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Multi-drug resistant bacteria from Kolkata Ganga river with heterogeneous MDR genes have four hallmarks of cancer cells but could be controlled by organic phyto-extracts

Asit Kumar Chakraborty

Department of Biotechnology and Biochemistry, Oriental Institute of Science and Technology (OIST), Vidyasagar University, West Bengal, Midnapore-721102, India.

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ABSTRACT

Abundant multi-drug resistant (MDR) bacteria from Kolkata Ganga River and Digha Sea were detected and characterized. Extended spectrum beta-lactamases (ESBL) superbugs (*Escherichia, Phenalkaligenes*, *Pseudomonas, Citrobacter, Stenotrophomonas*) were found everywhere in water. Such MDR-bacteria were resistant to at least four antibiotics and showed four hallmarks of cancer cells: (a) MDR genes in plasmids similar to diversified oncogenes, (b) active mutations producing ESBL and inhibitor resistant similar to GTP-bound Gly->Val mutant Ha-ras oncoprotein, (c) Activation of *bla*CTX-M/TEM-SHV, *acrAB, tetA* and *cmr* genes with high copy number and expression, similar to over expressed retroviral oncogenes and (d) high amount of small plasmid-like DNAs apart from large conjugative plasmids similar to high copies of chromosomes and miRNA in tumour cells. An improved MDR-Cure organic phyto-extracts (*Cassia fistula*, *Suregada multiflora*, *Syzygium aromaticum, Cinnamomum zeynalicum, Aristolochia indica*, etc) cured skin and nail infections; and also cleared superbug infection in rat model. PubMed and GenBank search indicated antimicrobial resistance (AMR) had created an acute problem in modern society worldwide and could be designated as 21st century pseudo antibiotic dark age. However, the author has presented data to support a view that Indian medicinal plants and spices has genuine potential for new drug discovery to control MDR bacteria which have highly contaminated in global water.

Keywords: Kolkata superbugs, Ganga river pollution, drug resistomes, MDR-cure, phyto-antibiotics.

E-mail: chakraakc@gmail.com. Tel: (91) 9339609268.

INTRODUCTION

Multi-drug resistant (MDR) microbial contamination and infections are seriously threatening world population (Amita et al., 2004; Okeke et al., 2005; Mekenna, 2013; Sander et al., 2014; Mataseje et al., 2016). An infection of the author's toe after microsurgery was persistent and at the same time a video was telecasted by STAR-Networks regarding an NDM-1 infection at South Kolkata (Kumarasamy and Kalyanasundaram, 2012) near author's residence. So the author was prompted to look if such MDR bacteria are indeed located in Kolkata water bodies. Such study indicated that South Kolkata streets after rain were contaminated with drug resistant bacteria and it appeared that rain water was also a good source of MDR-bacteria (Chakraborty, 2015). Traditional medicines were active in India before the discovery of antibiotics in late 1920s and beyond as were described in Sanakrit books like Charaka Samhita, Sashruta Samhita and Veda (Sastri, 1956). Major roles of Indian medicinal plants and spices in medicine were described in Chiranjib Bonoushadhi Bengali book (vol-I, ISBN 81-7066-606) and

the author made various organic extracts to cure nail infections which highly could be seen in Kolkata during rainy season. However, plant extracts became inactive on storage at room temperature for six months and more stable active principles for drug formulation became necessary. Indian spices had long history for antibacterial activities and search indicated that darchini and labanga stored at room temperature had antibacterial activities and thus a change of composition in MDR-Cure was necessary as described later. Pubmed and GenBank search however indicated that the situation of MDR calamity had reached every home of Earth and pharmaceutical companies were reluctant to invest in new drug discovery against MDR-bacteria (Chakraborty, 2016c; Okeke et al., 2005).

It was proved that thousands of MDR genes like diverged beta-lactamases (*bla*CTX-M, *bla*NDM1, *bla*KPC, *bla*OXA), drug modifying (*aac*, *aad, aph, arr*) and drug efflux (*acr, mex, env, tet, mcr)* genes were accumulated in conjugative plasmids of MDR-bacteria and also moved to chromosome to increase mdr genes dose (Paul et al., 2015; Laxminarayana, 2014; Sun et al., 2014; Zgurskaya et al., 2015; Chakraborty, 2016c). The situation is so daunting; hundreds of PCR reactions should be performed for single patient to know the antimicrobial resistance (AMR) status before starting combination therapy which is costly and also some time toxic. Further, prolong antibiotics therapy usually generates drug resistance in bacteria in the patient and repeated dose of many antibiotics should be avoided. Hopefully, third generation cephalosporins (cefotaxime, ceftrioxane) and carbapenems (meropenem, imipenem) resistant bacterial contamination were still very low (>5% and >2% respectively) in the environment. Still MRSA, NDM1, KPC2 types infections are hard to treat in India (Diestra et al., 2008). We described here molecular characterization of MDR bacterial species and their inhibition by modified MDR-cure, a phyto-extracts from combination of *Cassia fistula*, *Suregada multiflora*, *Syzygium aromaticum, Cinnamomum zeynalicum* etc (Sahoo et al., 2011; Nascimento et al., 2000).

