Title

Antibiotic resistance and molecular characterization of *Shigella* isolates recovered from children less than 5 years in Manhiça, Southern Mozambique.

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Running title: Characterization of *Shigella* spp in Mozambican children

Abstract

The objective of the study was to assess antibiotic resistance and molecular epidemiology of *Shigella* isolates from a case-control study of diarrhea, conducted from 2007-2012 in children less than 5 years in Manhiça district, southern Mozambique. All isolates were tested for antimicrobial susceptibility using disc-diffusion method. PCR was used to detect different molecular mechanisms of antibiotic resistance. Serotyping was performed using specific antisera. Clonal relationship for *S. flexneri* and *S. sonnei* was assessed by Pulsed-Field Gel Electrophoresis (PFGE). Of the 67 analyzed *Shigella* isolates, 59 were diarrheal cases and 8 controls. *S. flexneri* (70.1%; 47/67), was the most frequent species followed by *S. sonnei* (23.9%; 16/67). The most prevalent *S. flexneri* serotypes were 2a (38.3%; 18/47), 6 (19.2%; 9/47) and 1b (14.9%; 7/47). High rates of antimicrobial resistance were observed for trimethoprim/ sulfametoxazole (92.5%; 62/67), tetracycline (68.7%; 46/67), chloramphenicol (53.7%; 36/67) and ampicillin (50.7%; 34/67). Multidrug resistance was present in 55.2% (37/67) of the isolates and was associated with 8.1% (3/37) case fatality rate. PFGE revealed 22 clones (16 for *S. flexneri* and 6 for *S. sonnei*), among which, P1 (31.9%; 15/47), P9 (17%; 8/47) and P2 (10.6%; 5/47) were the most prevalent *S. flexneri* clones. In conclusion, *S. flexneri* was the most prevalent species with MDR isolates mainly belonging to three specific clones (P1, P9 and P2). The case fatality rate observed only for MDR isolates is a matter of concern indicating the need for appropriate treatment.

Key words: *Shigella* spp, antibiotic resistance, molecular epidemiology.

1. Introduction

According to the World Health Organization (WHO), diarrheal diseases remain as one of the most common and major causes of infant mortality in children in developing countries. Despite advances in the understanding of the disease and management strategies, globally 0.75 million children die annually as a consequence of diarrhea [1] and 165 million cases of dysentery annually were estimated to occur due to *Shigella* [2]. However, more recent estimations place *Shigella* disease burden around 90 million episodes and 108,000 deaths per year [3] which is still high. The wide spread of disease can be explained by the low infectious dose of the bacterium (10-100 cells), the direct person-to-person transmission, contaminated food and water transmission, and its low susceptibility to stomach acids [4]. In terms of clinical impact, *Shigella* is the enterobacteria showing the most serious outcome with fever, abdominal cramps and blood and mucus in the stools. The contribution of vectors, such as houseflies, has also been assessed and may contribute to the spread of the disease [5].

The role of *Shigella* spp. as causing childhood diarrhea in developing areas is probably the most recognized context in the epidemiology of shigellosis. A recently published 3-year, multicenter, prospective, matched case-control study of moderate-to-severe diarrhea (the Global Enteric Multicenter Study - GEMS) [6], evaluated the cause and impact of diarrheal diseases in over 22,000 children under 5 years from seven different sites of Asia and Africa including Mozambique. The study confirmed *Shigella* spp. among the top five important pathogens associated with childhood diarrhea [6]. Furthermore, several other studies have reported *Shigella* associated diarrhea in children living in areas with poor resources [7–9]. In developed countries, *Shigella* have been isolated especially during outbreaks of gastroenteritis following ingestion of contaminated food or water [10,11].

Four species of *Shigella* with 50 serotypes are currently recognized namely, *S. dysenteriae* (15 serotypes), *S. flexneri* (15 serotypes), *S. boydii* (19 serotypes) and *S. sonnei* (1 serotype) [12], among them, *S. flexneri* and *S. sonnei* are responsible for endemic forms of disease whereas *S. dysenteriae* 1 accounts for devastating epidemics [13]. Although the epidemiology of *Shigella* may vary from region to region, *S. sonnei* is being predominant in developed countries while *S. flexneri*

in those less developed [14]. However, a change in trend has been reported from developing countries, where *S. flexneri* serotypes have been replaced by *S. sonnei* in areas undergoing economic development and improvements in hygiene [15,16] which supports the importance of continuous surveillance to track disease epidemiology changes.

