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Virulence factors and mechanisms of antimicrobial resistance in *Shigella* strains from periurban areas of Lima (Peru)

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Abstract

The study was aimed to describe the serotype, mechanisms of antimicrobial resistance, and virulence determinants in *Shigella* spp. isolated from Peruvian children. Eighty three *Shigella* spp. were serogrouped and serotyped being established the antibiotic susceptibility. The presence of 12 virulence factors (VF) and integrase 1 and 2, along with commonly found antibiotic resistance genes was established by PCR. *S. flexneri* was the most relevant serogroup (55 isolates, 66%), with serotype 2a most frequently detected (27 of 55, 49%), followed by *S. boydii* and *S. sonnei* at 12 isolates each (14%) and *S. dysenteriae* (4 isolates, 5%). Fifty isolates (60%) were multi-drug resistant (MDR) including 100% of *S. sonnei* and 64% of *S. flexneri*. Resistance levels were high to trimethoprim-sulfamethoxazole (86%), tetracycline (74%), ampicillin (67%), and chloramphenicol (65%). Six isolates showed decreased azithromycin susceptibility. No isolate was resistant to nalidixic acid, ciprofloxacin, nitrofurantoin, or ceftriaxone. The most frequent

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Transparency declarations

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resistance genes were *sul*2 (95%), *tet*(B) (92%), *cat* (80%), *dfrA1* (47%), *bla*$_{OXA-1}$ *like* (40%), with *intl*1 and *intl*2 detected in 51 and 52% of the isolates, respectively. Thirty-one different VF profiles were observed, being the *ipa*H (100%), *sen* (77%), *virA* and *icsA* (75%) genes the most frequently found. Differences in the prevalence of VF were observed between species with *S. flexneri* isolates, particularly serotype 2a, possessing high numbers of VF. In conclusion, this study highlights the high heterogeneity of *Shigella* VF and resistance genes, and prevalence of MDR organisms within this geographic region.

Keywords

shigellosis; *Shigella* serotypes; antimicrobial resistance; Ipa; enterotoxins; autotransporters

Introduction

Shigella spp., a member of the Enterobacteriaceae genus that has the ability to invade and replicate within the colonic epithelium, is considered a major cause of dysentery. Despite a decreasing role in contributing to childhood mortality over last years, it is still estimated that around 28000 children younger than 5 years of age die every year due to shigellosis (Lanata *et al.*, 2013). Oral rehydration and antimicrobial therapy are recommended treatments for this illness; however, recent reports have determined that the rate of antimicrobial resistance for *Shigella* spp. is increasing (Ahmed *et al.*, 2006; Pons *et al.*, 2013; Sire *et al.*, 2008). In fact, antibiotic resistance is becoming a progressive worldwide problem including South America (Bastos *et al.*, 2011; Lima *et al.*, 1995; Suarez *et al.*, 2000). For these reasons, the World Health Organization has targeted the development of a vaccine for *Shigella* as a high priority (Steele *et al.*, 2012). However, for the best vaccine coverage and effectiveness, a vaccine will need to cover all relevant serogroups and serotypes that are prevalent around the world. In this line, a recent multicenter study developed at different Asian and African sites also determined the serotypes that are needed to guarantee an effective vaccine (Livio *et al.*, 2014).

Regarding Peru, a recent study analyzing 403 *Shigella* isolates from Peruvian amazon children noted a high prevalence of antimicrobial resistance to include those antibiotics designated as first-line therapy. For instance, 79% of these 403 isolates were resistant to trimethoprim-sulfamethoxazole, 73% were resistant to ampicillin, 69% were resistant to erythromycin and 16% were resistant to azithromycin. Additionally, the appearance of quinolone resistance in 5% of isolates was also reported (Kosek *et al.*, 2008). Azithromycin is considered a promising alternative treatment for *Shigella* spp. and other Enterobacteriaceae (Pons *et al.*, 2013, Retsema *et al.*, 1987), and is currently used for treatment of infectious diarrhea in Peru. However, azithromycin-resistant *Shigella* strains have been reported (Howie *et al.*, 2010). Meanwhile, quinolones are currently the treatment of choice for shigellosis, although increasing resistance has been described in different geographical areas (Pons *et al.*, 2013, Ashkenazi *et al.*, 2003; Mamishi *et al.*, 2009; Mensa *et al.*, 2008).

Several mechanisms of antibiotic resistance have been described in *Shigella* spp. These mechanisms may be classified within two main categories: those related with chromosomal mutations (Mensa *et al.*, 2008 Ghosh *et al.*, 1999) and those which possess the potential to be transferred among microorganisms; often plasmid encoded or based within structures as transposons or integrons (Mandomando *et al.*, 2009; Navia *et al.*, 2005; Pan *et al.*, 2006; Peirano *et al.*, 2005; Yah *et al.*, 2010). Similarly the damage caused by this bacterium is associated with the presence of virulence factors, which also may be located in the chromosome or in transferable structures. Thus, currently at least 5 genomic islands, SHI-1 to 3, SHI-O and SRL, carrying virulence factors, SRL also carrying antibiotic resistance genes, have been described (Schroeder and Hilbi, 2008). Additionally, virulence plasmids (pINV) contain genes involved with cellular invasion (Schroeder and Hilbi, 2008; Yang *et al.*, 2005; Thong *et al.*, 2005) and play an important role in the virulence process and in the passage of the bacterium from cell to cell (Barrantes and Achi, 2009).

When *Shigella* comes in contact with epithelial cells the type III secretion system (T3SS) is activated causing the release of effector proteins such as IpaA, IpaB, IpaC, IpaD, IpgB1, IpgD and VirA. Three of them (IpaB, IpaC and IpaD), are considered key virulence factors in *Shigella* spp. because they have both effector functions, essential for host cell invasion and intracellular survival, but also control the secretion and translocation of other effector proteins (Schroeder and Hilbi, 2008). These proteins help the polymerization and depolymerization of actin, facilitating bacterial invasion of the host cell (Schroeder and Hilbi, 2008; Barrantes and Achi, 2009; Ashida *et al.*, 2007). After cell invasion, *Shigella* releases other effectors such as IcsB, which protects the bacteria from being recognized and trapped by the host cell autophagy machinery (Schroeder and Hilbi, 2008). Additionally, this bacterium produces other proteins such as VirA, which facilitates entry and intracellular motility by the degradation of microtubules (Schroeder and Hilbi, 2008).

