the French National Reference Centre for *Escherichia coli*, *Shigella* and *Salmonella* collection to survey the frequency of Stx-producing *Shigella* species isolated from French travellers returning from the Caribbean. Approximately 21% of the isolates tested were found to encode and produce Stx. These isolates included strains of *S. flexneri* 2a, *S. flexneri* Y, and *S. dysenteriae* 4. All of the travellers who were infected with Stx-producing *Shigella* had recently travelled to Haiti, the Dominican Republic, or French Guiana. Furthermore, whole genome sequencing showed that the toxin genes were encoded by a prophage that was highly identical to the phage that we identified in our previous study. These findings demonstrate that this new *stx*-encoding prophage is circulating within that geographical area, has spread to other continents, and is capable of spreading to multiple *Shigella* serogroups.

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Introduction

Shiga toxins (Stxs) are cytotoxins that act by inhibiting eukaryotic protein synthesis, eventually leading to host cell death [1]. Stxs are classified as AB_5 toxins on the basis of their structure [2]. They consist of an enzymatically active A subunit, which shows RNA N-glycosidase activity, and a B pentamer, which is responsible for binding of the toxin to glycolipid receptors on the target cell surface. After binding, Stx enters a mammalian cell by endocytosis, and eventually traffics to the endoplasmic reticulum, where the A subunit is proteolytically cleaved to an inactive, A2 subunit and an active A1 subunit, which binds to and inactivates the host cell ribosome [3]. Infections with bacteria that produce Stx can cause haemorrhagic colitis and lead to more serious complications such as haemolytic–uraemic syndrome, a potentially deadly condition [4].

Although Stxs are commonly made by *Shigella dysenteriae* serotype 1 and Stx-producing *Escherichia coli* (STEC), *stx* genes have recently been found in other *Shigella* species [5–7].

S. dysenteriae 1 produces the prototypical Stx, which is encoded on the chromosome within a defective bacteriophage [8]. Stx is secreted by *S. dysenteriae* 1 via an unknown mechanism. In STEC, the Stx family is composed of two different branches, Stx1 and Stx2, which contain many subtypes and variants that are antigenically related [9]. Stxs in the Stx1 family are nearly identical to *S. dysenteriae* 1 toxin, whereas subtypes from the Stx2 family share approximately 50% homology with Stx [10,11]. In contrast to *S. dysenteriae* 1, the toxin genes in STEC are encoded by lambdoid prophages, and toxin release occurs through lytic induction of the prophage [12–14].

In a previous study, we analysed 26 clinical isolates from US public health department laboratories of *Shigella flexneri* 2a that produce and release Stx [6]. The toxin genes in these isolates are carried by a new *stx*-converting phage, ΦPOC-J13. These *S. flexneri* isolates were identified on the basis of their shared pulsed-field gel electrophoresis (PFGE) pattern in the CDC PulseNet database. Additionally, of the patients who reported foreign travel, ~60% had recently visited the island of Hispaniola (Haiti and the Dominican Republic), suggesting that the emergence of these strains is associated with that region.

Here, we further investigated this link between infection with Stx-producing *Shigella* and travel to Hispaniola by surveying the occurrence of *stx*-encoding *Shigella* species in French travellers returning from the Caribbean. Approximately 50–60% of all *Shigella* isolates from France and its overseas 'départements' (administrative subdivisions) are reported to the French National Reference Centre for *Escherichia coli*, *Shigella* and *Salmonella* (FNRC-ESS), located at the Institut Pasteur, Paris, France. The collection includes all serogroups of *Shigella*, and epidemiological data (date and site of isolation, gender, age, and international travel history) are recorded for each case. We utilized the FNRC-ESS collection of *Shigella* species from French travellers who had reported recent travel to the Caribbean to screen for *stx*. The findings reported here support our hypothesis that emergence of Stx-producing *Shigella* is occurring within Hispaniola, show that the *stx*-converting phage responsible has spread to other *Shigella* species, and demonstrate that Stx-producing *Shigella* has spread globally.

