Original Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

BIOFILM FORMATION AND ELECTRON MICROSCOPIC STUDIES IN MULTIDRUG RESISTANT CLINICAL ISOLATES OF *SHIGELLA SPS* ISOLATED FROM INFANT'S STOOL, NORTH PART OF KARNATAKA, INDIA

PRABHURAJESHWAR C AND KELMANI CHANDRAKANTH R*

Medical Biotechnology and Phage Therapy Laboratory, Department of Post Graduate Studies and Research in Biotechnology, Gulbarga University, Gulbarga-585 106, Karnataka, India

ABSTRACT

Diarrhoea and acute gastroenteritis are among the leading causes of illness and death in infants and children throughout the world, more than ever in developing countries and also a commending pathogen forming biofilms on many surfaces and latterly, increased resistance scarcity was recommended to be acquired in biofilm environ. The intent of study, to determine the biofilm formation of clinical *Shigella* isolates and their morphological alters. The present study work involved: 32 multi drug resistance *Shigella* isolates. Directive of *Shigella* isolates to form biofilms were screened by test tube and micro titre plate method. Biofilm formation prospective was resolute by growing the tested strains containing tryptic soya broth (TSB) medium with glucose and antibiotic supplement at different incubation periods. Morphological outcome of antibiotic stress isolates were also studied through electron microscopy. The propensity of all articulated *Shigella* isolates to form biofilms was professed. Base on biofilm-positive phenotype, the strains were classified as high, moderate and weak biofilm formation was distinguished. In two in-vitro screenings: Micro titre plate method is profoundly sensitive method for detection biofilm producing *Shigella* isolates as compare to test tube method. As drug resistance is a vital mess in Shigellosis, it is foremost to intercept the colonization of the organism by recommending peculiar practices to obviate biofilm formation. Electron microscopic studies relentless the morphology of cell, variability of thickness in cell wall and show up an adaptive response of the *Shigella* isolate to the ciprofloxacin antibiotic stress and lead to their drug resistance

KEYWORDS: **Biofilm, Scanning Electron Microscopy, Transmission Electron Microscopy, Fluoroquinolones, *Shigella* species,Shigellosis.**

KELMANICHANDRAKANTH R
Medical Biotechnology and Phage Therapy Laboratory,Department of Post Graduate Studies and Research in Biotechnology,*Gulbarga University, Gulbarga-585 106, Karnataka, India*

***Corresponding author**

INTRODUCTION

Shigella gastroenteritis is an intestinal and bowel infection caused by bacteria from the *Shigella* family. Other names for *Shigella* gastroenteritis include shigellosis, is a global human health problem. It is one of the major epidemic diseases in the history of mankind and most common killing diseases of children all over the world. It has been estimated that 91million worldwide contract shigellosis each year and among them, 1.1 million die.[1] About 4, 10,000 (40%) of these deaths occur among Asian children.[2] Shigellosis is endemic in many developing countries like India and the most important cause of bloody diarrhoea worldwide and is responsible for 5-10% of diarrheal illness.[3] Common symptoms include diarrhoea that may contain blood, mucus or pus, abdominal cramps, nausea and vomiting. In developing countries, there are 163.2 million annual cases and 69% of all patients are children under the age of 5 years,[1] to cause infection and high mortality, the bacteria have to be eaten, either directly through physical contact with a person with the illness or indirectly attributed to the lack of clean water, poor sanitation, malnutrition and the cost of antibiotic treatment. After incubating (bacteria multiplying) in the body for around one to three days, the infection causes the characteristic symptoms. These symptoms last for about four to seven days Antimicrobial resistance in hospitals and community has been posing a great public health problem in North Karnataka region of India 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 5, 32,031. Among the four species of *Shigella*, *Shigella dysentriae* and *Shigella flexnerii* were more predominant one [4], 10.1% of *Shigella* infection accounted in Davangere district[5] and where as decreased susceptibility to antimicrobials among the *Shigella* species (3.7%) in Manipal region[6] Contaminated water and poor hygienic is regarded as an important vehicle for transmission of various entero-pathogens. Bacteria live in habitats of frequently changing conditions and have evolved very sophisticated responses to adapt to environmental changes. These responses lead frequently to the activation and/or repression of a number of genes, such as those responsible of the adhesion and biofilm formation, to adapt cell physiology or metabolism to new conditions. Numerous studies to date indicate that human infections are, in large part, caused by the ability of bacteria to develop surface attached polymicrobial communities known as biofilms[7,8,9]. Microbial biofilms consist of groups of bacterial cells adherent to a surface and enclosed within a self produced extracellular matrix[10]. Adaptation to surface attached growth within a biofilm is accompanied by significant changes in gene and protein expression, as well as metabolic activity[11, 12] which confers resistance to antimicrobial therapy[13, 14] and host mechanisms of clearance.[15, 16] Many pathogenic bacteria have been observed to predominantly exist as biofilms, in both natural environments and within infected tissues as polymicrobial communities.[7, 9, 17-19] Importantly, biofilm formation is implicated as a significant factor involved in a number of chronic human infections.[7, 20, 21] Electron microscopy offers the unique ability to examine the characteristics of surface structure of biomaterials at relatively high resolution and proves particularly useful in the examination of the effect of antibiotics that act on the bacterial cell wall and also biological responses to biomaterials such as cell attachment and changes in morphology.[22,23] Overall, 32 multi drug resistant *Shigella* isolates were screened by Microtitre plate method or tissue culture plate and test tube method for resolving the biofilm production and also assess the reliability of these detection methods. So this present study aimed to evoke the effect of antibiotic stress on the morphology of fluoroquinolone resistant *Shigella* isolates were appraised by electron microscopic study and also evaluate the efficiency of biofilm formation at 1% of glucose level and minimal inhibitoric antibiotic concentration, of all four species of multidrug resistant *Shigella* isolates isolated from stools.