Currently, data on virulence factors of *Shigella* strains from Peru is limited. The aim of this study was to characterize a collection of *Shigella* strains isolated from children less than 2 years of age in periurban communities of Lima, Peru to help establish the serotype distribution, patterns and mechanisms of antimicrobial resistance, as well as their virulence profile.

Materials and Methods

Samples

Bacterial strains were isolated and characterized from a community-based randomized double-blind placebo controlled trial that compared bovine lactoferrin versus placebo for prevention for diarrhea in children (Ochoa *et al.*, 2013). All children were enrolled at 12-18 months and followed for 6 months with daily home visits. Overall 1235 diarrhea episodes were registered. The study was approved by Institutional Review Boards of the University of Texas Health Science Center in Houston and Universidad Peruana Cayetano Heredia in Lima.

Bacterial Isolates

Shigella isolates belonging to the first two years of the clinical trial were analyzed. In all cases *Shigella* isolates were identified by conventional biochemical and serotyping methods (Ochoa *et al.* 2013). When more than one *Shigella* strain by diarrhea episode was obtained, only the first isolated was considered. A total of 83 *Shigella* spp. were recovered: 69 samples from diarrhea cases and 14 from healthy children (without diarrhea or other gastrointestinal symptom one week before and after the stool sample collection). However, only 71 isolates (45 *S. flexneri*; 12 *S. boydii;* 10 *S. sonnei* and 4 *S. dysenteriae*) which were able to growth from the frozen stock underwent molecular analysis. *Escherichia coli* ATCC 25922, *S. flexneri* ATCC 12022, *E. coli* O42, *S. flexneri* 2a, and control strains carrying specific antibiotic resistance determinants and virulence genes donated by the Center for Biomedical Research of La Rioja - Spain (CIBIR) and from the internal collection of the Centre de Recerca en Salut Internacional de Barcelona (CRESIB) were used as quality control.

Serotypification

Shigella strains were serogrouped by agglutination with serogroup specific antisera (Denka-Seiken, Tokyo, Japan). Furthermore each serogrouped *Shigella* isolate were typed by agglutination with type-specific antisera (Denka-Seiken, Tokyo, Japan).

Analysis of clonal relations

The clonal relationships for 56 isolates (30 *S. flexneri*; 12 *S. boydii*; 10 *S. sonnei* and 4 *S. dysenteriae*) were established by Pulsed Field Gel Electrophoresis (PFGE) as previously described (Navia et al., 1999). PFGE profiles were compared using the fingerprinting software InfoQuest™ FP v.4.5 (Bio-Rad, Hercules, CA). The Dice coefficient was used to analyze the electrophoretic patterns, with clustering by the unweight pair-group method with arithmetic mean (UPGMA) with 1% tolerance and 1% of optimization in band position differences (Pons et al., 2015). Clonal groups were considered when the similarity levels were ≥85% (Erjnaes et al., 2006).

Antimicrobial susceptibility

Antibiotic susceptibility testing was performed by the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). The isolates were tested against the most commonly used antimicrobial agents: ampicillin (10 μg), ceftriaxone (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), azithromycin (15 μg), tetracycline (30 μg), nitrofurantoin (300 mg), nalidixic acid (30 μg) and ciprofloxacin (5 μg). In the case of azithromycin, because of the absence of an established breakpoint, Minimal Inhibitory Concentration (MIC) was also determined by the agar dilution method according to CLSI guidelines (CLSI, 2011) on all isolates with an inhibitory halo ≤ 15 mm (Ochoa, *et al.*, 2009). Multi-drug resistance (MDR) was defined as resistance to 3 or more unrelated classes of antibiotics. For analysis purposes intermediate and resistant isolates were considered together.

Detection of genes encoding virulence factors

Twelve virulence factors were sought by PCR (Table 1). In all cases, the DNA extraction was performed by the thermal shock lysis technique, and the PCR was performed in a 20 μL reaction mixture containing, 0.25 mM of each dNTP, 4 μL of 5× colorless buffer 2.4μL of 25mM MgCl2 (GoTaq® Promega, Madison, USA), 0.5 U of Taq polymerase (GoTaq® Promega, Madison, USA) and 2μL of DNA template. The reaction products were run on 1.5% agarose gels and stained with Sybr Safe (Invitrogen, Eugene, USA).

Determination of Molecular Mechanisms of Antimicrobial Resistance

The presence of transferable antibiotic resistance mechanisms was sought by conventional PCR in isolates exhibiting full or intermediate resistance to β-lactam, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole or macrolides. Additionally, in strains with azithromycin halo ≤ 15 mm, the presence of point mutations in the *rplD, rplV* and *rrlH* genes was also determined by PCR as previously reported (Table 1). Also, the presence of genes encoding *int1* and *int2* was sought by PCR (Table 1). In all cases the reaction products were visualized as above.

Statistical Analysis

The $\chi 2$ test or Fisher's exact test were used as appropriate, p values <0.05 were considered significant.

Results

Serogroups and serotypes

The 83 strains of *Shigella* spp. were distributed as follows: 55 (66%) were *S. flexneri*, 12 (14%) were *S. boydii*, 12 (14%) were *S. sonnei* and 4 (5%) *S. dysenteriae.* The most frequent serotypes for each serogroup were serotype 2a for *S. flexneri* (27 out of 55 isolates; 49%), serotype 10 for *S. boydii* (3 isolates, 25%) and serotype 2 for *S. dysenteriae* (50%). Regarding *S. sonnei*, 7 isolates (58%) showed the phase I. (Table 2). Eight out of the 14 control isolates were *S. flexneri* (5 belonging to the serotype 2a, and the remaining 3 being serotype 4a, 4b and 6 respectively), 4 *S. boydii* and 2 *S. sonnei*.

Clonal relationships

The study of the clonal relationships showed that in a general manner *S. dysenteriae* and *S. boydii* isolates were clonally unrelated (Data not show). Among *S. boydii* were described 10 different pulsotypes. All these pulsotypes possessing 1 isolate each, excepting two pulsotypes; one of them comprising those isolates belonging to the serotype 2, and the other 2 out of 3 isolates classified as serotype 10; In the case of *S. flexneri*, were described 23 different pulsotypes, one of them comprising 6 *S. flexneri 2a* isolates, other 2 *S. flexneri 2a* isolates, and the remaining 21 comprising only a single strain. Meanwhile, 9 out of 10 *S. sonnei* isolates had an identity level higher than 85%. Thus, we considered the isolates to be closely related or possibly clonal.