MATERIALS AND METHODS

Water collection and drug sensitivity assay

Water from Ganga River was collected at 7 am to 11 am on Monday from Babu Ghat and Howrah Station area. About 100 µl of water was spread onto 1.5% Luria Bartoni-agar plate containing different antibiotics at 5 to 50 µg/ml. Further a Beta-lactam cocktail (50 µg/ml ampicillin, 25 µg/ml Feropenem, 10 µg/ml Cefotaxime and 5 µg/ml Imipenem) was used to screen *bla* genes activated superbugs. MDR bacteria were selected in agar-plate containing ampicillin+ streptomycin+ chloramphenicol+ tetracycline or ciprofloxacin at 50, 50, 34 and 20 µg/ml respectively (Chakraborty, 2015). As imipenem resistant bacteria were present low (0.2 cfu/ml water) and a modified method was followed. 2 ml 5X LB medis was added into 10 ml Ganga River water and imipenem was added at fc 2 µg/ml and was incubated 24 h to get imipenem resistant bacterial population. Antibiotics were purchased from HiMedia and stored at 20 to 50 mg/ml at -20°C. Antibiotic papers were also purchshed from HiMedia according to CLSI standard. Antibiotic papers are: Met-10 µg (methicillin), CAZ-30 µg (ceftazidime), AT-50 (aztreonam), COT-25 µg (Cotrimoxazole), LOM-15 µg (lomofloxacin), VA-10 µg (vancomycin), AK-10 µg (amakacin), LZ-10 µg (linezolid), TGC-15 µg (tigecycline) and IMP-10 µg (imipenem). The antibiotic solutions were made as follows: ampicillin 50 mg/ml in water, tetracycline 20 mg/ml in ethanol, chloramphenicol 34 mg/ml in ethanol, ciprofloxacin 50 mg/ml in water, cefotxime 25 mg/ml in water, and streptomycin 50 mg/ml in water (Clinical and Laboratory Institute, 2015).

Electron microscopy procedure

Bacteria was grown in filtered Luria-Burtini media (10 g NaCl + 10 g bacto-tryptone + 5 g peptone/L water pH 7.0) for overnight at 37°C in shaker-incubator or 72 h in case of bacterial spore. EM was performed at the Main Instrument Facility of Indian Institute of Chemical Biology, Kolkata, India. The 10 µl of bacterial suspension at concentration 10^6 cfu/ml was loaded onto carbon coated copper grid and stained for 30 s with 1% phosphotungstic acid in water. The grid was dried for 10 min at room temperature under light illumination and photograph was taken in a TECNA1 Transmission Electron Microscope (FE1 Inc. Netherland) at 6000 to 8000x.

Isolation of bacterial genomic DNA

Genomic DNA was isolated by Proteinase-K-SDS method following extraction with phenol-$CHCl_3$-isoamyl alcohol (25:24:1) and ethanol precipitation (Maniatis et al., 1982). 1.5 ml over night culture was spun at 5000 rpm and the bacterial pellet was dissolved in 50 µl TE buffer (10 mM TrisHCl PH 8.0 + 1 mM EDTA) and 25 µl 10% SDS and 5 µl. of 20 mg/ml proteinase-K were added and was incubated for 2 to 4 h until clear solution occurred. Then added 25 µl 5 M sodium chloride (NaCl) and 100 µl $CHCl_3$: isoamyl alcohol (25:1) and was shaked slowly by up and down. Then centrifuged at 8000 rpm for 10 min and 99% ethanol was added into upper aqueous solution. Then centrifuged at 10000 rpm for 10 min and the DNA pellet was dissolved in 50 µl TE buffer. 1 µl RNase A was added and incubated for 15 min at 37°C and extracted with phenol-$CHCl_3$-isomyl alconol (25:24:1) and was precipitated with 1/9 vol of 3 M sodium acetate pH 5.2 and 2 volumes of 99% ethanol. The DNA pellet was finally dissolved in 50 µl TE buffer.

Isolation of bacterial plasmid DNA

The plasmid DNA was isolated from overnight culture using Alkaline-Lysis method. Simply, to bacterial pellet 100 solution I was added and vortexed. Then 200 µl of cold solution II added to make transparent solution and then 150 µl cold of solution III was added and mixed well. After 10 min, the solution containing huge white coloured precipitate of chromosomal DNA-cell debries wee removed by centrifugation at 10000 rpm for 10 min. To clear solution then added 1 ml 99% ethanol and centrifuged at 10000 rpm for 10 min at 4°C. Plasmid DNAs from four such preparation were combined and the tRNAs were removed by Rnase A treatment as above and finally plasmid DNA was dissolved in 50 µl TE buffer and was stored at -20°C. 0.8% agarose gel lecetrophoresis in 1x TAE buffer at 50 V for 4 to 6 h was performed to see the plasmid DNAs after staining in 0.5 µg/ml ethidium

Table 1. Primers used in this study.

Name	Sequence of the primers	Temp (°C)	Size
P27F	5'-AGA GTT TGA TCC GAA CGC T-3'	62	1.4 kb
P1392R	5'-TAC GGC TAC CTT GTT ACG ACT TCA-3'	65	
cmrF	5'-TTC GTT AGT CTG CCG TTG CT-3'	56	323 bp
cmrR	5'-ATC GCT GGC AAA CAG GGT TA-3'	57	
blaVIM-F	5'-CAG ATT GCC GAT GGT GTT TGG-3'	57	519 bp
blaVIM-R	5'-AGG TGG GCC ATT CAG CCA GA-3'	61	
tetF	5'-CTT CGC TAC TTG GAG CCA CT-3'	57	910 bp
tetR	5'-GCA GAC AAG GTA TAG GGC GG-3'	57	
acrAB-F	5'-ATG CTC TCA GGC AGC TTA GCC-3'	59	.1 kb
acrAB-R	5'-TGT CAC CAG CCA CTT ATC GCC-3'	59	

Source: Chakraborty (2015).

bromide and UV illumination (Chakraborty, 2015; Doumith et al., 2012).

