

Phenotypic Identification and Molecular Characterization of *Shigella* sp from Diarrhoeal Patients' Stool Samples in Nigeria

Isawumi Abiola¹, Oluduro Anthonia Olufunke¹, Moses Ikechukwu Benjamin¹ and Ariyo Adenike Bosede²

¹Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria, 220005

²Biological Science, faculty of Science, Federal University, Otuoke

E-mail: aoluduro2003@yahoo.co.uk.

Telephone: +2348069379885.

Abstract: Shigellosis is a worldwide health concern especially in developing countries with poor sanitation and has contributed to the increase in the mortality rate of infants. The study reports the antibiotic resistance profiles and characterization of resistance (*gyrA*, *blaCTX*) and virulence (*Stx1*) genes in *Shigella* sp recovered from stool samples of diarrhoeal patients in Nigeria. One hundred and forty-eight diarrhoeal patients' stool samples collected from various health institutions in Ile-Ife, Osun State, Nigeria were examined for the presence of *Shigella* sp. Isolation was done on *Salmonella-Shigella* agar plates at 37°C and isolates identity confirmed by conventional biochemical tests. Antibiotic susceptibility of isolates was by Kirby-Bauer's disc diffusion technique and isolation of plasmid DNA in the multiple antibiotic resistant (MAR) isolates was carried out. Molecular detection of resistance (*gyrA*) and virulence (*Stx*) genes was by polymerase chain reaction. A total of 58 *Shigella* sp comprising *S. sonnei* (37.9%), *S. boydii* (37.9%), *S. flexneri* (13.8%) and *S. dysenteriae* (10.3%) were recovered. Susceptibility to antibiotics was in varying proportions. All isolates were resistant to ceftriazone and resistance was mostly to nitrofurantoin (96.5%), augmentin (94.8%) and amoxicillin 82.2%). Generally, 80% of the *Shigella* sp were multiple antibiotic resistant (MAR) types, displaying 22 various MAR patterns. Plasmid DNA bands of varying sizes (1.17-23.13 kb) and fluoroquinolone resistance (*GyrA* -270bp) genes were harboured by all the representative MAR isolates. None of the representative MAR isolates contained the beta-lactam resistance (*BlaCTX*) gene. Shiga toxin (*Stx*-400bp) was detected in only one of the isolates. The recovery of multiple antibiotic resistant *Shigella* sp harbouring large size plasmid DNA and fluoroquinolone resistance gene is of great health consequence.

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Introduction

Shigella was recognized as the etiologic agent of Shigellosis in 1898 by Kiyoshi Shiga, a Japanese scientist and was adopted as a genus in the 1950 (Kim *et al.*, 2002). *Shigella* exists as a single organism and causes disease in primates (Ryan *et al.*, 2004). It is only naturally found in humans and apes (Potter, 2006). During infection, it typically causes bacillary dysentery with diarrhoea as one of the major symptoms (Mims *et al.*, 1993).

Shigellosis is a worldwide health concern especially in developing countries with poor sanitation, lack of personal hygiene and use of contaminated water supplies. Its effects have contributed to the increase in the mortality rate of infants (WHO, 2004). Estimation by the World Health Organization (WHO, 2004) indicates that the world population suffered from 4.5 billion incidences of diarrhoea causing 1.8 million deaths annually. Approximately 99% of the cases occurred in developing countries, where poor hygiene and limited access to clean drinking water promote the spread of enteric diseases. Malnutrition and the lack of

appropriate medical intervention contribute to the high mortality rate, especially for young children.

It has been reported that species of the genus *Shigella* are among the bacterial pathogens most frequently isolated from patients with diarrhoea. A total of 5 to 15% of all diarrhoeal episodes worldwide can be attributed to an infection with *Shigella* (Kotloff *et al.*, 1999). The emergence of multidrug-resistant *Shigella* stains and a continuous high disease incidence imply that shigellosis is an unsolved global health problem (Sansone, 2005). Since it is an acute intestinal infection, the symptoms can range from mild watery diarrhoea to severe inflammatory bacillary dysentery characterized by strong abdominal cramps, fever, and stools containing blood and mucus. According to Venkatesan *et al.* (2006), the disease is usually self-limiting but may become life-threatening if patients are immune compromised or if adequate medical care is not available.