MATERIALS AND METHODS

Bacterial Isolates

The clinical isolates used in this study were isolated from infant's stools. They were identified upto species level on the basis of colonial and Gram's stain morphology, carbohydrate fermentation, indole test and final confirmation was performed with specific polyvalent antisera (Deben Diagnostics Ltd, Ipswich, Suffolk, IP3 9SX, United kingdom). Among 43 previously isolated *Shigella* isolates[4], thirty two multi drug resistant isolates were considered for assessment in the present study. For each study; an overnight culture was diluted in fresh Tryptic soya broth and further incubated to assure exponential growth conditions.

Susceptibility testing

Resistance level and synergistic activity of previously isolated all *Shigella* isolates to various antibiotics (Hi-Media Private Ltd., Mumbai, India) were determined by the microdilution method as described by the Clinical and Laboratory Standards Institute. *Shigella dysentriae* 13313 and *Shigella flexneri* 12022 obtained from American type culture collection (ATCC) were used as the reference and standard control organism in all the susceptibility procedures.

Biofilm formation assay of Shigella isolates

A qualitative analysis of biofilm formation study was determined by test tube method and Microtitre or tissue culture plate method. This biofilm assay test was carried out for all positive multidrug resistant *Shigella* isolates (32) which consisted of different groups. These methods were originally standardised according to the Mohamed.[24]

Test tube Method (TM)

Samples were inoculated on Xylose-Lysine Deoxycholate agar for the detection of *Shigella Sps* and other species identification was done by colony morphology and staining. A quiet modified qualitative assessment of biofilm formation was resolved and estimated as previously described by spectrophotometric assay.[25] Tryptic soya broth (10ml)

was inoculated with loopful of microorganisms from overnight culture plates and to each tube an additional amount of 2 ml of Tryptic Soy broth with 1% glucose was added and incubated for 24 hrs at 37°C (this test has been done in composite with TSB+ 1% Glucose and Antibiotic concentration at 64µg/ml). The grown tubes were decanted and washed with phosphate buffer saline (PBS, pH 7.2) to eliminate the unbound bacteria and dried. To evaluate the formation of biofilm, remaining attached bacteria were fixed with 2 ml of 99% methanol. After 5-10 minutes the tubes were emptied and left to dry. Dried tubes were stained with 2 ml of crystal violet (2%). Excess stain was removed and tubes were washed with deionised water. Tubes were then dried in inverted position and Tubes were air dried. The Biofilm formation was regarded positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not symbolic of biofilm formation. Dye attached to cells was dissolved in 1.5 ml of 33% glacial acetic acid. Optical density (OD) of each tube was resolute at intervals of 18, 24 and 48 hrs at 490 nm. The blank was determined by measuring OD of tube filled with PBS and positive control was determined by measuring OD of tube with pure culture. The amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong.

Microtiter Plate Assay Method (MTP)

The microtiter plate assay method is most widely used and considered as excellence test for detection of biofilm formation. Isolates from fresh agar plates were inoculated in Tryptic soya broth broth supplemented with 1% glucose [24] and incubated for 18 hr at 37°C (this plate test has been done in combination with TSB+ Glucose+ Antibiotics (64µg/ml) with different incubation periods: 18 and 72hours and its synergetic effect) and the culture was diluted 1 in 100 with fresh broth and Individual wells of sterile, polystyrene, 96 well-flat bottom micro titre plate's (Corning Inc., Corning, NY, USA) wells were filled with 0.2ml of aliquots of the diluted cultures and only broth served as control to check the sterility and non-specific binding of media. The microtiter plates were incubated for 18 hr and 24 hr at 37^{0}C without shaking. After incubation, content of each well was gently removed by tapping the plates. The wells were washed three - four times with 0.3ml of distilled water, dried in an inverted position and stained with 0.3ml of crystal violet (2%) for 45 min with adequate shaking. After stained, stain was rinsed off by through washing with deionised water and plates were kept for drying. Quantitative analysis of biofilm production was performed by adding 0.3ml of ethanol-acetic acid (95:5, v/v) to destain the wells. 0.1ml from each well was transferred to a new microtiter plate and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 490 nm using a microtiter plate reader (iMark Microplate Reader S/N 12883 Biorad Pvt Ltd India). Each assay was performed in triplicate. Uninoculated medium was used as a control to determine background OD. The mean OD_{490} value from the control wells was subtracted from the mean OD_{490} value of tested wells.

Scanning Electron Microscopy

As it is difficult to observe small changes in cell morphologies of bacteria under the light microscope, SEM was used in the present investigation to examine the minor changes in cell morphology of the populations that have adapted to antibiotic stress. The preferred *Shigella sps* were grown in TSB media with increasing fluoroquinolone's (ciprofloxacin) concentrations as described earlier. The bacterial cells from each culture were retrieved by centrifugation at 6000 rev/min and the cells were washed twice with potassium phosphate buffer (50 mM, pH 7.0). Bacterial cells were then fixed by drown in 2.5% glutaraldehyde in potassium phosphate buffer (50 mM, pH 7) for overnight at 4°C. Then the specimens were washed twice with buffer and dehy-drated by ethanol series (v/v) ranging from 30%, 40%, 50%, 60%, 70%, 80% and 90% to 100% and stored in 100% ethanol. For SEM, the specimens were dried to critical point, coated with gold and inspected with an S-200C scanning electron microscope[15]. Results were correlated with standard *Shigella* ATCC 12022 culture and control selected *Shigella sps*. The cell volumes were directly measured from SEM photographs to compute the cell volume beside subsequent equation: $V\ (\mu m^3) = \pi/4W^2L + \pi/3W^2L$ Where W and L stand for the width and length, correspondingly, of the central part of the cylindrical cell and R was the equatorial radius of the spheroid caps at both ends of the cylinder. Normal cellular volumes are calculated by using 30 individual bacteria per population. Cells presenting divisions, rupture or elongation into a filament were not incorporated.