PCR and DNA sequencing

16S rDNA gene colour Sanger's di-deoxy sequencing was performed by SciGenom Limited, Kerala, India). PCR amplification was performed using 1 unit Taq DNA polymerase, 0.25 mM dXTPs, 1.5 mM $MgCl_2$, 20 ng DNA template for 35 cycles at 95°C/30" (denaturation)-52°C/50" (annealing)-72°C/1.5' (synthesis). The product ran on a 1% agarose gel in 1X TAE buffer at 50 V for 2 to 4 h and visualized under UV light and photograph was taken. The primers for 16S rRNA amplification and mdr genes are given in Table 1. NCBI BLAST analysis was performed for bacterial specific gene analysis (www.ncbi.nlm.nih.gov/blast) and data was submitted to GenBank (Chakraborty et al., 1993).

Preparation of organic phyto-extract (MDR-cure)

The barks of *Suregada multiflora* (Ban-Naranga) and *Cassia fistula* were collected on July 2016 from medium sized tree at Midnapore district of West Bengal. *Syzygium aromaticum* - flowerbuds (labanga spice) and *Cinnamomum zeynalium*-Bark (darchini) were purchased from grocery stores at Kolkata and Midnapore, respectively. *Chenopodium album*-whole shrub was collected from South Kolkata bazaar on January 2017. Each 10 g dried and grinded plant and spice parts (*Suregada multiflora*, *Cassia fistula*, *Syzygium aromaticum*, *Chenopodium album*, *Cinnamomum zeynalicum, Aristilochia indica*) was suspended in 40 ml ethanol-ethylacetate and overnight extracts were mixed and concentrated 5 to 10 times (MDR-cure) and 50 µl used for Kirby-Bauer agar hole assay (Sastri, 1956; Dubey et al., 2005; Duglia, 2011). The crude plant extract was purified by aqueous differential precipitation by adding equal volume of water to increase purity. Further, thin layer chromatography (TLC) was performed using methanol, acetone and acetic acid as mobile phase for 2 to 4 h. Organic molecules were seen and recovered by UV shadowing and was eluted in ethanol from silica-gel. Mass spectrometry (Mass), NMR (Nuclear Magnetic Resonance spectrometry) and FTIR (Furier Transformed Infra Red spectrometry) are in progress to characterize active chemicals (Chaudhri, 1996; Cowan, 1999; Singh et al., 2013; Korcan et al., 2013; Daglia, 2011; Chakraborty, 2015).

RESULTS

Contamination of superbugs in the environment of Kolkata

The water from Ganga River, Kolkata streets and famous railway stations after rain in monsoon seasons (June to July) gave very distinct drug resistant colonies in presence of 50 µg/ml of ampicillin and amoxicillin with 3500 to 5500 cfu/ml of water (Figure 1). The cfu/ml of water was reduced to five fold in case of tetracycline and azithromycin at 20 and 50µ/ml, respectively, whereas reduced to 49 fold with 34 µg/ml chloramphenicol and 50 µg/ml streptomycin. In presence of beta-lactamase inhibitors cavulinic acid and sulbactam, cfu/ml further reduced to ~50 cfu/ml of water. Similarly inclusion of three antibiotics tetracycline, ampicillin, chloramphenicol, streptomycin in combination produces only 10 to 20 cfu/ml water. Further, imipenem resistant species were found rare with only 0.2 to 0.3 cfu/ml of Ganga River water. The results indicated that everywhere had MDR-bacteria and 30 to 40% was ampicillin and amoxycillin resistant as well as <1 to 2% were superbugs (MDR but % XDR was low and no PDR was detected). As for example, imipenem resistant bacterial species were present extremely low or ~0.003%. According to law, MDR bacteria must be resistant to at least three different groups of antibiotics. So the percentage of MDR-bacteria like resistant to three drugs, ampicillin, streptomycin and

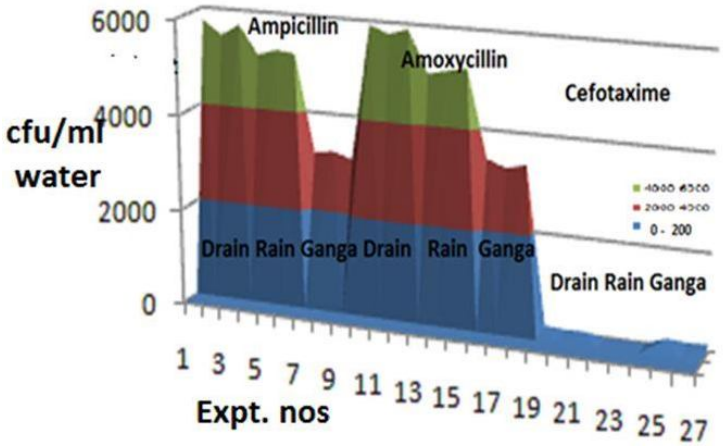

Figure 1. Contamination of MDR bacteria in Kolkata. 100 µl water from Ganga river, rain or Kolkata drain were spread onto LB+1.5% agar in presence or absence of antibiotics (ampicillin, amoxicillin or cefotaxime) and incubated at 37°C for 24 h and naked eye visible colonies were counted (Chakraborty, 2015).