Materials and methods

Bacterial strains and growth conditions

Shigella strains were grown in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ, USA) at 37°C with aeration, or on Tryptic Soy Broth plates containing 1.5% agar and 0.025% Congo red (Sigma-Aldrich, St Louis, MO, USA). *E. coli* K-12 strain MG1655 was grown in Luria–Bertani broth and on Luria–Bertani agar plates.

Taxonomic identification of isolates

Shigella 'species' identification was confirmed with conventional methods, and serotyping was performed by slide agglutination assays with a complete set of antisera allowing recognition of all described *Shigella* serotypes [15]. The results of whole genome sequencing confirmed identification of the isolates.

PCR analysis of *Shigella* clinical isolates

DNA was extracted from the *Shigella* clinical isolates with the InstaGene matrix kit (Bio-Rad, Hercules, CA, USA), and screened by PCR for *stx* with the previously described primers Lin 5′ and Lin 3′, which detect *stx* and its variants [16,17]. Subsequent PCR with primers Lin 5′ and VT1b allowed detection of most variants of stx_1, and a PCR with primers Lin 5′ and stx2-R allowed detection of most variants of stx_2. Similarly, DNA extracts were screened for stx_2 with primers Lin 5′ and stx2-R [16,18]. Strains that were positive for *stx* by PCR were further subtyped according to the consensus international methods described in Scheutz *et al.* [9]. The PCRs were carried out with a PCR Taq DNA polymerase kit (Applied Biosystems/Roche, Foster City, CA, USA).

Cell lysates from the *stx*-encoding isolates were analysed by PCR with primer pairs Stx1R2/Phage_stxR2 and Phage_stx1F2/Stx1F2 to show that *stx* was phage encoded [6]. The insertion site of the phage into locus S1742 or a homologous gene was determined by PCR with primers for the upstream region of S1742 and an early phage gene (primers S1742_up and Stx_phage_up), and by amplifying a late phage gene and the downstream region of S1742 with primers Stx_phage_dn and S1742_dn [6]. These PCRs were carried out with PCR Master Mix 2X according to the manufacturer's specification (Fermentas, Pittsburgh, PA, USA).

Cytotoxicity assay

Whole cell lysates and supernatants from the *stx*-encoding isolates were tested in a Vero cell cytotoxicity assay as previously described [6].

Determination of plaque-forming units

Phage particles were isolated from overnight supernatants and absorbed onto *E. coli* MG1655 as previously described [6]. Plaque plates were incubated overnight at 37°C before plaque-forming units were enumerated. The plaques observed were verified to be due to an *stx*-encoding phage by spotting 50 μL of phage prepared from overnight supernatants onto a soft agar overlay of MG1655. After overnight incubation at 37°C, the zone of clearing from where the phage preparation was spotted was removed and analysed by PCR with primers stx1-det-F1 and stx1-seq-R1 to detect *stx* [9]. To confirm that the PCR product from the overlay was not due to bacterial

contamination, supernatant from an *stx*-positive, non-phage-producing strain was spotted, and was shown to be *stx*-negative.

Whole genome sequencing and analysis

Genomic DNA was extracted from overnight cultures with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Sequencing libraries were prepared with either the TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA, USA) or the Nextera DNA Sample Prep Kit (Illumina). DNAs were sequenced on the Illumina MiSeq Platform, generating paired-end 250-bp reads in sufficient quantity to provide over ×35 coverage for each genome. Raw reads were trimmed and draft genome sequences were assembled *de novo* with CLC Genomics Workbench v6.5.1 or v7.0.4 (CLC bio, Boston, MA, USA). In most cases, the entire phage harbouring *stx* was contained on one contig; otherwise, two contigs were bioinformatically joined to obtain the entire phage sequence, and this was then verified by mapping the reads onto the phage sequence.

