Guidelines

For Monitoring of River Hygiene

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List of abbreviations used

AMR: Anti-Microbial Resistance

API: Active Pharmaceutical Ingredients

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistance Genes

BHU: Banaras Hindu University

CPCB: Central Pollution Control Board

GDP: Gross Domestic Product

GPS: Global Positioning System

KB: Kirby Bauer Disc diffusion assay

Kg: Kilogram

LB: Luria Bertani

MDR: Multi-Drug Resistant

NWQMP: National Water Quality Monitoring Programme

PPC: Pollution Control Committee

RB: RightBiotic Assay

SDG: Sustainable Development Goal

SPCB: State Pollution Control Board

STP: Sewage Treatment Plant

USD: United States Dollar

UTI: Urinary Tract Infection

WASH**:** Water, Sanitation, and Hygiene

WHO: World Health Organization

WWTP: Waste Water Treatment Plant

Guidelines for monitoring of River and Waterbody Hygiene

1.0 Introduction

Water is the most important components for survival of life and constitutes 70% of the total body composition. Adequate availability of water of required quality is pre-requisite for survival and quality of human life. Due to its cardinal importance in health and overall survival, "Water, Sanitation, and Hygiene (WASH)" is one of the sustainable development goals (SDG 6th) defined by the United Nations. Apart from its use as an essential component for survival, water is required for agricultural and industrial activities, and power generation, thus holding a central role in the global food supply, economic prosperity and survival of all living organisms on the planet earth [1]. Importance of clean & adequate water is apparent from the fact that all major civilizations of the ancient times have developed and flourished near banks of major river, in India and across the world, a trend that subsequent human generations continue to follow [2]. Thus rivers are the life-line for any civilization. As a result of the exponentially growing human activities, most world rivers are now being impacted, including by long range transport of pollutants. These changes are regarded as a global water quality issue. River surveillance activities have gained tremendous importance, particularly under the pressure of national and international regulations, the most important being the drinking water standards of the World Health Organization, WHO (1994).

Threats to the health of an aquatic ecosystem can come from many sources. Sometimes this source is a natural disturbance. However, more often it is due to human activity. Usually human-created pollutants comprise the major disturbance to a lake, river, or stream. Agricultural fertilizers are also a concern because they can cause an ecological problem in the form – eutrophication. Eutrophication is the process in which a body of water becomes too low in oxygen because of too high levels of nitrogen and phosphate. Pollution is not readily evident, and lakes, rivers and streams which seem fine on the surface may be collapsing without providing any visible signs of decay. ***This makes water testing very crucial for preserving the health of the aquatic ecosystem in general.*** Regular checks of several factors in a lake, river, or stream can allow us to identify threats and minimize their damage. Knowing exactly what is wrong with an aquatic ecosystem can help us in pinpointing the source of the problem/s. While high levels of toxic substances, mutagens, or heavy metals would indicate industrial pollution,

high levels of nitrogen and phosphorus most commonly indicate that the problem is overuse of fertilizer on yards & fields.

Pollution of major rivers across the world has become a major cause of concern in past few decades. Large rivers stretches are polluted mainly due to open defecation, discharge of untreated /partially treated sewage, discharge of industrial wastewater containing chemicals and pharmaceutics, use of antimicrobial pesticides for crops, biomedical waste generated in hospitals and clinics and from animal husbandry industries [3]. The settlement and continuous operations of industries require an adequate and lasting supply of water to carry out various industrial operations. These industries generate a huge amount of wastewater after completion of various processes and discharge the generated wastewater into the environment, mostly in the water bodies located in vicinity of the functioning industries. As per the national guidelines, industries must treat their wastewater using sophisticated industrial effluent treatment plants before releasing it into the environment [4]. Unfortunately, it has been observed that Indian industries often discharge untreated or partially treated water into the nearby water bodies or rivers leading to severe water pollution and water toxicity. Continuous discharge of untreated water into rivers gradually pollutes the water and makes it useless for drinking, agriculture and further industrial use [5]. The Central Pollution Control Board (CPCB) in association with State Pollution Control Boards (SPCBs)/Pollution Control Committees (PPCs) is monitoring the quality of water bodies at 2500 locations across the country under National Water Quality Monitoring Programme (NWQMP) and their observations show that organic pollution is the predominant cause of water pollution in India. Based on the magnitude of organic pollution, CPCB in 2008 identified 150 polluted river stretches which was increased to 302 in 2015.

Aquatic environments harbor diverse freshwater bacterial communities which may be subjected to anthropogenic pressures, while domestic wastewaters receive direct loads of antibiotics and pathogenic bacteria from human excretion [6]. The nature of these environments allows them to function as ‘hotspots’ for resistance through selection of Antibiotic Resistant Bacteria (ARB) and the circulation of Antibiotic Resistance Genes (ARGs) through the stimulation of horizontal gene transfer between members of the microbiome/s. Antibiotics and other Active Pharmaceutical Ingredients (APIs) are released into the environment by pharmaceutical industries and API manufacturers leading to contamination of surrounding soil

and water bodies [7]. A high concentration of antibiotic residue in water bodies then becomes a fertile ground for emergence of Multi-Drug Resistant (MDR) microbes called "Super-Bugs". The emergence of such super-bugs poses a great threat to human health as infections caused by MDR bacteria are very difficult to treat leading to high incidence of mortality. Bacteria develop antibiotic resistance in the presence of residual levels of antibiotics, and these ARB are in turn able to spread their ARG to other bacteria through mechanisms such as horizontal gene transfer, mediated by mobile genetic elements (e.g., plasmids, integrons) or co-selecting agents such as biocides and toxic metals [8].

In initial days, most of the antibiotics were obtained naturally by various fungi and bacterial species. However, the present manufacturing of antimicrobials is highly dependent on fermentation and synthetic/semi-synthetic routes. Moreover, the microbes used in the production of antibiotics are genetically modified to increase the yield of antibiotics. The release of these genetically modified strains in the environment further exacerbate the environmental hazard posed by pharmaceutical companies. According to an estimate, production of 1,000 kg penicillin G produces 35,000 kg of biological sludge, 10,000 kg of wet mycelium, 1,200 liters of solvents, and 56,000 litres of fermentation media indicating a serious challenge to the environment if the waste is released into the environment without due care and proper treatment [8].

An estimated global production of antimicrobial agents is approximately 100,000 tons and a whopping 63% (63,200 tons) of antibiotics are used in livestock industry. Asia is one of the largest producers of antimicrobial agents and APIs and several studies have confirmed presence of antibiotics in river water and other water bodies. The overall concentration of antibiotic reaching the wastewater systems due to human waste is much lower in comparison with localized discharge from various manufacturers because antibiotics are used by a small fraction of the population at any given time. Moreover, the concentration of antibiotic residues present in the wastewater also gets reduced during the treatment process if effective treatment standards have been followed [8]. In contrast, point discharge of antibiotics by industries can lead to very high concentration of antibiotic residues in water bodies and surrounding areas [8].

A case in sight is the story of Hyderabad, a global hub for generic drug manufacturing with several pharmaceutical industries located in Patancheru-Bollarum corridor. A study has reported presence of the highest concentration of antibiotic ciprofloxacin at 28 and 31 mg/l on

two consecutive days in water bodies located in Patancheru area, Hyderabad [5]. This concentration of ciprofloxacin is 1,000 times higher than the concentration required to kill bacteria [5]. Lübbert et al [9] reported antibiotic residues from 28 environmental sampling sites in the sewers of industrial area in Hyderabad, India. Presence of drugs such as ciprofloxacin, norfloxacin, cetrizine, terbinafine, citalopram and enoxacin have also been reported in water samples taken from wells, lakes and discharge from the effluent treatment plants [10]. Presence of antibiotics has also been reported from other major Indian rivers such as Kaveri, Vellar and Tamiraparan, where water of Kaveri river was found to contain carbamazepine and the highest levels of Triclosan in surface waters were observed in Tamiraparani River [11]. The study also reported presence of parabens in river water [11] and clearly established that insufficiently treated wastewater from the pharmaceutical industries have led to a severe contamination of ground and surface water bodies highlighting the urgent need to improve the situation with paramount priority to reduce the deleterious long term consequences of pharmaceutical drugs and antibiotics [10] on local biodiversity. Antibiotic-resistant genes even to high-end antibiotics were detected in Mutha river flowing through Pune, India, with 30-times higher concentration in the sediments near the city, originating from domestic and municipal sewage waste [12]. While isolation of *Enterococci*, a commensal of human gut, was possible from river sources at several places, the rate of vancomycin-resistant *Enterococci* ranged between 22-100% from banks of river Gomti [13]. Since hospitals are the places with highest level of antimicrobial consumption, their effluent waters are expected to be the richest source of resistant bacteria and their genes. Mutiyar and Mittal [14] have reported the alarming extent to which residues of fluoroquinolones, sulphonamides and tinidazoles were recovered from a hospital effluent in India. The high cost associated with regular monitoring of antimicrobial levels in pharmaceutical wastewater makes it a low-priority objective for India [8] and this potentially 'AMR-rich' municipal wastewater is finally discharged into the nearby water bodies.