tetracycline was also as low as only 0.2%. Then we have tested the pure rain water (collected on 4th floor roof keeping a 50 ml plastic tube inside a 500 ml beaker) and it has also very similar numbers of bacteria indicating as the major source of bacterial contamination on streets, ponds and sea (Figure 1). This means if any superbug escaped from clinics to environment by physical calamity like storm, tide, flood or earth quake and then bacterial spore could spread anywhere by wind and would fall during rain affecting mass populations. Old city like Kolkata has damage sewage system and floods everywhere of the city during monsoon causing nail or skin infections (Chakraborty, 2015). The experiment indicated that rain water contains a mixture of bacterial populations having different genotypes and drug sensitivities. We have isolated few strains of gram-negative superbugs (KA1, KR1, DG1, KC1, KT1, and KG1 that are resistant to at least three different groups of drug (e.g. ampicillin, streptomycin, cefotaxime, azithromycin, ciprofloxacin, tetracycline or chloramphenicol). The nomenclature is given as follows: “K” means Kolkata origin, KA1 means ampicillin selected 1st then mdr-selected; KR1 means rain source and mdr-selected, KC1 means 1st chloramphenicol selected then mdr selected; KT1 means 1st tetracycline selected then mdr-selected, KG1 means from Ganga river water. Mdr-selection usually mean LB-agar plate contains a mixture of four antibiotics at 50 to 100 µg/ml concentration (ampicillin, streptomycin, ciprofloxacin, azithromycin) (Chakraborty, 2015). The bacterial counts in water sources from open drain, rain water and Ganga River at Kolkata were compared and surprisingly high incidence (~40%) of penem drug resistant bacteria were found everywhere. There were low incidence of 3rd generation cephalosphorin resistant bacteria in Ganga and drain water but absent in rain water (Figure 1). This suggests 3rd generation cephalosporins still could be used in Kolkata for common bacterial infection. Most of the bacteria were *Escherichia* rod and flagellates as demonstrated by electron microscopy and also could form circular spores (Figure 2). Surprisingly, KG-1, KT-1 or KC-1 strains are resistant to 12 Hi-Media antibiotic strips according to CLSI standard (Chakraborty, 2015; Hombach et al., 2012; Wright, 2010) and also highly resistant to vancomycin, co-trimoxazole and methicillin (Figure 3).

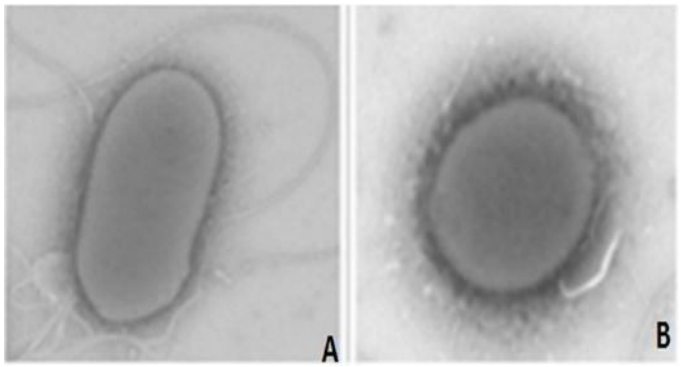

Figure 2. Electron microscopy of MDR Escherichia coli KR-1. Overnight culture was grown in LB media. For spore formation such ON culture was kept at 4°C for few days. For TEM, 10 µl bacterial suspension (10^6 cfu/ml) was spread onto a carbon coated copper grid (GSCu400C, ProSci Tech) and was stained for 30 sec with 1% phosphotungstic acid in water (pH 7.0). The grid was dried for 10 min at RT under light illumination and photograph was taken in a TECNA1 Transmission Electron Microscope (EEI Inc. Netherland) at 6000x (Chakraborty, 2015).