The *stx*-encoding prophage sequences were extracted from the genomic assemblies of the isolates investigated and aligned to the ΦPOC-J13 phage reference sequence (GenBank accession KJ603229) with the Mauve algorithm within the MegAlign Pro module of the Lasergene software package (DNASTAR, Madison, WI, USA). Phylogenetic analysis of identified single-nucleotide polymorphisms (SNPs) was conducted with SplitsTree 4 [19], by use of the neighbour-net algorithm and untransformed *p* distances.

PFGE

PFGE was performed according to the protocol developed by the CDC (http://www.cdc.gov/pulsenet/pathogens/index.html), with *Salmonella enterica* serotype Braenderup H9812 as the control strain. Agarose-embedded DNA was digested with 50 U of XbaI (Roche Diagnostics, Indianapolis, IN, USA) for at least 2 h in a water bath at 37°C. The restriction fragments were separated by electrophoresis in 0.5X TBE buffer (Invitrogen, Carlsbad, CA, USA) at 14°C for 18 h with a Chef Mapper electrophoresis system (Bio-Rad) and pulse times of 2.16–54.17 s. The gels were stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC, USA), and DNA bands were visualized with UV transillumination (Bio-Rad). PFGE results were analysed with BioNumerics Software v6.6 (Applied-Maths, Kortrijk, Belgium), and banding pattern similarity was compared by use of a 1.5% band position tolerance.

Nucleotide sequence accession numbers

The Whole Draft Genome sequences have been deposited at DDBJ/EMBL/GenBank under the accession numbers listed in Table 1.

Results

A review of records between 1994 and 2008 revealed 67 *Shigella* isolates submitted to the FNRC-ESS that had been obtained from patients who had reported recent travel to Haiti or the Dominican Republic. Of the 67 isolates, 51 were tested for *stx* by PCR. The remaining 16 isolates were either not found in the collection or were not viable. Four randomly selected *Shigella* isolates from French Guiana, a French overseas 'département' in South America, plus one isolate from a traveller returning from French Guiana were also included in the analysis. The isolates included all serogroups of *Shigella* (*Shigella boydii*, *S. dysenteriae*, *S. flexneri*, and *Shigella sonnei*). Of the 51 isolates from patients for whom travel to either Haiti or the Dominican Republic had been reported, 11 were found to be *stx*-positive. This included nine *S. flexneri* 2a isolates, one *S. dysenteriae* 4 isolate, and one *S. flexneri* Y isolate. An *S. flexneri* 2a isolate from a traveller returning from French Guiana was also *stx*-positive; however, the four randomly selected French Guiana isolates were negative for *stx* by PCR. Additionally, all of the isolates analysed were found to be stx_2-

TABLE 1. *stx*-positive *Shigella* isolates from French travellers

Isolate	Species	Year of isolation	Reported travel	Age (years)[a]	Gender	GenBank accession no.
BS1021	*S. flexneri* 2a	2003	Haiti	1–5	Female	LAHV01000000
BS1022	*S. flexneri* 2a	2004	Dominican Republic	15–64	Male	LAHW01000000
BS1023	*S. flexneri* 2a	2005	Dominican Republic	8	Male	LAHX01000000
BS1024	*S. flexneri* 2a	2005	French Guiana	15–64	Male	LAHY01000000
BS1025	*S. flexneri* 2a	2008	Haiti	13	Male	LAHZ01000000
BS1041	*S. flexneri* 2a	1999	Dominican Republic	4	Female	LAIA01000000
BS1042	*S. flexneri* 2a	2005	Dominican Republic	4	Female	LAIB01000000
BS1043	*S. flexneri* Y	2005	Haiti	39	Male	LAIC01000000
BS1044	*S. flexneri* 2a	2005	Dominican Republic	1–5	Female	LAID01000000
BS1045	*S. flexneri* 2a	2007	Dominican Republic	31	Male	LAIE01000000
BS1046	*S. flexneri* 2a	2008	Dominican Republic	4	Female	LAIF01000000
BS1047	*S. dysenteriae* 4	2008	Haiti	50	Male	LAIG01000000

[a]For some patients, the exact age was not recorded, but was rather described as a range.

