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Determination of minimum inhibition concentration of bacteria (*E. coli* and *S. aureus*) in surface water and wastewater against ciprofloxacin

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Due to the increase in pharmaceuticals and antibiotics in surface water and wastewater, it is giving a place for the bacteria to become antibiotic resistant to a particular antibiotic or to a group of antibiotics by the mutation of their genes. In this study, minimum inhibition concentrations correspond to 50% inhibition (IC_{50}) of two bacteria, *E. coli* and *S. aureus* were tested. The bacteria were collected from surface water and wastewater at different locations of Kolkata and Howrah. Among the fluoroquinolone antibiotics, the concentration value of ciprofloxacin has been detected in its highest contamination level, so the IC_{50} values of bacteria were tested against this antibiotic. While checking for the IC_{50} values for bacteria against ciprofloxacin, it was found that the *E. coli* and *S. aureus* isolated from wastewater and surface water were found to be 26 times and 30 times more resistance against pure *E. coli* and *S. aureus*. Along with this, bacteria present in wastewater was found to be more resistant than the bacteria found in surface water.

Keywords: *E. coli*, *S. aureus*, bioassay, minimum inhibition concentration, ciprofloxacin.

Introduction

Due to the increase in concentration of pharmaceuticals including antibiotics in wastewater and surface water, the bacteria are getting multi-drug resistant by the mutation of their genes[1]. These antibiotics are not only used for human medication but are also used in various poultry productions, sub-therapeutic levels in livestock, in aquatic culture for prevention of diseases and growth of marine life. Because of its extensive use and its wide spread occurrence, the antibiotic resistance has spread almost around every part of the world in surface water, wastewater, and drinking water. Development of antibiotic resistant pathogenic strains due to long term exposure of various antibiotics has been reported by several research groups[2]. Long term exposure of antibiotics lead to the selection pressure among bacterial strains that radically enhances bacteria containing resistance genes[3]. As a result of which, the antibiotics exposure to different environmental conditions leads to the development of antibiotic resistance bacteria.

It was found that 80% of the normal healthy persons excreta contained antibiotic resistant bacteria[4]. 245 nos. of *E. coli* and 167 *S. aureus* species were found to be multi-drug resistant in an untreated hospital wastewater[5]. Again, *E. coli and S. aureus* were isolated from various food stuffs and its resistance were checked against various antibiotics[6]. Among fluoroquinolone antibiotics, ciprofloxacin (CIP) has been detected at a very high concentration of 31,000 µg/L in a wastewater discharged by drug manufacturing companies at Patancheru near Hyderabad, India[7]. Not only in India, the antibiotic loading has spread all over the world which is giving rise to the antibiotic resistance bacteria all over the world. The presence of multi-drug resistant bacteria *E. coli* and *S. aureus* in untreated hospital wastewaters as well as in wastewater treatment plant in South East Queensland, Australia was analyzed[3]. The antibiotic concentration was found to be present in micrograms per litre in surface water, wastewater and ground water on some parts of Kolkata and Howrah.

In this study, the minimum inhibition concentrations of two bacteria *E. coli and S. aureus* was determined against ciprofloxacin antibiotic. For the determination of IC_{50} values, wastewater and surface water were collected from different

parts of Kolkata and Howrah and isolation of bacteria were done by selective media.

Materials and methods

(A) Reagents and glass wares used:

All Borosil glass wares such as test tubes, conical flasks, small beakers, large beakers, petri dishes (75 mm diameter), measuring cylinders, spreaders etc. were autoclaved prior to use. The pipette controller of 10 mL and 1 mL along with tips, microplates, paraffin wax and cotton were also used. Side instruments such as loops, spirit lamps, test tube stand were also used. MUG agar, Hi-Media (28.1 g in 1000 mL) and Mannitol Salt Agar, Hi-Media (111 g in 1000 mL) were used for isolation of *E. coli* and *S. aureus* respectively. For the culture of bacteria, Lactose Broth, Hi-Media (13 g in 1000 mL) was used. Ciprofloxacin (CIP) hydrochloride injection (200 mg/100 mL, 99.6%) was purchased from Frank Ross, Kolkata, West Bengal.