In addition to the diversity of *Shigella*, there is an increasing rate of antibiotic resistance for the most common used therapy (ampicillin, tetracycline, chloramphenicol, sulfonamides, sulphamethoxazole-trimethoprim and nalidixic acid) putting a serious threat for patient management. This scenario leads to the use of more expensive new-generation antibiotics such as fluoroquinolones though resistance has also been reported [17]. The rapid emergence of multidrug-resistant (MDR) strains is largely due to their ability to acquire and disseminate exogenous genes associated with mobile genetic elements such as, transposons, integrons, plasmids, and other genomic islands [18]. Thus, the ability to recognize and characterize multidrug resistant clones, will favor the development of preventive measures for infection control. Although *Shigella* infections has been previously reported in Mozambique [19], including data from the Global Enteric Multicenter Study (GEMS) [12], the molecular epidemiology and the impact of multidrug resistance in patient outcome of these infections remain unknown. Therefore, we hereby present analysis of the trends of antibiotic resistance and clonal relatedness of *Shigella* isolates from children aged less than 5 years in Manhiça District enrolled as part of the GEMS study from December, 2007 to November, 2012. This information will allow us to evaluate if there is a particular clone showing antibacterial resistance circulating in the area of study.

2. Material and methods

2.1.Study area

The study was conducted by the Manhiça Health Research Centre (Centro de Investigação em Saúde de Manhiça – CISM) in Manhiça district, a rural area of Maputo province in southern Mozambique. Since 1996, CISM has been running a Health Demographic Surveillance System (HDSS) for vital events and migrations in the population living within the study area covering approximately 95,000 inhabitants. In 2014, the study area was expanded to the whole District currently covering 183,000 inhabitants. Each person living within the DSS study area is issued a unique Permanent Identification Number that describes the geographic location, household

number and personal number within the household. A full description of the geographic and socio-demographic characteristics of the study community has been detailed elsewhere [20].

2.2.Bacterial isolates and study population

All isolates analyzed were collected from a case-control study of diarrhea conducted by CISM at Manhiça district from December 2007 to November 2012 with the main objective to estimate the burden and etiology of moderate-to-severe diarrhea (MSD) in children less than 5 years of age to guide future interventions. Cases of less severe diarrhea were also included from 3rd November 2011 to 2nd November 2012. Age in months was stratified in three strata (0-11, 12-23 and 24-59). Clinical and epidemiological data were collected over the study period. Cases of diarrhea were all children under 5 living within the HDSS area presenting to Manhiça district hospital with symptoms of diarrhea, defined as three or more loose stools in the last 24 hours. Study clinicians assessed each child with diarrhea for eligibility. To be included, the episode had to be new (onset after ≥7 diarrhea-free days), acute (onset within the previous 7 days), and fulfill at least one of the following criteria for moderate-to-severe diarrhea: sunken eyes (confirmed by parent or caretaker as more than normal); loss of skin turgor (abdominal skin pinch with slow [≤2 s] or very slow [≥2 s] recoil); intravenous hydration administered or prescribed; dysentery (visible blood in loose stools); or admission to hospital with diarrhea or dysentery [6]. For each child with diarrhea, one to three healthy control children (no story of diarrhea in the previous 7 days, matched by age, sex and neighborhood) were randomly selected using the HDSS database and enrolled within 14 days of presentation of the corresponding index case. Case fecal samples were collected within 12 hours of registration of the diarrheal episode, and control samples within 14 days after case enrolment. Once collected, samples were kept in a cool box until processed. Children were not enrolled if unable to produce fecal samples within the established first 12 hours or prior to the initiation of antibiotics. Each fecal specimen comprised a whole stool specimen (in screw top fecal specimen cups carried in Styrofoam boxes with cold packs), a fecal swab in Modified Cary Blair medium in a plastic screw top test tube, and a fecal swab in buffered glycerol saline in a screw top test tube [21]. Additionally, if antibiotics were to be given to patients before stool was produced, we obtained two rectal swabs for bacterial culture pending passage of the whole stool for the remaining assays. Fecal specimens were platted on specific culture media for detection of bacterial pathogens according to standard methods. *Shigella* species were identified by colonies morphology

