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

M. D. Gray¹, D. W. Lacher², S. R. Leonard², J. Abbott², S. Zhao², K. A. Lampel², E. Prothery³, M. Gouali³, F.-X. Weill³ and A. T. Maurelli¹

¹) Uniformed Services University of the Health Sciences, Bethesda, 2) US Food and Drug Administration, Laurel, MD, USA and 3) Institut Pasteur, Unité des Bactériennes Pathogènes Entériques, Centre National de Référence des Escherichia coli, Shigella et Salmonella, Paris, France

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.

Clinical Microbiology and Infection published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases.

Keywords: Dominican Republic, Haiti, Shiga toxin, Shigella

Original Submission: 3 February 2015; Revised Submission: 1 May 2015; Accepted: 2 May 2015
Editor: F. Allerberger
Available online 14 May 2015

Introduction

Shiga toxins (Stxs) are cytotoxins that act by inhibiting eukaryotic protein synthesis, eventually leading to host cell death [1]. Stxs are classified as AB₅ 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 pulse-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.

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 stx1, and a PCR with primers Lin 5’ and stx2-R allowed detection of most variants of stx2. Similarly, DNA extracts were screened for stx2 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 its variants [16,17]. Similarly, DNA extracts were screened for stx2 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 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 stxl-det-F1 and stxl-seq-R1 to detect stx [9]. To confirm that the PCR product from the overlay was not due to bacterial

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.
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 TrueSeq 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 for 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-

---

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

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Year of isolation</th>
<th>Reported travel</th>
<th>Age (years)*</th>
<th>Gender</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS1021</td>
<td>S. flexneri 2a</td>
<td>2003</td>
<td>Haiti</td>
<td>1–5</td>
<td>Female</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1022</td>
<td>S. flexneri 2a</td>
<td>2004</td>
<td>Dominican Republic</td>
<td>15–64</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1023</td>
<td>S. flexneri 2a</td>
<td>2005</td>
<td>Dominican Republic</td>
<td>8</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1024</td>
<td>S. flexneri 2a</td>
<td>2005</td>
<td>French Guiana</td>
<td>15–64</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1025</td>
<td>S. flexneri 2a</td>
<td>2008</td>
<td>Haiti</td>
<td>13</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1041</td>
<td>S. flexneri 2a</td>
<td>1999</td>
<td>Dominican Republic</td>
<td>4</td>
<td>Female</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1042</td>
<td>S. flexneri 2a</td>
<td>2005</td>
<td>Dominican Republic</td>
<td>4</td>
<td>Female</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1043</td>
<td>S. flexneri 2a</td>
<td>2005</td>
<td>Haiti</td>
<td>39</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1044</td>
<td>S. flexneri 2a</td>
<td>2005</td>
<td>Dominican Republic</td>
<td>1–5</td>
<td>Female</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1045</td>
<td>S. flexneri 2a</td>
<td>2007</td>
<td>Dominican Republic</td>
<td>31</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1046</td>
<td>S. flexneri 2a</td>
<td>2008</td>
<td>Dominican Republic</td>
<td>4</td>
<td>Female</td>
<td>LAHV01000000</td>
</tr>
<tr>
<td>BS1047</td>
<td>S. dysenteriae 4</td>
<td>2008</td>
<td>Haiti</td>
<td>50</td>
<td>Male</td>
<td>LAHV01000000</td>
</tr>
</tbody>
</table>

*For some patients, the exact age was not recorded, but was rather described as a range.*
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 CD50/mL values for the clinical isolates ranged between $1.5 \times 10^2$ and $5 \times 10^5$, 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 the 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: 12203958.12457349

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 I was included as a positive control. CD50/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.
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
clinical information on the isolates, the health consequences of infection with Stx-producing Shigella remain unclear. However, infections with Shigella dysenteriae type 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.

Acknowledgements

We thank all of the corresponding laboratories of the FNRC-ESS network. We thank I. Carle, M. Lejay-Collin, C. Ruckly, S. Darnell and R. Fernandez for their excellent technical assistance. The FNRC-ESS is co-funded by the Institut de Veille Sanitaire. The Unité des Bactéries Pathogènes Entériques belongs to the Integrative Biology of Emerging Infectious Diseases Laboratory of Excellence funded by the French Government’s Investissement d’Avenir program (grant no. ANR-10-LABX-62-IBEID). This work was also supported by grant R01A124656 from the National Institute of Allergy and Infectious Diseases. The results included herein were previously presented at the 49th US–Japan Conference on Cholera and Other Enteric Bacterial Infections and the 2015 Mid-Atlantic Microbial Pathogenesis Meeting. The opinions or assertions contained herein are the private ones of the authors, and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

References