The four species of the genus *Shigella* that is Responsible for Shigellosis include *Shigella dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. The low infectious inoculum, (as few as 10 organisms)

render *Shigella* sp highly contagious. The predominant serogroups of *Shigella* sp occurring in a region also appears to be related to the socio-economic development; and evidence also indicates that the severity of shigellosis is related to the infecting serogroup. *Shigella dysenteriae* known as Shiga bacillus has been recognized as the most virulent, because it can produce Shiga Toxin with 12 serotypes and the major cause of epidemic dysentery for nearly 100 years (WHO, 2004). Diarrhoeal infections are caused by ingesting certain bacteria, viruses or parasites that may be spread by water, food, utensils, hands, flies; which eventually leads to passage of watery stools usually at least three times in 24 h period. Generally, *Shigella* invades the host through the M-cells in the gut epithelial of the large intestine using a Type III secretion system acting as a biological syringe to injects protein into cells which triggers bacterial invasion and the subsequent lysis of vacuolar membranes resulting in Shigellosis and the emergence of multidrug-resistant *Shigella* strains and a continuous high disease incidence imply that shigellosis is an unsolved global health problem (Sansonet, 2005).

The progressive increase in antibiotic resistance among enteric pathogens in developing countries has been reported (Egah *et al.*, 2003). This might be due to environmental factors, geographic differences or different patterns of antibiotic usage (Farshad *et al.*, 2006). The present study was therefore, designed to isolate and characterize *Shigella* sp in diarrhoeal patients' stool samples in Ile-Ife, Nigeria, and provide relevant information in the choice of antibiotics for the treatment of shigellosis in the study area.

Materials and Methods

Collection of Samples

One hundred and forty-eight (148) diarrhoeal patients' stool samples were collected between April, 2011 and March, 2012 at Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC), Ile-Ife and in some other Health Institutions within Ile-Ife, Nigeria. Samples collected in clean universal sampling bottles were immediately transported to the laboratory for bacteriological analysis. The stool samples were enriched in Selenite F broth, incubated at 37°C for 24 hours and streaked on *Salmonella-Shigella* agar plates, and incubated at 37 °C for 48 h. Pure colonies were obtained by successive streaking on *Salmonella-Shigella* agar plates. The identity of isolates was confirmed by conventional biochemical tests with reference to Bergey's Manual of Determinative Bacteriology.

Antibiotic Susceptibility Test

Antibiotic susceptibility of the *Shigella* isolates was done by the Kirby-Bauer's antibiotic disc

diffusion method. The disc containing the antibiotics augmentin (30µg), amoxicillin (25µg), nitrofurantoin (200µg) pefloxacin (5 µg), tetracycline (30 µg), ciprofloxacin (10µg), ofloxacin (5µg), ceftriazone (30 µg), gentamicin (10 µg) and cotrimoxazole (25 µg) were firmly placed on Mueller-Hinton agar (HIMEDIA lab. Ltd Vadhani) plates previously seeded with standardized inoculums (10⁶cfu/ml). The plates were then incubated at 37°C for 24 h. The diameter of the zones of inhibition were measured to the nearest millimeter and interpreted according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2009).

Isolation of Plasmid DNA

Plasmid DNA extraction was carried out on the representative MAR isolates using TENS buffer (Tris 25mM, EDTA 10mM, NaOH 0.1N and SDS 0.5%) and separated by 0.8% (w/v) agarose gel in Tris-acetate-EDTA buffer containing ethidium bromide (20 ml of 50 X TAE and 6.0 µl of 10 µg/ml ethidium bromide per litre). Plasmid DNA bands were visualized by UV light illuminator and photographed with a Leicaflex SL-camera.

Molecular detection of resistance (*gyrA* and *blaCTX*) genes

Twenty isolates that were resistant to two major classes of antibiotics (Fluoroquinolones and Beta-lactams) were selected for the molecular detection of *gyrA* and *blaCTX* genes respectively. Primers (*gyrA*-1) 5'-CGT TGG TGA CGT AAT CGG-3', (*gyrA*-2) 5'-CCG TAC CGT CAT AGT TAT-3' and (*blaCTX*-1) 5'-ATG TGC AGY ACC AGT AAR GTK ATG GC-3' and (*blaCTX*-2) 5'-TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3' (R, Y, K, S are wobbles base pairs) were used