(B) Sample collection:

The study area included a total of 34 sampling locations for surface water and wastewater as marked in Fig. 1.8 and 12 nos. of surface water sample were collected from Kolkata and Howrah respectively. Similarly, 8 nos. and 6 nos. of wastewater samples were collected from Kolkata and Howrah respectively. The samples were collected in sterilized polyethylene containers and carried to the laboratory in ice boxes and stored at 4°C until further use.

(C) Instruments:

The bacteriological hood or Laminar UV-Hood was used for performing the culture, isolation and bioassay for the two bacteria taken into account (*E. coli and S. aureus*). Bacteriological incubator was used for the isolation and culture of the respective bacteria where temperature was fixed at 37°C. UV-Vis Spectrophotometer (Systronics India Ltd. Model: DD2203) was used for checking the absorbance value with respect to 0.5 McFarland solution. The optical density (OD) values of the samples in bioassay was measured by microplate reader (Thermo Scientific, MULTISKAN GO).

(D) Experimental procedure:

For conducting bioassay, first the samples (wastewater and surface water) are collected from various places. MUG agar and Mannitol salt agar are used to isolate *E. coli* and *S. aureus* strains respectively. The Agar media was sterilized

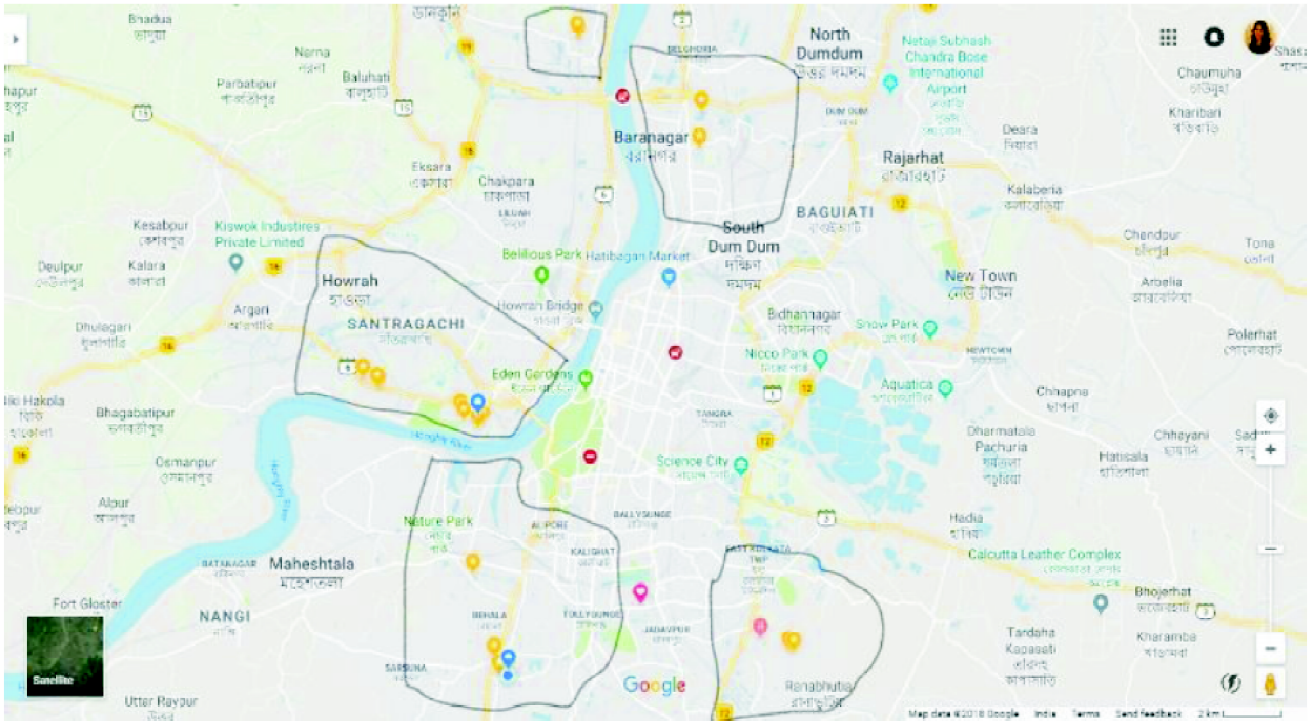

Fig. 1. Location map of sites for sample collection.