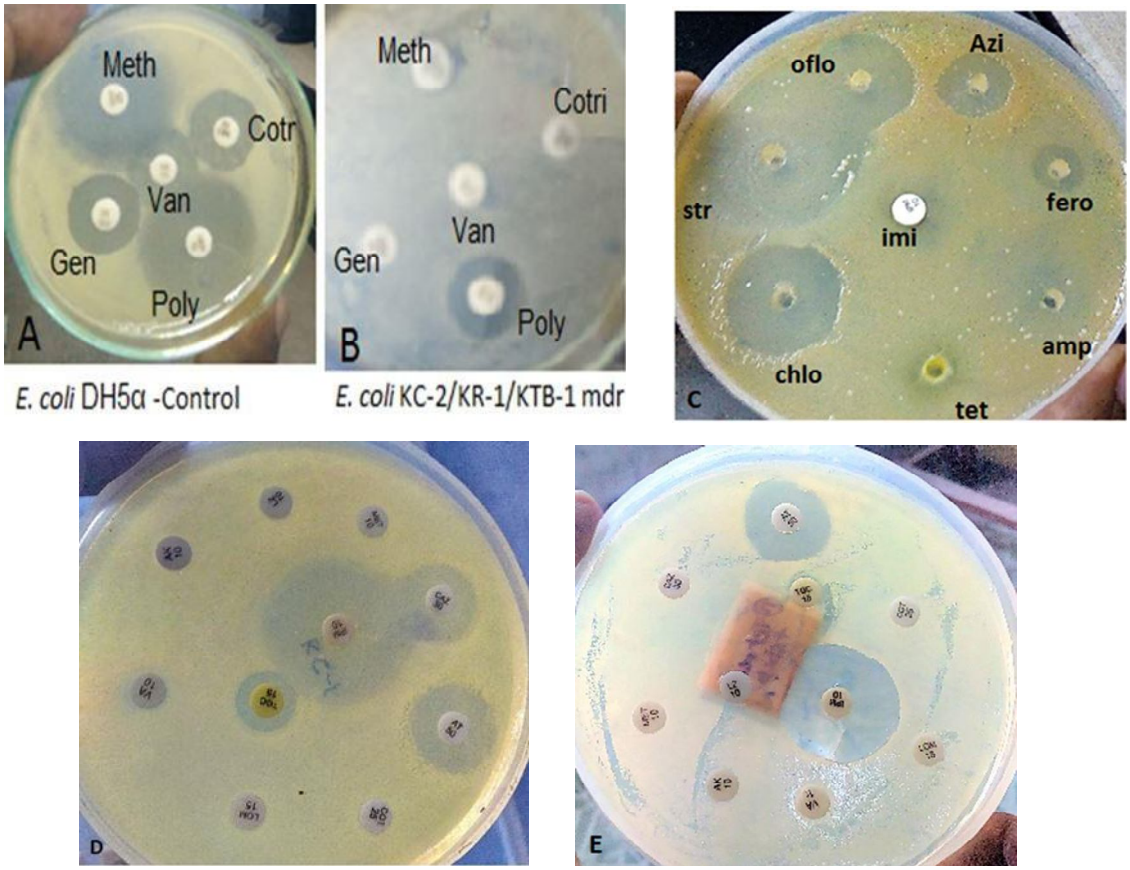

Figure 3. Antibiotic paper disc (HiMedia) assay according to CLSA guidelines. *E. coli* DH5α bacteria was used as control (panel A) and MDR *Escherichia coli* different strains of KR1, KC-2, and KTB-1 all appeared resistant to methicillin, gentamycin, vancomycin and co-trimethaxole but sensitive to polymyxin-B. All isolates were resistant to ampicillin, amoxicillin, cefotaxime, tetracycline, streptomycin and neomycin but few sensitive to ciprofloxacin and chloramphenicol (Chakraborty, 2015). A GI-12 imipenem resistant isolate was markedly sensitive to tetracycline, chloramphenicol, streptomycin (panel C) but other GI-8 imipenem resistant isolate was resistant to methicillin, linezolid and vancomycin but sensitive to amikacin and tigecycline (data not shown). *Citrobacter* sp. KC-1 was very resistant to lomofloxacin, vancomycin, amikacin, and linezolid but sensitive to tigecycline, aztreonam and imipenem (panel D). Similarly, newly isolate KG-12 was resistant to tetracycline, chloramphenicol, methicillin, lomofloxacin, amikacin, vancomycin, linezolid, and tigecycline but sensitive to aztreonam and imipenem (panel E).

Determination of genus by 16S rRNA gene sequencing

We have isolated genomic DNA from pure culture and have performed 16S rRNA gene sequencing for Genus and species determination by NCBI BLAST analysis. In Figure 4 representative area of chromatogram of colour di-deoxy sequencing were presented which detected MDR bacteria as *Escherichia coli, Pseudomonas aeruginosa, Citrobacter* sp., *Enterococcus* sp., *Paenalkaligenes* sp. and *Stenotrophomonas* sp. Interestingly, such analysis detected a 6bp deletion in *Paenalkalines* sp rRNA gene at nt 1131 position as compared to *Escherichia* and *Pseudomonas* which had 6bp deletion at position nt 89 (not seen here). The sequences were deposited to GenBank with accessions nos. KY769875 to KY769883.

Determination of antibiogram of MDR bacteria

Antibiogram was prepared for few selective bacterial clones as described in Table 2. The *Escherichia* species were very much resistant to penicillins as well as vancomycin, and gentamycin but appeared sensitive to polymyxin-B. Most bacteria are resistant to chloramphenicol, tetracycline, streptomycin and cotrimethazole. Imipenem resistant species were present suggesting NDM-1 and KPC-2 were also spreading in Kolkata as described previously (Paul et al., 2013). An *E.*