Presence of antibiotics in river water is a global concern and Yellow River, Hai River and Liao River in northern China have also been found contaminated with several types of antibiotic residues like Ciprofloxacin, Norfloxacin, Oxytetracycline and Ofloxacin [15, 16]. Indeed, authors of another recent study led by University of York, estimated presence of 14 antibiotics in rivers of 72 countries across six continents found that 65% of the total of 711 sites tested positive for presence of antibiotics with some of them at concentrations as high as 200 times of the safe

level range, from 20-32000 ng/L (as per antimicrobial resistance, 'AMR', industry alliance standards), depending on the antibiotic. Ciprofloxacin most frequently exceeded safe levels, surpassing the safety threshold at 51 places. Moreover, high-risk sites were typically adjacent to wastewater treatment systems and waste or sewage dumps. Some of the world's most iconic rivers were sampled, including the Chao Phraya, Danube, Mekong, Seine, Thames, Tiber and Tigris. The team found that safe limits were most frequently exceeded in Asia and Africa, but sites in Europe, North America and South America also had high level emphasizing the important role of the natural environment in the antimicrobial resistance problem. In India the rate of isolation of *E. coli* resistant to third generation cephalosporin was 25, 70 and 95% when the inlet to the treatment plant was domestic water alone, domestic waste along with hospital effluent and hospital effluent alone, respectively [17]. Of the 283 E. coli isolates from the south Indian river Cauvery in Karnataka, 100% were resistant to third generation cephalosporin [18]. The groundwater and surface water that are used for drinking and recreational purposes have been reported with17% rate of *E. coli*, resistant to third generation cephalosporin, in central India [19], 07 % in north India (Kashmir) [20], 50% in east India (Sikkim) [21] and 100% in south India (Hyderabad) [9].

In light of these facts and the i) growing burden of antimicrobial resistance and ii) emergence of 'Super Bugs' it is advisable to conduct hygiene survey/s at all the points selected by CPCB for NWQMP in rivers/waterbodies of the country. For comparison of data collected and generated across the country it is advisable to conduct these surveys using **uniform guidelines and test methods deployed for 'River /water body hygiene survey** and also test suspected discharge points for presence of pathogenic antimicrobial resistant bacteria. The employed test method should be user-friendly, affordable and lab and trained-personnel independent. The proposed guidelines have several phases as delineated below and are expected to yield significant information about prevalence of pathogenic antimicrobial resistant bacteria in rivers/water bodies. As the nature of waste water is dictated by local geographical conditions, industrial discharge/s and waste water treatment practices WWTP), it is suggested that concerned stakeholders conduct detailed gap analysis to enable and include related action plans for conducting 'River/water body Hygiene Surveys' for ensuring compliance to Hon'ble NGT order dated 5.04.2019 in OA 426/218.

2.0 Recognition Phase

In this era of increasing incidence of AMR, the recognition and identification of causative factors for rising antibiotic and other API pollution in rivers and other water bodies, across the globe is of paramount importance [8, 22]. In this phase samples from various sites listed below will be tested for presence of bacteria load, different types of pathogenic bacteria present and their antibiotic sensitivity pattern to a pre-decided panel of front line antibiotics:

1. Industrial wastewater discharge sites
2. Inlet-site for STP plant
3. Outlet-site from STP post treatment
4. Several sites along individual river flow path where regular monitoring of water quality is done by CPCB, SPCB and PCC

2.1 Identification of contaminated water bodies

Increased incidence of antimicrobial resistant bacteria when observed during regular monitoring of water bodies (under the NWQM program) will help in identification of "hot-spots" for antibiotic pollution, emergence of AMR and assist in locating the lapses in wastewater discharge from industry and/or inappropriate/incomplete treatment of urban waste that is being discharged into nearby river/s and other surface water bodies.

2.2 Geographical details of the river and contaminated hot spots

It is recommended that complete geographical data be maintained for each tested site. This should include

i) Global Positioning System (GPS) for locations tested
ii) Complete and clear digital map
iii) Presence of waste-water treatment plants in the vicinity of the identified site
iv) Identification of discharge of untreated or inadequately treated biomedical waste and waste from hospitals and treatment facilities
v) Identification of nearby localities adversely affected by contamination

2.3 Hydrological and catchment description of the river and/or water body

This includes total water flow in the river/stream (both monsoon and non-monsoon periods), sources of water-flow in the river/stream, total effluent discharged into the stream (both treated & untreated), categorization of effluents based on the industry (pharmaceutical, chemical, other), and any other relevant information helpful in identifying the source of contamination by antibiotics and other drugs. Details of total sewage directly discharged at a given location (both treated and untreated), total solid-waste generation in the city/locality, data on categorization of solid-waste into categories, e.g. (hazardous, biodegradable, non-biodegradable, etc.), and existing guidelines used to manage/treat both industrial effluent and solid-waste would be helpful in identifying gaps in the existing processes and would be useful in developing a roadmap for an action plan for addressing the risk posed by growing incidence of AMR microbes.

2.4 Maintenance of digital record

All the information collected must be made available publically on the website of the Central Pollution Control Board, the concerned State Pollution Control Board and Pollution Control Committee, and these must be updated periodically as per CPCB norms.

Representative Table format for recording details of samples collected

Sl. No.	Sample collection Site	Coordinates
1		
2		
3		

Not: More rows may be added as per site and state specific requirement

2.5 Sources and implications of contaminated river/water body

For centuries, rivers have supplied water for drinking, irrigation, fisheries, animal husbandry and other anthropogenic activities in their vicinity and immediate surroundings. In recent decades, river waters have got polluted to extreme levels due to uncontrolled discharge of sewage, effluents of chemicals and pharmaceutical industries and disposal of solid and hazardous waste into the river stream. As a case study in sight an estimated, 600 million liters of untreated domestic sewage is discharged into Musi every day [23, 24]. Several global studies have demonstrated that antibiotic-rich effluent from pharmaceutical industries and hospitals exacerbate presence of antibiotic residues in water bodies and promote the propagation of antimicrobial resistant microbes. Solid wastes generated from the city (biomedical waste, e-

waste, plastic and hazardous waste) also contribute to pollution, especially contributing to presence of pathogenic bacteria and heavy metal contaminants in river water.

3.0 Methods

3.1 Site selection and sample collection

Currently, every state board carries out water-quality monitoring under the NWQMP at selected locations in their respective states. It is advisable that SPCBs and PPCs must select all those sites for identification of anti-microbial resistant pathogenic bacterial strains. Water samples from the selected sites should be collected in clean, sterile water-storage containers and stored in pre-cooled ice gels to prevent bacterial growth during transportation. Once the sample reaches the designated laboratories[1], RightBiotic assay as per the manufacturer's instructions can be performed to identify the bacterial load, type and antibiotic sensitivity/resistance pattern.

3.2 Identification of Bacterial load and type

As mentioned above, RightBiotic[2] system with the accompanying kit is sufficient to estimate the bacterial load, bacterial identification and anti-microbial resistance pattern[3] of pathogenic bacterial species present in water. However, it is pertinent to note that RightBiotic may report "Mixed Culture" if more than two bacterial types are present in the water sample.

