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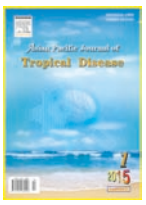

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Prevalence and antibiotic susceptibility pattern of fluoroquinolone resistant *Shigella* species isolated from infants stool in Gulbarga district, Karnataka, India

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ABSTRACT

Objective: To resolve the incidence rate of *Shigella* species and emergence of multiple drug resistance to cephalosporins and fluoroquinolones generation drugs in Gulbarga district and nearby area is a matter of concern for the better management of Shigellosis in children.
Methods: The study work involved all infants with acute diarrhoea, ranging 1 month to 5 years of age. Total 334 stool samples were collected during 2013-2014. Incidence, phenotypic and genotypic (16S rRNA) characteristics and antimicrobial resistance were determined for emerging antibiotics against *Shigella* strains were studied.
Results: Total 43 (12.87%) positive *Shigella* species retrieved from 334 stool samples, *Shigella dysentriae* (48.83%), *Shigella flexneri* (32.55%) were the predominant, followed by *Shigella sonnei* (18.60%) and no case of *Shigella boydii* (0%). Among the *Shigella* isolates 32 (74.41%) were MDR strains, confer the high resistance rate like 100%, 95.33%, 87.5% and 85% to different class of antibiotics studied.
Conclusions: This study illustrates the appearance of the results of long-term supervision and antimicrobial resistance in clinical *Shigella* strains. Data collected as part of the examination will be involved for developing treatment, allocate for the community and to present control report with which to discriminate outbreak strains in the future.

1. Introduction

Shigellosis is a bacterial infection of the intestinal tract caused by bacteria of the genus *Shigella* and manifested by diarrhoea. This is more serious than the common "stomach flu". *Shigella* species was the second most common bacterial agents causing diarrhoea after *Escherichia coli* (*E. coli*)[1]. Shigellosis is endemic to many developing countries and also occurs in epidemics causing considerable morbidity and mortality. The main symptom of this infection is bloody diarrhoea and the minimum infective dose is as low as 10-100 bacterial cells due to relative resistance to stomach acid. Shigellosis is a major public health concern worldwide, especially in developing countries[2,3]. The infection is most frequent and is highest among children between the ages of 01-05 years, the elderly and the immune compromised[4].

A more recent annual estimate of the shigellosis burden is at approximately 90 million incidences and 600 000 deaths worldwide. Two-thirds of all cases and most of the deaths occur among children under 0-5 years of age[5]. *Shigella* infections are very contagious; transmission of the disease is primarily by person-to-person contact through contaminated hands[6]. Outbreaks in children typically occur under conditions involving close physical contact, such as those encountered in day-care centres, nursing homes, custodial institutions, cruise ships, aboriginal reservations and crowded refugee camps, with poor hygiene practices and contaminated food or water serving as the vehicle for infection[5,7-9]. Mild illness of Shigellosis causes loose, watery diarrhoea. More severe infection causes high fever, stomach cramping, vomiting and diarrhoea with blood or mucus in the stool.

The genus *Shigella* consists of four major serological groups A through D according to their O-polysaccharide antigens corresponding to the four species, respectively[10]. In current taxonomy, these four serological subgroups are recognised as four species within the genus *Shigella*: *Shigella dysentriae* (*S. dysentriae*), *Shigella flexneri* (*S. flexneri*), *Shigella boydii* (*S. boydii*) and *Shigella sonnei* (*S. sonnei*). The four distinct species can be differentiated on the basis of serogrouping and biochemical analysis[11].

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Shigellosis is endemic in many developing countries like India and an important cause of bloody diarrhoea worldwide. Antimicrobial resistance in hospitals and community has been posing a great public health problem in North Karnataka region over the years. In local Gulbarga district and surrounding region of Gulbarga, diarrhoea has been estimated to be responsible for approximately 11%-13% of all childhood illnesses, with a population of about 532 031. Among the four species of *Shigella, S. dysentriae* (48.83%), *S. flexneri* (32.55%) had been the predominant one and followed by *S. sonnei* (18.60%) isolated during July 2013 to March 2014. From April 2014 sudden increase in the number of patients admitted with dysentery was not noted.