upon culturing in MacConkey and XLD following by slide agglutination with specific antisera (Denka Seiken Co., Ltd., Tokyo, Japan). Quality control was performed at the Centers for Vaccine Development (CVD), University of Maryland, USA. At least one enteropathogen was identified in 79% (2968/3757) and two or more microbial agents in 41% (1540/3757) of the analyzed stool samples. Overall, S*higella* was isolated in 2.1% (79/3757), of which 67 were characterized in the present study. Forty-nine (73.1%) were recovered from moderate-severe-diarrhea (MSD) and 18 (26.9%) from less-severe-diarrhea (LSD). The proportion of cases of diarrhea among the analyzed isolates was 88.1% (59/67) and 11.9% (8/67) for controls. In addition, the isolates were mostly from older children aged 24-59 months (47.8%; 32/67) followed by 12-23 months (41.8%; 28/67) and 0-11 months (10.4%; 7/67). Mortality was reported in 3 children (5%; 3/60) of known outcome.

2.3.Antimicrobial susceptibility and resistance mechanisms

Antimicrobial susceptibility was assessed by disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) [22] for chloramphenicol (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), amoxicillin plus clavulanic acid (30 µg), gentamicin (10 µg), tetracycline (10 µg), ceftriaxone (30 µg), cefotaxime (30 µg), tobramycin (10 µg), trimethoprim-sulfametoxazole (25 µg). Multidrug resistance was defined as the presence of resistance in at least three unrelated class of antibiotics. Molecular determinants of resistance were screened by PCR using specific protocols for β-lactams (*TEM* [23], *SHV, OXA-1* [24], *CTX-M* [25]), tetracycline (*tetA*, *tetB*, *tetD*), chloramphenicol (*cmlA*, *floR*) and trimethoprim/sulfametoxazole (*dfrIa*, *dfr8, dfr12*) [23]. For DNA extraction, the isolates were recovered from -70°C freezer, streaked on MacConkey agar plates and incubated for 18-24 hours in a 37ºC incubator. A loop full of bacterial colonies was suspended into 500µl of distilled water and boiled for 10 minutes in a heat block and then centrifuged at 10,000 xg for 10 minutes. The supernatant was used as DNA template for PCR. DNA amplification was performed into 0.2ml Eppendorf tubes in 25µl reaction volume. Master Mix was prepared by adding 12.5 µl of PCR master mix 2X (Qiagen Multiplex PCR kit), 10 µM of each primer (0.5 µl), 2.5µl of Q-solution 1X, 2 µl of PCR water (nuclease free) and 2µl of DNA template. The amplified product was visualized in 2% agarose gel after staining with ethidium bromide. Sequencing of PCR products

was performed at Macrogen (https://dna.macrogen.com/eng/). DNA sequences were assigned using the BLAST nucleotide sequence search tool.

2.4.Clonal relatedness studies

The relationship of the isolates was evaluated by Pulsed-Field Gel Electrophoresis (PFGE). PFGE analysis was based on the PulseNet standardized PFGE protocol [26]. *Shigella* plugs were prepared with 1% agarose (Lonza Incert agarose, cat. 50123, Lonza Rockland, ME, USA) and digested with *Xba*I (New England Biolabs) restriction enzyme according to the manufacturer's instructions. Plugs of Salmonella enterica H9812 (Biorad) was used as positive control for all PFGE assays. Electrophoresis in agarose gel Biorad 1% (Certified Megabase Agarose, cat. 161-3109, Biorad laboratories, Inc, Hercules, USA) was performed with the CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA) in total volume of 2 L 0.5X TBE buffer previously cooled to 14°C in the electrophoresis chamber. The program was at 14°C, 200V (6 V/cm^2), 20 h, with switch times of 5–35 s. Gels were stained with Syber safe and visualized under UV light. Restriction patterns were analyzed through the infoQuest^{TM} FP software v.4.5 (Biorad laboratories, Inc, Hercules, USA) with 1% Dice coefficient tolerance. Isolates with at least 85% of similarity were considered as the same profile and clones were defined as PFGE clusters containing more than one isolate.

2.5.Data analysis

Laboratory data were entered to Microsoft excel file and combined to clinical information. Statistical analyses were performed using STATA software, version 14.1. Proportions were compared using the chi-square test or Fisher's exact test, as appropriate.