Antibiotic resistance phenotypes

Overall, the highest rates of antimicrobial resistance among *Shigella* isolates were to trimethoprim-sulfamethoxazole (84%), tetracycline (74%), ampicillin (67%), and chloramphenicol (65%) (Table 3). None of the isolates was resistant to nalidixic acid, ciprofloxacin, nitrofurantoin, or ceftriaxone. A total of 50 strains (60%) were MDR (100% of *S. sonnei*; 64% of *S. flexneri*, 17% of *S. boydii*, and 25% of *S. dysenteriae*). Six isolates (7%) showed an azythromicin diameter inhibition halo lower than 15 mm with correlating MIC levels of 4-8 μg/ml to azithromycin. Antimicrobial resistance levels were significantly higher for ampicillin, chloramphenicol and tetracycline in *S. sonnei* isolates compared to other *Shigella* serogroups ($p < 0.05$) (Table 3). *S. flexneri* isolates belonging to the serotypes 4a and 4b were susceptible to all tested agents except trimethoprim-sulfamethoxazole, while those belonging to the serotype 2a had significantly higher levels of resistance ($p < 0.05$) to ampicillin, tetracycline and chloramphenicol than the remaining *S. flexneri* together. No differences in resistance rates were associated to diarrhea or control isolates.

Antibiotic resistance mechanisms

The most common mechanism of resistance to β-lactam agents was the presence of bla_{OXA-1} like genes which were detected in 18 isolates (12 *S. flexneri* of which 9 were serotype 2a), followed by bla_{CARB} like genes, which were presents in 11 isolates, and bla_{TEM} like detected in 4 isolates. No isolate tested positive for either the bla_{SHV} or bla_{OXA-2} like genes but 5 isolates possessed both a bla_{CARB} like gene plus an bla_{OXA-1} like gene. Sixteen *S. flexneri* and 4 *S. sonnei* (24% out of the analyzed ampicillin-resistant isolates) did not present any of the mechanisms of resistance sought. Tetracycline resistance was mainly associated to the presence of the *tet*(B) gene (Table 4) which was detected in 40 out of 44 resistant isolates analyzed (including 2 of the 3 intermediate ones). The *tet*(A) gene was detected in one case concomitantly with the *tet*(B) gene. Chloramphenicol resistance was mainly linked to the presence of *cat* genes (37 out of 43 resistant and intermediate isolates; 86%). Regarding trimethoprim-sulfamethoxazole, the *sul2* (54 out of 57 resistant isolates; 95%) was widely present in sulfonamide resistant isolates, while *dfrA*1 like genes, involved in the trimethoprim resistance was detected in 47% of the isolates. In the isolates exhibiting a diameter inhibition halo ≤ 15 mm to azithromycin, 2 strains (1 *S. dysenteriae* and 1 *S. sonnei*) had the amino acid substitution P80S in the *rplV* gene with the *S. sonnei* also harbouring the *mph*(A) gene (Table 4). No mutations were observed in the *rplD* and *rrlH* genes and no other azithromycin resistance related gene was found. Finally, the integrase encoding genes were also detected: *intl*1 (48%), and *intl*2 (45%) (Table 4). No differences in antibiotic resistance mechanisms were associated to diarrhea or control isolates

Virulence related genes

Overall the most frequently detected virulence genes were: *ipa*H (100%), *sen* (77%), *virA* and *icsA* (75%). The “antivirulence” factors *ompT* and *cadA* were not found in any isolate (Table 5). A high heterogeneity in the combination of virulence factors was observed. Thus, 31 different virulence factors profiles were observed (Table 6), with the most frequent patterns being that of profile J and profile I represented by 8 isolates each (all of them being *S. flexneri* 2a, except 1 *S. flexneri* Y with profile I). The remaining profiles only include 1 to

3 isolates, except profile G (7 isolates), profile C (6 isolates) and profile F (4 isolates) (Table 6).

In general, the *S. flexneri* isolates, especially those belonging to the serotype 2a, possessed more virulence factors than other serogroups. Described further, the analyzed 23 *S. flexneri* 2a, all had the *ipa*H gene, 22 (97%) had the *sigA* gene, 21 (91%) had the *sat* gene, 20 (87%) had the *sepA*, *vir*A, *icsA*, *pic*, *set1*A, *set1*B genes and 17 isolates (74%) possessed the *ipg*D gene, while remaining virulence factors were present in less than 50% of *S. flexneri* 2a isolates (Table 5).

Regarding differences between species, the *sat* gene was mainly detected in *S. flexneri* isolates (41 out of 45; 91%), while it was absent in *S. sonnei* and only present in 3 out of 12 (25%) *S. boydii*. Similarly, the *set1A* and *set1B* genes, encoding the toxin ShET1, were only found in *S. flexneri* isolates being also concomitantly found with the *pic* gene, which additionally was detected in 5 *S. boydii* and 1 *S. dysenteriae*. Moreover, all *set1A* and *set1B* positive isolates also presented with the *sigA* gene, although the *sigA* gene was detected in the absence of *pic*, *setA*, and *setB* genes in 4 *S. flexneri*, 4 *S. boydii*, 10 *S. sonnei* and 1 *S. dysenteriae*, but concomitantly with the *pic* gene in another 6 *S. boydii* and 1 *S. dysenteriae*. When other associations were sought between virulence factors it was observed that the *virA* and *icsA* genes were concomitantly present in *S. flexneri* isolates, while the combination *ipg*D, *ics*A was found in the remaining species. No differences were found in the number and specific association of virulence factors among diarrhea and control strains.

DISCUSSION

Shigellosis is a common cause of bacterial diarrhea and a significant public health problem endemic throughout the world. In this study, 83 *Shigella* strains were analyzed, and identified by serogroup and serotype. Of the 4 serogroups detected, including a variety of serotypes for each serogroup, *S. flexneri* serotype 2a was the most common, accounting for 49% of all *S. flexneri* isolates. This high percentage of the serotype 2a has also been observed in other studies in Peru (Kosek *et al.*, 2008) and in other countries (Livio *et al.*, 2014).

