CHARACTERISATION OF CLINICAL SHIGELLA ISOLATES, USING SEROTYPING AND ANTIMICROBIAL RESISTANCE PROFILES IN ZIMBABWE.

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Key words: antimicrobial resistance, speciation, extended spectrum beta-lactamase (ESBL).
ABSTRACT

Objective: The study was conducted to determine the species distribution and antimicrobial susceptibility patterns of *Shigella* species obtained from clinical samples (faeces, urine & blood). **Design:** This was a cross sectional analytical study.

Settings: The study was conducted between August 2016 and May 2017 at the National Microbiology Reference Laboratory (NMRL), Harare and National University of Science and Technology (NUST), Applied Biology and Biochemistry Department in Zimbabwe.

Isolates: A total of 123 stored *Shigella* isolates were obtained from the NMRL and all isolates were confirmed as *Shigella* using serology and PCR. Speciation of *Shigella* isolates was done using serology. Antimicrobial susceptibility testing was performed on all the isolates using the disc diffusion assay and antibiotic resistance genes were detected using conventional PCR. The isolates stored at NMRL were collected from different provinces around Zimbabwe. Results: *S. flexneri* was found to be the most prevalent species 87(71%) followed by *S. sonnei* 18(14%), *S. boydii* 11(9%) and *S. dysenteriae* 7(6%). A total of 115 isolates (93.5%) were resistant to two or more drugs suggesting wide spread multi-drug resistance (MDR). Twenty six multi-drug resistance patterns were observed. The most common patterns (resistance to trimethoprim-sulfamethoxazole, tetracycline, ampicillin and chloramphenicol) were exhibited by 28(24.3%) isolates for both combinations. *Shigella* was mainly isolated from stool samples: 112[*S. flexneri* (89.7%), *S. sonnei* (94.4%), *S. dysenteriae* (90.9%) and *S. boydii* (8%)] as compared to urine (4.6%, 5.6%, 9.1% and 0%) and blood (5.7%, 0%, 0%, 0%) respectively. Fourteen isolates (11.4%) were positive for the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes or a combination. **Conclusion:** The observed high multi-drug resistance could hamper treatment of shigellosis in Zimbabwe.
INTRODUCTION

Acute gastroenteritis is one of the leading causes of illness and death in infants, children, immune-compromised and aged individuals throughout the world, especially in developing countries [29]. *Shigella* infection is regarded as a global public health concern, causing diarrhoea in both the developed and developing regions. The WHO estimates that 165 million diarrhoeal cases occur each year globally of which 163 million cases occur in developing countries [14, 28]. About 1.1 million deaths occur each year with the highest incidences in children less than 5 years of age [48]. Many African countries are infested with shigellosis with fatalities reported in Eritrea, Ethiopia and Nigeria [30, 7 and 51]. The prevention of *Shigella* infections has proven difficult due to the low inoculum that is needed to cause disease and inadequate empirical therapy options secondary to antimicrobial resistance [18, 19]. *Shigella* serogroups distribution may differ from country to country and within a region but its spread is exacerbated by the low infectious dose of 10 to 100 bacteria plus the ease with which person to person and faecal-oral transmission occurs [48].

Shigellosis can be managed by treatment with antibiotics to manage infection and eradicate faecal excretion of bacteria thus preventing further disease transmission. The bacterium’s progressive acquisition of resistance to widely-used antimicrobials makes infection management difficult [8, 31, and 48]. Antimicrobial susceptibility testing information on *Shigella* is of great importance for correct drug treatment of the bacteria and to check for multi-drug resistant bacterial strains. Wang *et al.*, [47] reported that massive usage of antimicrobial agents in livestock production and human disease has increased and favoured the survival of multi-drug resistant (MDR) pathogens. Misuse of antibiotics has been linked to the proliferation survival of multi-drug resistant pathogens [8]. The World Health Organisation has also called for the development of candidate vaccines against *Shigella* [51]. In considering the efficacy of a *Shigella* vaccine, reliable estimates of the burden of disease in target endemic areas is necessary. There is also need to consider the age
specific incidence data, risk factors, distribution of the various serogroups and subtypes of Shigella causing infection [7].