and allowed to cool at room temperature; after which 10 mL of the solution was poured in petri dish and allowed to solidify. 150 to 200 μL of the sample water was poured and spread through spreader uniformly throughout the agar media followed by sealing it properly with paraffin wax. The sealed petri dishes were then kept in bacteriological incubator at 37°C for 24 h (for *E. coli*) and 48 h (for *S. aureus*). Well distinguished colonies formed in the media were picked up for culture of respective two strains. Susceptibility testing of the selected antibiotics was performed by broth dilution method. Inoculum for susceptibility testing was prepared by dissolving a loopful of each bacterial isolate in 0.9 sterile saline solutions (lactose broth; standard solutions were prepared by dissolving 1.3 g in 100 ml distilled water) and placed in 24 h and 48 h of incubation at 37°C for *E. coli* and *S. aureus* respectively; then the turbidity was measured. The turbidity was compared with 0.5 McFarland standard solutions. Thereafter, the bio-assay was performed using 96 well plates. The inoculums (100 μL) and antibiotic standards (100 μL) were added to 96 well microplates and incubated for 24 h at 35°C. The optical density was measured by microplate reader (Thermo Scientific, MULTISKAN GO). Optical density was converted to inhibition of *E. coli and S. aureus* growth using the following equation:

$$I(\%) = \left(\frac{OD_{625,pos} - OD_{625,exp}}{OD_{625,pos} - OD_{625,neg}} \right) \times 100\%$$

where, I is the percent inhibition, OD_{625} is the optical density at 625 nm; pos, neg, and exp indicate the positive growth control, negative growth control and experimental samples, respectively.

To determine inhibition concentration corresponds to 50% inhibition of bacterial growth Hill's equation was used[8]. The equation is given as follows:

$$I(\%) = I_{min} + \left(\frac{I_{max} - I_{min}}{1 + \left(\frac{IC_{50}}{C} \right)^{H}} \right)$$

where, I_{max} is the maximum inhibition, I_{min} is the minimum inhibition, IC_{50} is the CIP concentration that results in 50% inhibition of *E. coli* growth, C is the CIP concentration in the well, and H is the Hill slope.

By analyzing the data with the above equation, IC_{50} and Hills slope were obtained by solving in Data Solver.

Results and discussion

(A) Resistance of E. coli in surface water of Kolkata and Howrah:

The surface water was collected from different locations of Kolkata and Howrah to examine the resistance of *E. coli* against ciprofloxacin. It was found that pond water near Parnasree, Behala and Satabdi, Garia had maximum IC_{50} values compared to other ponds of South Kolkata. This might be due to the fact that, the pond water near Parnasree and Satabdi involved all types of activities such as washing of clothes, cleaning, bathing, throwing of wastes etc. throughout the years. The bacteria present in that pond were subjected to such adverse conditions that their genes were mutated and were resistant enough to the antibiotics having high inhibition concentration of 551.07 μg/L and 570.53 μg/L, respectively which were 22.04 and 22.82 times more resistance than pure *E. coli*. The IC_{50} for pure *E. coli* was determined to be 25 μg/L.

Other pond water at James Court, Sakherbazar and Nayabad, Garia, the IC_{50} values were less which indicated that these ponds were involved in less domestic activities. The bacteria were less resistant to the antibiotics having 6.86 and 5.05 times more resistance than pure *E. coli*. The minimum inhibition concentration of *E. coli* in pond water, Barisha was found to be 71.78 μg/L which was 2.87 times more resistance than pure *E. coli*. It can be concluded that since this pond was not involved in any sort of activity at that location, the bacteria present were less resistant to antibiotics. Considering North Kolkata, pond water at Netaji Colony, had few activities having an IC_{50} value of 217.85 μg/L which was 8.71 times resistance. Whereas lake at Bonhooghly had IC_{50} values of 100.72 μg/L indicating 4.02 times resistance which was less compared to other ponds. Hence it can be seen that except two pond water at Parnasree, Behala and Nayabad, Garia, the bacteria at other ponds were less resistance towards ciprofloxacin antibiotic compared to the above two ponds.