Classically *S. boydii* has been mainly reported in samples from the Indian subcontinent and remains uncommon in other areas (Niyogi, 2005). In a recent multicenter report by Livio *et al* (2014), *S.boydii* accounted for only 5.5% of the *Shigella* recovered at all sites, with Bangladesh having the lowest prevalence at 3.9% and India with the maximum prevalence at 11.0%. Nonetheless, although recent reports shows a low prevalence in some South American countries such as Brazil or Chile (Bastos and Loureiro, 2011; Hamilton-West *et al.*, 2007; Peirano *et al.*, 2006), other countries such as Argentina have reported a prevalence rate of 7.7% for *S. boydii* (Rolfo *et al.*, 2012). Our prevalence of 14% for *S. boydii* is comparable to the prevalence rates seen within the Indian subcontinent, being also in accordance with other studies developed in different Peruvian areas (Kosek *et al.*, 2008; Fernández-Prada *et al.*, 2004).

Recently it has been proposed that a vaccine which would cover the O antigen of *S. flexneri* belonging to the serotypes 2a, 3a and 6 plus that of *S. sonnei* will provide coverage of around 88% of current shigellosis cases (Livio *et al.*, 2014). This also assumes cross protection against *S. flexneri* 1a, 1b, 2b, 3b, 4a, 4b, 5a, 5b, 7b, X and Y. However, the aforementioned study considers data obtained from different African and Asian countries, but not from Latin America. In our case, 83-87% of present isolates would have been under the predicted umbrella of this type of proposed vaccine.

Another recent study (Szijártó *et al*, 2013) has shown that the use of avirulent *S. flexneri* serotype 2a strain, lacking major immune determinants, including O antigens, resulted in heterologous protection against *S. flexneri* serotype 6 and *S. sonnei*, through the development of antibodies against shared minor antigens. If the results obtained could be extended to other *Shigella* spp. serotypes, this could present an approach to develop a broader-spectrum *Shigella* vaccine.

The present data showed a high diversity of circulating *S. flexneri* and *S. boydii* strains in the area, while all but one *S. sonnei* were related phylogenetically. Regarding *S. flexneri* our results are in agreement with previous studies developed in Peru and Chile (Fernandez-Prada *et al.*, 2004) that showed the presence of different circulating strains in periurban areas of Lima. In the case of *S. boydii*, the heterogeneity of strains is also highlighted by the presence up to 6 different serotypes, with a maximum of 3 isolates each.

Of special relevance is the detection of 3 cases of diarrhea in which a *S. sonnei* phase II was recovered. *S. sonnei* phase II is considered avirulent, having lost its virulence plasmid and consequently also its virulence (Sansonetti *et al*, 1981). A possible explanation may be the total or partial lost of the virulence plasmid during subcultures or storage, as has been previously described (Sasakawa *et* al., 1986). However, a recent study (Tajbakhsh *et al.*, 2012) undertaken to determine the relevance of *Shigella* infections among patients admitted with acute diarrhea or gastroenteritis in Iran showed that the 25 *S. sonnei* isolates recovered were phase II.

Antimicrobial resistance is becoming a major concern all over the world with reported rates of MDR *Shigella* strains increasing worldwide (Pons *et al.*, 2013, Kosek *et al.*, 2008; Ashkenazi *et al.*, 2003). This study supports these findings with 67% of the isolates being multi-drug resistant. Of present isolates, *S. sonnei* had the highest level of MDR at 100% with all isolates resistant to ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. This finding is different from that reported by Navia *et al.* (2005), where *S. flexneri* was described as having the highest rate of MDR isolates. Additionally, the high level of chloramphenicol resistance found among *S. sonnei* isolates is in disagreement with a series of reports developed in different geographical areas, including the South American region (Pons *et al.*, 2013; Mandomando *et al.*, 2009; Navia *et al.*, 2005; Hamilton-West *et al.*, 2007). However, these results may be explained by the fact that almost all *S. sonnei* belongs to the same clonal group as has been aforementioned.

Interestingly, despite high levels of quinolone-resistance in Peru of other Enterobacteriaceae, either pathogenic or commensals (Mosquito *et al.*, 2012; Pons *et al.*,

2014), and the emergence of quinolone resistance in *Shigella* spp. in other different geographical areas (Pons *et al.*, 2013; Ashkenazi *et al.*, 2003; Mamishi *et al.*, 2009; Mensa *et al.*, 2008), no quinolone-resistant *Shigella* strain was found in this study. Thus, quinolones appear to be a good option in this region for the treatment of shigellosis, although increased use may encourage the development of quinolone resistance and should be monitored.

Despite testing for the most frequently described β-lactamases, more than 40% of the ampicillin resistant isolates did not present any of them. This phenomenon has been previously described in the same area in a study designed to determine the levels and mechanisms of antibiotic resistance in *E. coli* strains (Mosquito *et al.*, 2012). Although other factors, such as a possible polymorphism affecting primers annealing regions may not be ruled out, both results together, suggest the presence of an unusual mechanism of ampicillin resistance spreading in the area. In this line of thought, in the area has also been observed the presence of unusual high levels of resistance to rifaximin mediated by the overexpression of efflux pumps (Gomes *et al.*, 2013b), and then, the possible role of overexpressed efflux pump, which might be related with the presence of environmental toxics, in the resistance to ampicillin may not be ruled out. On the other hand, several isolates possess more than one established mechanism of ampicillin resistance. This fact showed the ability of *Shigella* spp. to acquire a diversity of β-lactam resistance determinants presents in the area.