Transmission Electron Microscopy

TEM was performed for morphological characterization of the cell wall ultra structure of selected *Shigella* isolates. Additionally, to detect and further characterize alterations of the cell surface *Shigella* isolates. Preparation and interrogation of fluoroquinolone resistance cells (*Shigella* isolates) by transmission electron microscopy were performed as described previously.[26] The cells were fixed in 2.5% gluteraldehyde in 3.1M phosphate buffer (pH 7.2) for 24 h at 4^{0}C and washed with PBS for 4 times each 45 minutes, then post fixed in 1% aqueous Osmium Tetroxide for 2 h later washed with deionised water for 6 times each 45 minutes, dehydrated in series of graded alcohols, infiltered and embedded in araldite 6005 resin or spur resin.[27] Incubated at 80^{0}C for 72h for complete polymerization. Ultra thin (60nm) sections were made with a glass knife on ultra microtome (Leica Ultra cut UCt-GA-D/E-I/00), mounted on copper grids and stained with saturated aqueous Urenyl acetate (UA) and counter stained with Reynolds lead citrate (LC). Viewed under TEM (Model: Hitachi, H-7500 from JAPAN) at required magnification as per the standard procedures at RUSKA lab's College of Veterinary Sciences, SVVU, Rajendranagar, Hyderabad, India. Cell wall thickness was calculated using of photographs taken at a final magnification of 30 kPa. To measure the cell wall thickness, selected *Shigella sps* (antibiotic stress) were considered and results were revealed as the mean ± SD.[28] Statistical calculations were contrived using the parametric data.

FIGURE 1
Screening of biofilm producers of Shigella isolates by Test Tube Method; Tube 1- Shigella isolate; Tube 2– Control; Tube 3–Antibiotic treated Shigella isolate.

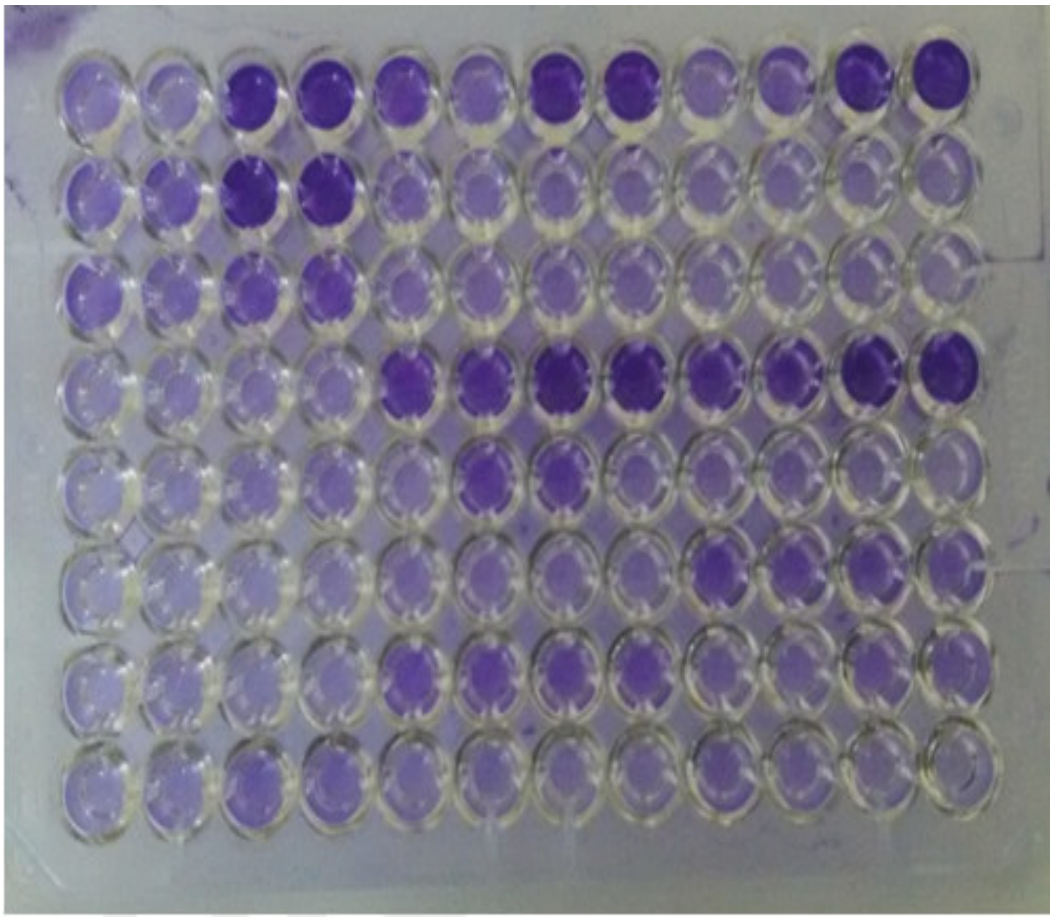
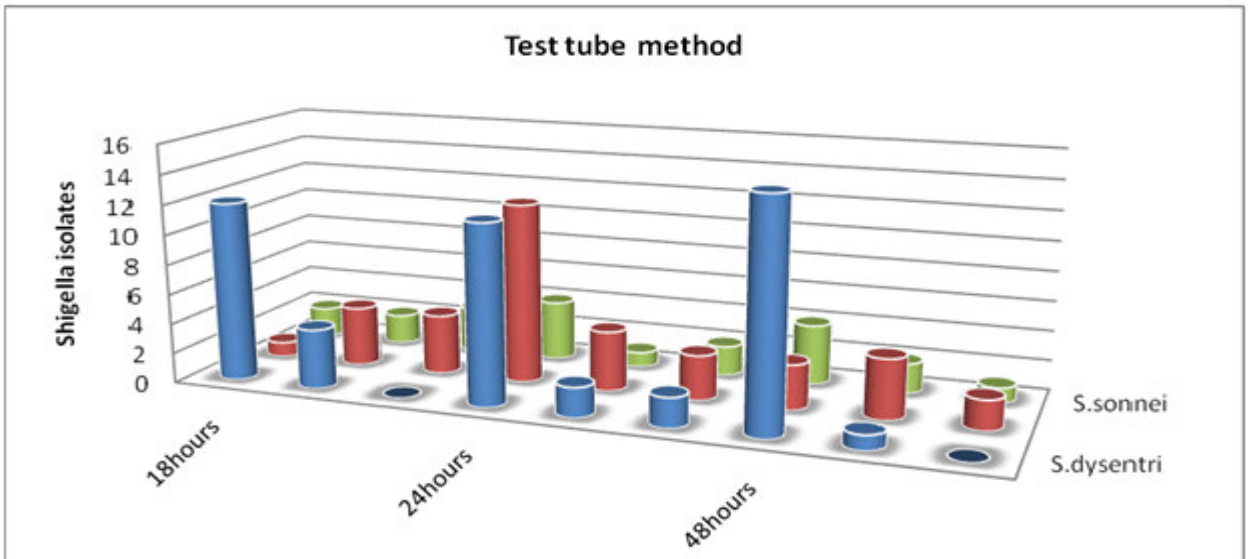


