# Whole genome sequencing identifies independent outbreaks of Shigellosis in 2010 and 2011 in La Pampa Province, Argentina

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**Abstract**

*Shigella sonnei* is an emergent cause of diarrheal disease in middle-income countries. The organism causes endemic disease and is also associated with sporadic outbreaks in susceptible populations. In 2010 and 2011 there were two suspected outbreaks of diarrheal disease caused by *S. sonnei* in La Pampa province in central Argentina. Aiming to confirm these as outbreaks and provide insight into the relationship of the strains causing these infections we combined antimicrobial susceptibility testing and pulsed field gel electrophoresis (PFGE) with whole genome sequencing (WGS). Antimicrobial susceptibility testing suggested the two events were unrelated; organisms isolated in 2010 exhibited resistance to trimethoprim sulphate whereas the 2011 *S. sonnei* were non-susceptible against ampicillin, trimethoprim sulphate and cefpodoxime. PFGE profiling confirmed the likelihood of two independent outbreaks, separating the isolates into two main XbaI restriction profiles. We additionally performed WGS on 17 isolates associated with these outbreaks. The resulting phylogeny confirmed the PFGE structure and separated the organisms into two comparatively distantly related clones. Antimicrobial resistant genes were common, and the presence of an OXA-1 was likely associated with resistance to cefpodoxime in the second outbreak. We additionally identified novel horizontally transferred genetic material that may impinge on the pathogenic phenotype of the infecting strains. Our study shows that even with a lack of supporting routine data WGS is an indispensible method for the tracking and surveillance of bacterial pathogens during outbreaks and is becoming a vital tool for the monitoring of antimicrobial resistant strains of *S. sonnei*.

79 **Report**

80 Dysenteric diarrhea caused by members of the bacterial genus *Shigella* (comprised of the
81 species *S*. flexneri, *S*. sonnei, *S*. boydii and *S*. dysenteriae) remains an on-going public health
82 issue in many industrializing countries. It is estimated that the global burden of disease
83 caused by *Shigella* spp. is ~125 million cases annually [1], the majority of these cases arise
84 in children aged under five years. The Global Enteric Multicenter Study (GEMS), a case-
85 control study of paediatric diarrheal disease conducted in Africa and Asia, found that
86 enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* were the two most common bacterial
87 agents of diarrhea in sub-Saharan Africa and South Asia [2,3]. Notably, *Shigella* spp. were
88 the most prevalent pathogen among children between 24 and 59 months old [3].
89
90 Of the four *Shigella* species *S. flexneri* and *S. sonnei* are responsible for the vast majority of
91 the global burden of disease. Traditionally, *S. sonnei* has been the predominant cause of
92 bacterial dysentery in industrialized countries, whereas *S. flexneri* has been considered to
93 be associated with endemic disease and travel to lower income countries [4]. However, this
94 trend is changing as *S. sonnei* is now emerging as a problem in lower middle-income
95 countries, seemingly replacing *S. flexneri* as the leading cause of dysentery in these locations
96 [5]. This trend has also been observed in parts of Latin America [6,7], roughly correlating
97 with improvements in sanitation, water quality and, potentially, a fall in passive immunity
98 against *S. sonnei* via a decline in other bacteria associated with poor water quality [8]
99
100 Argentina is a middle-income country in South America with endemic Shigellosis [9], the
101 number of officially reported cases of Shigellosis in 2014 was 4,116. This represents a
102 comparatively high proportion of the 6,200 cases of confirmed the cases of bacterial
103 diarrhea in 2014. *Shigella* outbreaks occur sporadically and rapidly and are frequently

104 associated with changes in antimicrobial susceptibility [10,11]. Aiming to better understand
105 the dynamics of Shigellosis in Argentina we gathered bacterial isolates from two suspected
106 outbreaks of Shigellosis investigated by the public health authorities in Argentina between
107 2010 and 2011. The two temporally independent events were attributed to *S. sonnei* and
108 occurred within the La Pampa province in the central region of the country. In this
109 retrospective study we combined available epidemiological data and microbiological data
110 with Pulsed Field Gel Electrophoresis (PFGE) and whole genome sequencing (WGS) of the *S.*
111 *sonnei* isolates from the suspected outbreaks in 2010 and 2011 to investigate their
112 relatedness. We also analysed the discriminatory ability of WGS compared to PFGE, the
113 current international gold-standard method for public health strain tracking.
114
115 In December 2009 the Gobernador Centeno hospital in the city of General Pico reported an
116 increased number of cases of diarrhea above the expected endemic rate, an outbreak was
117 suspected. The first *Shigella* (confirmed by standard microbiological methods to be *S.*
118 *sonnei*) was isolated on the 7th January 2010; the last culture confirmed case of *S. sonnei* was
119 on the 26th February 2010. The cases were distributed throughout (i.e. no apparent case
120 clustering) General Pico. There were 26 reported cases, of which detailed microbiological
121 data was available on nine. Of the 26 reported cases, 13 were in children aged between 0-5
122 years, and 13 cases were aged between 6-69 years (median: five years); 10 cases were
123 female and 16 were male. No epidemiological association was recorded between cases,
124 apart from a potential cluster in a single household (n=4 cases). The most common disease
125 presentations were diarrhea with blood and mucus (13/26; 50%) and diarrhea with blood
126 without mucus (11/26; 42%). Of the nine available *S. sonnei* organisms isolated during the
127 potential 2010 outbreak, all had an identical antimicrobial susceptibility patterns by disc
128 diffusion [12], exhibiting susceptibility against ampicillin, ciprofloxacin, nitrofurantoin,