From studies conducted in Asia and sub-Saharan Africa on Shigella, there has been the emergence of multidrug resistance to different antibiotics including ampicillin, trimethoprim-sulphamethoxazole and nalidixic acid [10, 16]. Drugs like fluoroquinolones, azithromycin and pivamidocillin have been found to be efficacious for the treatment of shigellosis in children and adults in sub-Saharan regions [28, 30]. In this study we sought to speciate and determine the antibiotic sensitivity profiles of clinical Shigella isolates associated with shigellosis in Zimbabwe. We also investigated the occurrence of the ESBL genes (BlaTEM, BlaSHV, and BlaCTX-M). There are several reports of ESBL-producing Shigella from India [10]. In the recent years, various ESBL-producing Shigellae were also reported from Korea (CTX-M-14) [42], Argentina (CTX-M-2) [46], Viet Nam (CTX-M-15, and CTX-M-24) [40], France, and Turkey [5]. Over the last few decades, Shigella has demonstrated unique ability to acquire horizontally-transferred genetic material, thereby making previously-efficacious drugs, like sulphonamides, tetracycline, ampicillin, and cotrimoxazole largely ineffective [41]. Despite the increasing resistance of bacteria to antibiotics reported worldwide [35] there is paucity of data on speciation and antibiotic resistance patterns of Shigella in Zimbabwe and Southern Africa. This study will therefore add to the available literature on the Shigella species.

MATERIALS AND METHODS

Samples

A total of 123 stored Shigella isolates were obtained from the National Microbiology Reference laboratory (NMRL, Harare). The isolates had been obtained from stool, urine and blood samples collected from participants suffering from diarrhoea over a 10 month period from August, 2016 to May, 2017. The samples were referred to the NMRL from different hospitals, clinics and private laboratories from several provincial and district hospital and clinics around Zimbabwe.
Resuscitation of isolates,

The stored isolates were processed according to standard Laboratory identification protocol. The isolates were initially thawed and isolated and subcultured on Xylose Lysine Deoxycholate (XLD) (Vermicon, Munich). Following 18 hours incubation at 37°C on Xylose Lysine Deoxycholate (XLD) (Vermicon, Munich) or MacConkey (Vermicom, Munich) plates were examined for non-lactose fermenting colonies (NLFc). The NLFc colonies (presumptive positive colonies) were sub cultured onto the following screening media; kligler iron agar (KIA), lysine (LIA), indole and simmon-citrate (Vermicom, Munich) and then incubated at 37°C. Three primary characteristics of a bacterium were detected by this media, including ability to ferment carbohydrates (lactose, sucrose, glucose), ability to produce gas, and non-production of hydrogen sulphide gas. Inoculation of the slope and butt medium was done using a small amount of growth from a resuscitated pure culture. Colonies were inoculated onto the media by stabbing the butt and streaking the surface of the slant slope in a zigzag pattern to the bottom of the tube. The tubes were incubated at 37°C to interpret according to standard methods. Test organism should react positive with indole test and negative with citrate test. All the 123 isolates were resuscitated successfully.

Speciation of Shigella isolates

Speciation was done on organisms that were positively identified as positive Shigella spp. using biochemical tests. The organisms were emulsified using 2-3 drops of normal saline, then antiserum to screen for Shigella were added to one drop of the emulsified organism. The antiserum and organism were mixed by rocking the slide for 30 seconds to one minute. Agglutination was recorded for all the antiserum Shigella sonnei Poly Phase I and II, Shigella boydii Poly C, Shigella flexneri Poly B and Shigella dysentriae Poly A (Mast, UK).
Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on Mueller Hinton agar (New England Biolabs, Midrand, South Africa) on all isolates using the disc diffusion assay. The following antibiotics were used: ciprofloxacin (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), tetracycline (30 μg), ampicillin (10 μg), cefpodoxime (30 μg) and trimethoprim-sulfamethoxazole (1.25 μg) (MAST, UK). The plates were incubated at 37°C for 24 hr, and inhibition zones were measured. The results were interpreted according to the CLSI [16] guidelines. *Escherichia coli* strain (ATCC 25922) was used for quality control.

Screening test for ESBLs

Isolates were screened for ESBL production by using disc diffusion of ceftazidime (CAZ) and cefpodoxime (CPD) placed on inoculated plates containing Mueller Hinton agar according to the CLSI, [16] recommendations. Co-resistance to ceftazidime and ceftriaxone by Shigella isolates was used as a phenotypic indicator for isolates capable of ESBL production. Isolates showing inhibition zone size of ≤11 mm with ceftazidime (30 μg) and cefpodoxime (30 μg), were suspected to be ESBL producers.