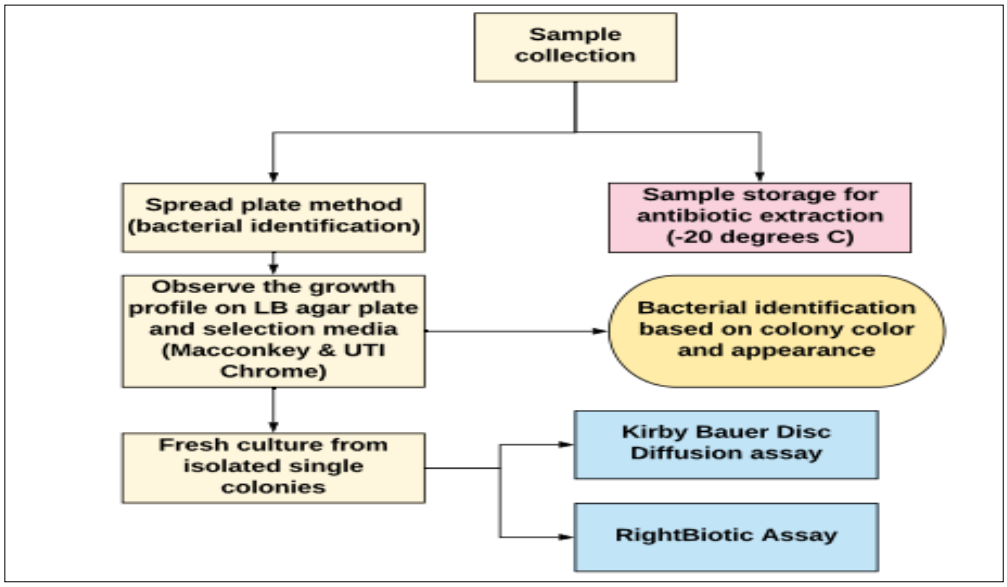

Fig. 1: Illustration of sample collection and steps for sample processing of water samples obtained from different sites

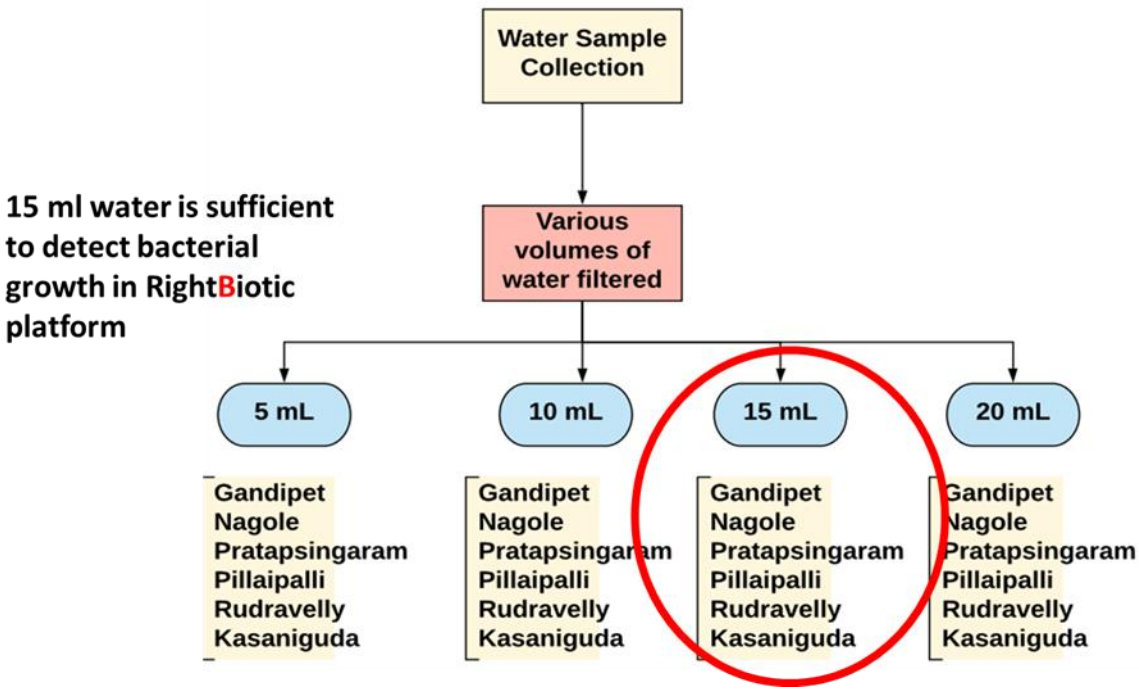

Fig. 2: Procedure used in "Qucik Hygeiene Survey of River Musi" carried out as as per the NGT order (OA No.426/2018)

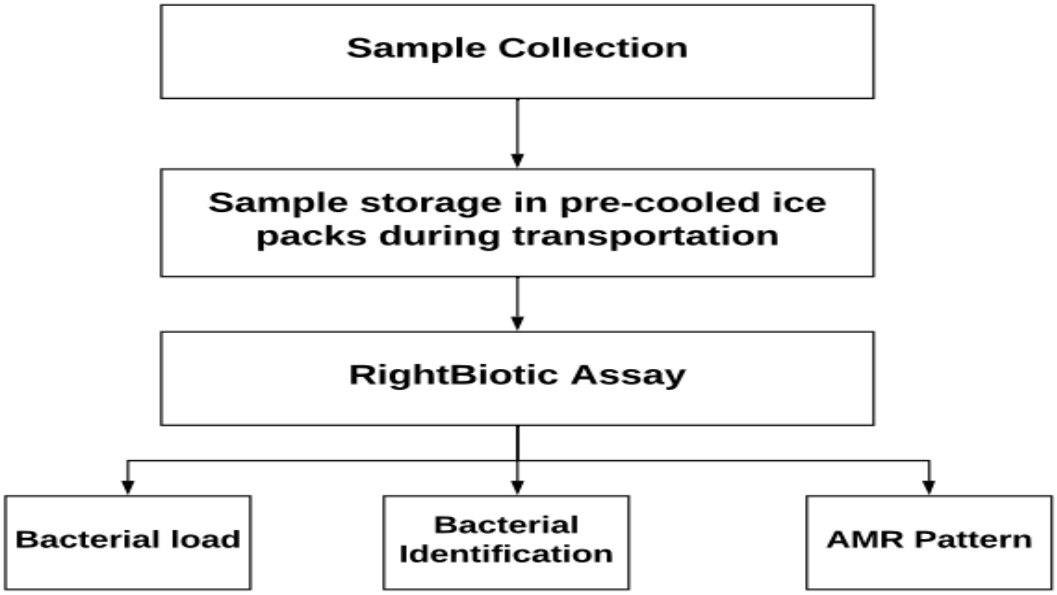

Fig. 3: Steps for sample collection and expected results from the RightBiotic assay

[1]Samples must be stored at 4°C, in case of delay in sample processing.
[2]RightBiotic provides bacterial load, bacterial identification and AMR pattern in 4 hrs.
[3]Antibiotic panels for AMR can be customized in RightBiotic system as per requirements.

4.0 Material required

- RightBiotic System: 01 Nos
- RightBiotic Kits: 01 / water sample
- Incubator: 01 Nos at 37^{o}C
- Sterile sample collection tubes: 01 / water sample
- Sample transporter: 01 Nos with capacity to carry 10-15 samples
- Chiller: 01 Nos

5.0 Significance of conducting River and/or water body hygiene surveys

Bacteria and fungi that cause infections in people and animals are becoming increasingly resistant to antimicrobials. There is a worrying trend that pathogens are developing resistance to antibiotics to a degree where last-resort antibiotics are no longer effective. This, in turn, has severe implications for public health and healthcare costs. With 700,000 people losing battle to AMR per year and another 10 million projected to die from it by 2050, AMR alone is killing more people than cancer and road traffic accidents combined together [6]. Economic projections suggest that by 2050, AMR would decrease Gross Domestic Product (GDP) by 2-3.5% with a fall in livestock by 3-8%, costing USD100 trillion to the world [7]. The global rise of AMR has attracted the attention of WHO. WHO launched a global action plan on AMR in 2015 with 5 strategic objectives, one of which was to strengthen knowledge of spread of AMR through surveillance and research. The One Health concept highlights the inter-relatedness among human and animal health, food and environment and fosters collaboration involving all stakeholders dealing with these spheres [25].

At the G20 in Hangzhou in September 2016, world leaders acknowledged the serious danger to public health, growth and global economic stability posed by AMR, a sentiment echoed at a special United Nations High-Level Meeting dedicated to the topic later on that month. However, despite this heightened sense of urgency, concrete action on tackling drug resistance remains slow and incomplete and many seasoned observers fear that we are doing too little, too late. Evidence is also piling up that global pharmaceutical companies – whose role, should be to cure sick people and channel resources into the development of new medicines, – are actually contributing to the spread of drug-resistant infections through pollution at their own production sites or those of their suppliers. A series of reports, including the present study, throw

light on this, the third major cause of AMR, by revealing how low quality production processes and the dumping of inadequately treated manufacturing waste could be fueling the worldwide spread of AMB and amplifying the already considerable impact of the excessive consumption of antibiotics in human medicine and their prolific use in livestock rearing.

Analysis of water samples in 2016, under the supervision of Dr. Mark Holmes from the University of Cambridge found high levels of drug resistant bacteria at sites in three Indian cities namely: Hyderabad, New Delhi and Chennai and our study confirms that the situation has not changed since then. As drug resistance in India's human and animal population continues to rise, overuse of antibiotics and inappropriate disposal of the same in the environment continue to pollute and either directly or indirectly, have become the "resistance hotspots".