The Polymerase Chain Reaction (PCR) composed of the following: 2.5µl of PCR buffer, 1.5µl of MgCl₂, 0.1 unit of Taq DNA Polymerase, 0.2µl of Primers (both forward and reverse), 1.5µl of Template DNA, and 0.5µl of dNTPs was employed. Sterile water was added to make a final volume of 25µl. All the samples were amplified in a programmable thermocycler (Eppendorf AG 2233, Hamburg, Germany) and was programmed with the following parameters: denaturation at 95°C for 3 min, 95°C for 30 s for 30 cycles, annealing at 42°C for 60 s for *gyrA* and 62 °C for 60 s, extension at 72°C for 1 minute and final extension at 72°C for 10 min for molecular detection of the *blaCTX* gene. A volume of 10 µl of amplified PCR products was subjected to electrophoresis at 80 V in horizontal gel containing 1.2 % agarose with Tris-borate buffer (45 mM Tris borate, 1 mM EDTA) for about 1h 30 min. A 100 base pair marker was used as reference. The gel was stained with ethidium bromide and photographed in an Ultraviolet-transilluminator.

Molecular detection of virulence Shiga Toxin (stx 1) gene

The primers employed for the detection of the virulence factor Shiga Toxin are (stx1-1) 5'-GTG GCA TTA ATA CTG AAT TGT CAT CA- 3' and (stx1-2) 5'-GCG TAA TCC CAC GGA CTC TTC-3'.

The Polymerase Chain Reaction (PCR) composed of 2.5µl of PCR buffer, 1.5µl of MgCl₂, 0.1 unit of Taq DNA Polymerase, 0.2µl of Primers (both forward and reverse), 1.5µl of Template DNA, and 0.5µl of dNTPs. Sterile water was added to make a final volume of 25µl. The reaction conditions were 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 51°C for 60 s, 72°C for 60 s and the last cycle at 72°C for 10 min.

Ten microliter (10µl) of the PCR products was analyzed by 1.2% of agarose gel after which the reaction in TE buffer was allowed to run at 80 V for 1h 30 min. The gel was stained with ethidium bromide and photographed in an Ultra violet-transilluminator.

Results

Shigella sp were recovered from 58 of the 148 diarrhoeal patients' stool samples analysed (Table 1). The isolates were identified as *S. sonnei* 22 (37.9%), *S. boydii* 22 (37.9%), *S. flexneri* 8 (13.8%) and *S. dysenteriae* 6 (10.3%). The antibiotic susceptibility profile of the isolates is presented in table 2. Susceptibility to antibiotics varied, 84.5% were sensitive to ofloxacin, pefloxacin (75.9%) and gentamicin (72.5%). Resistance to ceftriazone, nitrofurantoin, augmentin and amoxicillin was high.

Table 1. Occurrence of *Shigella* isolates in the faecal samples of the diarrhoeal patients

Isolates	Frequency (%)
<i>S. sonnei</i>	22 (37.9%)
<i>S. boydii</i>	22 (37.9%)
<i>S. flexneri</i>	8 (13.8%)
<i>S. dysenteriae</i>	6 (10.3%)

Table 2. Antibiotic susceptibility profile of *Shigella* isolates

Antibiotics	Frequency (%)	
	resistance	Susceptible
Nitrofurantoin (200µg)	96.5	3.5
Tetracycline (30 µg)	44.9	55.1
Ceftriazone (30µg)	100	0
Gentamicin (10µg)	27.5	72.5
Cotrimoxazole (25µg)	34.4	65.6
Ofloxacin (5µg)	15.5	84.5
Amoxicillin (25µg)	82.8	17.2
Ciprofloxacin (10µg)	36.3	63.7
Augmentin (30µg)	94.8	5.2
Pefloxacin (5µg)	24.1	75.9

Forty-six (79.3%) of the isolates were MAR types and exhibited 22 multiple antibiotic resistance patterns. Resistance to four different antibiotics (30.9%) was the most frequent (Table 3). Antibiotic phenotype 'AUG, CRO, NIT' was the prominent resistance pattern observed.