DNA isolation

Three to four well isolated colonies were inoculated into 150-μl sterile nuclease free water and boiled for 20 minutes at 100 °C in a water bath. The solution was allowed to cool to room temperature. Bacterial cells were spun for 9 minutes at 10000 g. The supernatant containing crude DNA was transferred to a new tube and diluted using nuclease free water to a DNA concentration of 5 ng/μl – 15 ng/μl. Quantification of crude DNA was done on the supernatant using a BioDoc analyse (Biometra, Germany). Prior to being used for PCR reactions.
Polymerase Chain Reaction for detection of ESBL genes

Conventional PCR was used for the genetic detection of three major ESBL genes, TEM, SHV and CTX-M. The primers used for each gene have been described in previous studies [35]. One microliter of each of the DNA samples were mixed with all the necessary components for amplification in a 0.2ml PCR tube (Perkin-Elmer, USA) in a 10µl reaction volume and run on a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, USA). The reaction mixture included 1µl Dream Taq buffer (10X concentration) (Thermo Scientific, USA), 0.2µl of deoxyribonucleotide triphosphate (dNTP) mix, 10mM (Thermo Fisher scientific, USA), 0.16µl of each of the forward and reverse primers, 25µM (Inqaba Biotech, South Africa) and 0.08 µl of Dream Taq DNA polymerase (Thermo Fisher scientific, South Africa) and made up to 10 µl with nuclease free water. A *Klebsiealla pneumoniae* isolate known to be positive for the assayed ESBL (Bla<sub>TEM</sub>, Bla<sub>SHV</sub> and Bla<sub>CTX-M</sub>) genes was used as a positive control Bla<sub>TEM</sub> (Genbank accession number KT818790), Bla<sub>SHV</sub> (KT818791) and Bla<sub>CTX-M</sub> (KT818792). The PCR profile was as follows: for CTX-M, SHV and TEM; denaturation 95°C for 2 minutes, 35 cycles (denaturation 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension 72°C for 1 minute) and the final extension at 72°C for 10 minutes. The PCR products were subjected to electrophoresis in a 0.8 % agarose gel and visualized with ethidium bromide staining; results were checked using the Uvipro silver gel viewer (Uvitec, UK).

RESULTS

Biochemical and serology tests

One hundred and twenty three isolates (100%) reacted positively with Kliger iron agar (acid butt, alkaline slope), Lysine iron agar (positive acid butt, alkaline slope) and were citrate negative. All the 123 isolates reacted positively with specific *Shigella* antisera for the different species. One hundred and twenty isolates were confirmed by agglutinating with specific antisera of *Shigella*. 
Speciation of Shigella isolates

After serology tests the following Shigella species were identified, 87(71%) S. flexneri; 18(14%) S. sonnei; 11(9%) S. boydii; 7(6%) S. dysenteriae (figure 1).

![Percentage species distribution of Shigella species](image)

**Figure 1:** Showing the species distribution of the *Shigella* isolates.

**Species distribution according to source of isolate.**

The most frequently isolated species were: 4(4.6%) S. flexneri followed by 17(94.4%) S. sonnei, 10(90.9%) S. dysenteriae and 7(8%) S. boydii in stool. In urine 1(9.1%) S. dysenteriae had the highest frequency followed by 1(5.6%) S. sonnei, 4(4.6%) S. flexneri and 0(0%) S. boydii. *S. flexneri* 5(5.7%) was the only species isolated from blood samples (Table 1).
**Table 1**: Distribution of species according to source of isolate

<table>
<thead>
<tr>
<th>Species</th>
<th>Urine N (%)</th>
<th>Stool N (%)</th>
<th>Blood N (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em></td>
<td>4(4.6%)</td>
<td>78(89.7%)</td>
<td>5(5.7%)</td>
<td>87</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>1(5.6%)</td>
<td>17(94.4%)</td>
<td>0(0%)</td>
<td>18</td>
</tr>
<tr>
<td><em>S. dysenteriae</em></td>
<td>1(9.1%)</td>
<td>10(90.9%)</td>
<td>0(0%)</td>
<td>11</td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td>0(0%)</td>
<td>7(8%)</td>
<td>0(0%)</td>
<td>7</td>
</tr>
</tbody>
</table>

