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

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54 **Abstract**

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

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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* (EPEC) 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 7<sup>th</sup> January 2010; the last culture confirmed case of *S. sonnei* was  
119 on the 26<sup>th</sup> 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 (3<sup>rd</sup> February 2011 to 30<sup>th</sup> 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.

154

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

171

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



179 with resistance against chloramphenicol (*catA1*), streptomycin (*strAB*), tetracycline (*tetB*)  
180 and sulphonamides (*sulII*). We also identified several AmpC  $\beta$ -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

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

220

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

227

228

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237

238 **Declaration of interests**

239 The authors declare no competing interests.

240

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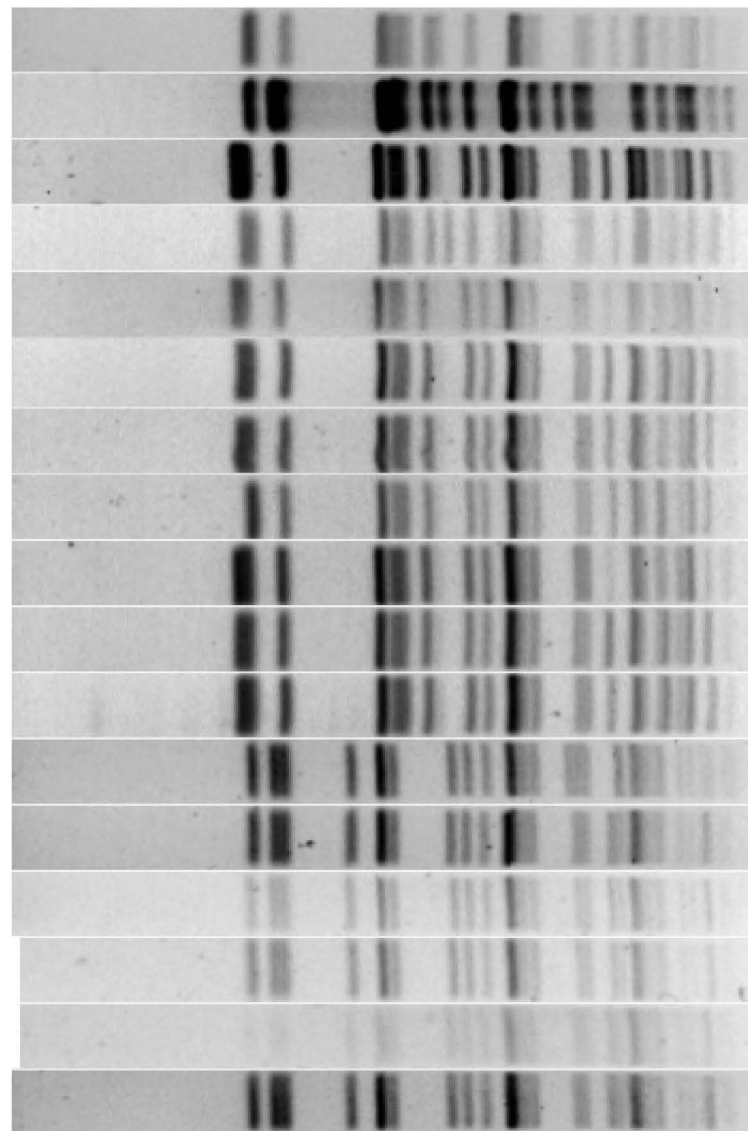
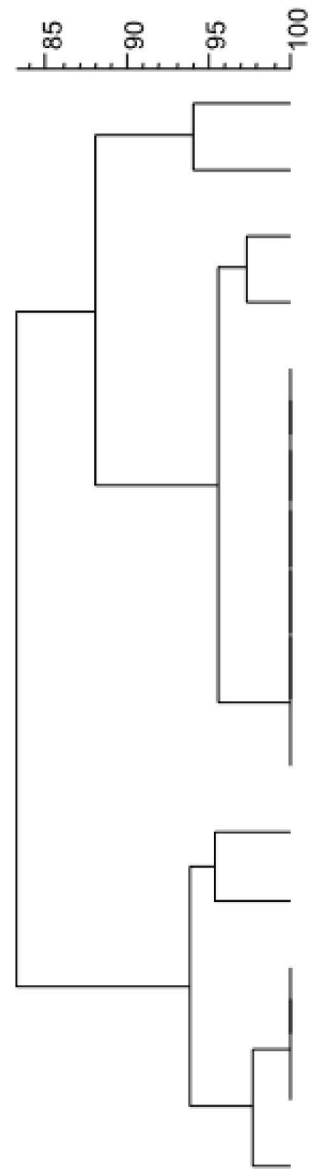
306 **Figure 1.** The relationship between *Shigella sonnei* isolated in independent outbreaks in La  
307 Pampa province, Argentina in 2010 and 2011

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

313

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

316 Unrooted maximum likelihood tree constructed using approximately 1,500 variable  
317 nucleotide sites from nine organisms isolated in 2010, seven isolates from 2010 and a single  
318 isolate from 2013 in La Pampa province, Argentina. *S. sonnei* Ss046 was added as the  
319 reference strain. Tree was viewed in microreact and nodes are labelled with the strain name  
320 and the year of isolation suffix collared according to susceptibility against cefpodoxime  
321 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