FIGURE 2
***Screening of biofilm producers of Shigella isolates by Microtitre Plate Assay method; Lanes 3-4, Strong producers of biofilm ; Lanes 5-6, Moderate producers of biofilm, Lanes; 9-10, Weak producers of biofilm*.**



	18hours			24hours			48hours		
S.dysentri	12	4	0	12	2	2	15	1	0
S.flexineri	1	4	4	12	4	3	3	4	2
S.sonnei	2	2	3	4	1	2	4	2	1

FIGURE 3

Number of Shigella isolates to their biofilm formation levels (strong, medium and weak) at different incubation periods (Blue colour- Shigella dysentriae, red colour- Shigella flexneri & green colour-Shigella sonnei).

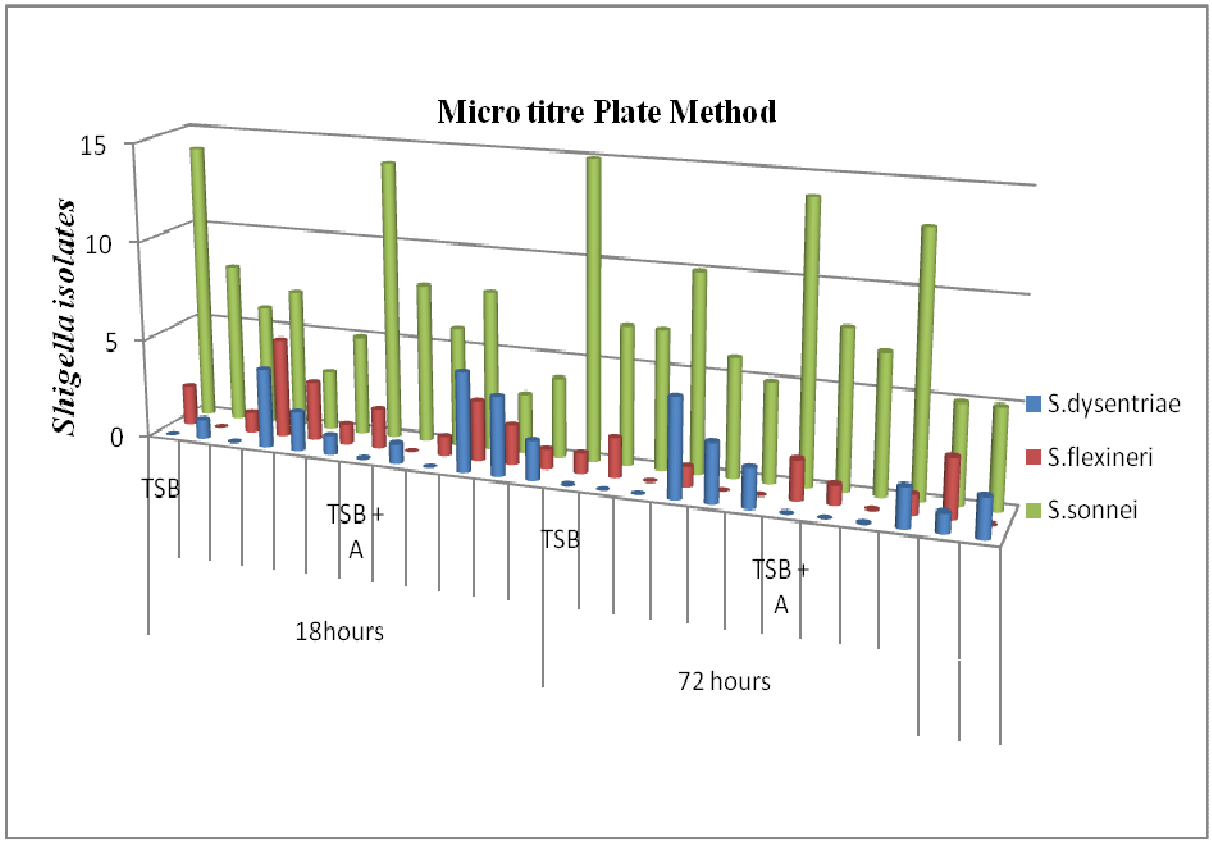


FIGURE 4

Number of *Shigella* isolates to their biofilm formation levels (strong, medium and weak) in combination with glucose and antibiotic supplements at different incubation periods (Blue colour- *Shigella dysentriae*, red colour- *Shigella flexneri* & green colour-*Shigella sonnei*).