Treatment of Shigellosis is a merger of rehydration and antibiotic therapy, this prompt treatment with effective antimicrobial agents shortens the duration of symptoms and car-riage and reduces the spread of infection. However, in India and other developing countries, antimicrobial resistance is an emerging problem among *Shigella* species and treatment options are becoming limited globally[12]. Reports of epidemic outbreaks of Shigellosis in India are frequent but studies of endemic (sporadic) cases are significant. The magnitude of the problem of *Shigella* infections in infants and children is thus not appropriately interpreted[13].

Over the decades, *Shigella* isolates resistant to multiple antimicrobial agents, such as ampicillin, tetracycline, sulphonamides and nalidixic acid have been reported from many developing countries, including India and Bangladesh[14,15], resulting in difficulties in the selection of accurate treatment. However, newer antimicrobial agents, such as ciprofloxacin, ceftriaxone, cefixime and azithromycin were found to be effective in treating multidrug-resistant *Shigella*–associated infections[14-17]. Recently, *Shigella* strains resistant to fluroquinolones like, ciprofloxacin, gatifloxacin and ofloxacin have emerged in India and Gulbarga district and its surrounding region of Karnataka state[18-20]. So this study is aimed to know the prevalence of Shigellosis in infants and its antimicrobial resistance pattern. We also examined the trends in antimicrobial resistance of *Shigella* isolates and correlated with previous data to suggest appropriative for provable antimicrobial therapy, possibly.

2. Materials and methods

2.1. Study region and population

This study was conducted at Shaha Children's Care Hospital and other diagnostic centres in Gulbarga district, Karnataka, India, between July 2013 and March 2014. This child care centre is the largest and rumoured in the Gulbarga district. Gulbarga city has seen a rapid increase in population size in recent years. The inadequate, lacking and poor infrastructure for waste disposal provides the favourable conditions for the spread and outbreak of diarrhoeal diseases. The main occupation of the people in the city includes business, commercial fishing, textile industry and employment in both public and private sectors.

2.2. Clinical sample collection

Total 334 stool samples were collected and examined for the presence of *Shigella* species over the period of this cross-sectional study. All studied infants ranged in the age from 1 month to 5 years. After collection of stool specimens in stool vials and rectal swabs were transferred in transport media (stored at 4 ˚C) within 1 h to the laboratory for analysis and processed within 24 h. All the infants were visited the hospital for the examination of: diarrhoea fever, diarrhoea together with vomiting and frequent passing. In some cases, dehydration was present. Before sample collection, the entire study population was registered with number codes.

2.3. Bacteriological study

All collected stool samples were tested for *Shigella* species. Fresh stool samples were enriched (approximately 1 g of each stool specimen was inoculated into 10 mL of *Shigella* broth, manually prepared) and broth was incubated for 18-24 h at 37 ˚C. After overnight incubation period, 0.1-0.5 mL of enriched *Shigella* broth was subcultured onto the xylose lysine deoxycholate agar and deoxycholate citrate agar and the plates were incubated for 18-24 h at 37 ˚C.

Suspected colonies on the primary or subculture plates resembling those of *Shigella* were selected for further identification by standard laboratory procedures.

All non-lactose fermenters were isolated and subjected to the biochemical test to identify the isolates. Identification of *Shigella* was done on the basis of oxidase test, mannitol fermentation test, indole test and triple sugar iron test. The performance and readings of the tests were quality controlled using the reference strain *S. flexneri* 12022, *S. dysentriae* 13313.

2.4. Serological test

Final confirmation of *Shigella* isolates (their species level identification) was performed with specific polyvalent antisera (Deben Diagnostics Ltd, Ipswich, Suffolk, IP3 9SX, United Kingdom) and results were interpreted according to manufacturer's guidelines.