Table 3. Multiple antibiotic resistance patterns of the Isolates

NUMBER OF ANTIBIOTICS	ANTIBIOTIC RESISTANCE PATTERNS	FREQUENCY (%)	TOTAL NUMBER OF ISOLATES (%)
2	AMX, CRO CRO, NIT AUG, CRO	1(1.72) 1(1.72) 1(1.72)	3 (5.16)
3	CRO, NIT, COT, AUG, CRO, NIT	1(1.72) 9(15.51)	10 (17.23)
4	AUG, CRO, NIT, PFX AUG, CRO, NIT, TET AUG, CRO, NIT, OFL AUG, CRO, NIT, COT AUG, CRO, NIT, CPX CRO, NIT, AMX, TET	1(1.72) 1(1.72) 2 (3.44) 2(3.44) 4(6.89) 6(10.34)	18 (30.99)
5	AUG, CRO, NIT, GEN, CPX CRO, NIT, COT, AMX, CPX AUG, CRO, NIT, CPX, TET CRO, NIT, COT, AMX, TET AUG, CRO, NIT, COT, CPX CRO, NIT, GEN, OFL, AMX	1(1.72) 1(1.72) 2(3.44) 2(3.44) 2(3.44) 1(1.72)	9 (15.48)
6	AUG, CRO, NIT, COT, CPX, TET CRO, NIT, GEN, AMX, TET, PFX AUG, CRO, NIT, GEN, COT, CPX AUG, CRO, NIT, COT, OFL, TET	1(1.72) 1(1.72) 1(1.72) 2(3.44)	5(8.60)
7	CRO, NIT, GEN, COT, AMX, CPX, TET	1(1.72)	1 (1.72)
	TOTAL	46	79.31

Table 4. Plasmid Profile of the MAR *Shigella* Isolates

Isolate Code	Number of plasmid haboured by the isolates	Estimated Molecular Sizes of Plasmid (kb)
SH1	3	23.13, 1.46, 1.17
SH2	3	23.13, 1.46, 1.17
SH3	3	23.13, 1.46, 1.17
SH4	3	23.13, 1.46, 1.17
SH5	1	23.13
SH6	3	23.13, 1.46, 1.17
SH7	3	23.13, 1.46, 1.17
SH8	3	23.13, 1.46, 1.17
SH9	3	23.13, 1.46, 1.17
SH10	3	23.13, 1.46, 1.17
SH11	3	23.13, 1.46, 1.17
SH12	3	23.13, 1.46, 1.17
SH13	3	23.13, 1.46, 1.17
SH14	3	23.13, 1.46, 1.17
SH15	3	23.13, 1.46, 1.17
SH16	3	23.13, 1.46, 1.17
SH16	3	23.13, 1.46, 1.17
SH17	3	23.13, 1.46, 1.17
SH18	3	23.13, 1.46, 1.17
SH19	3	23.13, 1.46, 1.17
SH20	3	23.13, 1.46, 1.17

AUG- Augmentin, CRO- Ceftriazone, NIT- Nitrofuratoin, GEN- Gentamicin, OFL- Ofloxacin, COT-Cotrimoxazole, AMX- Amoxicillin, CPX- Ciprofloxacin, TET- Tetracycline, PFX- Pefloxacin

The molecular weights of plasmid DNA haboured by the multiple antibiotic resistant isolates are presented in Table 4. All but one of the representative MAR *Shigella* isolates profiled for plasmid DNA showed multiple plasmid bands of molecular weights ranging from 1.171 to 23.130 kb. The gel electrophoresis showing the plasmid DNA fragments is depicted by figure 1.

Figure 2 shows the fluoroquinolone determinant resistance gene in the MAR *Shigella* isolates. The GyrA (270bp) gene were detected in all the 20 representatives' fluoroquinolone (ciprofloxacin and ofloxacin) resistant isolates meanwhile, only one of the isolates haboured shiga-toxin (stx) gene (Figure 3). All the twenty beta-lactam (amoxicillin and augmentin) resistant *Shigella* isolates screened did not harbour BlaCTX gene.