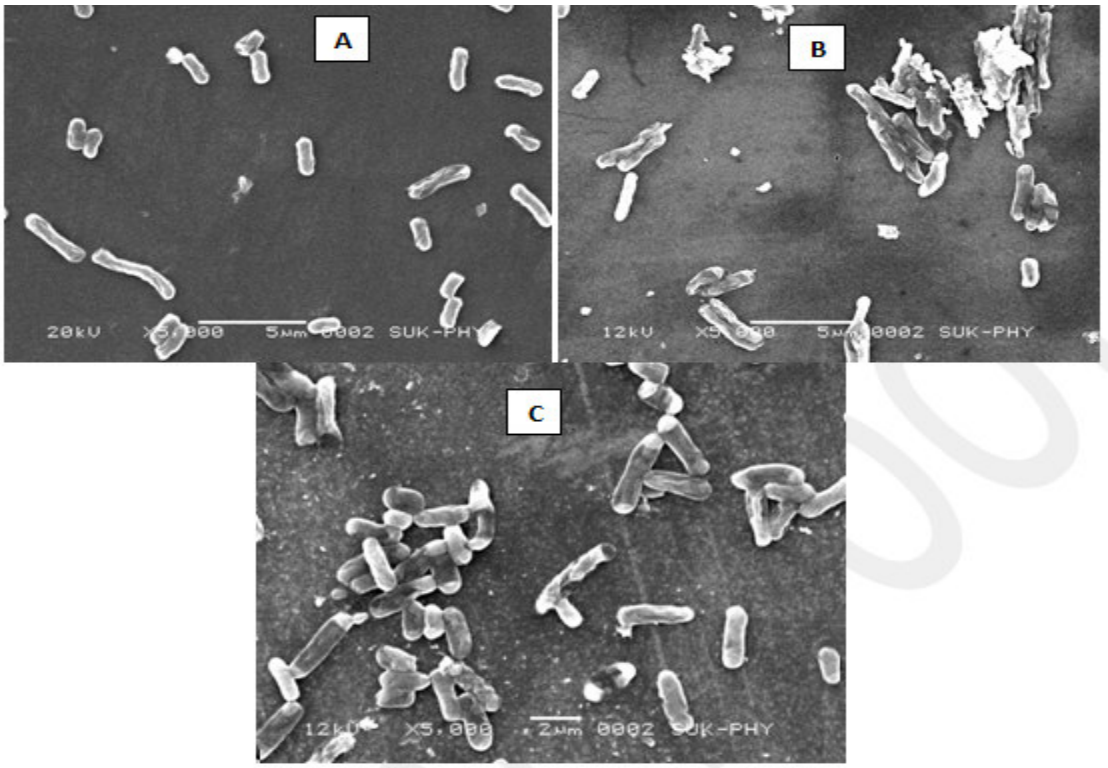


FIGURE 5

Scanning electron micrographs of Shigella isolates (A) Control isolate of Shigella without antibiotic (238) (B) Standard Shigella flexneri Strain ATCC 12022 (C) Shigella isolate (238) treated with antibiotic concentration of 64µg/ml.

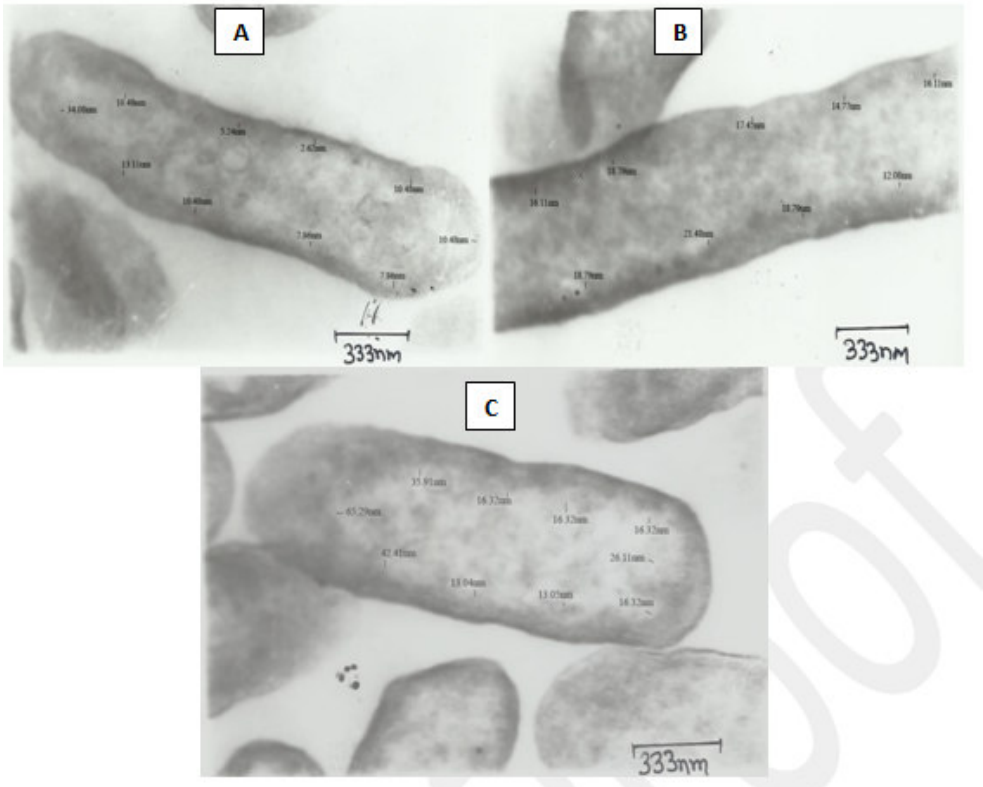


FIGURE 6

Transmission electron micrographs of Shigella isolates (A) Standard Shigella flexneri Strain ATCC 12022 (B) Control isolate of Shigella (238) (C) Antibiotic (ciprofloxacin) treated Shigella isolate (238) at magnification of 57900X.