129 fosfomycin, naladixic acid and cefpodoxime and non-susceptibility against trimethoprim
130 sulphate.
131
132 The second potential outbreak occurred the city of Castex, also in the province of La Pampa
133 (60 Km from General Pico) in the summer of 2011 (3rd February 2011 to 30th March 2011).
134 No supporting epidemiological data were available for this potential outbreak and six *S.*
135 *sonnei* were isolated. An equal proportion of males and females were infected and the
136 patient age range was 5-26 years (median: eight years). The antimicrobial susceptibility
137 profile of the organisms demonstrated that all organisms were non-susceptible against
138 ampicillin, trimethoprim sulphate and cefpodoxime. Additional laboratory testing suggested
139 that all organisms in this second potential outbreak exhibited AmpC production.
140
141 To confirm the likelihood of outbreaks and to investigate the temporal and spatial
142 relationship between organisms we performed PFGE after XbaI digestion on 17 available
143 isolates from 2010 (n=9) and 2011 (n=7) and one additional contextual strain isolated in
144 General Pico in 2013 using standardized PulseNet protocols as previously described (Figure
145 1) [13]. The XbaI PFGE generated nine differing restriction patterns that could be grouped
146 into two major groups (ARJ16X01.0086 and ARJ16X01.0318) that correlated precisely with
147 their year of isolation; ARJ16X01.0086 is the most frequent pattern described in Argentina.
148 These major restriction patterns differed by six fragments and had 85% pattern similarity.
149 Additional PFGE with BlnI (again following standardized PulseNet protocols [13]) methods
150 of on a limited subsample confirmed this grouping, signifying independent outbreaks likely
151 caused by two differing clones of *S. sonnei* that could be distinguished by their antimicrobial
152 susceptibility patterns. These cases clusters were additionally confirmed using the SatScan
153 function in WHONET software.

PFGE does not provide sufficient resolution for phylogenetic inference, to understand the genetic relationship between the organisms from the two outbreaks we performed WGS on nine *S. sonnei* isolated in 2010, seven *S. sonnei* isolated in 2011 and the single contextual isolate from 2013. We identified single nucleotide polymorphisms (SNPs) in comparison to a Chinese reference strain (Ss046, accession number CP000038[14]), as previously described [11,15,16], and constructed a maximum likelihood phylogeny of the 18 strains, identifying approximately 1,500 variable nucleotide sites (Figure 2). Our data confirmed that the outbreaks were associated with two differing, distantly related clones of *S. sonnei*. The first clone (-10 suffix in Figure 2) was comprised of the 2010 isolates; six were highly related, with three remaining isolates in the same group but located on longer branches. The second clone (-11 suffix in Figure 2) contained all six isolates from 2011; these isolates were almost identical, containing less than 10 nucleotide substitutions across their genomes. An additional isolate from 2011 (1193-11) lay outside this group and was deemed not to part of the same clonal outbreak. Notably, the 2011 clone could be distinguished by non-susceptibility against cefpodoxime (yellow nodes in Figure 2) (Data accessible and viewable at http://microreact.org/project/EkJeuWfx-).

We next assembled the genome sequences from the two independent outbreaks to identify additional horizontally transferred genetic material that may be associated with each of the clones and to classify the genes associated with changes in antimicrobial susceptibility. We found that the -10 clone contained a *dfrA1* gene, which is associated with resistance against trimethoprim sulphate. Further, we identified genes associated with resistance to additional antimicrobials that were not tested, including streptomycin (*strAB*), tetracycline (*tetAR*) and sulphonomides (*sulII*). The -11 clone also contained a *dfr* gene (A5) and genes associated