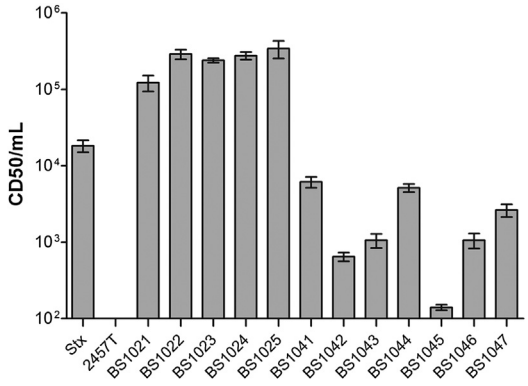

FIG. 1. *stx*-encoding *Shigella* species from French travellers release a functional toxin. Overnight supernatants were serially diluted ten-fold in medium, and applied to Vero cells to test for toxicity. Stx from *Shigella dysenteriae* 1 was included as a positive control. CD_{50}/mL is defined as the reciprocal of the dilution of Stx that kills 50% of Vero cells. Data represent an average of three independent experiments.

negative. Limited clinical data were available; however, the patients presented with symptoms of an intestinal infection characteristic of shigellosis. Travel, isolation date and patient information for the 12 *stx*-positive isolates are shown in Table 1.

The 12 *stx*-positive isolates were further characterized by determining whether they produced a functional toxin. Supernatants from overnight cultures of the *stx*-encoding *Shigella* isolates were cytotoxic to Vero cells as compared with an *stx*-negative laboratory strain of *S. flexneri*, 2457T (Fig. 1). The CD_{50}/mL values for the clinical isolates ranged between ~1.5 × 10^2 and ~5 × 10^5, demonstrating that all of the *stx*-positive isolates produced and released toxin. As the toxin genes are typically encoded by lambdoid prophages, we also monitored for the presence of viable phage progeny in overnight supernatants by performing a plaque assay with the *E. coli* indicator strain MG1655. Eight of the isolates formed plaques on MG1655. Viable phage progeny were not detected from three *S. flexneri* 2a isolates (BS1022, BS1045, and BS1046) and the *S. flexneri* Y isolate (BS1043). Failure of supernatants from these four isolates to produce plaques on MG1655 may be due to resistance of MG1655 to the phage or to mutations resulting in a defective phage (see sequencing analysis below).

Although the plaque assay demonstrated that the isolates were making viable phage progeny, we wanted to confirm that the plaques observed were due to an *stx*-encoding phage. In order to harvest enough DNA, we used a phage-spotting assay on MG1655, and performed PCR analysis from the zone of clearing. All of the isolates that formed plaques on MG1655 formed a zone of clearing and were *stx* positive, except for BS1047 (data not shown). Although it is not completely clear why BS1047 was capable of forming plaques when incubated with MG1655 in liquid culture but did not produce a zone of clearing in the spotting assay, differences between the two assays could probably explain this.

In our previous study, we designed primers based on the ΦPOC-J13 sequence to show that the *stx* genes in all 26 of the isolates analysed in that study were flanked by phage sequence. We utilized those primers to analyse the 12 *stx*-positive isolates from the French travellers, and found that their *stx* locus was also surrounded by a phage sequence similar to ΦPOC-J13 (data not shown). Additionally, our previous analysis showed that ΦPOC-J13 is inserted in the *S. flexneri* chromosome at locus S1742, which encodes a putative oxidoreductase. On the basis of our primer sequences designed from ΦPOC-J13, we determined that the phage in each of the 12 *stx*-positive French isolates had also inserted at locus S1742 or a homologous gene (data not shown).