TABLE 1

Screening of 32 Shigella isolates for detection of biofilm formation by Test Tube method with supplement of glucose and antibiotics at different incubation periods.

Biofilm formation (OD_{570nm})	No. Of Isolates and Incubation period								
	18 hours			24 hours			48 hours		
	S. dysentriae (n=16)	*S. flexneri* (n=09)	*S. sonnei* (n=7)	*S. dysentriae* (n=16)	*S. flexneri* (n=09)	*S. sonnei* (n=7)	*S. dysentriae* (n=16)	*S. flexneri* (n=09)	*S. sonnei* (n=7)
Strong (>0.240±0.022)	14 (87.5%)	08 (88.9%)	03 (42.9%)	14 (87.5%)	05 (55.6%)	02 (28.6%)	15 (93.8%)	07 (77.8%)	07 (100%)
Moderate (0.120±0.020)	02 (12.5%)	01 (11.1%)	04 (57.1%)	01 (6.3%)	02 (22.2%)	02 (28.6%)	01 (6.3%)	02 (22.2%)	--
Weak (<0.012±0.012)	--	--	--	01 (6.3%)	02 (22.2%)	03 (42.9%)	--	--	--
In combination with antibiotic (ciprofloxacin 64µg/ml)									
Strong (>0.240±0.022)	12 (75%)	01 (11.1%)	02 (28.6%)	12 (75%)	02 (22.2%)	04 (57.1%)	15 (93.8%)	03 (33.3%)	04 (57.1%)
Moderate (0.120±0.020)	04 (25%)	04 (44.4%)	02 (28.6%)	02 (12.5%)	04 (44.4%)	01 (14.3%)	01 (6.3%)	04 (44.4%)	02 (28.6%)
Weak (<0.012±0.012)	--	04 (44.4%)	03 (42.9%)	02 (12.6%)	03 (33.3%)	02 (28.6%)	--	02 (22.2%)	01 (14.3%)

TABLE 2

Screening of 32 Shigella isolates for detection of biofilm formation by Micro titer Plate method in combination with glucose and antibiotic at different incubation periods.

Biofilm formation (OD_{490nm})	No. of Isolates and Incubation period at 18 hours											
	TSB			TSB+G			TSB+A			TSB +A+G		
	S. dysentriae (n=16)	*S. flexneri* (n=09)	*S. sonnei* (n=07)	*S. dysentriae* (n=16)	*S. flexneri* (n=09)	*S. sonnei* (n=07)	*S. dysentriae* (n=16)	*S. flexneri* (n=09)	*S. sonnei* (n=07)	*S. dysentriae* (n=16)	*S. flexneri* (n=09)	*S. Sonnei* (n=07)
High (>0.240±0.022)	--	01 (11.1%)	--	04 (25%)	02 (22.2%)	01 (14.3%)	--	01 (11.1%)	--	05 (31.3%)	04 (44.4%)	02 (12.3%)
Moderate (0.120±0.020)	02 (12.5%)	--	01 (14.3%)	05 (31.3%)	03 (33.3%)	01 (14.3%)	02 (12.5%)	--	01 (14.3%)	03 (18.75%)	02 (22.2%)	01 (14.3%)
Weak (<0.012±0.012)	14 (87.5%)	08 (88.9%)	06 (85.7%)	07 (43.%)	03 (33.3%)	05 (71.4%)	14 (87.5%)	08 (88.9%)	06 (85.7%)	08 (50.0%)	03 (33.3%)	04 (25.0%)
Incubation period at 72 hours												
High (>0.240±0.022)	--	--	--	05 (31.25%)	03 (33.3%)	02 (28.6%)	--	--	--	02 (12.5%)	01 (11.1%)	02 (28.6%)
Moderate (0.120±0.020)	01 (6.25%)	02 (22.2%)	--	01 (6.25%)	--	--	02 (12.5%)	01 (11.1%)	--	01 (6.25%)	03 (33.3%)	--
Weak (<0.012±0.012)	15 (93.75%)	07 (77.8%)	07 (100%)	10 (62.5%)	06 (66.7%)	05 (71.4%)	14 (87.5%)	08 (88.9%)	07 (100%)	13 (81.25%)	05 (55.5%)	05 (71.4%)

TABLE 3

Effect of antibiotic (ciprofloxacin) on cell size of the Shigella isolates

Shigella Isolates	Width (μm^3)	Length (μm^3)	Radius (μm^3)	Volume (μm^3)
Shigella isolate 238	0.849±0.007	3.03±0.049	0.424±0.004	2.33±0.076
Shigella isolate 238 + antibiotic treated (64µg/ml)	1.02±0.013	3.53±0.048	0.51±0.006	3.72±1.20
Shigella standard strain	0.50±0.007	1.52±0.018	0.25±0.003	0.381±0.008

Each mean value of average 30 cells size based on the Scanning electron microscopy photos.

RESULTS

Biofilm formation has been reported in all strains of *Shigella sps.* Cognate with the infection of biomedical devices.[18,29-31] Evaluation to perceive the diagnosis and pathogenesis of these infections have been obsessive upon the adherence of microorganisms to surfaces. In the present study overall 32 multi drug resistant clinical isolates of *Shigella Sps.* (including: *Shigella dysentriae* (n=16), *Shigella flexnerii* (n=9) and *Shigella sonnei* (n=07)) were tested by two *in-vitro* screening methodology for the propensity to form biofilm.