**Antibiotic susceptibility assay**

*Shigella* isolates studied showed different antimicrobial susceptibility patterns towards the nine antibiotics tested (Table 2). *S. boydii* was noted to be resistant to 5 antibiotics with the highest resistance shown against chloramphenicol (85.7%). *S. flexneri* was resistant to 4 antibiotics with the highest being against chloramphenicol (78.4%). *S. sonnei* was resistant to 6 antibiotics and the highest resistances were noted against chloramphenicol and tetracycline with 77.8% respectively. *S. dysenteriae* was resistant to 6 antibiotics and the highest resistance was noted for trimethoprim-sulfamethoxazole and chloramphenicol with 81.8% respectively.
Table 2: Antibiotic resistance profiles of different *Shigella* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>CIP</th>
<th>CPD</th>
<th>TMX</th>
<th>NAL</th>
<th>CAZ</th>
<th>TET</th>
<th>AMP</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. boydii</em> (n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>7(100%)</td>
<td>7(100%)</td>
<td>1(14.3%)</td>
<td>5(71.4%)</td>
<td>6(85.7%)</td>
<td>2(28.6%)</td>
<td>7(100%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>1(14.3%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>1(14.3%)</td>
<td></td>
</tr>
<tr>
<td>Resistance</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>5(71%)</td>
<td>2(28.6%)</td>
<td>1(14.3%)</td>
<td>5(71.4%)</td>
<td>0(0%)</td>
<td>6(85.7%)</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. flexneri</em> (n=87)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>82(94.3%)</td>
<td>82(94.3%)</td>
<td>25(28.7%)</td>
<td>64(73.6%)</td>
<td>75(86.2%)</td>
<td>18(20.7%)</td>
<td>12(13.8%)</td>
<td>16(18.4%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>4(4.5%)</td>
<td>4(4.5%)</td>
<td>6(6.9%)</td>
<td>7(8.1%)</td>
<td>7(8.1%)</td>
<td>5(5.7%)</td>
<td>8(9.2%)</td>
<td>3(3.4%)</td>
</tr>
<tr>
<td>Resistance</td>
<td>1(1.2%)</td>
<td>1(1.2%)</td>
<td>56(64.4%)</td>
<td>16(18.3%)</td>
<td>5(5.8%)</td>
<td>64(73.6%)</td>
<td>67(77%)</td>
<td>68(78.2%)</td>
</tr>
<tr>
<td><em>S. sonnei</em> (n=18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>18(100%)</td>
<td>18(100%)</td>
<td>9(50%)</td>
<td>13(72.2%)</td>
<td>14(77.8%)</td>
<td>4(22.2%)</td>
<td>14(77.8%)</td>
<td>2(11.1%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>1(5.6%)</td>
<td>3(16.7%)</td>
<td>2(11.1%)</td>
<td>0(0%)</td>
<td>2(11.1%)</td>
<td>2(11.1%)</td>
</tr>
<tr>
<td>Resistance</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>8(44.4%)</td>
<td>2(11.1%)</td>
<td>2(11.1%)</td>
<td>14(77.8%)</td>
<td>2(11.1%)</td>
<td>14(77.1%)</td>
</tr>
<tr>
<td><em>S. dysenterie</em> (n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>10(90.9%)</td>
<td>10(90.9%)</td>
<td>2(18.2%)</td>
<td>6(54.5%)</td>
<td>10(90.9%)</td>
<td>9(81.8%)</td>
<td>3(27.3%)</td>
<td>2(18.2%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>2(18.2%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Resistance</td>
<td>1(9.1%)</td>
<td>1(9.1%)</td>
<td>9(81.8%)</td>
<td>3(27.3%)</td>
<td>1(9.1%)</td>
<td>2(18.2%)</td>
<td>8(72.7%)</td>
<td>9(81.8%)</td>
</tr>
</tbody>
</table>

**KEY:** AMP, Ampicillin; TET, Tetracycline; C, Chloramphenicol; NAL, Nalidixic acid; CIP, Ciprofloxacin; CAZ, Ceftazidime; TMX, Trimethoprim-sulfamethoxazole; CPD, Cefpodoxime.

**Multi-drug resistance (MDR) profile of *Shigella* species**

Multidrug resistance (MDR) is defined as insensitivity or resistance of a microorganism to the administered antimicrobial medicines (which are structurally unrelated and have different molecular targets) despite earlier sensitivity to it [43]. A total of 115 *Shigella* (93.5%) were resistant to two or more drugs (Table 3). The most prevalent resistotype was TET-AMP-C and TMX-TET-AMP-C which exhibited 24.3% prevalence. It was also noted that there were...
ten isolates that exhibited a multi-drug resistance to more than three antibiotics which is a cause of concern.