Our PCR analyses and the travel link to Hispaniola suggested that the phage in the *Shigella* isolates from the French travellers is also ΦPOC-J13. To investigate the similarity to ΦPOC-J13, whole genome sequencing was performed on all 12 isolates. The DNA sequences of the *stx*-encoding prophages from the 12 isolates were aligned and compared with ΦPOC-J13 (GenBank accession KJ603229) (Fig. 2a). The set of 13 prophages were nearly identical in sequence, with only 13 SNPs being identified among them, mostly within hypothetical or putative protein-encoding regions. Three insertion/deletion sites were observed in isolates BS1024, BS1041, BS1045, and BS1046. In addition to the 13 SNPs and three insertion/deletion sites, four IS elements were observed among the isolates investigated (Fig. 2a). Isolate BS1022 contained an IS1 element in an intergenic region and an IS2 element in a 2022-bp gene encoding a tail fibre protein. Isolate BS1046 also contained an IS2 element inserted in a large, 8.4-kb hypothetical protein-encoding gene. Finally, isolate BS1047 contained an IS2 element within a 546-bp putative tail fibre adhesin-encoding gene. It is uncertain what affect these mutations may have on Stx production and the production of infectious phage particles; however, it is possible that they may account for the varying results that were observed in our assays above.

The 13 SNPs identified were used to construct a phylogenetic tree of the relationships among the *stx*-encoding prophages from the 12 isolates investigated and ΦPOC-J13 (Fig. 2b). The resulting unrooted phylogeny places the prophage from isolate BS1022 (minus the two IS elements) as the potential founder, with eight of the prophages being only one SNP different from the BS1022 prophage sequence. The prophages from isolates BS1047 and ΦPOC-J13 are the most divergent, with three and four SNP differences from the BS1022

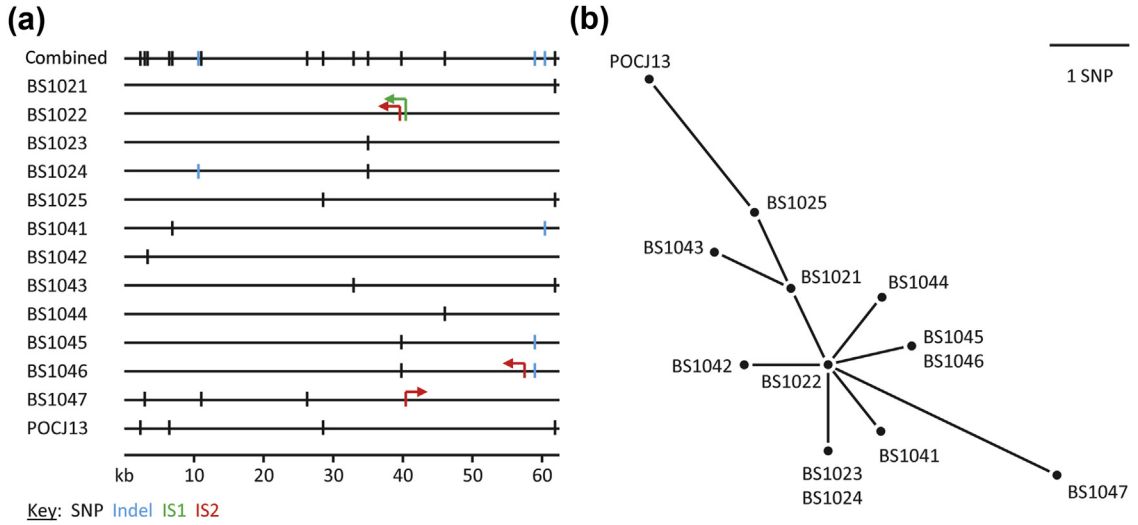

FIG. 2. *stx*-encoding prophage sequence similarity. (a) Single-nucleotide polymorphisms (SNPs), insertion/deletion (indel)sites and IS elements within the prophage sequences of the isolates investigated. SNP locations are indicated by vertical black lines, and indel sites by blue lines. The locations and orientations of the IS1 and IS2 elements are indicated by the green and red arrows, respectively. (b) SNP-based phylogenetic relationships of the prophages.

prophage, respectively. These findings indicate that all of the Stx-producing *Shigella* species isolated have acquired the same phage.