Considering South Howrah surface water, high IC_{50} values were obtained at ponds of Chunabhati and Podara having maximum resistance. The pond water at Bacharapara and Jorapukur, Chunabhati had the highest IC_{50} value of

504.84 μg/L and 670.06 μg/L respectively. This is due to the fact these ponds had all activities such as bathing, washing, throwing garbages etc. in huge amounts throughout the years which resulted in the mutation of genes in bacteria thereby making them 20.19 and 26.80 times more resistance compared to pure *E. coli.*

Considering two ponds in Podara, IC_{50} values of 329.37 μg/L and 262.68 μg/L respectively which were 13.17 and 10.50 times resistance. Considering the surface water inside the IIEST Shibpur campus, it was found that pond water at First Gate near IIEST, Neem Jhil pond water, pond water near Hydraulics Lab and Goligan Ganga pond water, IIEST Shibpur had less IC_{50} values as compared to the pond waters of Chunabhati and Podara. The IC_{50} values of pond water, First Gate, IIEST was 104.83 μg/L, Neem Jhil pond water was 86.94 μg/L, Hot pond water, Hydraulics Lab was 115.75 μg/L and Goligan ganga pond water was 61.21 μg/L having 4.19, 3.47, 4.63 and 2.44 times more resistance than pure *E. coli*. The lower resistance is due to the fact that these ponds were inside the IIEST Shibpur campus and hence care and precaution was taken to keep these ponds clean. The ponds were kept under maintenance and were guarded by fencing on all sides of the pond. Bidisha pond of IIEST Shibpur was also maintained but the IC_{50} value obtained was higher than the other ponds of the campus. The IC_{50} value was 159.43 μg/L which was 6.377 times more resistance. This might be due to the fact that, because of the flow of water from Hooghly River while high tide into the Bidisha pond water and thereby bacteria of Hooghly River water gets mixed with Bidisha pond water resulting in an increase in antibiotic resistance. The pond water at Koley market had minimum IC_{50} values of 101.98 μg/L as the pond was well maintained.

Considering North Howrah Surface water, few activities were observed in the pond at Uttarpara and Bali Dhanga with IC_{50} values of 241.28 μg/L and 179.03 μg/L which were 9.65 and 7.16 times more resistance.

Hence, it can be concluded that the surface water of North and South Kolkata and Howrah are equally polluted with an average IC_{50} value of 236.32±188.31 μg/L. The ponds are not maintained and there is an increase in activities thereby making bacteria highly resistance to the antibiotics.

(B) Resistance of S. aureus in surface water of Kolkata and Howrah:

Having the same activities of the above described location and ponds, *S. aureus* was also found resistant to the antibiotics. The pond water at Parnasree, Behala and Satabdi, Garia had the highest IC_{50} values of 533.52 μg/L and 461.04 μg/L respectively which were 13.33 and 11.52 times more resistance than pure *S. aureus*. The IC_{50} for pure *S. aureus* was determined to be 40 μg/L. The pond water at James Court, Sakherbazar and Nayabad, Garia also had the higher IC_{50} values of 106.18 μg/L and 177.22 μg/L which was much lesser than the above ponds and were 2.65 and 4.43 times resistance. The pond water at Barisha had no activities and hence the IC_{50} values of *S. aureus* was also less as 87.73 μg/L which was 2.49 times resistance.

Taking North Kolkata into consideration, no *S. aureus* was obtained at the ponds near Netaji Colony and Bonhooghly.