The national pollution index (Comprehensive Environmental Pollution Index, or CEPI), which has been in place since 2009, has been used to determine the environmental status of industrial areas across India, including the Patancheru-Bollaram cluster, which has been classified as "critically polluted". With Governments around the world scrambling to contain the devastating and very costly damage that AMR is already wreaking on public health systems worldwide, urgent action must be taken to address every single man-made source of resistance, whether of human, animal, or industrial origin. If any one of these sources is left unaddressed, we will soon lose the fight against AMB and AMR will continue to thwart all measures to treat infectious diseases. Fig. 9 clearly shows that the growing resistance to antibiotics does not spare any class of pathogenic bacteria, potentially capable of causing untreatable infections.

The World Health Assembly on May 23, 2019 passed a resolution urging member states to strengthen infection prevention and control measures, including i) water sanitation and hygiene; ii) enhance participation in global antimicrobial surveillance system; iii) ensure prudent use of quality-assured antimicrobials; and iv) support multi-sectoral annual self-assessment survey. **Antibiotic resistance is rampant in India with a label of India being 'the AMR capital of the world'** [26]. A study by scientists at BHU, Varanasi showed that a large number of bacteria in River Ganga are resistant to commonly used antibiotics. As per the 'scoping report on AMR in India' (2017), under the aegis of Government of India, among the Gram-negative bacteria, more than 70% isolates of *E. coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* and nearly half of all *Pseudomonas aeruginosa* were resistant to fluoroquinolones and third

generation cephalosporins. Although the resistance to drug combination of piperacillin-tazobactum was still <35% for *E. coli* and *P. aeruginosa*, the presence of multiple resistance genes including carbapenemases made 65% *K. pneumoniae* resistant. Among the Gram-positive organisms, 42.6% of *Staphylococcus aureus* were methicillin-resistant and 10.5% of *Enterococcus faecium* were vancomycin-resistant. Rates of resistance among *Salmonella typhi* and *Shigella* species were 28-82%, respectively, for ciprofloxacin, 0.6-12% for ceftriaxone and 2.3-80% for co-trimoxazole. For *Vibrio cholerae*, resistance rates to tetracycline varied from 17-75% in different parts India [27].

Some of the major health challenges faced by human populations settled in the catchment area of polluted river as reported in published literature [23, 28] are summarized below:

1. Pathogenic infections due to bacteria, viruses and other parasites (e.g. hookworm) are common due to continued usage of contaminated water
2. Skin rashes and allergic reactions are frequently observed in individuals living in the area
3. Large scale fish death and significant loss in biodiversity has been reported in the past due to a very high degree of water pollution
4. Miscarriages in livestock have been reported from several catchment areas of contaminated river
5. Several multi-drug resistance bacterial strains have been isolated from more than one river in India and many other countries.
6. Heavy metal contamination can cause liver and kidney problems. Heavy metal contaminated water also raises the risk for various cancers such as gallbladder, skin, and lungs.
7. Presence of hexavalent chromium in wastewater from industries can cause stomach cancer and higher incidence of lung and gastrointestinal cancer.

Major health consequence of various pathogenic strains have been summarised in Table 1.

Sr. No.	Bacterial species (Frequency)	Adverse Health Consequences
1	*E. coli*	• *E. coli* is a common gram-negative bacterium that colonizes the gut of warm-blooded animals and its presence in water indicates faecal contamination. *E. coli* also enters into water from cattle rearing [29, 30].

		• Various strains of *E. coli* produce toxins and can cause diarrhoea, colitis, and even death in some cases [29]. • Contamination of drinking water with Shigella-toxin producing *E.coli* can lead to waterborne disease outbreaks [30, 31].
2	*Klebsiella* species	• *Klebsiella* is a gram-negative bacterium and member of the family *Enterobacteriaceae*. *K. pneumonia* is one of the common bacterial pathogenic species [32]. • *Klebsiella* species can reach water bodies via faecal contamination or nutrient rich waste water from certain industries such as pulp industry or sugar-cane industries [33]. • *Klebsiella* species are commonly present in the environment. Some of the common sources of *Klebsiella* in nature are soil, water, or vegetation. • *Klebsiella* species are responsible for hospital acquired infections (nosocomial infections) and *Klebsiella* from water may reach to hospital environment [33].
3	*Enterococcus* species	• *Enterococcus* species are an indicator of faecal contamination in water. Other possible sources are agricultural runoffs and sewage [34]. • Infection with *Enterococcus* species can cause nosocomial infections, Urinary Tract Infection (UTI) and infections of wounds [35].
4	*Staphylococcus* Species	• The presence of *Staphylococcus* species in water indicates the presence of organic matter and other organic pollutants [36]. • *Staphylococcus* species can cause wound infections, food spoilage and other chronic health conditions [36].
5	*Pseudomonas* species	• *Pseudomonas* species can survive in both high-nutrient rich environment and low-nutrient rich environments [37]. • *Pseudomonas* can reach water bodies via sewage pollution and can cause pneumonia, UTI, meningitis and gastrointestinal problems. It is also a major cause of septicaemia [37].
6	*Shigella*	• *Shigella* is one of the major causes of water-borne diarrhoea • Its presence in water indicates faecal contamination [33].
7	*Salmonella*	• *Salmonella* infections cause fever, gastroenteritis, vomiting, and nausea [33]. • The pathogenic strains of *Salmonella* can enter into water via faecal discharge and entry from livestock and wild animals.
8	*Vibrio Cholera*	• *Vibrio Cholera* causes cholera [33]. • It enters into water through faecal contamination [33].
9	*Acinetobacter*	• *Acinetobacter* is an occasional pathogenic organism [33]. • It is commonly found in soil and sewage [33].

Emphasis on recognizing and implementing appropriate wastewater treatment strategies

Hospital wastewater can be a source of antimicrobial-resistant microbes. Current regulations for hospital waste disposal were developed before the risk of environmental contamination related to antimicrobial-resistant microbes and antimicrobials were considered. Moreover, industries located in the catchment of any river are treating the effluent in their own Effluent Treatment Plants and sending the treated effluent to a common effluent treatment plants (CETPs) as per their membership. The treated effluent water from CETPs is discharged through pipeline to the nearest STP for further treatment. However, ***it is well established that antibiotic removal efficiencies depend on their chemical properties and the waste-water treatment processes used.*** Ghosh et al [38] reported detectable levels of antibiotics in STP discharge/s, and add that only some antibiotics can be removed in significant proportion by STPs. It can thus be concluded that the elimination of pharmaceuticals in conventional WWTPs is presently considered insufficient, and a number of substances of concern for the aquatic environment have been identified in STP effluent in several countries.

There is no denial that rivers like Musi are one of the most polluted rivers in the country and has been reported to have very high concentration of antibiotics in it's water. The higher antibiotic residues can promote the emergence of drug-tolerant bacterial strains in river water. Musi is now categorized under "Priority-I" list for pollution control [39]. It is important to highlight here that a recent study by IIT-Hyderabad has shown the presence of various fluoroquinolones in river Musi at 18 locations [40]. The study indicates that water treatment at Amberpet CETP is unable to remove antibiotics present in the sewage and other effluents and is one of the major causes for antibiotics detected in Musi downstream of Amberpet CETP. Thus, defining the root source of antimicrobial-resistant bacteria detected in a given wastewater influent is a big knowledge gap in our current understanding even though it is of paramount importance. **Undertaking regular river water hygiene survey, using uniform guidelines and test methods, is the most critical next step to regular water quality monitoring and this will also identify suspected discharge points for adopting appropriate remedial methods to avoid development of Antimicrobial Resistant Bacteria which can transfer antimicrobial resistance genes creating pathogenic superbugs.**