**Table 3: Resistotypes of *Shigella* isolates.**

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Number of isolates</th>
<th>*Percentage %</th>
<th>Resistotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>24.3</td>
<td>TMX-TET-AMP-C</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>24.3</td>
<td>TET-AMP-C</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>8.7</td>
<td>TMX-TET-AMP</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>5.2</td>
<td>TMX-NA-TET-AMP-C</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>5.2</td>
<td>AMP-C</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>4.3</td>
<td>TMX-TET-C</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>4.3</td>
<td>TET-AMP-C</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>3.5</td>
<td>TMX-CAZ-TET-AMP-C</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>2.6</td>
<td>NA-TET-AMP-C</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>2.6</td>
<td>TMX-TET-C</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>2.6</td>
<td>TMX-AMP-C</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>2.6</td>
<td>TMX-C</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>2.6</td>
<td>TET-C</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>1.7</td>
<td>NA-TET-AMP</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>1.7</td>
<td>CAZ-TET-AMP</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0.86</td>
<td>NA-CAZ-TET-AMP-C</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0.86</td>
<td>TMX-NA-TET-AMP-C</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>0.86</td>
<td>TMX-NA-TET-C</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>0.86</td>
<td>CPD-NA-CAZ-C</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>0.86</td>
<td>NA-CAZ-TET-C</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>0.86</td>
<td>TMX-NA-CAZ</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>0.86</td>
<td>TMX-CAZ-AMP</td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>0.86</td>
<td>NA-AMP-C</td>
</tr>
</tbody>
</table>
Distribution of TEM, SHV and CTX-M genes in *Shigella* species

Co-resistance to ceftazidime and cefpodoxime by *Shigella* was used as a phenotypic indicator for isolates capable of ESBL production. Out of the 123 *Shigella* isolates, 14 (11.4%) were phenotypically positive for ESBL production. The 14 isolates were tested for the presence of ESBL genes TEM, SHV and CTX-M using PCR. All 14 ESBL producing *Shigella* isolates carried the *bla*<sub>CTX-M</sub> (Fig 2), *bla*<sub>SHV</sub> (Fig 3), and *bla*<sub>TEM</sub> (Fig 4) genes or a combination. Of the 14 *Shigella* isolates a total of 12 (75%) were positive for the *bla*<sub>TEM</sub> genes, followed by 9 (56.3%) *bla*<sub>SHV</sub> and 3 (18.8%) *bla*<sub>CTX-M</sub> (Table 4).

**Table 4:** Distribution of TEM, SHV and CTX-M genes in *Shigella* isolates.

<table>
<thead>
<tr>
<th>Detected Gene(s)</th>
<th>Number of Isolates</th>
<th>Species (n=no. species)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTX-M</strong></td>
<td>3 (18.8%)</td>
<td><em>S. flexneri</em> (n=3)</td>
</tr>
<tr>
<td><strong>SHV</strong></td>
<td>9 (56.3%)</td>
<td><em>S. flexneri</em> (n=7); <em>S. boydii</em> (n=1) and <em>S. dysenteriae</em> (n=1)</td>
</tr>
<tr>
<td><strong>TEM</strong></td>
<td>12 (75%)</td>
<td><em>S. flexneri</em> (n=9); <em>S. sonnei</em> (n=2) and <em>S. boydii</em> (n=1)</td>
</tr>
<tr>
<td><strong>CTX-M &amp; SHV</strong></td>
<td>3 (18.8%)</td>
<td><em>S. flexneri</em> (n=3)</td>
</tr>
<tr>
<td><strong>CTX-M &amp; TEM</strong></td>
<td>3 (18.8%)</td>
<td><em>S. flexneri</em> (n=3)</td>
</tr>
<tr>
<td><strong>SHV &amp; TEM</strong></td>
<td>7 (43.8%)</td>
<td><em>S. flexneri</em> (n=6) and <em>S. boydii</em> (n=1)</td>
</tr>
<tr>
<td><strong>CTX-M, SHV &amp; TEM</strong></td>
<td>3 (18.8%)</td>
<td><em>S. flexneri</em> (n=3)</td>
</tr>
<tr>
<td><strong>None</strong></td>
<td>4 (25%)</td>
<td><em>S. flexneri</em> (n=4)</td>
</tr>
</tbody>
</table>