In South Howrah, the pond water at Bacharapara, had the lower value of IC_{50} of 95.87 μg/L which was 2.39 times resistance. But higher IC_{50} values of pond water at Jorapukur, Chunabhati was obtained as 508.54 μg/L which were 12.71 times resistance than pure *S. aureus*. This might be due to the fact that the *S. aureus* might have been present for a very long time in pond water at Jorapukur and due to the maximum activities in it, the bacteria got resistant to the antibiotics whereas *S. aureus* might not have been present in pond water in Bacharapara from years and so the inhibition concentration of bacteria was low. The two ponds in Podara also had the higher IC_{50} values for *S. aureus* as 355.63 μg/L and 476.71 μg/L which were 8.89 and 11.91 times more resistance than pure *S. aureus*. The surface water of IIEST Shibpur had lower IC_{50} values for *S. aureus* and were same as IC_{50} values of *E. coli*. The IC_{50} values of *S. aureus* in pond water at First Gate, IIEST was 76.02 μg/L, Neem Jhil pond water was 73.70 μg/L, the pond water at Hydraulics Lab was 146.94 μg/L and Goligan Ganga pond water was 143.07 μg/L which were 1.90, 1.84, 3.67 and 3.57 times more resistance than pure *S. aureus*.

It is seen that both *E. coli* and *S. aureus* had more or less same inhibition concentration for the same antibiotics and had same resistance. But Bidisha Lake had higher IC_{50} values of 541.00 μg/L for *S. aureus* which was 13.52 times more resistance and was higher than IC_{50} value for *E. coli*. This might be due to the fact that since at high tides, water from Hooghly River flows into the pond and *S. aureus* present in that water might have become more resistant to antibiotics than *E. coli* present in it. The pond water at Koley, Shibpur

had IC_{50} value of 99.82 μg/L which was lower because of the good maintenance of the pond. Considering North Howrah surface water, both ponds at Uttarpara and Bali Dhanga had IC_{50} values of 202.85 μg/L and 97.69 μg/L which were 5.07 and 2.44 times more resistance than pure *S. aureus* and was same as observed in IC_{50} values of *E. coli*.

Hence, it is concluded that, *E. coli* and *S. aureus* were found to have almost same IC_{50} values for the same sample of surface water with an average value of 236.32±188.31 μg/L and 237.40±182.99 μg/L resulting that both bacteria are equally resistant to ciprofloxacin antibiotic except in sample of Bidisha pond water.

(C) Resistance of E. coli in wastewater of Kolkata and Howrah:

The *E. coli* present in wastewater was collected from various drains and were analysed for IC_{50} values. Wastewater collected from the drain at Sakherbazar and James Residency apartment had an IC_{50} value of 202.85 μg/L and 197.96 μg/L indicating that the bacteria are 8.11 and 7.90 times more resistant to pure *E. coli* and the bacteria present have developed antibiotics resistant genes. The drain at Taratala had an IC_{50} value of 763.14 μg/L indicating 30.32 times more resistance than pure *E. coli.* The bacteria present at that locality have gained high resistance to antibiotics. This might be due to the fact that the drain water at Taratala gets the wastewater from the hospital located besides it. The wastewater from hospital might contain huge amount of antibiotics which are released into the drain for years, thereby making the bacteria resistant enough against ciprofloxacin. The drain water from Behala thana had IC_{50} value of 418.01 μg/L which was 16.72 times more resistance than pure *E. coli.* The wastewater collected from the drain at Sakherbazar received water from various market places like fish, vegetable, poultry etc. Since antibiotics are also used in fish and poultry markets, these antibiotics are released which gets collected at the drains.

Considering two drains of Garia, which was situated besides Garia metro station to bus stand drain (Drain 1) and other drain being 1 km away from new Garia station (Drain 2) had higher IC_{50} values of 450.92 μg/L and 384.74 μg/L which were 18.03 and 15.38 times more resistance than pure *E. coli*. This might be due to the fact that wastewater from Garia metro to bus stand have open urinals which is being used by passengers. Apart from this, drain may get its water from the urinals or toilets from metro station through years, making the bacteria present there having higher resistance to the antibiotics.