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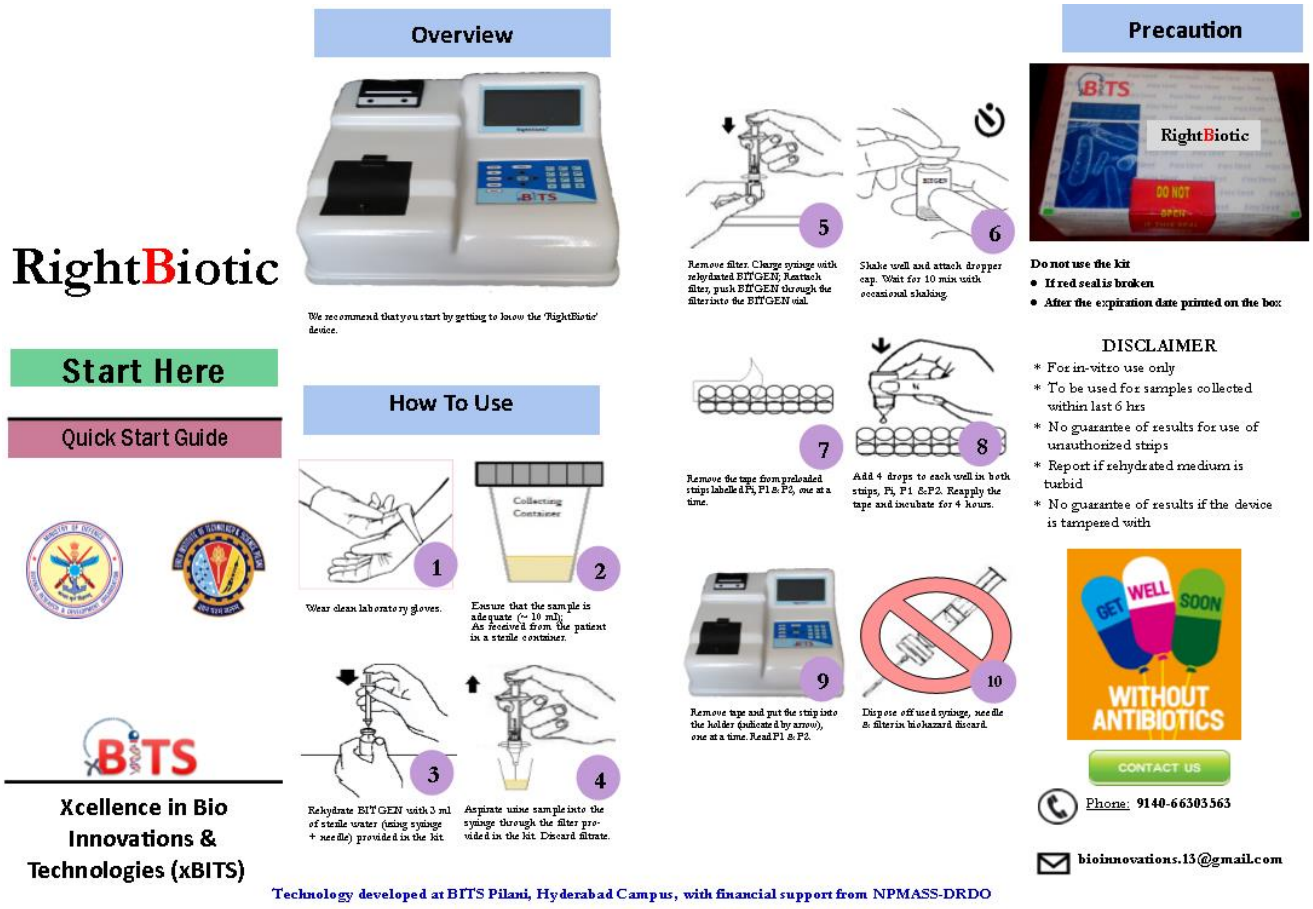


Technology developed at BITS Pilani, Hyderabad Campus, with financial support from NPMASS-DRDO

Pictorial presentation of method for RightBiotic Assay

Rapid Antibiotic Sensitivity Testing System

Kit to be used with the reader

Annexure B

PROCEDURE TO USE THE RIGHTBIOTIC MACHINE

Sl. No	Option 1	Option 2
1	Switch on the machine (switch located at the back of the machine near the power cord lead)	
2	The machine will run a self-test and then wait for the prompt "insert QC strip". Insert the provided QC strip and press enter.	
3	After the QC strip has been read the machine displays (on the screen) "QC Strip validation OK".	
4	Remove the QC strip and press enter	
5	The machine is now on its home screen	
6	Press Test button to enter PID (Patient ID) screen	
7	Press Test button again to add new patient ID and then press enter again	
8	The status will get updated as PID is entered on this ID screen; more new patient ID's can be added by repeating steps 6 & 7.Upto 20 PIDs can be added and if you need to add more you will have to delete some older ones	
9	Select the desired PID by up and down keys on the keypad	
10	Press Enter and the following message will be displayed on the screen for chosing options 1. Run fresh Pi panel 2. Run incubated Pi panel 3. Run final Pi and antibiotic panels	
11	Press 1 to run fresh Pi panel	
12	The platform for loading strip will come out and prompt on screen will ask for blanking; **place the empty strip holder and press "Enter" for blanking.**	
13	After blanking is complete the machine will prompt to "Insert fresh Pi panel"; insert the strip and press enter	
14	After reading the strip the platform will come out, remove the strip and machine displays "Remove panel and incubate Pi panel for 2 hours"	
15	Status gets updated as "Fresh Strip done"	
16	After 2 hours of incubation is over go to ID screen and select the patient ID already entered and status updated as "Fresh strip done". Press Enter and press 2 from keypad to run incubated Pi panel	After 4 hours of incubation is over go to ID screen and select the patient ID already entered and status updated as "Fresh strip done". Press Enter and press 3 from keypad to run final Pi and antibiotic panels.
17	The machine will prompt for blanking again, repeat step 12 After the blanking is complete the platform for loading strip will come out and machine will prompt to "Insert Incubated Pi panel"	The machine will prompt for blanking again, repeat step 12 After the blanking is complete the platform for loading strip will come out and machine will prompt to "Insert Final Pi panel"
18	Insert the Strip and press Enter	Insert the Strip and press Enter
19	The result for 2 hours will be printed as UTI Gram-ve/Gram+ve and the machine will prompt "Incubate panel P1 and P2 OR P3and P4 for 2 hours"	After reading of Final Pi is completed a screen will be displayed showing different antibiotic panels, two panels will be displayed as Yes on the basis of Pi reading.

20	After reading the strip the platform will come out, remove the strip and machine displays either “Incubate panel P1 and P2 for 2 hours” or “Incubate panel P3 and P4 for 2 hours”	Press Enter and the machine will prompt to “ Insert Panel-P3”, insert the selected antibiotic panel on the strip reader platform and press Enter
21	The status will get updated as “Incubated Strip done”	After reading the first antibiotic strip the strip reader platform will come out, machine will prompt to “Insert Panel P4”
22	**After 4 or 5 hours (2+2 or 3+2) of incubation is over go to ID screen again and select the patient ID as selected in previous steps; press Enter and press 3 from keypad**	The strip reader platform will come out remove Panel P1 and insert Panel P2 and press Enter
23	Repeat Step 12	After the reading is completed the strip reader platform will come out again, remove the strip and press Enter
24	The machine prompts “Insert final Pi panel”.	The final result will be displayed on the screen and will also be printed by thermal graphic printer
25	The platform for strip reader comes out, insert the strip and press Enter	Now the ID screen doesn’t have the ID for which final report has been given.
26	After reading of final Pi is completed a screen will be displayed showing different antibiotic panels, two panels will be displayed as yes on the basis of Pi reading.	You can read the next sample now
27	Press Enter and the machine will prompt to “ Insert Panel-P1”, insert the selected antibiotic panel on the strip reader platform and press Enter	
28	After reading the first antibiotic strip the strip reader platform will come out, machine will prompt to “Insert Panel P2”	
29	The strip reader platform will come out remove Panel P1 and insert Panel P2 and press Enter	
30	After the reading is completed the strip reader platform will come out again, remove the strip and press Enter	
31	The final result will be displayed on the screen and will also be printed by thermal graphic printer	
32	Now the ID screen doesn’t have the ID for which final report has been given.	

Adopted from references 41-45

Annexure C

Title	HOW TO PERFORM THE RIGHTBIOTIC ASSAY

Requirements-:

Sr. No	Provided in the kit	Not provided in the kit
1	Syringe	Wastewater Sample (10ml)
2	Water vial	Gloves
3	BITGEN media vial	Data collection sheet
4	Syringe filter	Cello tape
5	8 well strips (3)	Tissue Paper
6	Dropper Cap	Marker

Procedure-:

1.1 Rehydrate media with 3 ml of sterile water with the help of syringe.

1.2 Filter the water sample by aspirating water in the syringe through filter attached; take off the filter and discard the filtrate in the same container.

1.3 Take media in the syringe, then attach the same filter which was used earlier and then filter the media drop wise and shake the vial by swirling it.