179 with resistance against chloramphenicol (*catA1*), streptomycin (*strAB*), tetracycline (*tetB*)
180 and sulphonomides (*sulII*). We also identified several AmpC β-lactamase genes potentially
181 explaining the non-susceptibility against cefpodoxime including CMY and the Extended
182 Spectrum Beta Lactamase (ESBL) gene, OXA-1. Further, in the -11 clone we identified and
183 assembled a large (>90 Kb) plasmid that exhibited substantial homology and synteny to the
184 recently described 96 Kb p12-4374_96 plasmid in *Salmonella* Heidelberg [17] (accession
185 number: CP012929). This plasmid, not previously described in *Shigella*, encoded a
186 multitude of potentially interesting functions including a conjugation system, a type IVb
187 pilus and the ethanolamine utilization protein, EutE.
188
189 Here we have combined traditional methods for tracking bacterial pathogens
190 (antimicrobial susceptibility testing and PFGE) and combined them with WGS to evaluate
191 two potential outbreaks of Shigellosis in a single province in 2010 and 2011 in Argentina.
192 Our data suggests that there were two independent outbreaks of *S. sonnei* induced diarrhea
193 in 2010 and 2011, finding that the organisms causing these case clusters were distantly
194 related to each other. This was somewhat unexpected given the geographical proximity of
195 these two locations and signifies that multiple clones of *S. sonnei* are likely circulating in
196 Argentina, several of which have outbreak potential. We found that the antimicrobial
197 susceptibility profile was sufficient to distinguish between these outbreaks, providing an
198 almost perfect temporal correlation with cefpodoxime resistance. This relationship was
199 further confirmed by PFGE, the current gold standard for strain tracking in such scenarios
200 in Argentina [18]. However, PFGE additionally over predicted the variability within the
201 genomic structures, identifying several banding patterns within the specific clones.
202
203 In this particular investigation WGS augmented the findings of the conventional approaches

and provided new insight into these outbreaks. Firstly, the phylogenetic inference, which has become standard for WGS of *S. sonnei* [11,15,16], permitted us an exquisite view of the relationship between and within the outbreaks, eventually confirming the two outbreaks. Further, assembly of the genome sequences identified the presence of the range antimicrobial resistance genes, predicting resistance to additional antimicrobials that were not susceptibility tested. These data permitted us to detect the ESBL gene OXA-1 [19], which we hypothesized to be associated with resistance against the third generation cephalosporin, cefpodoxime. ESBL genes are becoming more commonly reported in *Shigella* in Asia [20]. Our data predict that this concerning phenomenon is additionally occurring in Latin America via differing determinants. Oral third generation cephalosporins are one of the current mainstays of treatment for Shigellosis in Argentina, we recommend further genomic surveillance in this region to detect circulating beta lactamase genes. The assembled genome sequences additionally identified further novel sequences encoding potential virulence associated loci that may have a phenotypic effect during infection. These novel genes require conformation and additional experimentation to confirm their role in disease.

Our study contains some limitations including a lack of strain diversity for better phylogenetic inference and a lack of epidemiological data. However, our investigation of these outbreaks represents a “real life” scenario, where limited data hamper contextualization. Here we show that even with a lack of supporting routine data WGS becomes an indispensible method for the tracking and surveillance of bacterial pathogens during outbreaks.

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**Declaration of interests**

The authors declare no competing interests.