Biofilm formation assay of Shigella species

Test tube method (TM)

In this permuted method, from the total 32 isolates were determined for biofilm formation at different incubation periods: 18, 24 and 48 hours. High biofilm producers were 14(87.5%), 08(88.9%) and 03(42.85%) at 18

hours, 14 (87.5%), 05 (55.5%) and 02 (28.6%) at 24 hours and 15 (93.75%), 07 (77.8%) and 07 (100%) at 48 hours, 02 (12.5%), 01 (11.1%) and 04 (57.1%) at 18 hours, 01 (6.3%), 02 (22.2%) and 02 (28.6%) at 24 hours and 01 (6.3%), 02 (22.2%) and 00 (00%) at 48 hours were moderate and non/weak biofilm producers at 18 and 48 hours, 01 (6.3%), 02 (22.2%) and 03 (42.9%) were weak biofilm formation at 24 hours incubation including all species of *Shigella* isolates [Fig. 1 & Table 1].Biofilm formation also determined in combination with antibiotic (Fluoroquinolone 64µg/ml), strong biofilm formation were 12 (75%), 01 (11.1%) & 02 (28.6%) at 18 hours, 12 (75%), 02 (22.2%) & 04 (57.1%) at 24 hours and 15 (93.8%), 03 (33.3%) & 04 (57.1%) at 48 hours and 04 (25%), 04 (44.4%) & 02 (28.6%) at 18 hours, 02 (12.5%), 04 (44.4%) & 01 (14.3%) at 24 hours and 01 (6.3%), 04 (44.4%) & 02 (28.6%) at 48 hours were moderate and 04 (44.4%) & 03 (42.9%) at 18 hours, 02 (12.5%), 03 (33.3%) & 02 (28.6%) at 24 hours and 02 (22.2%) & 01(14.3%) isolates were acknowledged as non/weak biofilm producers, proportionately [Fig. 1, 3 & Table 1].

Microtitre plate assay method (MTP)

In microtitre approach, from total 32 multidrug resistant isolates were tested for biofilm formation for all species of *Shigella* isolates for disparate combination (Tryptic soya broth + glucose +antibiotic (64µg/ml) + synergitic effect of glucose + antibiotic) used at different incubation periods: 18 and 72 hours. 01(11.1%), 04 (25%), 02 (22.2%), 01 (14.3%), 01 (11.1%), 05 (31.3%), 04 (44.4%) and 02 (12.5%) isolates were presented strong (OD_{490} ≥ 0.5), 02 (12.5%), 01 (14.3%), 05 (31.3%), 03 (33.3%), 01 (14.3%), 02 (12.5%), 01 (14.3%), 03 (18.8%), 02 (22.2%) and 01 (14.3%) were moderate (OD_{490} ≥ 0.2 to< 0.5) and 14 (87.5%), 08 (88.9%), 06 (85.7%), 07 (43.8%), 03 (33.3%), 05 (71.4%), 14 (87.5%), 08 (88.9%), 06 (85.7%), 8 (50.0%), 03 (33.3%) and 04 (25%) were non/weak biofilm producers (OD_{490} ≥ 0 to <0.2) subsequently after 18 hours incubation period. After 72 hours of incubation, 05 (31.25%), 03 (33.3%), 02 (28.6%), 02 (12.5%), 01 (11.1%) and 02 (28.6%) isolates were exhibited strong, 01 (6.3%), 02 (22.2%), 01 (6.3%), 02 (12.5%), 01 (11.1%), 01 (11.1%) and 03 (33.3%) were moderate and 15 (93.8%), 07 (77.8%), 07 (100%), 10 (62.5%), 06 (66.7%), 05 (71.4%), 14 (87.6%), 08 (88.9%), 07 (100%), 13 (81.3%), 05 (55.6%) and 05 (71.4%) correspondingly after 72 hours incubation period as delineated in the Fig. 2, 4 and table 2.

Scanning Electron Microscopy (SEM) study

The upshots of cell morphology of fluoroquinolone resistant *Shigella sps* were inspected by SEM evinced that in the occupancy of ciprofloxacin (64µg/ml), the cells evolved their morphology on the subject of different concentrations of antibiotic. In the absence of ciprofloxacin the cell morphology of control *Shigella* isolate 238 were reputedly regular [Fig. 5A]. However standard ATCC culture unveiled no altered in their cell morphology [Fig. 5B]. Anyhow standard culture 238 showed no changes in their cell morphology [Fig. 5C] but enlarged, mal-formed and rough surfaced was noticed in the antibiotic treated *Shigella sps* with a concentration of 64µg/ml [Fig. 5, Table 3].

Transmission Electron Microscopy (TEM) study

The transmission electron microscopic approach affirmed that, in-vitro effect of ciprofloxacin (*Shigella* isolates shown baseline resistance of 64µg/ml MIC were administered to chemical fixation prior to study) on the cell morphology: especially cell size and thickness of the cell wall of *Shigella sps*.TEM images formed that there was momentous variations in the thickness of the bacterial cell wall. The control strain of *Shigella* isolate 238 observed the cell wall thickness of 17.15±0.6 nm [Fig. 6B], the standard strain *Shigella flexneri* ATCC 12022 were shown 11.61±0.45nm (Fig. 6A) correspondingly, where as cell wall thickness was increased higher in size of 26.11±0.8nm at magnification 57900X for antibiotic treated *Shigella species* as shown in the figure [Fig. 6C].