Taking North Kolkata wastewater into consideration, the drain at Baranagar and at T. N. Chatterjee Road had higher IC_{50} values of 535.63 μg/L and 765.66 μg/L which were 21.42 and 30.62 times more resistance than pure *E. coli*. The municipal drain water is discharged at that locality and the outlet of drain at Baranagar receives water from ISI (Indian Statistical Institute) and from other complexes located besides the ISI. The wastewater collected from T. N. Chatterjee Road is 10 min walking from the above place. So, it can be said that the same municipal wastewater and wastewater from ISI and other companies are getting mixed and hence bacteria are having higher resistance to antibiotics.

Considering South Howrah wastewater, the drain at Lebu Khali math, Chunabhati had IC_{50} value of 349.26 μg/L because the drain received all the wastewater from the residential area and hence the bacteria had developed 13.97 times more resistance to the antibiotic. The main road drain in Chunabhati received all the municipal wastewater and hence the IC_{50} values was 381.66 μg/L which was 15.26 times more resistance than pure *E. coli*. The municipal drain consists of all the wastewater coming from residential and other institutions which leads to increase in antibiotic resistant bacteria. Municipal wastewater was also collected from the drain at Podara, situated at the main road where the IC_{50} values obtained was 180.33 μg/L which was 7.21 times more resistance, whereas, wastewater collected from the drain, 10 feet beside Podara Main Road was found to have IC_{50} value of 549.79 μg/L which was much higher than the wastewater collected from the main road drain. This might be due to the fact that, the wastewater received by the drain 10 feet away from main road may receive other wastewaters from other drains and gets mixed with it, thereby having an increase in higher IC_{50} values.

The drain water at First Gate, IIEST Shibpur had IC_{50} value of 160.46 μg/L which was 6.41 times resistance. The drain received wastewater from hostels and housing drain. There was no source of any wastewater coming from hospital wastes or municipal wastes, so the bacteria present in that wastewater were less resistant to antibiotics. But the drain water at College Ghat, Howrah had higher IC_{50} values of 651.36 μg/L which was 26.05 times more resistance than

pure *E. coli*. The wastewater received in College Ghat, Howrah included huge amount of wastes coming from residential and commercial areas, thereby leading to an increase in IC_{50} of *E. coli*.

It is seen that the bacteria present in wastewater had higher IC_{50} values than surface water except for few samples where both surface water and wastewater had same IC_{50} values. The average value of IC_{50} for *E. coli* in wastewater was 427.96±205.71 μg/L. This might be due to the fact that surface water which were not at all maintained had been used for bathing, washing and other activities for years and hence due to these activities, the bacteria present in surface water have been exposed to various antibiotics, thereby having an increased IC_{50} values and increased resistance compared to pure *E. coli.*

(D) Resistance of S. aureus in wastewater of Kolkata and Howrah:

The wastewater collected from drain at Sakherbazar receiving from various market places like fish, vegetable and poultry to examine the resistance of *S. aureus* against ciprofloxacin antibiotic. The IC_{50} values of *S. aureus* was found to be 215.13 μg/L which was 5.37 times more resistant and almost same result was obtained incase of *E. coli*. *S. aureus* was found to be more resistant than *E. coli* having IC_{50} value of 263.82 μg/L obtained from the drain of James Residency apartment. The bacteria were 6.59 times more resistance than pure *S. aureus*. The drain at Taratala received wastewater from hospital which had lower IC_{50} values for *S. aureus* as compared to IC_{50} of *E. coli*. The minimum inhibition concentration of *S. aureus* was found to be 137.85 μg/L which was 3.44 times more resistance indicating that *E. coli* was subjected to higher exposure of antibiotics and hence developed higher resistance, whereas, *S. aureus* experienced lesser exposure to antibiotics resulting in lower IC_{50} values. The drain water at Behala Thana had higher IC_{50} values of 335.39 μg/L which was 8.38 times more resistance. The *E. coli* also had similar values for resistance to the antibiotics which indicates that both *E. coli* and *S. aureus* were equally subjected to the antibiotics resulting in higher IC_{50} values in both cases. The two drains at Garia had different IC_{50} values for *S. aureus*. The wastewater collected from Garia metro station to bus stand drain (Drain1) had IC_{50} values of 129.40 μg/L, whereas the other wastewater collected from 1 km away from drain (Drain 2) had IC_{50} value of 705.68 μg/L which were 17.64 times resistance. This might be due to the fact that *S. aureus* present in Drain 1 might have been exposed to antibiotics for very short period of time resulting in lower IC_{50} values, whereas, *S. aureus* at Drain 2, might have been exposed to antibiotics for longer period and hence there was increased IC_{50} values. Considering North Kolkata wastewater, both the drains at Baranagar and T. N. Chatterjee Road had lower IC_{50} values for *S. aureus* as compared to higher values of IC_{50} values for *E. coli*. The IC_{50} values obtained for *S. aureus* was found to be 144.56 μg/L and 83.74 μg/L which were 3.61 and 2.09 times resistance. This might be due to the fact that *S. aureus* may have been exposed to the antibiotics over short period of time, and hence their IC_{50} values were less as compared to long exposure of *E. coli* to the antibiotics at the same place.