2.5. Molecular identification by 16S rRNA

In the present study the determination of the 16S rRNA sequencing has been employed as a tool in arriving at identification and confirmation of bacterial strains by sequencing of 16S rRNA isolated from clinical (stool) samples. This method is rapid and reliable technique for molecular identification. An isolated *Shigella* isolate sequence was commercially sequenced at "Chromous Biotech Pvt ltd, Bangalore. Crude sequence obtained by sequencing the amplified product was characterized. Initially, the sequence was submitted for the BLASTn search[21] at NCBI server (www.ncbi.nlm.nih.gov/nblast.html) and the homologous hits were studied. Such hits sequences across the species were retrieved and subjected for multiple alignment using ClustalW at EBI server. Based on the scores of multiple alignment the dendogram was generated and visualised using PHYLIP 3.6 version and studied for the evolutionary distance with other similar sequence retrieved from different species.

2.6. GenBank accession number

The partial sequence of the 16S rRNA gene of both strains kcps77 and pck237 have been deposited in the International Nucleotide Sequence Database, that is, in the National centre for Biotechnology Information (NCBI).

2.7. Antimicrobial susceptibility test

Antibacterial susceptibility testing in the clinical laboratory is the most often performed test using the disk diffusion method[22]. The method was originally standardised according to the Clinical Laboratory Standard Institute[23] and quality assurance guidelines of World Health Organization (WHO). In order to monitor drug resistance, clinical and public health officials depends on accurate performance and appropriate reporting of the results of these susceptibility.

2.7.1. Disc diffusion susceptibility test

The Antibiotic susceptibility was tested by disc diffusion method by emulsifying 4-5 colonies of *Shigella* culture into tryptic soya broth of optical density at 0.2 at 600 nm. Thus, prepared inoculums were spread over Muller-Hinton agar with sterile cotton swab and the antibiotic discs were placed at their equal distance and incubated at 37 °C for 24 h. The potency of antibiotic disc was used as per CLSI (2010) guidelines.

2.7.2. Antimicrobial agents

Reference samples of various antimicrobial agents and amino glycosides of known potency were kindly supplied by the manufacturers, as follows: penicillin, ciprofloxacin ofloxacin, gatifloxacin, gentamycin, ampicillin, amikacin, nalidixic acid, ceftriaxone were procured commercially from Hi-media, Mumbai Pvt Ltd, India and stored at 4 °C until use.

3. Results

Over the study period of 9 months, a total of 334 stool specimens were investigated from the paediatric age group (1-5 years), admitted in a paediatric ward, with a record of fever, frequent passing of loose stools with blood and pus.

Shigella species were isolated from 43 (12.87%) of the 334 stool samples, among the *Shigella* isolates, the most common and predominant subgroup was *S. dysentriae* (48.83%), *S. flexneri* (32.55%), followed by *S. sonnei* (18.60%) and *S. boydii* (0%) subsequently (Table 1).

Table 1

Prevalance of *Shigella* subgroups isolated from infants with diarrhoea. [*n* (%)].

Shigella species	Prevalance
S. dysentriae	21 (48.83)
S. flexneri	14 (32.55)
S. sonnei	8 (18.60)
S. boydii	0
Total (*n* = 334)	43 (12.87)

3.1. Age group-wise assessment for the isolation of Shigella of diarrheal infants

Among the culture-confirmed episodes of Shigellosis, 43 (12.87%) occurred in infants; 22 (51.16%) infants were under the age of 1 month to 1 year, 17 (39.53%) infants were under the age of 1–3 years, 3 (6.92%) infants were under the age of 3-5years and 1 (2.32%) infants were 5 years above. Male infants were predominantly affected, 26 (60.46%) and 17 (39.53%) female. The maximum diarrheal cases in infants involved in this study, attended wide peak among the age group ranging 1 month to 1 year, followed by the age group between 1 to 3 years (Table 2).