DISCUSSION

Acute dysentery is a common disease in many developing countries, whereas in these countries diarrhoea is repeatedly attained due to poor hygiene and contaminated water. In our previous study *Shigella dysentriae* and *Shigella flexnerii* strains were the most deluges and predominant one in our region.[4] Out of all total *Shigella* isolates, most of isolates were eminently resistance to fluoroquinolone's class of antibiotics. Technological expansions have furnished certain assistances for human growth, both in industry and medical fields. Eradication of pathogen microorganisms by antimicrobial agents and usage of many indwelling medical devices in order to increase the function of any defective organ, are some significant advances which can be given as examples to these benefits.[32] Granting all these favourable things, merging in antimicrobial resistant micro-organisms upturning every passing day and as a denouement; perpetual nosocomial infections in hospitals, mortality and extensive economical impairment come true. Therefore, investigation of mechanisms played a role in drug resistance and fighting against them, have great place in preventing these casualties. One of these mechanisms playing a role in persistent infections is known as biofilm formation.[33] This present part of study exhibits a momentous existence of biofilm formation in bacterial infections. It is noticeable from the review that biofilm formation is imperative in bacterial pathogenesis. Biofilms play a crucial part in colonization during infection, affording a moment for the bacteria to evolve drug resistance. It is convincing that the expanding practice of antibiotics and embed devices in hospitals devote to the enhancement of attributes that cooperate biofilm development in clinical pathogens[34] and formation of biofilm by clinical isolates of *Shigella sps* was construe as a virulence factor decisive for colonization of prosthetic devices in humans.[35] In our current exercise the modified test tube method, total 32 multi drug resistance *Shigella* isolates (including different species of *Shigella*) were checked for biofilm formation. The biofilm formation resolved at different incubation periods i.e. 18, 24 and 48hrs exhibited, *S. dysentriae* and *S. flexneri* were predominantly strong biofilm producers at all three incubation periods and *S.*

sonnei were shown 100% strong production during 48 hrs incubation, *S.flexneri* was shown good at all three incubation periods and *S. sonnei* were consider as highest moderate biofilm formation during 18 and 24hrs of incubation period whereas *S. flexneri* & *S. sonnei* were predominantly non/weak biofilm formation only at 24hrs of incubation period. Biofilm formation in combination with antibiotic *S.dysentriae* were presented best, where as *S. flexneri* & *S. sonnei* were shown good strong biofilm formation during all three incubation periods, *S.flexneri* shown predominantly moderate biofilm formation compare to the *S.dysentriae* &*S.sonnei* and *S.flexneri* were exhibited good non/weak biofilm formation in all the three incubation periods compare to the *S.dysentriae* & *S.sonnei* as shown in Figure 3.In micro titre plate of biofilm assay: *S.dysentriae* & *S.flexneri* were shown strong biofilm formation in combination with TSB+glucose and TSB+antibiotic+glucose as compared to TSB & TSB+antibiotic. All three *Shigella* isolates were shown moderate biofilm formation in combination with TSB+glucose & TSB+antibiotic+glucose as compared to TSB & TSB+antibiotic and the same isolates shown non/weak biofilm formation in combination with TSB, TSB+antibiotic as compared to the TSB+glucose & TSB+antibiotic+glucose during the 18 hrs of incubation period.All the isolates of *Shigella* were shown positive strong biofilm formation in combination with TSB+glucose & TSB+antibiotic+glucose as compared to TSB & TSB+antibiotic, *S.dysentriae & S.flexneri* shown positive moderate biofilm formation in combination with TSB, TSB+antibiotic & TSB+antbiotic+glucose and all three *Shigella* isolates were positive for non/weak biofilm formation in combination with only TSB as compared to TSB+glucose, TSB+glucose & TSB+antibiotic+glucose consistently during 72 hrs of incubation period [Fig. 4]. In modified micro titer plate method, extended incubation for 72hrs could take to a superior differentiate between strong, moderate and non/weak biofilm producing *Shigella isolates* and biofilm formation as compared with 18 hrs of incubation period.

CONCLUSION

In the present observation, the test tube method corresponds together with the micro titer plate method for strongly biofilm producing isolates, but it was onerous to differentiate between weak and biofilm negative isolates due to the wavering in detected results by distinctive viewers accordingly, high changeability was noted and allocation in biofilm positive and negative was crucial by tube method. In accord with the preceding reports, test tube method cannot be praised as general screening test to diagnose biofilm producing isolate.[6] We achieve that this data demonstrate that the microtitre plate method is most sensitive, authentic and uniform screening of biofilm formation and can provide as a predictable quantitative tool for definitive biofilm producing *Shigella* isolates. As drug resistance is a vital mess in Shigellosis, it is foremost to intercept the colonization of the organism by recommending peculiar practices to obviate biofilm formation. At present, there is also necessity to define the reservoirs for colonization and the passage of transmission of Shigellosis, after all only scanty curative options prevail for treatment of Shigellosis. Electron microscopic study profess that cell morphology and varying thickness in cell wall of ciprofloxacin treated cells of *Shigella* isolate afforded strong deposition that the existence concentration of ciprofloxacin is stressful for the bacterial populations, represented by the large size. The inflation in cell size shortens the proportionate contact surface and therefore weakens the fix adequate surface for organic (antibiotic) compounds. Thus, bigger cells can stand for the stress conditions superior than natural (regular) cells of the same species. Current SEM study concedes that bacteria have emerged an acclimatise response to the antibiotic stress and have promote drug resistance. This prospective disquieting situation as fluoroquinolone's generation antibiotic is one of the few catalysts needed to treat patients with Shigellosis infection.

ACKNOWLEDGMENTS

The authors are (Kelmani Chandrakanth Revanasiddappa and Prabhurajeshwar Chidre) profusely thankful to the Department of Biotechnology, Gulbarga University, Gulbarga for providing facilities for pursuing the research work at the Department and to Shaha Children's Care Hospital and Diagnostic Centres for providing stool samples.

CONFLICT OF INTEREST STATEMENT

We declare that no conflict of interest.

ETHICAL APPROVAL

This Medical Biotechnology and Phage Therapy Laboratory, Department of Biotechnology approved ethical clearance by Institutional Clearance Certificate (IECC) for *in-vitro* and *in-vivo* studies.

AUTHORS CONTRIBUTION

First author is responsible for carrying out the research work, data analysis and optimization of experimental work and Corresponding author is responsible for research planning executing and providing valuable inputs and in writing manuscript.

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