1.4 Attach the dropper cap and wait for 10 minutes.

1.5 Remove the tape from each strip.

1.6 Load 3-4 drops of media in each well of all strips (P_1, P_2, P_3, P_4 and P_i).

1.7 Reapply the sealing tape back on the strips and incubate at 37ºC for 4 hours.

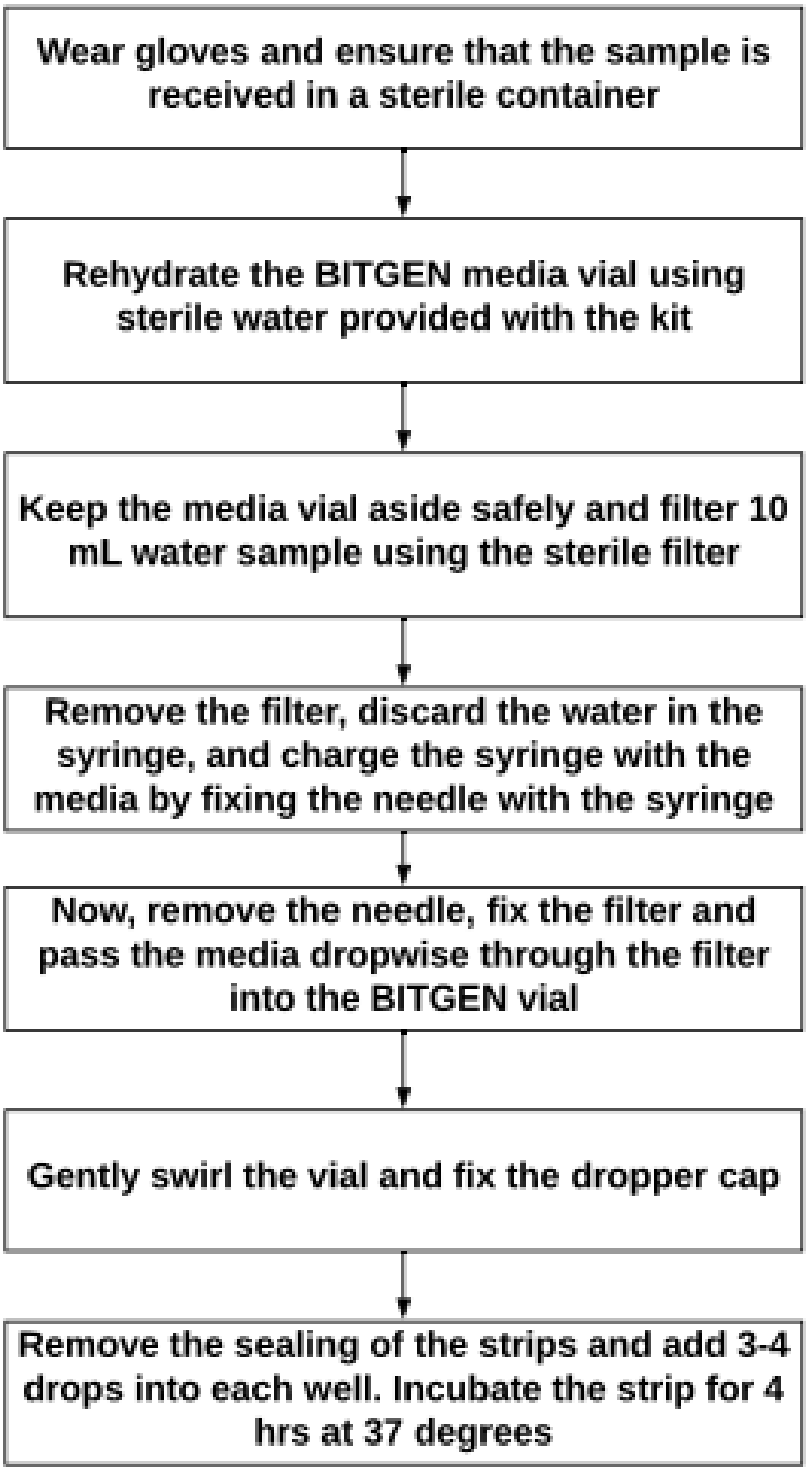
1.8 Switch on the RightBiotic machine, remove the tape and put the strip into the strip holder.

1.9 Get the printout of the results or transfer to a laptop through a USB or to an android phone through a wireless module.

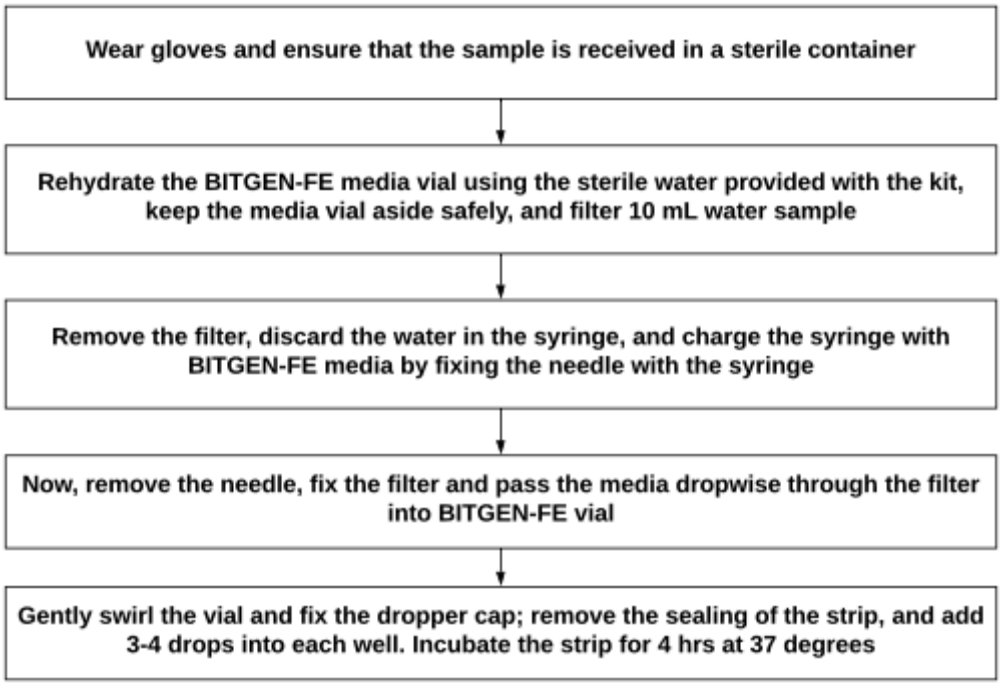
1.10 Record the results as a hard copy also.

Annexure D

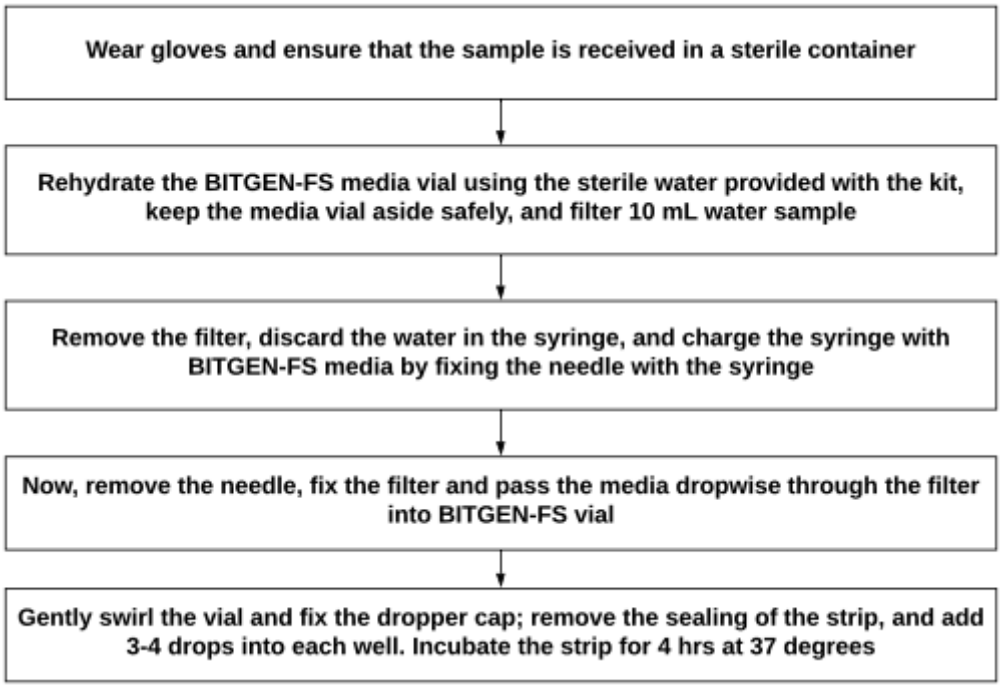
Flow chart for carrying out the RightBiotic assay



Protocol for identification of fecal coliform using RightBiotic



Protocol for identification of fecal *Streptococci* using RightBiotic



Annexure E

Findings and Conclusions from the Quick Hygiene Survey of Musi River

As can be seen from the Figure below below 6 out of 9 sampling sites had one or more bacteria and some had upto four different types of bacteria, all known to cause human diseases. Three sites found to show no presence of bacteria were:

1. Musi at Wadapally (Before confluence)
2. Krishna at Wadapally (Before confluence)
3. Musi + Krishna at Wadapally (After confluence)

These sites were also found to have a pH around 8 to 8.5 and a high concentration of dissolved Ammonia and phosphates probably coming from the increased agricultural runoff, containing both fertilizers and pesticides from the paddy cultivation area near these sites.