Table 2

Sex and age distribution of 43 confirmed cases of Shigellosis.

Shigella species	Sex determination of the positive cases		Age group			
	Male infants	Female infants	1 month < 1 years	1 < 3 years	3 < 5 years	> 5 years
S. dysentriae	12	9	12	6	2	1
S. flexneri	8	6	6	8	1	-
S. sonnei	6	2	4	3	-	-
Total 43 (12.87%)	26 (60.46%)	17 (39.53%)	22 (51.16%)	17 (39.53%)	3 (6.97%)	1 (2.32%)

3.2. Periodic variance of Shigella strains

Month wise assortment of the Shigellosis showed wide infection rate during September and October over the period of isolation. Between 2013-2014, a narrow infection were also observed during the months of March and April. Cases of Shigellosis were found to be low during the winter seasons (Figure 1).

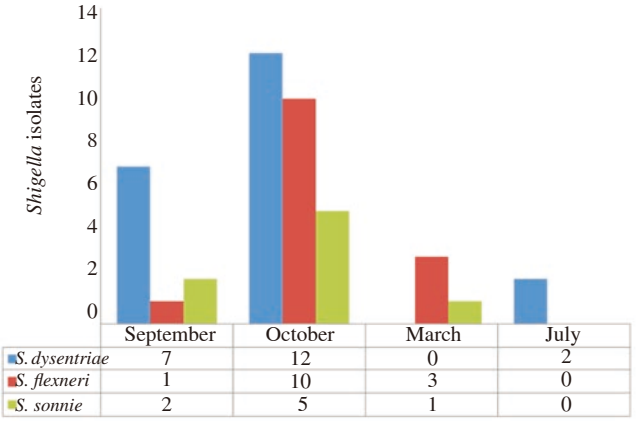

Figure 1. Seasonal variation of *Shigella* isolates during 2013-14 at Gulbarga district, Karnataka.

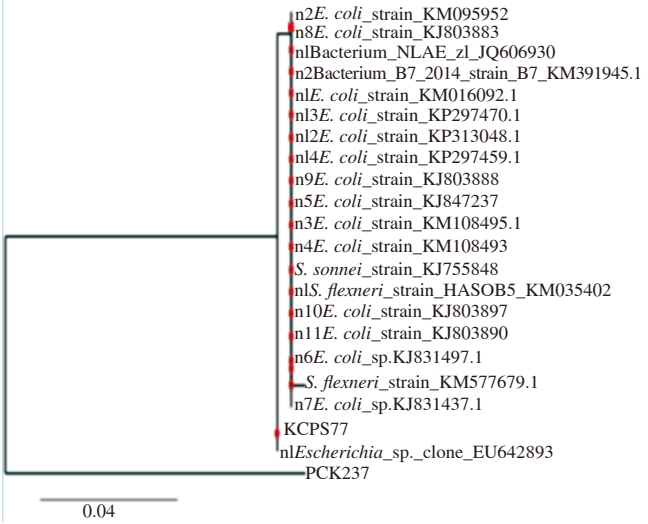

Figure 2. Phylogenetic relationships of *Shigella* species based on maximum-likelihood analysis of 16S rRNA.

3.3. Molecular Identification of Shigella isolates

The *Shigella* strain kcps77 and pck237 was identified by 16S rRNA sequencing, after the initial analysis at NCBI and RDP II (http//:rdp.cme.msu.edu) websites, the relevant sequences were downloaded and phylogenetic analysis was done and tree was constructed using ClustalW software and showed the phylogenetic relationship of the isolates (Figure 2). In analysis of the sequence showed 99% sequence similarity with the sequence reported on *Shigella* spp. and consequently the isolate kcps77 and pck237 were phylogenetically similar to *Shigella* species and *E. coli*. Phylogenetic studies indicate that the *Shigella* is more appropriately treated as subgenus of *Escherichia*. (*E. coli* O157:H7). The 16S rRNA isolate sequence is deposited in the GenBank with the accession number KM369962 & KP017572 (Figure 2).