Macrolide resistance may be related with the presence of specific chromosomal mutations at *23S rRNA*, *rplV* (encoding the L4 ribosomal protein) and/or *rplD* (encoding the L22 ribosomal protein) genes, as well as with the presence of different transferable mechanisms of macrolide resistance (Gomes *et al.*, 2013a; Howie *et al.*, 2010). Although, the L22 amino acid substitution P80S has not yet been described to help confer macrolide resistance, it is located near the macrolide binding pocket at the nascent peptide exit tunnel, and might affect the ribosomal conformation in this area making azithromycin binding difficult. Further studies are needed to elucidate this fact. Regarding, the *mph*(A) gene, it was previously described in different members of Enterobacteriaceae family, including *S. sonnei* isolates recovered during different outbreaks (Howie *et al.*, 2010; Bounghar-Bourtchai *et al.*, 2008; Sjölund-Karlson *et al.*, 2013) where the *mph*(A)-positive isolates showed MIC levels ≥ 64 μg/ml, quite higher than present *mph*(A)-positive strain. This difference may be explained by the diverse genetics environments in which *mph*(A) may be located, its expression levels, as well as specific genetic backgrounds. It was not surprising to see this gene present in Peru given that the *mph*(A) gene ranks among one of the most frequent macrolide resistance encoding genes detected in Enterobacteriaceae. In this setting, where the prevalence is low for this transferable mechanisms of azithromycin resistance and the described low frequency of selection of chromosomal mutations (Gomes *et al.*, 2013a), together with notably lower MIC levels suggests that azithromycin is still a viable treatment option in Peru. In fact, azithromycin, and also furazolidone, are considered as alternative treatment for shigellosis within this region (Ecker *et al.*, 2013; Erdman *et al*, 2008; MINSA, 2006). Regarding furazolidone resistance, it has been described as related with the presence of punctual mutations at the *nfs*A and/or *nfs*B genes (Whiteway *et al.*, 1998) and to the best of our knowkledge no transferable mechanism of resistance has been described. No CLSI

breakpoints for furazolidone are available (CLSI, 2011), then we used nitrofurantoin, which is a member of the same antibiotic class, determining that the isolates were highly susceptibility to this antimicrobial agent.

Class 1 integron is the most common integron found in clinical *Shigella* spp. isolates with the presence of integrases 1 or 2 being reported with varying frequencies (Peirano *et* al, 2005, Toro *et al.*, 2005). In this study, the presence of *int1* and *int2* occurred at similar frequencies, despite the slight species differences found. However, it is of interest to note the low number of *sul1* genes detected that are usually associated with class 1 integrons. This is in accordance with that observed by Peirano *et al* (2005), which detected only 2 out of 109 sulphonamide resistant *Shigella* spp. isolates with the *sul1* gene and also with the presence of atypical class 1 integrons lacking the *sul1* gene (Pan *et al.*, 2006; Zhu *et al.* 2011). Thus, present results might be related to the carriage of this atypical class 1 integron.

The non-detection of *ompT* and *cadA* genes is not surprising, once in *Shigella* spp. are considered as deleted or inactivated (Day *et al.*, 2001*;* Maurelli *et al.*, 1998; Schroeder and Hilbi, 2008). Nevertheless, a report of 2009 by Li *et al.* (2009) showed the presence of *cadA* in *S. boydii* serotype 11 and *S. dysenteriae* serotype 1, both serotypes absent in our series.

Both the *ompT* and *cadA* genes encode antivirulence factors; with OmpT interfering with the action of IcsA (Nakata *et al.*, 1993) and *cadA* gene, encoding for the product cadaverin, which acts as an inhibitor of *Shigella* enterotoxins (Maurelli *et al.*, 1998). The absence of these genes enhances the pathogenicity of *Shigella* spp.

The present data showed the presence of a few isolates in which the *pic* gene was present when *set1A* and *set1B* were absent, as has also been described previously in *S. sonnei* (Yang *et al.*, 2005). As these genes are encoded in the same genetic locus in the sense (*pic* gene) and antisense (*set1A* and *set1B*) strands (Yang *et al.*, 2005), these data suggests the presence of inactive *pic* genes due to internal deletions or sequence alterations, or the presence of a different *pic* gene variant. Additionally, the *sig*A gene was present in *S. flexneri* isolates that also had *set1A* and *set1B* genes detected suggesting the presence of the pathogenicity island SHI-1, which carry all of these genes (Schroeder and Hilbi, 2008). As account with the *pic* gene, the *sigA* gene was also found in the absence of remaining SHI-1 carried genes in several isolates. This fact has also been previously described in *S. boydii*, *S. sonnei* and *S. dysenteriae* by Yang *et al* (2005), most likely representing a second location for this gene.

In general, the *S. flexneri* isolates, especially those belonging to the serotype 2a, tended to possess a high number of virulence factors. This fact is in disagreement with the consideration that *S. dysenteriae* is the most virulent of the 4 *Shigella* serogroups (Yang *et al.*, 2005).

A statistical difference in the prevalence of the sat gene between *S. flexneri* and *S. sonnei* has been described (Ruiz *et al.*, 2002). Moreover, differences in the geographic prevalence of the *sat* gene were observed. Thus, a prevalence of 71% in the Latin American *S. flexneri* isolates has been showed, which also is in agreement with the present results. Similarly, previous studies (Livio *et al.*, 2014; Noriega *et al.*, 1995; Vargas *et al.*, 1999) showed that

ShET-1 is more frequent in *S. flexneri* than in other *Shigella* spp., being of special relevance in those *S. flexneri* isolates belonging to the serotype 2a, and rarely found in other *S. flexneri* serotypes. Additionally, the SHI-1 pathogenicity island which carries both the *pic* and *sigA* genes are also more frequently detected in *S. flexneri* 2a. These data suggest that particularly the *S. flexneri* 2a strains not only possess high levels of multi-drug resistance but also tend to be more likely to have multiple virulence factors within their genome compared to other *Shigella* strains. However, neither specific relation between the numbers of analyzed virulence factors not presence of specific one was related with the healthy or diarrhea status of the children. This fact might be explained by differences in the expression of these factors, or by the presence/absence of other non-analyzed virulence factors. Moreover, a possible role of acquired immunity may not be ruled out.

The main limitation of the present study is that the expression of the sought genes has not been determined. Thus, although not probable, it may not be ruled out that some of these genes remained inactive, given that PCR detection does not unequivocally signify functionality.

This study presents an in-depth delineation of the virulence characteristics and resistance mechanisms of *Shigella* isolates from a Latin America country highlighting the high heterogeneity of virulence factors and prevalence of MDR organisms within this geographic region. In order to maintain effective treatments for shigellosis both continuous surveillance for emerging antimicrobial resistance to commonly used antibiotics to treat these infections needs to occur within each distinct geographic area, as well as to assist the development of an effective vaccine that covers the most predominant serotypes causing infection within South America.