The other six sites as per the water monitoring done by TSPCB not only had high content of total dissolved solids but also a high load of total coliform and fecal coliform bacteria. A high bacterial load was also observed on LB agar spread plates. Further, five out of the nine sites, namely, Nagole, Pratapsingaram, Pillaipalli, Rudravelly and Kasaniguda had higher diversity pf pathogenic bacteria and overall load all through the 10-week long study period. Gandipet alone showed a fluctuating bacterial population and on four out of six sampling days no pathogenic bacteria could be isolated from the samples collected from this site.

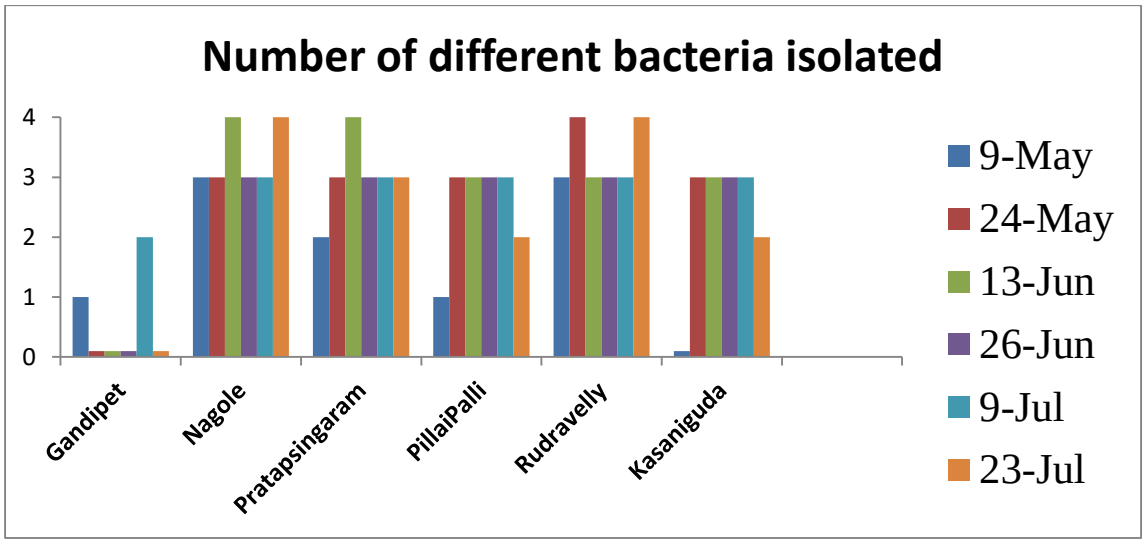


Figure showing number of bacterial genera obtained at various places on different dates

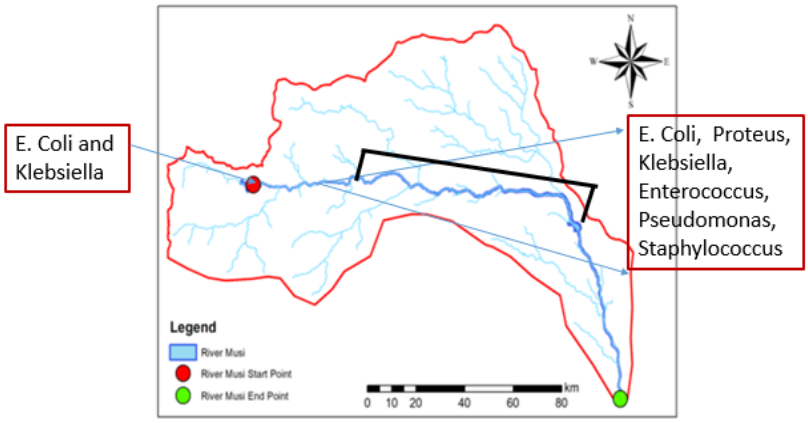
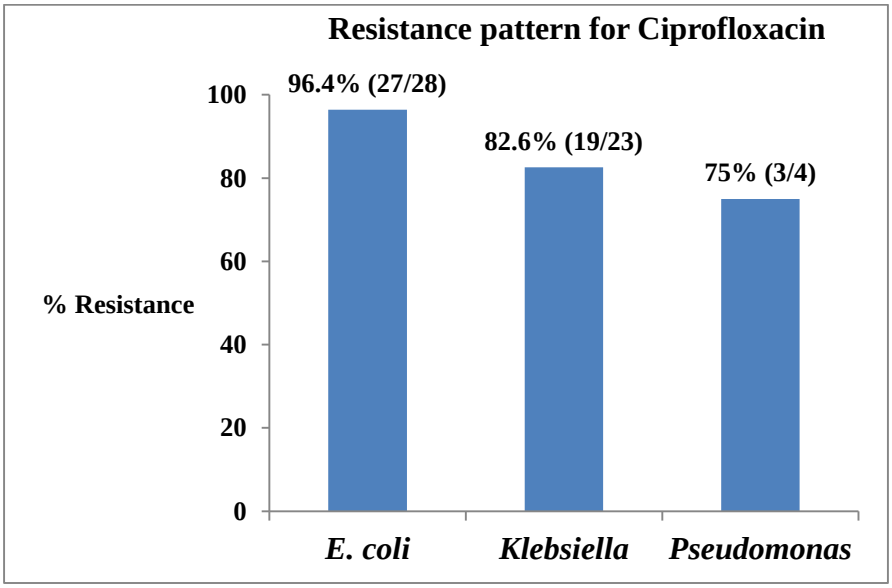


Figure showing types of pathogenic bacteria isolated from upstream & downstream sites on Musi river

It is noteworthy to mention that these sites had frothing waters, discolouration and deposit of dyes or other metal ions on the rocks and other solid structures in the water. In light of earlier reports of high concentration of Ciprofloxacin [5, 43] being reported in the Musi river water resistance to this particular antibiotic was analyzed for all gram negative bacteria isolated during the course of this study (Fig. 7). Out of 56 strains, 49 bacterial strains were resistant to Ciprofloxacin. The AMR pattern in the present study bolsters the assumption that higher antibiotic discharge in water bodies has direct implication in increasing the incidence of ciprofloxacin resistant pathogenic bacteria in Musi river.



Resistance for Ciprofloxacin among gram-ve bacteria isolated during May to July 2019

It is alarming that all pathogenic bacteria isolated from the 54 water samples over the 10-week period were found to be multidrug resistant, showing resistance to more than 4 out of fourteen antibiotics tested and *staphylococcus* and *pseudomonas* strains from multiple sites showed resistance to as many as 13 out of 14 antibiotics, signalling XDR features. From analyses of AMR pattern it is evident that water samples from 6 out of the 9 sites tested had high bacterial load, multiple known bacterial pathogens and all the isolated bacteria showed multi-drug resistance to minimum 4 and maximum 13 antibiotics out of the panel of antibiotics tested (14 for gram negative and 14 for gram positive bacteria). As can be seen from figure given below, the resistance pattern covered all classes of antibiotics including the narrow and broad spectrum antibiotics, and fourth generation Cephalosporins and Fluoroquinolones. The bacterial load present at various sites corroborated with the quality of water as reported by TSPCB, with sites of "class E" water quality having the highest multidrug resistant bacteria.

The antibiotic resistance of the isolated pathogenic bacteria assessed by the conventional Kirby Bauer Disc diffusion assay (KB) and RightBiotic Assay (RB) were compared**. From the results summarised in Table below, it is evident that RB assay can be used for rapid and quick hygienic survey of river and other surface waters.** Based on volume standardization experiments just 15ml water sample was found to be enough for RB assay.