3.4. Antibiotic susceptibility test among Shigella strains

The antibiotic susceptibility test was carried out for all the 43 positive *Shigella* isolates against the 18 antibiotics consisted different groups (Figure 3). 32 (74.41%) were MDR strains among the total *Shigella* isolates. *Shigella* isolates have showed significantly high rate antibiotic resistance against various antimicrobial drugs widely used in Gulbarga district, Karnataka.

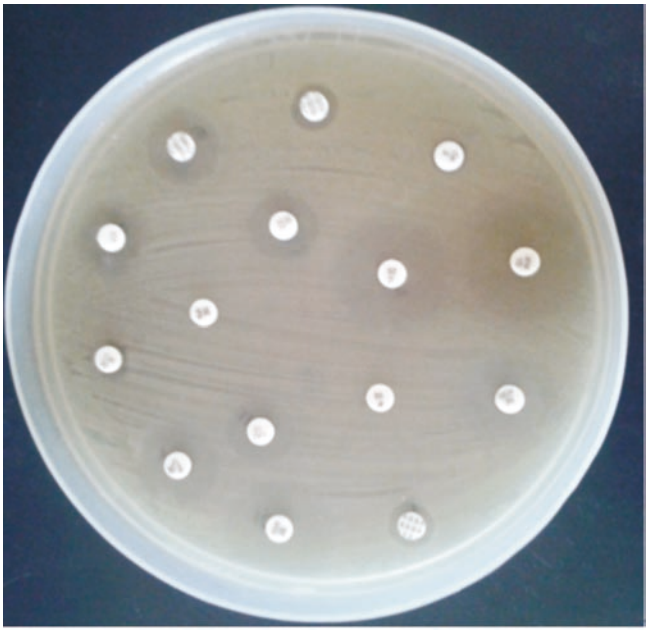

Figure 3. Antibiotic susceptibility test of *Shigella* isolates by disc diffusion method

Among the four species of *Shigella*, *S. dysentriae* showed high resistance rate (100%) to ampicillin, tetracycline, amikacin, penicillin, Nalidixic acid and gatifloxacin, co-triamaxazole (87.5%), cephalosporins (85%) and fluoroquinolones (95.33%) antibiotics. *S. flexneri* showed 100% resistance to tetracycline, cefepime, ciprofloxacin, ceftriaxone and ceftazidine, followed by *S. sonnei* showed 100% resistance to only cephalosporins class of antibiotics (Table 3).

Table 3
Drug resistance pattern of *Shigella* species by disc diffusion method.

Antimicrobial agents	No.(%) of MDR *Shigella* isolates resistant to:			
	S. dysentriae (n = 16)	*S. flexneri* (n = 9)	*S. sonnei* (n = 7)	All *Shigella* isolates
Ampicillin	16 (100.00%)	7 (77.78%)	6 (85.71%)	29 (90.62%)
Amikacin	16 (100.00%)	5 (55.55%)	4 (57.14%)	25 (78.12%)
Gentamycin	13 (81.25%)	6 (66.67%)	2 (28.57%)	21 (65.62%)
Imepenem	14 (87.50%)	6 (66.67%)	2 (28.57%)	22 (68.75%)
Tetracycline	16 (100.00%)	9 (100.00%)	5 (71.42%)	30 (93.75%)
Cephalexin	14 (87.50%)	8 (88.89%)	7 (100.00%)	29 (90.62%)
Cefepime	13 (81.25%)	9 (100.00%)	7 (100.00%)	29 (90.62%)
Ciprofloxacin	15 (93.75%)	9 (100.00%)	6 (85.71%)	30 (93.75%)
Ceftriaxone	13 (81.25%)	9 (100.00%)	7 (100.00%)	29 (90.62%)
Co-triamaxazole	14 (87.50%)	8 (88.89%)	6 (85.71%)	28 (87.50%)
Ceftazidime	14 (87.50%)	9 (100.00%)	7 (100.00%)	30 (93.75%)
Nalidixic acid	16 (100.00%)	7 (77.78%)	6 (85.71%)	29 (90.62%)
Cephotaxime	14 (87.50%)	8 (88.89%)	7 (100.00%)	29 (90.62%)
Chloromphenicol	14 (87.50%)	5 (55.55%)	5 (71.42%)	24 (75.00%)
Penicillin	16 (100.00%)	8 (88.89%)	6 (85.71%)	30 (93.75%)
Ofloxacin	15 (93.75%)	8 (88.89%)	6 (85.71%)	29 (90.62%)
Gatifloxacin	16 (100.00%)	8 (88.89%)	5 (71.42%)	29 (90.62%)
Oxacillin	13 (81.25%)	6 (66.67%)	4 (57.14%)	23 (71.87%)