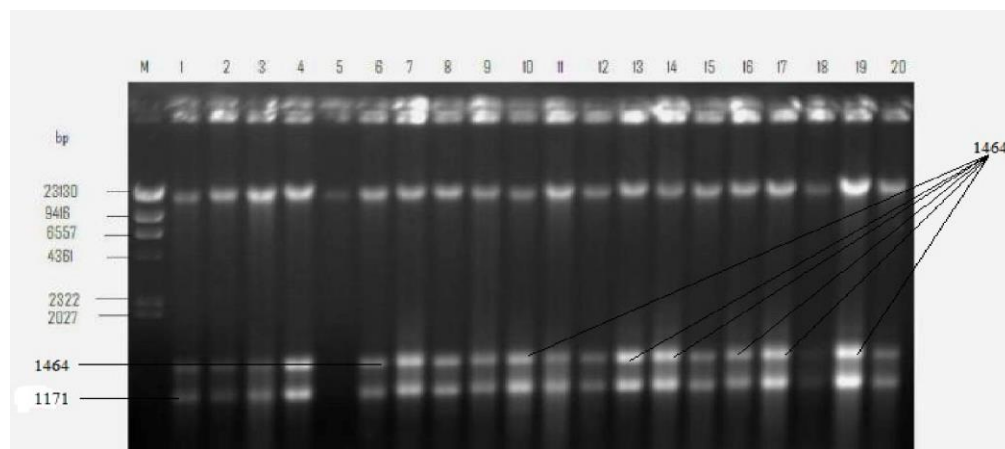


Figure 1: The gel electrophoresis of the plasmid DNA fragments. Lane M: DNA marker (Hind III digest) 23.1 Kb, Lanes 1-20- *Shigella* isolates.

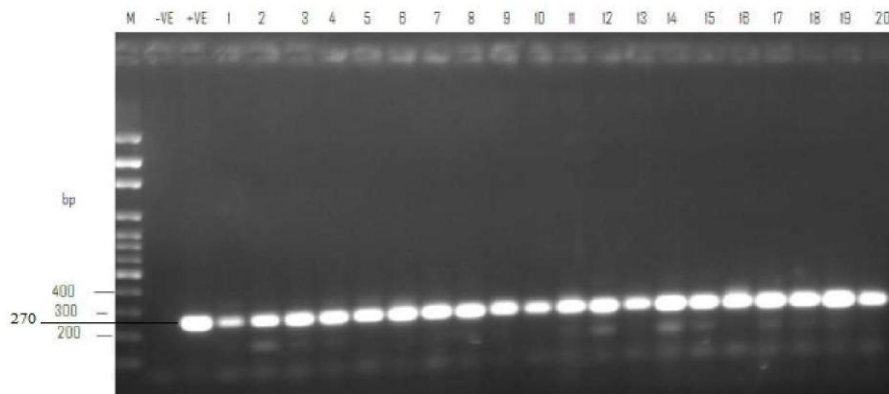


Figure 2: The detection of the gyrA gene in *Shigella* isolates obtained from stool samples of diarrhoeal patients. Lane M (100 bp DNA marker), Lane - (PCR supermix without DNA), Lane + (*Shigella dysenteriae*) and Lanes 1-20 are gyrA gene of the *Shigella* isolates.

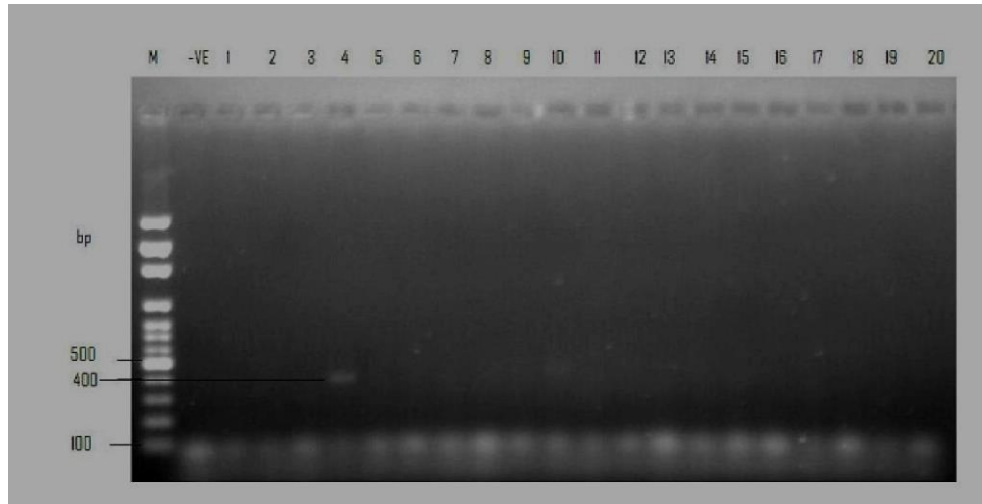


Figure 3. The detection of Shiga-Toxin (stx) gene in multiple antibiotic resistant *Shigella* isolate obtained from stool samples of diarrhoeal patients.