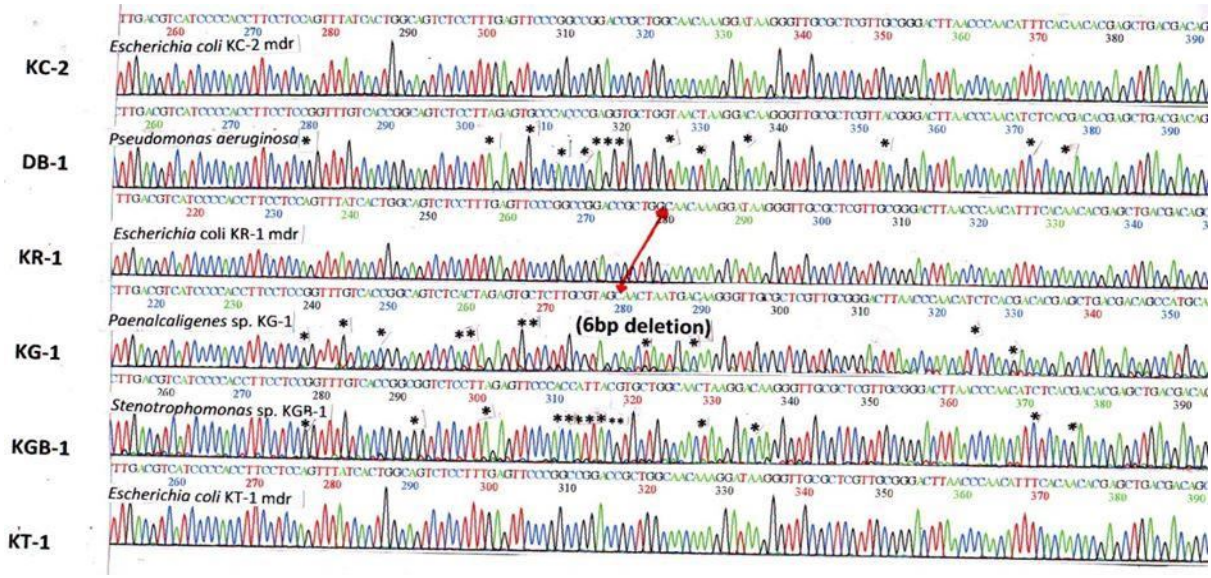

Figure 4. 16S rRNA gene profiling of isolated MDR-bacteria. Genomic DNAs were isolated from 1.5 ml overnight culture by SDS-Proteinase-K method following phenol-$CHCl_3$-isoamyl alcohol extraction and RNase treatment. 20ng DNA was amplified using universal primers and then di-deoxy colour sequencing was performed by SciGenome (Kerala, India). Reverse primer mediated sequencing pattern (~140bp) was shown here comprising 1197-1057 (reverse strand) rRNA gene of *E. coli* (accession no, AE014075, region nt. 235000-237000). BLAST analysis determined MDR bacteria as *Escherichia coli* KC-2/KT-1/KR-1 mdr, *Pseudomonas aeruginosa*-DB-1 mdr, *Paenalkaligenes* sp KG1 mdr and *Stenotrophomonas* sp KGB-1 mdr (accession nos. KU769875-KU769883).

Table 2. Antibiogram of the isolated and characterized MDR-bacteria from Kolkata.

Bacteria / Source	Accession no	Major drug resistance patterns
Escherichia coli KC-1 mdr (Ganga river)	KU898253	Methicillin, Tetracycline, Streptomycin, Ciprofloxacin, Cotromoxazole, Lomofloxacin, Vancomycin, Amikacin, Linezolid,
Escherichia coli KR-1_mdr (Kolkata rain)	KY769883	Methicillin, Cefotaxime, Aztreonam, Azithromycin , Chloramphenicol, Tetracycline, Vancomycin, Amikacin, Streptomycin, Cotrimoxazole,
Pseudomonas aeguginosa DB-1 mdr (Kolkata street)	KY769875	Metthicillin, Cefotaxime, Ceftrioxane, Azithromycin, Chloramphenicol, Vancomycin,
Escherichia coli KC-2_mdr (Kolkata street)	KY769878	Methicillin, Tetracycline, Amikacin, Vancomycin, Cotrimoxazole, Streptomycin, Azithromycin
Escherichia coli KT-1 _mdr (Kolkata street)	KY769881	Metthicillin, Cefotaxime, Tetracycline, Ciprofloxacin, Chloramphenicol, Azithromycin, Gentamycin, Vancomycin, Amikacin, Linezolid
Escherichia coli KT-2_mdr (Ganga river)	KY769882	Metthicillin, Vancomycin, Lomofloxacin, Cotrimoxazole, Azithromycin, Amikacin
Phenalkaligenes sp. KG-1_ mdr (Ganga river)	KY769879	Neomycin, Gentamycin, Azithromycin, Ciprofloxacin, Polymyxin, Vancomycin,
Stenotrophomonas sp. KGB-1_mdr (Ganga river)	KY769880	Methicillin, Azithromycin, Tetracycline, Streptomycin, Cotrimoxazole, Vancomycin,
Escherichia coli KTB-1_mdr (Kolkata street)	KY769877	Methicillin, Tetracycline, Cotrimoxazole, neomycin, Streptomycin, Azithromycin
Pseudomonas aeruginosa DG-2_mdr (Digha sea)	KY769876	Cefotaxime, Ciprofloxacin, Tetracycline, Streptomycin, Cotrimoxazole, neomycin

In vitro assay were performed (in LB media) to check the ampicillin, tetracycline, chloramphenicol, streptomycin, ciprofloxacin and azithromycin drug sensitivity. HiMedia CSLM antibiotic paper disc also used to determine the drug resistance as demonstrated in Figure 3. All MDR bacteria are ampicillin and amoxicillin resistant but imipenem sensitive.

coli ET-1 clinical MDR bacteria (isolated at Bankura Medical College, West Bengal) was however sensitive to streptomycin and vancomycin contrary to environmental *Escherichia* species. *Stenotrophomonas* sp. KGB-1 was very sensitive to vancomycin, polymyxin-B and co-trimethazole but resistant to ampicillin, feropenem, tetracycline, ciprofloxacin and azithromycin (Sandez et al., 2014). KG-8, KC-2 and KG-12 were extremely drug resistant to tlomofloxacin, amakacin, linezolid and tigecycline drugs those were described in many superbugs pathogenesis. This implied a real superbug contamination in holy Ganga River water that was consumed raw in religious occasions in India.