Considering South Howrah wastewater, the drain water at Lebu Khali math and main road drain water of Chunabhati had higher exposure to antibiotics thereby having an increased IC_{50} values of 476.29 μg/L and 348.27 μg/L respectively, which were 11.90 and 8.70 times more resistance than pure *S. aureus*. The *E. coli* also had same IC_{50} values as that of *S. aureus*. The municipal wastewater collected from main road drain, Podara had higher IC_{50} value of 236.79 μg/L which were 5.91 times resistance. The IC_{50} of *E. coli* was also found same as *S. aureus*. But the IC_{50} value of wastewater collected from drain 10 feet away from main road of Podara had higher value of 967.61 μg/L which was 24.19 times more resistance than pure *S. aureus.* This indicates that *S. aureus* present in this locality have been exposed to higher concentration and to longer extent leading to higher IC_{50} values.

The drain water of First Gate, IIEST Shibpur had IC_{50} value of 189.53 μg/L which was 4.73 times resistance. The drain at College Ghat had higher IC_{50} value of 923.81 μg/L which was 23.09 times more resistance than pure *S. aureus*. This indicates that *S. aureus* bacteria have also been exposed to higher concentration of antibiotic for longer duration thereby having a higher IC_{50} value. This shows that *S. aureus* has also become resistant towards antibiotics with an average IC_{50} value of 368.42±293.46 μg/L.

Hence, we can conclude that both *E. coli* and *S. aureus* have developed resistance against ciprofloxacin with minimum inhibition concentration (IC_{50}) for surface water and wastewater as shown in Table 1.

Table 1. Minimum inhibition concentration (IC_{50}) of *E. coli* and *S. aureus* in surface water and wastewater

Source	IC_{50} of *E. coli*	IC_{50} of *S. aureus*
Surface water (Kolkata and Howrah)	236.32±188.31	237.40±182.99
Wastewater (Kolkata and Howrah)	427.96±205.71	368.42±293.46

Conclusion

From the study, it is found that both *E. coli* and *S. aureus* have developed resistance of more than 20 times in case of surface water and wastewater. Both *E. coli* and *S. aureus* had the same inhibition concentration of the same sample, as they have been exposed to the same extent except at some places. Wastewater had higher IC_{50} values than surface water. This is due to the fact that different types of wastewater were discharged from Municipal Corporation, wastes coming from institutions, hotels, hospitals, household areas, fish markets, and poultry farms etc. where the bacteria have been exposed to antibiotics for longer duration. But there are ponds where IC_{50} values were same as wastewater because of the fact that these ponds have not been taken care of and not being maintained, thereby increasing activity in that pond lead to pollution increase and also higher IC_{50} values for both *E. coli* and *S. aureus*. The ponds which were under maintenance had lower IC_{50} values which showed that the bacteria *E. coli* and *S. aureus* were less resistant to the.

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