3. Results

3.1.Species prevalence and antibiotic resistance

Among the 67 analyzed isolates, 3 different *Shigella* species were found, being *S. flexneri* (70.1%; 47/67), the most dominant followed by *S. sonnei* (23.9%; 16/67), two *S. boydii* (3%) and two (3%) isolates which species could not be identified. The most prevalent *S. flexneri* serotypes were 2a (38.3%; 18/47), 6 (19.1%; 9/47) and 1b (14.9%; 7/47). Antimicrobial resistance was observed for

both cases and controls with high rates for trimethoprim/ sulfametoxazole (92.5%; 62/67), tetracycline (68.7%; 46/67), chloramphenicol (53.7%; 36/67) and ampicillin (50.7%; 34/67). *S. flexneri* was responsible for 70.1% (47/67) of the overall antibiotic resistance compared to 29.9% (20/67) for other species altogether. Thirty-seven (55.2%) were multidrug resistant found in both cases (91.8%; 34/37) and controls (8.1%; 3/37). Additionally, the majority of MDR isolates were from *S. flexneri* (47.7.7%; 32/67) compared to 7.4% (5/67) for other species (Table 1). The isolates remain susceptible to third generation cephalosporin (ceftriaxone and cefotaxime), quinolones (nalidixic acid and ciprofloxacin) and aminoglycosides (gentamicin and tobramycin) (data not shown). Overall, 82.1% (55/67) of the isolates possessed at least one gene conferring antibiotic-resistance, being the mostly found the *dfrIa* gene (59.7%; 40/67) followed by *OXA-1* (47.8%; 32/67), *dfr8* (41.8%; 28/67) and *tetB* (40.3%; 27/67) (Table 2). No *TEM*, *SHV*, *cmlA* and *dfr12* genes were detected in the analyzed isolates.

3.2. Epidemiological study

An overview of PFGE fingerprinting and strain information for individual isolates is illustrated in the Table 3. Fig. 1 shows the dendogram with PFGE image of *S. flexneri* and *S. sonnei* isolates. Twenty-two clusters were found, 16 for *S. flexneri* and 6 for *S. sonnei*. All *S. flexneri* isolates except 9, considered as singletons, belonged to 7 different clones. Regarding to *S. sonnei*, 4 clones were identified and 2 isolates were singletons. The predominant *S. flexneri* clones were P1 (31.9; 15/47), P9 (17%; 8/47) and P2 (10.6%; 5/47). In the case of *S. sonnei* isolates, they were mostly represented by P1 and P5 with 25% of the isolates for each clone (4/16).

In terms of evolution of the clones across the years, *S. flexneri* P1 clone was found through the whole study period (except 2007), P9 from 2009-2012 and P2 was only detected in 2011 and 2012. In contrast to *S. flexneri*, the distribution of *S. sonnei* clones was irregular however the main clones (P1 and P5) were mostly detected in 2012. Clonality among MDR isolates was observed as all MDR *S. flexneri* isolates were clustered in different clones (94.1%; 32/34) except two isolates (strain 310058 and 303291). Similar finding was observed for *S. sonnei* in which all MDR isolates were clonal. Furthermore, all died children (5%; 3/60) were MDR carries. Interestingly, the most commonly found MDR pattern was the combination of antibiotics including CHL-TET-AMC-AMP-SXT (72.9%; 27/37). Then, the same combination excluding amoxicillin-clavulanic acid

(CHL-TET-AMP-SXT) was found in 10.8%; (4/37) of the isolates, and, in the same proportion, the combination of CHL-TET-SXT followed by CHL-TET-AMC-AMP (8.1%; 3/37).

4. Discussion

In the present study, we characterized a collection of *Shigella* spp recovered from a case-control study of diarrhea in children less than five years in Manhiça District, Southern Mozambique from 2007 to 2012. Our findings demonstrate that the main burden of *Shigella* infections is from children aged 1-5 years, with the dominance of *S. flexneri* compared to other species. This observation supports the idea that *Shigella* species are geographically stratified based on the level of economic development in a given country [16] being *S. flexneri* the primary infectious species in developing countries whereas *S. sonnei* rates increase with economic development [16,27,28]. Currently, *S. flexneri* is predominant through Southeast Asia and Africa [12,29,30]. Additionally, the high prevalence of *S. flexneri* serotype 2a correlates with its clinical importance as the main target *Shigella* vaccine [31]. It has been postulated that a combination of four components which includes *S. flexneri* serotypes 2a, 3a, 6 and *S. sonnei* could provide an overall coverage of 88% through direct protection and cross protection among *S. flexneri* serotypes based on shared type and group-O antigen determinants [12]. This observation is in concordance with our findings where these serotypes together with *S. flexneri* 1b were the most prevalent.