* negative for molecular detection of all assayed ESBL genes.
Most isolates had a combination of genes detected that is 7 for the $bla_{SHV \& TEM}$ and 3 for the $bla_{CTX-M \& SHV}$, $bla_{CTX-M \& TEM}$ and $bla_{CTX-M, SHV \& TEM}$ cluster. The most commonly detected ESBL gene in the *Shigella* isolates was the $bla_{TEM}$ cluster with 75%. For the combination the $bla_{SHV \& TEM}$ was the most prevalent 43.8%. For the CTX-M gene the serotype *S. flexneri* was the only one that exhibited the gene. *S. flexneri* was also noted to exhibit the SHV gene and one isolate of *S. boydii* was also positive of the SHV gene. The TEM gene was present in different species; (9) *S. flexneri*, (2) *S. sonnei* and (1) *S. boydii* (Table 4).

**Figure 2:** Detection of CTX-M genes in *Shigella* isolates (MW- molecular weight marker 1kilo bases, 9, 10 and 12 are *Shigella* isolates).

**Figure 3:** Detection of SHV genes in *Shigella* isolates (M- molecular weight marker 1kilo bases, C- positive control 5, 7, 9, 10 and 11 are *Shigella* isolates).
Figure 4: Detection of TEM genes in *Shigella* isolates (M- molecular weight marker 1kilo bases, C- positive control, 2, 3, 4, 7, 8, 11 *Shigella* isolates). The isolate numbers 2 and 13 were *S. sonnei* and isolate number 14 was *S. boydii*.

**DISCUSSION**

*S. flexneri* was the most frequently isolated serotype in the study with a prevalence of 71% (Fig 1) which is comparable to studies done in Ethiopia (2009) (87.5%) [46], and South Africa (2009) (86%) that had similar prevalence. The prevalence of a particular species of *Shigella* differs in various geographical regions [41]. Kotloff *et al.*, [22] reported *S. flexneri* as the main serogroup found in developing countries (60%), followed by *S. sonnei* (15%), and *S. dysenteriae* (6%) and *S. boydii* (6%) [41]. In another study *S. dysenteriae* and *S. flexneri* were found to be the predominant species in developing countries, with *S. sonnei* accounting for most cases of shigellosis in developed countries and *S. boydii* predominating in Asia, mainly in India [40]. In another study on distribution of *Shigella* species and their antimicrobial patterns carried out in Africa, Asia and South America, *S. flexneri* was isolated predominately from Ethiopia [7], Kenya [7], Eritrea [24], Ghana [25], Pakistan [41], China [40], and Nepal [20]. Findings of predominant species have important implications for treatment and prevention strategies. In most developing countries, *S. flexneri* is the predominant *Shigella species* isolated from stool of patients with infectious diarrhoea [17].
Nevertheless, some studies suggest *S. flexneri* is more prevalent in tropical countries [7]. Prevalence of *S. dysenteriae* (Serotype A) and *S. boydii* (serotype C) was low in the present study (fig 1); this is consistent with previous reports [19; 35] of *Shigella* isolates from the public healthcare system in South Africa. The low prevalence could be because serogroup A is commonly associated with large dysentery outbreaks [38]; however, *S. boydii* (serogroups C) is generally the least frequently isolated of *Shigella* strains. It is typically associated with individuals who have travelled to endemic areas as equally observed in other studies [35, 4]. However, it is not unusual for one serogroup to replace another in the community from time to time. The comparative dominance of serogroups fluctuates with time, hygienic conditions and differences in population [13]. However, incidences of these serotypes in the healthcare system can be ascertained by longer surveillance studies. The prevalence of *Shigella* species in Ethiopia [6] and other parts of the world, seems to alternate mainly between *S. flexneri*, and *S. sonnei*, as these remain the most active agents of shigellosis. These findings are consistent with our findings were *S. flexneri* was the predominant species, however in the Ethiopian study the distribution was slightly different as the order of prevalence was *S. flexneri* (54%), *S. dysenteriae* (22.4%), *S. sonnei* and *S. boydii* [6]. Another study done in Gonder, Ethiopia found *S. flexneri* (72.2%), *S. dysenteriae*, *S. boydii*, and *S. sonnei* [34]. A study done by Beyene et al. [7] in Jimma and Addis Ababa found (68.9%) *S. flexneri*, (9.8%) *S. boydii*, and (21.3%) *S. sonnei* [7] and another study from Ghana by Opintan et al. [24] found (70.8%) *S. flexneri*, (16.7%) *S. dysenteriae*, (8.3%) *S. sonnei* and (4.2%) *S. boydii* [24]. No *S. dysenteriae* was reported from the study conducted at Jimma and Addis Ababa, Ethiopia in 2006 [7] which is in contrast with findings in our study. The difference in the pattern of species distribution may be due to ecological or geographical differences, study time or human host differences.