PCR analysis of MDR genes in isolated MDR-bacteria

We have started to detect the specific MDR genes in isolated MDR bacteria guided from antibiogram data. *Escherichia* species were found activated *bla*CTX-M/NDM-1/TEM, *acr*AB, *mcr*, *tet* genes (Figure 5). The data appeared simple and commensurate with other published data but high expression of drug efflux genes like acrAB and tetA/C (Figure 5, lanes 4 and 5) in Kolkata MDR strains were not reported previously that supported by view that no antibiotics would work as all antibiotics will be kick out from bacterial cell cytoplasm keeping bacteria alive even high dose of antibiotic taken by patients (Wright, 2010). We have very recently detected the presence of NDM-1 beta-lactamase gene in Kolkata superbugs which further suggested that penicillin, cephalosporins, carbapenem, cavulinic acid, sulbactam would not work to cure such infection (Figure 3, panel C).

Mechanism of MDR genes activation and creation

The published data suggested (Table 3) that very large plasmids were created due to combination of conjugative plasmids with 10 to 15 *mdr* genes and many *Tpn* genes. Question arises why such big plasmids created and how many of plasmids were present in MDR bacteria? When isolated plasmid DNA was digested with different restriction endonuclease (EcoRI, PstI) as shown in Figure 6, panel A, it gave a smear indicating heterogeneous plasmids present. When plasmid DNA and genomic DNA ran palse field gel electrophoresis a huge small plasmid-like DNA were seen and genomic DNA contaminated with large plasmids (Figure 6, panel B). Further, we detected large plasmids in standard 0.8% agarose gel when ran longer at 25 V for 8 h (mini gel) from MDR bacteria isolated from Ganga River and Digha Sea (Figure 6, panel C). All these data indicated that huge transposon-tagged DNA were present in MDR bacteria to create new genes and it indeed very hard to separate fragmented genomic DNA and large plasmid DNA by conventional

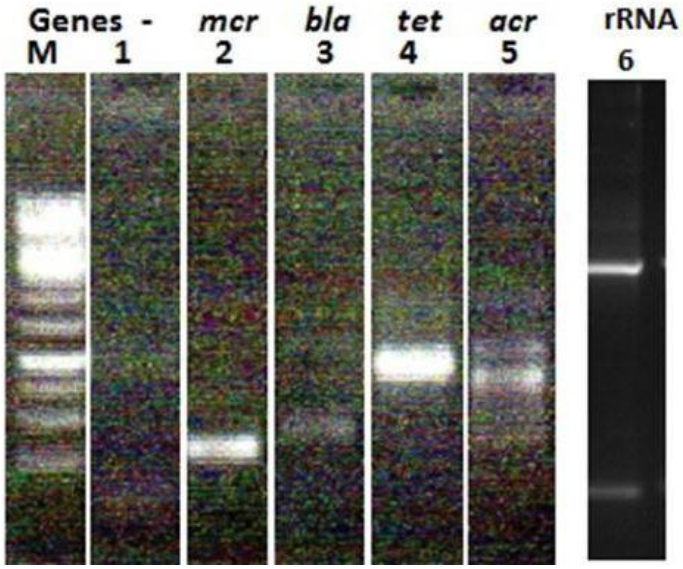

Figure 5. PCR analysis of MDR genes in E. coli KT-1 mdr. To determine the presence of MDR genes in high molecular weight plasmids, we isolated genomic and plasmid DNA and performed PCR reactions using gene specific probes. *bla*VIM gene is beta-lactamase gene, *mcr* gene is proton drug efflux (Mdt), *tet* is tetracycline drug efflux and *acr*AB is tripertriate proton drug efflux protein, all highly activated in MDR bacteria in Kolkata. *bla*CTX-M is highly activated and blaNDM1 is moderately activated in Kolkata superbug (in progress). Multi-copies 16S rRNA gene primers were used as control. Plasmid DNA gave poor result except *mcr* gene indicating genomic DNA may contaminated with large plasmids but chromosomal localization of such genes could happen.

molecular biology methods, So large amount of DNA amplification or fragmentation was a prerequisite change in creating MDR bacteria (Chakraborty, 2015), Further, all MDR plasmids did accumulated transposase, integrase and recombinase genes supporting such view (Table 3).

Search for anti-bacterial extract from Indian medicinal plants

50 µl of different plant extract was loaded onto 5 mm hole of LB-agar plate spread with 150 µl over night culture of *Escherichia coli* DH5α and incubated for overnight at 37°C to see zone formation as indicator of anti-bacterial activity. We tested 100 different plant species like *Terminalis arjuna*-bark, *Mangifera indica*-bark, *Azadirachita indica*-bark, *Sesbania sesban*-whole plant, *Clerodendrum infortunatum*- leaves, *Marsilea quadrifolia*-leaves, *Amarphophallus campanulatus*- leaves, *Cordia diotoma*- stem *Curcuma longa*- rizome, and *Suragada multiflora*- Bark, *Terminalia arjuna*- Bark, *Cinnamomum zeynalium*- Bark, *Chenopodium album*-whole shrub, *Syzygium aromaticum*- flowerbuds, *Cassia fistula*-bark, *Mangifera indica*-bark, *Brassica campestries*- whole

Table 3. MDR Genes activation in plasmids.