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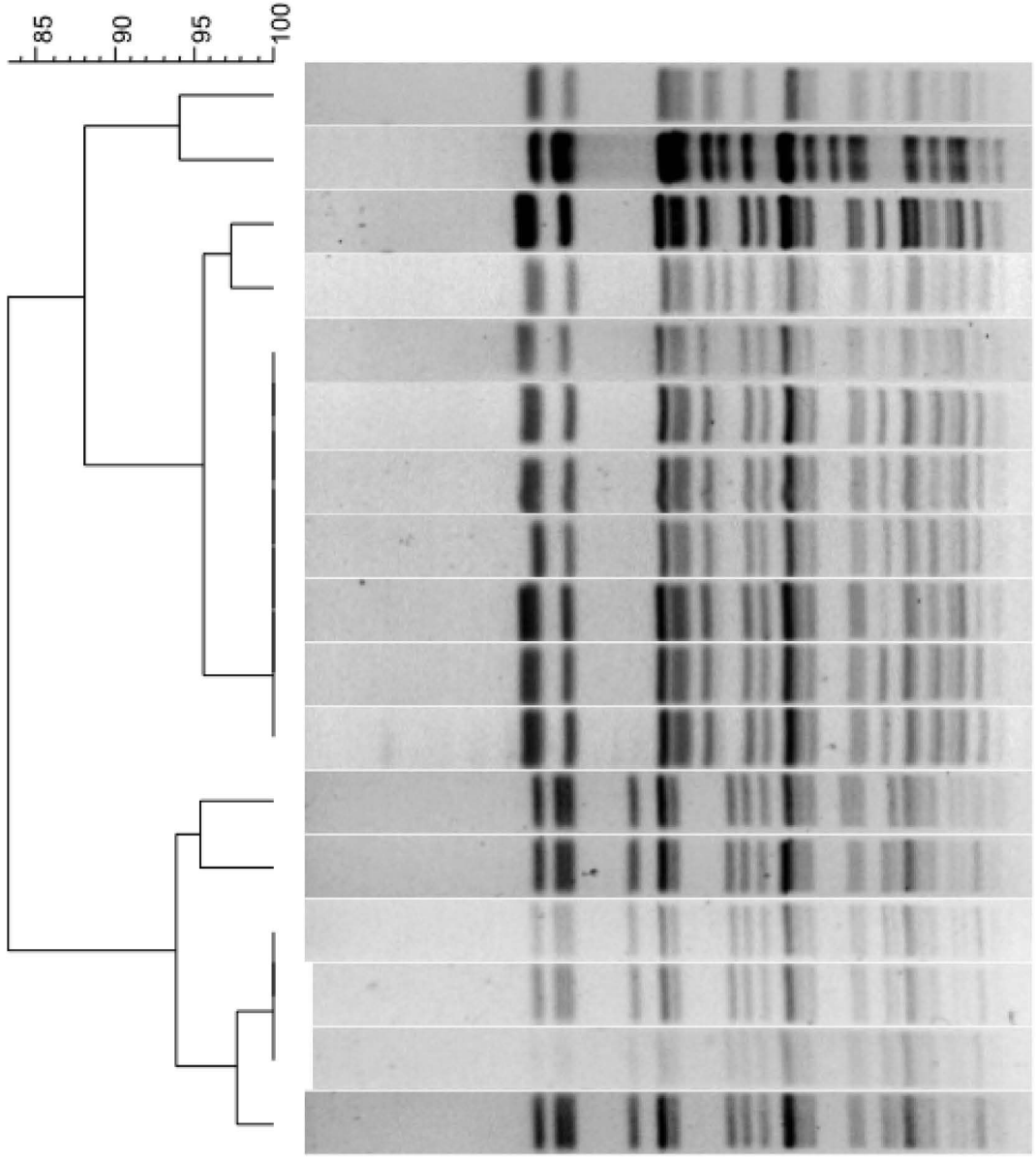
**Figure 1**. The relationship between *Shigella sonnei* isolated in independent outbreaks in La Pampa province, Argentina in 2010 and 2011

Dendogram based on PFGE profile after XbaI digestion of *Shigella sonnei* isolated from stool samples in 2010 (General Pico) and 2011 (Castex). One additional isolate from 2013 (General Pico) was included as contextual strain. Information regarding the strain ID, the date of isolation and the digestion pattern (according to PulseNet Latinoamerica) are provided.

**Figure 2**. The phylogenetic relationship of *Shigella sonnei* isolated in independent outbreaks in La Pampa province, Argentina in 2010 and 2011

Unrooted maximum likelihood tree constructed using approximately 1,500 variable nucleotide sites from nine organisms isolated in 2010, seven isolates from 2010 and a single isolate from 2013 in La Pampa province, Argentina. *S. sonnei* Ss046 was added as the reference strain. Tree was viewed in microreact and nodes are labelled with the strain name and the year of isolation suffix collared according to susceptibly against cefpodoxime susceptibility (orange; susceptible, yellow, non-susceptible).




| Sample ID | Isolation date | XbaI pattern |
|---|---|---|
| SS1193/11 | 2011-06-13 | ARJ16X01.0271 |
| SS843/13 | 2013-04-03 | ARJ16X01.0410 |
| SS393/10 | 2010-02-03 | ARJ16X01.0083 |
| SS409/10 | 2010-02-08 | ARJ16X01.0084 |
| SS392/10 | 2010-01-27 | ARJ16X01.0086 |
| SS397/10 | 2010-01-26 | ARJ16X01.0086 |
| SS400/10 | 2010-01-27 | ARJ16X01.0086 |
| SS401/10 | 2010-01-27 | ARJ16X01.0086 |
| SS404/10 | 2010-02-02 | ARJ16X01.0086 |
| SS405/10 | 2010-01-27 | ARJ16X01.0086 |
| SS408/10 | 2010-02-02 | ARJ16X01.0086 |
| SS995/11 | 2011-03-30 | ARJ16X01.0334 |
| SS996/11 | 2011-03-30 | ARJ16X01.0245 |
| SS480/11 | 2011-02-03 | ARJ16X01.0318 |
| SS481/11 | 2011-02-04 | ARJ16X01.0318 |
| SS482/11 | 2011-02-23 | ARJ16X01.0318 |
| SS994/11 | 2011-03-22 | ARJ16X01.0338 |


SS405-10
SS409-10
SS401-10
SS408-10
SS392-10
SS404-10
SS393-10
SS397-10
SS400-10
SS1193-11
Ss046
SS843-13
SS995-11
SS996-11
SS480-11
SS994-11
SS481-11
SS482-11