Lane M-100 bp DNA marker, Lane -ve (PCR supermix without DNA), Lanes 1-20 - the *Shigella* isolates, Lane 4 (Presence of stx gene of size 400bp)

Discussion

The recovery of *Shigella* sp from the stool samples analysed indicates that the organism is one of the etiological agents implicated in diarrhoeal in the study area. This corroborates studies by Moez *et al.* (2003), Delappe *et al.* (2003) and Fulla *et al.* (2005) who reported the isolation of *Shigella* sp from stool samples of diarrhoeal patients.

This study revealed high susceptibility rate of the *Shigella* isolates to ofloxacin (79.3%) and pefloxacin (75.8%). This finding is in agreement with the result of the study done by Lee *et al.* (2001) who reported similar antibiotic susceptibility trend in Korea during the last two decades. This present investigation showed that the *Shigella* isolates tested with various antibiotics were resistant to more than one class of antibiotic used. The high rate of resistance to amoxicillin, nitrofurantoin, ceftriazone and augmentin is suggestive of misuse of these antibiotics.

The level of resistance to the commonly used antibiotics, tetracycline and amoxicillin was similar to studies conducted elsewhere in the world (Subekti *et al.*, 2001; Voogd *et al.*, 1992). Tetracycline and amoxicillin are widely used in the study area to treat shigellosis, but in the present study, 44.9% of the *Shigella* isolates developed resistance to tetracycline and 82.8% to amoxicillin, and in such cases, ciprofloxacin and ofloxacin should serve as suitable alternative. In other studies, overall resistance to tetracycline, amoxicillin and pefloxacin was quite low (Voogd *et al.*, 1992). The results of the present study suggest a reconsideration of the empiric use of some commonly prescribed antibiotics for the treatment of Shigellosis.

The management of shigellosis is therefore a major economic and health problem due to globally emerging antibiotic resistant strains of *Shigella* (Replogle *et al.*, 2000; Lee *et al.*, 2001; McIver *et al.*, 2002). The high susceptibility of *Shigella* isolates to ofloxacin and pefloxacin as reported in this study is an advantage for practitioners in the treatment of shigellosis in the study area.

About 80% of the isolates were multiple antibiotic resistant types, which is in consonance with the detection of multiple-resistant strains of *Shigella* sp in Africa (Egah *et al.*, 2003), Asia (Lee *et al.*, 2001), England and Wales (Cheasty *et al.*, 1998).

In this study, ninety-five percent (95%) of the isolates were found to have multiple plasmids DNA with similar band patterns. Tacket *et al.* (1984) and Litwin *et al.* (1991) also reported multiple plasmid bands in *Shigella* sp with similarities in band patterns. In this study, it can be suggested that antibiotic resistance determinants are carried by plasmids even though blaCTX gene that encodes the beta-lactamase enzyme was not found in the representative beta lactam antibiotic resistant isolates screened. The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer (plasmid mediated resistance), and β -lactamase gene expression may be induced by exposure to β -lactams (Drawz and Bonono, 2010). The determination of plasmid profile has been shown to be a powerful tool in epidemiological studies. Plasmid profile may aid in differentiation of strains, identifying a source of infection, or evaluating the efficiency control measures (Litwin and Ryan, 1991).

Resistance to ciprofloxacin and ofloxacin (fluoroquinolones) in the *Shigella* sp isolated in this study can be explained by the presence of a gyrA gene. Gyrase A gene was detected in the entire representative isolates screened which is in consonance with the reports of Taneja (2007) and Dhodapkar *et al.* (2008) which reported the presence of gyrA gene in *Shigella* sp isolated from stool samples. The presence of shiga toxin (stx1) gene, though in only one of the representative isolates screened is worth noting because it's an index of virulence. The essential mechanism of virulence of *Shigella* sp resides in its ability to enter susceptible epithelial cells, induce apoptosis in infected macrophages and this virulence factor inhibits protein synthesis, ultimately causing cell death (Clerc *et al.*, 1986).

Conclusion

Shigella species may be considered as one of the important etiological agents of diarrhoea with a high rate of antibiotic resistance profile in the study area. The high prevalence of multiple antibiotic resistant *Shigella* isolates is highly consequential in terms of therapeutic management of shigellosis. There is a need for greater attention to the appropriate use of antibiotics, health education of food handlers, the establishment of hygienic measures to prevent or decrease transmission, and the development of new effective drugs that can be safely use to combating Shigellosis.

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