Shigellosis is becoming an increasingly significant public health problem due to development of multiple antimicrobial resistances, frequently resulting in treatment failure, leading in turn to health complications and deaths [8]. Shigellosis is a major public health problem in
developing countries. Antimicrobial therapy with fluoroquinolones is recommended to shorten the course of disease and fecal shedding [36]. Various studies have reported an increase in antimicrobial resistance amongst different species of *Shigella* against commonly used drugs, including ampicillin, tetracycline, chloramphenicol and co-trimoxazole [40]. Antimicrobial agents as effective options for shigellosis treatment are becoming limited due to globally emerging drug resistance. Multiple resistant strains have occurred in India [5] and Africa [8].

In this study, *S. flexneri* showed antimicrobial resistance to ampicillin (77%), chloramphenicol (78.2%), tetracycline (73.6%), and trimethoprim-sulfamethoxazole (64.4%). In a study in Brazil by Achi et al. (2016) [2] similar resistance patterns, resistance to (70%) ampicillin, (68%) chloramphenicol, (73%) tetracycline, and (61%) trimethoprim-sulfamethoxazole were observed. Also, in a study done in Ethiopia [7] *Shigella* spp were resistant to (90.1%) trimethoprim-sulfamethoxazole. In the United States, the most common resistance patterns among 620 *Shigella* isolates 86% of which were *S. sonnei*, were resistance to ampicillin (77%), streptomycin (54%), trimethoprim-sulfamethoxazole (37%), sulfamethoxazole (32%), and tetracycline (31%) [15]. A similar resistance pattern to our current study was reported from India [10]. Data from developing countries, such as Chile, indicate that most *Shigella* spp. were resistant to ampicillin (82%), co-trimoxazole (65%), tetracycline (53%), and chloramphenicol (49%) [20]. Reports from Bangladesh, where shigellosis is highly endemic, show a similar resistance pattern to our current study [9]. Outbreaks caused by multi-resistant *S. dysenteriae* type 1, including strains resistant to nalidixic acid, have also been reported [31]. The study from Bangladesh was consistent with the present study finding in which all the serogroups exhibited a significant resistance to ampicillin (40.2%), co-trimoxazole (65.4%), tetracycline (60.3%), and chloramphenicol (80.9%) [44]
Nowadays, recommended therapy for people infected with *Shigella* includes fluoroquinolones [10, 26], azithromycin [3], and third generation cephalosporins [31]. In a study conducted by the Zimbabwe, Bangladesh, South Africa dysentery study group, ciprofloxacin taken twice daily for 3 days achieved clinical cure rates similar to those for the same dosage given for 5 days in children with *Shigella* infection [7]. However, it has been observed that *S. sonnei* readily acquires resistance to ampicillin and cephalosporins through conjugative resistance-plasmids carrying resistance cassettes to beta-lactamase [37]. In *S. sonnei* and *S. flexneri*, chromosomal mutations can confer resistance to quinolones [22]. Continuous monitoring of antimicrobial susceptibilities of *Shigella* spp. through a surveillance system is thus essential for effective therapy and control measures against shigellosis.