The Stx-producing *S. flexneri* 2a isolates from our previous study were identified in PulseNet on the basis of their shared PFGE pattern, JZXX01.0357. To determine whether these new isolates shared the same signature pattern, PFGE was performed on seven of the isolates from the French travellers. None of the patterns matched JZXX01.0357 (BS937). Moreover, each of the seven PFGE patterns was different (Fig. 3). Only one of the isolates (*S. flexneri* 2a, BS1045) had a pattern number already in the PulseNet Database, JZXX01.1361. We used this pattern to search for matches, and found two isolates that were 100% matched: one isolate from the Massachusetts state laboratory, and one isolate from the Maryland state laboratory.

Discussion

Overall, 12 of 56 (~21%) *Shigella* isolates analysed from the FNRC-ESS collection of French travellers returning from the Caribbean were found to produce Stx. This finding reinforces our hypothesis that the emergence of *stx*-encoding *Shigella* species is originating from Haiti and the Dominican Republic. Furthermore, the finding that the isolates from the French travellers did not share the same PFGE pattern with each other, or with the previously published Stx-producing *S. flexneri* 2a strains, highlights the importance of using multiple approaches to identify these new strains of *stx*-encoding *Shigella*.

This is the first report of this new *stx*-encoding phage harboured in different *Shigella* species and serotypes, indicating that ΦPOC-J13 and homologous *stx*-encoding phages are capable of spreading to multiple *Shigella* species. Because we have limited

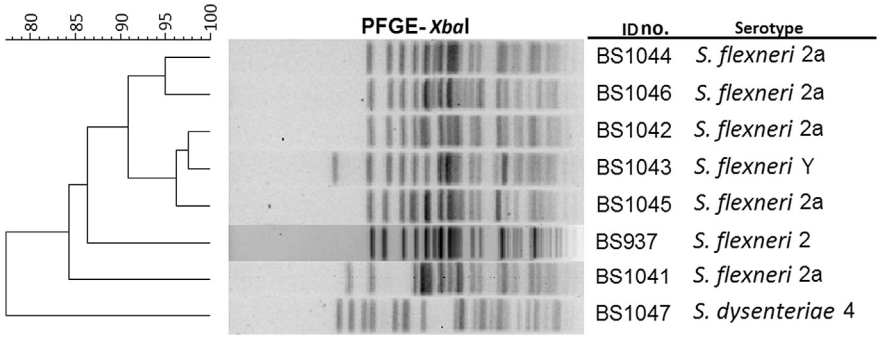

FIG. 3. Dendrogram of pulsed-field gel electrophoresis (PFGE) analysis (XbaI) of Stx-producing *Shigella*. The PFGE patterns from seven of the Stx-producing *Shigella* isolates from French travellers (BS1044, BS1046, BS1042, BS1043, BS1045, BS1041, and BS1047) are compared with that of BS937 (PulseNet PFGE pattern JZXX01.0357).

clinical information on the isolates, the health consequences of infection with Stx-producing *Shigella* remain unclear. However, infections with *S. dysenteriae* 1 and STEC result in complications of haemolytic–uraemic syndrome in ~10% of cases [20]. Therefore, these new Stx-producing *Shigella* isolates have the potential to cause more severe disease than is typically associated with non-*S. dysenteriae* type 1 infections.

Finally, we have now identified Stx-producing *Shigella* in both French and US travellers who had recently visited the island of Hispaniola. We have also isolated *stx*-positive *Shigella* from Haitian children in Haiti (manuscript in preparation). It is still uncertain what environmental factors have contributed to the emergence of these species in that region. However, our findings imply that travellers are capable of spreading these *Shigella* strains globally. It is impossible to predict the extent of international spread of Stx-producing *Shigella*. Nonetheless, one could speculate that, as the strains spread, they may become capable of persisting in the ecosystems of other regions, and that the *stx*-encoding phage may spread to other *Shigella* species in those regions. If either of those phenomena occurs, infections with Stx-producing *Shigella* may become more prevalent worldwide.

Transparency declaration

The authors have no conflicts of interest to disclose.

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