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Table 1
Primers and PCR conditions used in the study

	Nucleotide sequence (5′→3′)	Annealing (°C / time)	Amplicon size (bp)	Ref.
Virulence				
ipaBCD	GCTATAGCAGTGACATG ACGAGTTCGAAGCACTC	63°C / 45 sec	500	Sharma *et al.*, 2010
icsA	TGATGGACTTTCTCCCTTGG CCGCTACCACCAAGAATCAT	55°C / 60 sec	220	Efremova *et al.*, 2004
ipaH	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	60°C / 20 sec	619	Toma *et al.*, 2003
ipgD	ATGCACATAACTAATTTGGGA TCTTATACAAATGACGAATACCC	60°C / 60 sec	1618	Niebuhr *et al.*, 2000
pic	ACTGGATCTTAAGGCTCAGGAT GACTTAATGTCACTGTTCAGCG	58°C / 60 sec	572	Boisen *et al.*, 2009
sat	ACTGGCGGACTCATGCTGT AACCCTGTAAGAAGACTGAGC	55°C / 90 sec	387	Ruiz *et al.*, 2002
sen	ATGTGCCTGCTATTATTTAT CATAATAATAAGCGGTCAGC	55°C / 90 sec	799	Vila *et al*, 2000
sepA	GCAGTGGAAATATGATGCGGC TTGTTCAGATCGGAGAAGAACG	58°C / 60 sec	794	Boisen *et al.*, 2009
setlA	TCACGCTACCATCAAAGA TATCCCCCTTTGGTGGTA	55°C / 90 sec	209	Vila *et al.*, 2000
setlB	GTGAACCTGCTGCCGATATC ATTTGTGGATAAAAATGACG	55°C / 90sec	147	Vila *et al.*, 2000
sigA	CCGACTTCTCACTTTCTCCCG CCATCCAGCTGCATAGTGTTTG	58°C / 60 sec	430	Boisen *et al.*, 2009
virA	CTGCATTCTGGCAATCTCTTCACATC TGATGAGCTAACTTCGTAAGCCCTCC	55°C / 90 sec	215	Villalobo *et al.*, 1998
Antivirulence				
cadA	TTCAAAAACATCGATAACGA ACGGTATGCACCGTGAAT	55°C / 60 sec	669	Li *et al.*, 2009
ompT	CCCGGGTCATAGTGTTCATC ATCTAGCCGAAGAAGGAGGC	60°C / 60 sec	559	Jonhson *et al.*, 2000
Resistance				
bla_{CARB}-*like*	AATGGCAATCAGCGCTTCCC GGGGCTTGATGCTCACTCCA	58°C / 30 sec	586	Cabrera *et al.*, 2004
bla_{OXA-1}-*like*	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	56°C / 60 sec	598	Cabrera *et al.*, 2004
bla_{OXA-2}-*like*	CGATAGTTGTGGCAGACGAA CCACTCAACCCATCCTACCC	55°C / 60 sec	550	Vila *et al.*, 1997
bla_{TEM}-*like*	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC	63°C / 65 sec	1150	Saenz *et al.*, 2004
bla_{SHV}-*like*	ATGCGTTATATTCGCCTGTG TTAGCGTTGCCAGTGCTCG	58°C / 30 sec	841	Cabrera *et al.*, 2004
dfrA1 *	GTGAAACTATCACTAATGG TTAACCCTTTTGCCAGATTT	55°C / 60 sec	474	Cabrera *et al.*, 2004
*dfrA7***	TTGAAAATTTCATTGATTG TTAGCCTTTTTTCCAAATCT	55°C / 60 sec	474	Cabrera *et al.*, 2004
sul1	TGGTGACGGTGTTCGGCATTC GCGAAGGTTTCCGAGAAGGTG	63°C / 30 sec	789	Saenz *et al.*, 2004

	Nucleotide sequence (5′→3′)	Annealing (°C / time)	Amplicon size (bp)	Ref.
sul2	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	59°C / 30 sec	722	Saenz *et al.*, 2004
cat	GGTGAGCTGGTGATATGG GGGATTGGCTGAGACGA	61°C / 30 sec	209	Mosquito *et al.*, 2012
cmlA	TGTCATTTACGGCATACTCG ATCAGGCATCCCATTCCCAT	95°C / 60 sec	455	Saenz *et al.*, 2004
floR	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	95° / 30 sec	868	Saenz *et al.*, 2004
tet(A)	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	57°C / 60 sec	937	Saenz *et al.*, 2004
tet(B)	CTCAGTATTCCAAGCCTTTG CTAAGCACTTGTCTCCTGTT	57°C / 30 sec	416	Saenz *et al.*, 2004
ere(A)	GCCGGTGCTCATGAACTTGAG CGACTCTATTCGATCAGAGGC	60 °C / 30 sec	420	Nguyen *et al.*, 2009
ere(B)	AGAAATGGAGGTTCATACTTACCA CATATAATCATCACCAATGGCA	52 °C / 60 sec	548	Sutcliffe *et al.*, 1996
erm(A)	TCTAAAAAGCATGTAAAAGAAA CGATACTTTTTGTAGTCCTTC	52 °C / 30 sec	533	Nguyen *et al.*, 2009
erm(B)	GAAAAAGTACTCAACCAAATA AGTAACGGTACTTAAATT	45 °C / 30 sec	639	Nguyen *et al.*, 2009
erm(C)	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	45 °C / 30 sec	642	Nguyen *et al.*, 2009
mef(A)	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	54 °C / 30 sec	345	Nguyen *et al.*, 2009
mef(B)	ATGAACAGAATAAAAAATTG AAATTATCATCAACCCGGTC	45 °C / 30 sec	1255	Liu *et al.*, 2009
mph(A)	GTGAGGAGGAGCTTCGCGAG TGCCGCAGGACTCGGAGGTC	60°C / 30 sec	403	Nguyen *et al.*, 2009
mph(B)	ATTAAACAAGTAATCGAGATAGC TTTGCCATCTGCTCATATTCC	50°C / 30 sec	868	Achard *et al.*, 2008
msr(A)	GCACTTATTGGGGGTAATGG GTCTATAAGTGCTCTATCGTG	58°C / 30 sec	384	Nguyen *et al.*, 2009
rplV	CGGTGGAAAGCGGAGACAAGAAGCC ACCAGTTTTGCGTCCAGTTCAGGCT	56°C / 45 sec	925	Gomes *et al.*, 2013a
rplD	GGCAAGAAAATGGCAGGTCAGATGG TTCCATCGCAGTAGACGCTTTTTCA	56 °C / 45 sec	846	Gomes *et al.*, 2013a
23S rRNA	TAAGGTAGCGAAATTCCTTGTCG TGATGCGTCCACTCCGGTC	61°C / 15 sec	755	Gomes *et al.*, 2013a
Other				
*int*1	GGGTCAAGGATCTGGATTTCG ACATGGGTGTAAATCATCGTC	63°C / 30 sec	483	Saenz *et al.*, 2004
int2	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	62 °C / 30 sec	788	Saenz *et al.*, 2004