ESBL-producing *Shigella* pose a distinctive test to clinical microbiologists, clinicians, infection control professionals and scientists tasked in finding new drugs. ESBL-producing strains are usually found in hospitals where antibiotic use is frequent and the patients are critical [40]. In spite of high prevalence of resistant strains, ESBL production associated with *S. flexneri* and *S. Sonnei* as previously reported in South Africa between 2003 to 2009 [42] was consistent with the findings of the current study. However, ESBL producing isolates have been observed elsewhere such as in Nigeria [5]. In this study \( n = 14/123 \) (11.4\%) of strains were resistant to beta-lactamase using phenotypic screening. In Europe, Asia, and Africa the most common plasmid-encoded \( \beta \)-lactamase in resistant Gram-negative bacteria, including *Shigella*, is TEM-1 [39]. This enzyme can hydrolyze a number of penicillin’s and cephalosporins and is easily transferred to other members of the Enterobacteriaceae family as well as to *Vibrio cholerae* [38]. In this study, three ESBL genes (CTX-M, SHV and TEM) were detected from three different *Shigella* serotypes namely *S. flexneri*, *S. sonnei* and *S. boydii* (Fig 2, 3 and 4). In the study *S. flexneri* was found to contain all the 3 ESBL genes followed by *S. sonnei* and *S. boydii* which had the genes SHV and TEM only (Table 4). The most prevalent gene was the TEM (9.8\%) followed by SHV (4.9\%) and CTX-M (2.4\%). In a study conducted in South Africa (2009), PCR and DNA sequencing revealed that 16 out of
the 20 isolates were positive for the \( \text{bla}_{\text{TEM}-1} \) β-lactamase gene, the most commonly encountered β-lactamase gene in members of the family Enterobacteriaceae, characterized by high hydrolytic activity against penicillins and early cephalosporins such as cephalothin and cephaloridine [12] which is consistent with the present study. The TEM-type β-lactamase gene was the most commonly identified ESBL via PCR analysis. These data are in contrast with worldwide data, which describe \( \text{bla}_{\text{CTX-M}} \) as the most common gene identified in members of the genus *Shigella* [21]. Members of the family Enterobacteriaceae that produce CTX-M-type β-lactamase are a major cause of disease outbreaks and sporadic infections worldwide [31]. The \( \text{bla}_{\text{CTX-M}} \) gene has been reported to co-exist on the same plasmid with the \( \text{bla}_{\text{TEM}} \) gene [37]; this correlates well with the results of the current study, which showed that all the isolates that possessed the \( \text{bla}_{\text{TEM}} \) gene also possessed the \( \text{bla}_{\text{CTX-M}} \) gene. Only six of the ESBL-producers from this study were PCR-positive for the SHV-type β-lactamase. This ESBL displays extended-spectrum activity towards oxyimino cephalosporins and aztreonam [12]. It is not uncommon for members of the family Enterobacteriaceae to carry multiple β-lactamase genes [21]. This was also true of the isolates described in the current study which carried multiple β-lactamase genes, including: \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{CTX-M}} \) (Table 4). It has been reported that carriage of multiple β-lactamase genes could lead to limited β-lactam therapeutic options [38]. Previously, ESBL producing clinical strains of *Shigella* spp. have been reported from developing countries like India [10], Brazil [35], Bangladesh [39] and others. In the past two decades both the isolation frequencies and the types of ESBLs have gradually increased. CTX-M, SHV and TEM type ESBLs are being increasingly reported in the *Shigella* spp. around the globe [17]. The ESBLs are detected most commonly in *Klebsiealla pneumoniae* and *Escherichia coli* but have been noted in other members of the Enterobacteriaceae family as well [40].
CONCLUSION

The results obtained from our study suggest that multiple drug resistance is widespread in clinical *Shigella* isolates. Our study could form a basis for further, more comprehensive, *in vitro* antimicrobial susceptibility studies on clinical *Shigella* in Zimbabwe. It is clear that this region shares the worldwide trend of increasing antimicrobial resistance. Sound surveillance based on competent and affordable microbiology is required to provide clear data on antimicrobial resistance and the species distribution, which in turn can enable revision of local treatment guidelines and fuel national and regional policies to contain antimicrobial resistance.

Contributors:

This work was carried out in collaboration between all authors. Authors KSK and JM designed the study, and carried out experimental work on the project. Authors MM, AT, TM carried out experimental work on the project. Author BSP assisted in the supervision and funding of the project. Author KSK developed the initial draft of the manuscript and all authors approved the final manuscript. All authors had full access to the data in the study. KSK and JM are the guarantors.

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REFERENCES

1. Abd-Elmege, M. District Laboratory Practice in Tropical Countries Part 2 Second

   resistance in Shigella from humans in England and Wales: recommendations for

3. Acikigiz, K, Levine, M, M, Hornick, R, B, Formal, S, B. CTX-M-3 Type Beta-
   Lactamase Producing Shigella sonnei isolates from Pediatric Bacillary Dysentery

   spectrum beta-lactamase in a Shigella sonnei clinical isolate: first report from