4. Discussion

Diarrhoea is a common problem in infants and children, but if not managed well, it can quickly turn deadly, more than 2.3 million children below the age of five die in India and Asian countries each year, about 334000 of which are attributed to diarrhoea (according to WHO). This is a needlness tragedy because the illness is easy to prevent and supervise. Children suffer from diarrhoea when they pass at least three loose or watery stools per day. This condition is due to gastro-intestinal infection caused by multifactorial bacterial pathogens and viral-parasites, where viral infection is more common. The infection with bacterial pathogens peak during the summer when there are suitable conditions such as humidity and high temperature which facilitate the bacterial growth and its propagation. It is responsible for about 40% of all hospital admissions due to diarrhoea among children under five worldwide.

Shigella species were the second most common and dominant bacteria isolated among infants presenting with bloody diarrhoea in Gulbarga district and nearby areas. The prevalence of Shigellosis among infected infants in our area was 12.87% which is similar and higher than the rate of infection reported earlier for India and other countries[24]. In our study, among 43 positive *Shigella* spp. *S. dysentriae* (48.83%), *S. flexneri* (32.55%) were found most common and predominant one followed by *S. sonnei* (18.60%) among the isolated *Shigella*. Male infants were predominantly affected 60.46% (26/43) than the female infants 39.53% (17/43) between the age 1-5 years; 1 month to 1 year infants were the more affected age groups.

Shigellosis due to *Shigella* strains resistant to multiple antibacterial drugs have rapidly expanded over the past 20 years in the South Asian region and have now spread widely to the Middle East Africa and Asian countries. The emergence and spread of drug resistance to newer and more potent agents used in treatment of Shigellosis is a major therapeutic challenge[25].

As the regular Shigellosis drugs have become outdated, cephalosporins and fluoroquinolones are the main treatment for *Shigella* infection. In the early days of fluoroquinolone treatment for gastrointestinal infections, ciprofloxacin and other fluoroquinolones

were found to be highly active *in vitro* and clinically effective in the treatment of infant's diarrhoea. However, a study performed during the period 2013-14 indicated that resistance is emerged against these drugs moderately, which is distressing[16,26].

The pattern of antimicrobial susceptibility testing of isolated *Shigella* spp. showed mostly multidrug resistance (74.41%) to the certainly available and cost-effective antibiotics namely, ampicillin, tetracycline, co-trimoxazole, chloromphenicol, penicillin, imepenem, amikacin, gentamycin, cephalosporins and fluoroquinolone class of different antibiotics.

In conclusion of the study, most of our diarrheal infants came from nearby (rural) area and the prevalence of antibacterial resistance in these infants was higher than city of Gulbarga district. In the rural infants, periodic disease may be allied to low hygienic management, this societal and expedient case and also the lack of cleaned water. Based on the sensitivity pattern, imepenem, gentamycin, amikacin and chloromphenicol will be the effective treatment for the Shigellosis.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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