Ref: Reference

*Amplify different *dfr* genes, including *dfrA1, dfrA5, dfrA15,dfrA 16.*

**Amplify different *dfr* genes, including *dfrA7, dfrA17*

Table 2

Serotype distribution of *Shigella* strains isolated from Peruvian children

S. flexneri (n: 55)		*S. boydii (n: 12)*		*S. sonnei (n: 12)*		*S. dysenteriae (n: 4)*	
Serotype	**n**	**Serotype**	**n**	**Phase**	**n**	**Serotype**	**n**
1a	2	1	2	I	7	2	2
1b	4	2	2	II	5	3	1
2a	28	4	2			5	1
3a	2	10	3				
3b	3	14	2				
4a	6	18	1				
4b	2						
6	2						
Y	3						
NT	2						
ND	1						

NT: Non typeable; ND: non-determined

Table 3

Number and percentage of antimicrobial resistant *Shigella* strains

Microorganism	serotype*	N	Amp		Sxt		Tet		Chl		Azm[1]	
			n	%	n	%	n	%	n	%	n	%
S. flexneri		**55**	**40**	**73**	**47**	**85**	**42**	**76**	**39**	**71**	**1**	**2**
	2a	28	25	89	26[a]	93	27[a]	96	24[c]	86	1	4
	4a	6	0	--	6	100	0	--	0	--	0	--
	Other	21	15	71	15	71	15[b]	71	15[a]	71	0	--
S. boydii		**12**	**3**	**25**	**9**	**75**	**6**[a]	**50**	**2**	**17**	**0**	--
S. sonnei		**12**	**12**	**100**	**12**	**100**	**12**	**100**	**12**	**100**	**4**	**33**
	I**	7	7	100	7	100	7	100	7	100	1	14
	II	5	5	100	5	100	5	100	5	100	3	60
S. dysenteriae		**4**	**1**	**25**	**3**	**75**	**1**	**25**	**1**	**25**	**1**	**25**
Total		**83**	**56**	**67**	**71**	**86**	**61**	**74**	**54**	**65**	**6**	**7**

Amp: Ampicillin, Sxt: trimethoprim-sulfamethoxazole; Tet: Tetracycline; Chl: Chloramphenicol; Azm: Azithromycin

No resistance to nalidixic acid, ciprofloxacin or ceftriaxone was found, and only 1 intermediate isolate to nitrofurantoin was detected.

1: Isolates which posses a azithromycin halo less than 15 mm, In all cases the MIC ranges between 4 and 8 µg/ml.

*Only those with at minimum of 5 isolates;

**In the case of *S. sonnei*, refers to phase

a: 1 intermediate isolate,

b: 2 intermediate isolates,

c: 6 intermediate isolates

Table 4

Presence of antibiotic resistance mechanisms of *Shigella* strains

Antibiotic family	Mechanism of resistance	*S. flexneri*		*S. boydii*		*S. sonnei*		*S. dysenteriae*		Total	
		n / N	%	n / N	%	n / N	%	n / N	%	n/N	%
β - lactams											
	bla_{TEM} *like*	2 / 31	6	1 / 3	33	1 / 10	10	0 / 1	--	4 / 45	9
	bla_{SHV} *like*	0 / 31	--	0 / 3	--	0 / 10	--	0 / 1	--	0 / 45	--
	bla_{OXA-1} *like*	12 / 31	39	1 / 3	33	5 / 10	50	0 / 1	--	18 / 45	40
	bla_{OXA-2} *like*	0 / 31	--	0 / 3	--	0 / 10	--	0 / 1	--	0 / 45	--
	bla_{CARB} *like*	7 / 31	23	2 / 3	66	1 / 10	10	1 / 1	100	11 / 45	24
	Non determined	16 /31	52	0 / 3	--	4 / 10	40	0 / 0	--	20 / 45	44
Tetracyclines											
	tet(A)	1 / 32	3	0 / 6	--	0 / 10	--	0 / 1	--	1 / 49	2
	tet(B)	30 / 32[a]	94	5 / 6[b]	83	9 / 10	90	1 / 1	100	45 / 49	92
	Non determined	2 / 32	6	1 / 6	17	1 /10	10	0 / 1	--	4 / 49	8
Phenicols											
	cat	24 / 30[c]	80	2 / 2	100	10 /10	100	1 / 1	100	37 / 43	86
	cmlA	0 / 30	--	0 / 2	--	0 / 2	--	0 / 1	--	0 / 43	--
	floR	0 / 30	--	0 / 2	--	0 / 2	--	0 / 1	--	0 / 43	--
	Non determined	6 / 30	20	0 / 2	--	0 / 2	--	0 / 1	--	6 / 43	14
Sulphamides											
	sul 1	0 / 36	--	0 / 8	--	1 / 10	10	0 / 3	--	1 / 57	2
	sul 2	34 / 36	94	8 / 8	100	9 / 10	90	3 / 3	100	54 / 57	95
	Non determined	2 / 36	5	0 / 8	--	1 / 10	10	0 / 3	--	3 / 57	5
Trimethoprim											
	dfrA1	22 / 36	61	5 / 8	62	0 / 10	--	0 / 3	--	27 / 57	47
	dfrA7	0 / 36	--	0 / 8	--	0 / 10	--	0 / 3	--	0 / 57	--
	Non determined	14 / 36	39	3 / 8	37	10 / 10	100	3 / 3	100	30 / 57	53