Adding to the diversity of *Shigella*, we also report a high level of antimicrobial resistance in the analyzed isolates to commonly used antibiotics (ampicillin, tetracycline, chloramphenicol and trimethoprim/sulfametoxazole), being *S. flexneri* much more resistant compared to all other species. This finding correlate with the global spreading of antibiotic resistance associated with the extensive use of these antibiotics in clinical practices [15]. In Mozambique, for example, trimethoprim/sulfametoxazole is still used as prophylaxis against HIV exposure. In addition, the high rates of antibiotic resistance among *S. flexneri* isolates observed in the present study compared to other species, have been also reported [19] and might suggest possible genetic differences among species. An important result shown in this study is the presence of MDR in isolates from the control group (community children with no symptoms of diarrhea); this is worrisome since they may act as reservoir for infection transmission. Most of them were *S. flexneri* (2 out of 3

MDR control isolates identified) which emphasize the high burden of *S. flexneri* in this rural Mozambique. In addition the observed case fatality rate of MDR isolates represents a serious public health concern and argues for rapid diagnostic for appropriate treatment.

Our finding also supports the importance of genes encoding resistance determinants in the spreading of MDR as showed by the occurrence of resistance mechanisms such as of *dfrIa*, *OXA-1* and *tetB* genes. Indeed, trimethoprim/sulfamethoxazole resistance is most commonly acquired through a plasmid-encoded variant of the dihydrofolate reductase while ampicillin resistance is mediated by beta-lactamases encoding genes such as *TEM-1* and *OXA-1* [28]. Moreover, the *tet*-like genes are responsible for most of tetracycline resistance [19]. However, in the present study, some resistant mechanisms could not be elucidated based on the screened genes, suggesting that other mechanisms seem to be contributing to the resistance phenotype, which is consistent with other reports [19,24]. Thus, additional studies are needed in order to go further in this determination. In contrast to other studies [17,32], our isolates remain susceptible to third generation cephalosporin (ceftriaxone and cefotaxime), quinolones (nalidixic acid and ciprofloxacin) and aminoglycosides (gentamicin and tobramycin) probably due to their infrequent use as alternative in this geographic location.

Although the high diversity, 60% of the *S. flexneri* isolates were represented by three dominant PFGE clusters composed mainly by the most frequent serotypes (1b, 2a and 6). The absence of dominant clones in *S. sonnei* can be explained in part due to the limited number of the analyzed isolates compared to *S. flexneri*. A good concordance between serotyping and PFGE genotyping was observed for *S. flexneri* 1b and 2b. All serotypes 1b belonged to the same PFGE profile, additionally most of 2a were grouped in a single cluster indicating minor genetic differences. Serotype 6 in contrast, showed more heterogeneity, with scattered isolates in different PFGE patterns. Similarly, isolates with the same antibiotic resistance profile were found in different PFGE and vice-versa; however, MDR isolates were more frequently found in clusters suggesting a possible clonal spreading. Indeed, PFGE profiles do not always accord with classic typing methods such as serotyping and antibiotic resistance, and PFGE is often usually a more discriminatory method. This was illustrated here where isolates of identical serotype and/or resistance pattern showed heterogeneity of DNA patterns by PFGE, which is similar to previous studies [34,35].

In summary, this is the first report of molecular epidemiology of *Shigella* associated-diarrhea in Mozambican children. Our findings suggest the presence of high diversity of *Shigella* spp causing diarrhea in Mozambican children with multidrug resistant isolates belonging to a limited number of clones. MDR isolates are likely to be more lethal indicating the need for appropriate treatment. The presence of MDR carriers in the community is an alert for the importance of community-based interventions for infection prevention/control. *S. flexneri* 1b was importantly found in our collection although not included as targeted vaccine serotype suggesting continuous disease surveillance to track changes over time.

Ethical approval

The isolates were collected as part of the Global Enteric Multi-Centre Study (GEMS) which was approved by the Institutional Review Board at the University of Maryland School of Medicine, USA and the National Bioethics Committee for Health of Mozambique and Hospital Clinic, University of Barcelona, Spain.

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Conflict of interest

The authors declare no conflict of interest

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