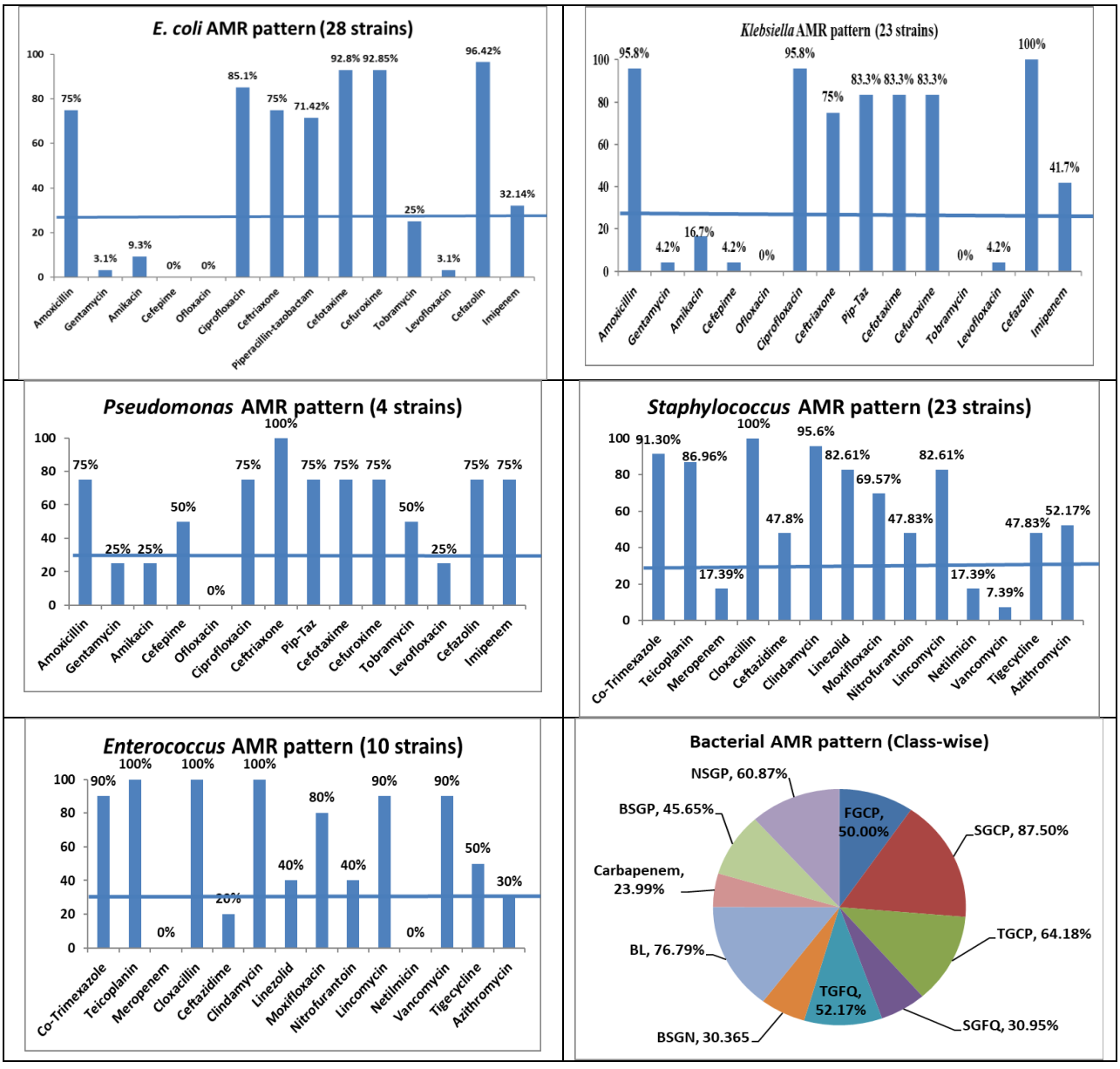
Table showing Sensitivity Data of RightBiotic in comparison with Kirby Bauer Method

Bacteria	**Match**	**Mismatch**
E. coli (n=28)	84.24 (342/406)	15.76 (64/406)
Staphylococcus (n=23)	67.19 (215/322)	27.61 (107/322)
Klebsiella (n=22)	83.06 (255/308)	16.94 (53/308)
Enterococcus (n=11)	72.73 (112/154)	27.27 (42/154)
Pseudomonas (n=4)	100.00 (56/56)	0.00 (0/56)
Total points	**1232**	**%**
Total study match	980	79.6
Total study mis-match	252	20.4

Conclusion

Every year, nearly 1 million people worldwide die from drug resistant infections. With this figure projected to climb to 10 million by mid-century, medical experts now put drug

resistance in the same bracket as the HIV/AIDS crisis, and are calling for a coordinated response from the international community to address the threat. In accordance with earlier reports from within India and other countries, the water samples from river Musi along its track through the city of Hyderabad were found to be of **very poor quality water**, had extremely **high bacterial loads**, and **alarming resistance pattern**s to almost all antibiotics and indicate an imminent public health disaster, waiting to happen.



Further Krishna river at Wadapally was found to be loaded with agricultural run off with a high pH of 8.5 and it is well known that excessive levels of nitrogen and phosphorus make their way into an aquatic ecosystem when they are washed off from fields and lawns by rainwater. This nitrogen and phosphorus work as fertilizers in the water causing an explosion of plant growth, including algae. Algae forms a thick mat on the surface of the water. Light cannot penetrate this mat of algae, and oxygen production is reduced by bottom-dwelling plants. Plants below the algae, as well as the algae, begin to die later in the season. The decomposers that consume these dying plants consume large amounts of oxygen, which is already diminished by the lack of light-inducing photosynthesis. Algal death can lead to an increase in the nutrient content of the water setting the stage for more bacterial species to thrive. In the present study, however, no bacteria were found in waters of Krishna, indicating presence of some toxic chemicals which could have inhibited the growth of bacteria at these sites. It is well established that microalgae from paddy fields produce strong broad spectrum antimicrobials and this can be another reason why no bacterial populations could be cultured from Krishna river water [46].

Need for urgent action plan

At the G20 in Hangzhou in September 2016, world leaders acknowledged the serious danger to public health, growth and global economic stability posed by antimicrobial resistance (AMR), a sentiment echoed at a special United Nations High-Level Meeting dedicated to the topic later on that month. However, despite this heightened sense of urgency, concrete action on tackling drug resistance remains slow and incomplete, and many seasoned observers fear that we are doing too little, too late. Evidence is also piling up that global pharmaceutical companies – whose role, should be to cure sick people and channel resources into the development of new medicines, – are actually contributing to the spread of drug-resistant infections through pollution at their own production sites or those of their suppliers. A series of reports, including the present study, have thrown light on this, the third major cause of AMR, by revealing how dirty production processes and the dumping of inadequately treated antibiotic manufacturing waste, is fuelling the worldwide spread of superbugs, amplifying the already large impact of the excessive consumption of antibiotics in human medicine and their prolific use in livestock rearing.

Analysis of water samples in 2016, under the supervision of Dr Mark Holmes from the University of Cambridge found high levels of drug resistant bacteria at sites in three Indian cities

namely: Hyderabad, New Delhi and Chennai and our study confirms that the situation has not changed since then. As drug resistance in India's human and animal populations continues to rise, the country's antibiotics manufacturing plants continue to pollute their surroundings and either directly or indirectly, have become the "resistance hotspots".

The national pollution index (Comprehensive Environmental Pollution Index, or CEPI), which has been in place since 2009, has been used to determine the environmental status of industrial areas across India, including the Patancheru-Bollaram cluster, which has been classified as "critically polluted". With Governments around the world scrambling to contain the devastating and very costly damage that AMR is already wreaking on public health systems worldwide, urgent action must be taken to address every single man-made source of resistance, whether of human, animal, or industrial origin. If any one of these sources is left unaddressed, we will lose the fight against AMR.

When it comes to tackling antibiotic resistance, addressing pollution from the manufacturing of antibiotics is a low-hanging fruit. There is growing recognition of this: long an ignored cause of AMR, it is now accepted by decision-makers and leading industry players alike that manufacturing discharges must be brought under control as a matter of urgency. **Quick hygiene surveys conducted using the RightBiotic technology platform** would be very helpful in providing real-time data on the nature of bacteria thriving in river water, industrial effluents, domestic waster after treatment, etc and help gather crucial evidence of emerging patterns of antimicrobial resistance in pathogenic bacteria at a very affordable cost—making large scale adoption feasible. Once the source of toxic chemicals including antibiotics in industrial effluent and domestic waste are mapped it will be easier for CPCB to adopt a remedial policy to improve the quality of water bodies and other reservoirs such as lakes etc and control the growing menace of widespread resistance to all antibiotics in use today.

Antibiotics are mainstay drug for treatment of infections in humans and livestock both, and their importance in human health cannot be emphasized enough.

It is a case requiring multi-sector intervention plan by all stakeholders under the leadership of Central and State Pollution Control Boards across the country to ensure the health of our rivers and through them the people of India.