Antibiotic family	Mechanism of resistance	*S. flexneri*		*S. boydii*		*S. sonnei*		*S. dysenteriae*		Total	
		n / N	%	n / N	%	n / N	%	n / N	%	n/N	%
Macrolides[1]	*mph*(A)	0 / 1	--	0 / 0	--	1 / 4	25	0 / 1	--	1 / 6	17
	mph(B)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	erm(A)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	erm(B)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	erm(C)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	mef(A)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	mef(B)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	msr(A)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	ere(A)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	ere(B)	0 / 1	--	0 / 0	--	0 / 4	--	0 / 1	--	0 / 6	--
	Non determined	1 / 1	--	0 / 0	--	3 / 4	--	1 / 1[1]	--	5 / 6	83[1]
Integrase											
	*int*1	21 / 42	50	1 / 11	9	9 / 10	90	1 / 4	25	32 / 67	48
	*int*2	23 / 42	55	5 / 11	45	1 / 10	10	1 / 4	25	30 / 67	45

n: Isolates in which was detected the mechanism of resistance. When an isolate presents more than one mechanism of resistance to the same antimicrobial agent, it is reported more than once, then total sum may be higher than 100%;

N: Total of non-susceptible isolates (resistant plus intermediate).

[a]: 2 intermediate isolates, both positives for the presence of the *tet*(B) gene.

[b]: 1 intermediate isolate in which no mechanism of resistance to tetracycline was determined;

[c]: 3 intermediate isolates presenting the *cat* gene.

[1]: Additionally, the amino acid substitution P80S was detected in 1 *S. sonnei* together *mph*(A) and 1 *S. dysenteriae*.

Table 5

Presence of virulence factors of *Shigella* strains

VF[1]			*S. flexneri* N: 45		*S. boydii* N: 12		*S. sonnei* N: 10		*S. dysenteriae* N: 4		Total N: 71	
Family	**Genes**	**Main Function[2]**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
T3SS* effectors												
	ipaH	Phagosome scape	45	*100*	12	*100*	10	*100*	4	*100*	71	*100*
	ipgD	Entry, host cell survival	31	*69*	9	*75*	5	*50*	3	*75*	48	*68*
	ipaBCD	Control of T3SS, phagosome scape	22	*49*	6	*50*	3	*30*	3	*75*	34	*48*
	virA	Motility,	36	*80*	10	*83*	4	*40*	3	*75*	53	*75*
SPATE†												
	pic	Mucinase	23	*51*	5	*42*	0	--	1	*25*	24	*34*
	sigA	Proteolytic toxin	27	*60*	10	*83*	10	*100*	2	*50*	49	*69*
	sepA	Protease, invasion	34	*76*	0	--	0	--	0	--	34	*48*
	sat	Proteolytic toxin	41	*91*	3	*25*	0	--	2	*50*	47	*66*
	icsA	Motility, intercellular spread	36	*80*	9	*75*	5	*50*	3	*75*	53	*75*
Enterotoxins												
	sen	Ion secretion	35	*78*	11	*92*	7	*70*	2	*50*	55	*77*
	set1A + set 1B‡	Ion secretion	23	*51*	0		0		0		23	*32*

[1]- VF: Virulence Factors;

[2]: The virulence factors may also develop other functions. N: Analyzed isolates; n: Positive isolates

*T3SS: Type Three Secretion System,

†SPATE=Serine Protease Autotransport of Enterobacteriaceae;

‡The *set1A* and *set1B* genes together, encoded the ShET-1 toxin. In all cases were found concomitantly.

Table 6

Virulence Profiles of *Shigella* strains

Serogroup	Serotype[1]	Case	No	*ipaH*	*ipaBCD*	*pic*	*setlA*	*setlB*	*sen*	*sat*	*sigA*	*sepA*	*ipgD*	*virA*	*icsA*	Profile	VF
S. flexneri																	
	la	D	1													A	2
	la	D	1													B	7
	lb	D	2													A	2
	lb	D	1													C	8
	lb	D	1													D	3
	2a	D	1													E	7
	2a	D	1													F	2
	2a	D	1													G	7
	2a	D	1													H	6
	2a	D	5													I	ll
	2a	C	2													I	ll
	2a	D	5													J	l2
	2a	C	3													J	l2
	2a	D	2													K	9
	2a	D	1													L	9
	2a	D	1													M	11
	3a	D	1													C	8
	3b	D	1													N	6
	3b	D	1													O	6
	4a	D	5													C	8
	4a	C	1													P	6
	4b	C	1													N	6
	4b	D	1													Q	6
	6	C	1													G	7
	6	D	1													R	7
	Y	D	1													S	6
	Y	D	1													I	11

Serogroup	Serotype[1]	Case	No	*ipaH*	*ipaBCD*	*pic*	*setlA*	*setlB*	*sen*	*sat*	*sigA*	*sepA*	*ipgD*	*virA*	*icsA*	Profile	VF
	Y	D	1	■	■	■	■	■		■	■	■		■	■	T	10
S. boydii																	
	1	C	1	■					■	■			■	■	■	N	6
	1	D	1	■	■	■			■		■		■	■	■	U	8
	2	D	1	■					■		■		■	■	■	V	7
	2	D	1	■		■			■		■					W	4
	4	C	1	■	■				■		■		■	■	■	G	7
	4	D	1	■		■			■		■		■	■	■	W	7
	10	D	1	■	■				■		■		■	■	■	G	7
	10	C	1	■		■			■		■		■	■	■	X	7
	10	C	1	■	■	■			■		■		■	■	■	U	8
	14	D	1	■	■				■		■		■	■	■	G	7
	14	D	1	■						■				■		Y	3
	18	D	1	■		■			■	■	■					Z	5
S. sonnei																	
	I	D	2	■	■				■		■		■	■	■	G	7
	I	D	1	■					■		■		■		■	AA	5
	I	D	1	■	■						■					BB	2
	I	D	1	■					■		■					CC	3
	I	C	2	■					■		■		■	■	■	V	6
	II	D	1	■					■		■					CC	3
	II	D	2	■							■					F	2
S. dysenteriae																	
	2	D	2	■	■				■	■			■	■	■	DD	7
	3	D	1	■	■	■					■		■	■	■	EE	6
	5	D	1	■							■					F	2

The presence of each VF is marked as a grey box

No: Number of isolates; VF: Number of virulence factors; D: Diarrhea, C. Control

[1]5 In the case of *S. sonnei* it is indicated the phase.