Accession number	Size (kb)	MDR Genes profiles with bla genes, drug modifying and drug efflux genes	GenBank year	Pathogenic bacterial name
AP012056	141	tetA,aac3'/6',cat, sul2, blaOXA1/CTXM15/TEM1, strB/A	2013	*K. pneumoniae*
KM877269	249	aad, hph, aac6', aac3', blaOXA1, catB, arr3, sul1	2015	*S. enterica*
LN555650	299	terF, sul1, strA, catB,blaACC1, aacA4, blaVIM1	2015	*S. enterica*
JN420336	267	blaNDM1, blaOXA1, aac6', qnrB1, catB, blaCTXM,	2012	*K. pneumoniae*
KC543497	501	Ter2, blaOXA10, MFS,, blaTEM-8, ble, catB8, aac	201 4	*P. aerogenosa*
CP009116	95	aph, blaTEM, aac3', MFS, dhfr, aad, arr2, blaNDM1	2014	*K. pneumoniae*
AP012055	250	blaNDM1, ccdA, ccdB, aadA2, catA1, qacA1	2013	*K. pneumoniae*
NC_019889	87	aac(3')-II, blaNDM1, sul1, MsrE, mphE	2014	*K. pneumoniae*
KF954759	73	blaKPC3, strB, aac(6'), chrB, ncrA/Y, srbA	2014	*K. pneumoniae*
NC_021087	26	blaGIM1, aacA4, aadA1, blaOXA-2, sul1	2015	*E. cloacae*
NG_035843	15	blaOXA30, catB3, arr-3, sul1, qnr, blaDHA1	2014	*E. coli*
NC_022078	317	ABC, merB, cat, aph*, aac3', cmr, tetA, blaKPC	2015	*K.pneumoniae*
CP011634	227	blaOXA, aad*, blaTEM, merC, sul1, aac	2015	*K. oxytoca*
HG530658	223	blaACC1, strA, aadA2, aac3'	2015	*E. coli*
NC_019375	180	blaVIM, aacA7, dhfr, ANT3', blaSHV5, sul1, aph3'	2014	*P. stuartii*
NC_022522	168	blaCTXM25, aacA4*, strB, strA, aadB, blaOXA21	2014	*S. enterica*
LC055503	160	blaSHV12, aac6', blaOXA10,aadA1, sul1, blaDHA1	2015	*K. pneumoniae*
HG941719	135	blaTEM/CTXM/OXA, aad, mphA, aac6', sull, tetA	2014	*E. coli*
KJ541071	44	sul1,blaOXA2, aadA/B, blaTEM, cat, blaGES5	2014	*E. coli*

Colour codes are: Green is Beta-lactamases, blue is drug acetyl/phospho/adenyl -transferases and red is drug efflux genes. AG acetyl transferases (*aac*), phosphotransferases (*aph*), adenyl transferases (*aad*/*ANT*), tetracycline efflux genes (*tet*A), chloramphenicol acetyl transferase (*cat*B), sulphamethazole/ trimethoprim resistance genes (*dhfr*, *sul*1/2), streptomycin phospho transferases (*str*A/B), rifampicin ribosyl transferase (*arr2*/3), tellurium/chromium/mercury resistance genes (*ter*2/F, *chr*B, *mer*B/C) respectively, bleomycin resistance gene (ble). * means multiple copies of the gene present. *bla*CTX-M-2/15/25 means different mutations compare to *bla*CTX-M-1. Enzymes aac3' and aac6' means 3' and 6' position of the drug that enzymes acetylate. Note that most types mdr genes were heterogeneous in sequences and sometime no similarities among themselves even perform same function (e. g. Beta-lactamases: NDM1, OXA-48, KPC-2, Amp-C, VIM-1, IMP-1, TOHO-1, ACC-1 have no similarities in amino acid sequences but all hydrolyse β-lactam drugs).

shrub, *Justicia gendarussa*- leaves, *Nyctanthes arbortriestis*-leaves, *Nerium indicum*–leaves and *Psidium guyava*-leaves etc few to name (Lee et al., 2014; Nascimento et al., 2000; Dubey et al., 2005). The process of such work was demonstrated in Figure 7 by flow diagram.

It appeared that *Cassia fistula*, *Suregada multiflora*, *Chenopodium album* and *Cinnamomum zeynalium* were very high activities as compared to others However, when the extract made from room temperature stored grinded plant parts at 6 and 12 months, a remarkable loss of activities were seen. This result was very sad as, we will face problem of raw material if any commercial process could be developed. Then we got some information from PubMed that Indian spices might contain good anti-bacterial activities (Chaieb et al., 2007; Fu et al., 2007; Elumalai et al., 2010; Noumedem et al., 2013). Result indicated that cloves and derchini indeed had good activities killing mdr bacteria but not elachi or black pepper and other spices. Cloves and darchini were stored long time at room temperature and that result indeed was encouraging. Such extract was remaining active after heating at 70°C for 10 min (data not shown). Cost reduction was an important step for commercialization and when we changed extraction medium from ethanol to methanol, dry weight of extracted material was increased but activity was reduced. When we added 50% water and the precipitate re-dissolved in ethanol, the activity restored. Thus column chromatography is essential but again infrastructure and high cost needed to characterize the active principles and to make pure MDR-Cure. So we did Thin Layer Chromatography and cut the illuminated fast moving upper band and such alkaloid type chemicals easily inhibited the MDR-bacterial species (Figure 7, panel 6). Mass, NMR and FTIR are in process to know chemical structures of the active principles. However, the MDR-Cure cured nail infections and also cleared the induced infections in rats and rats survived longer than 3 months (data not shown, Chakraborty, 2015).

DISCUSSION

After the discovery of penicillins and tetracyclines in 1930s, thousands of antibiotics were developed to cure

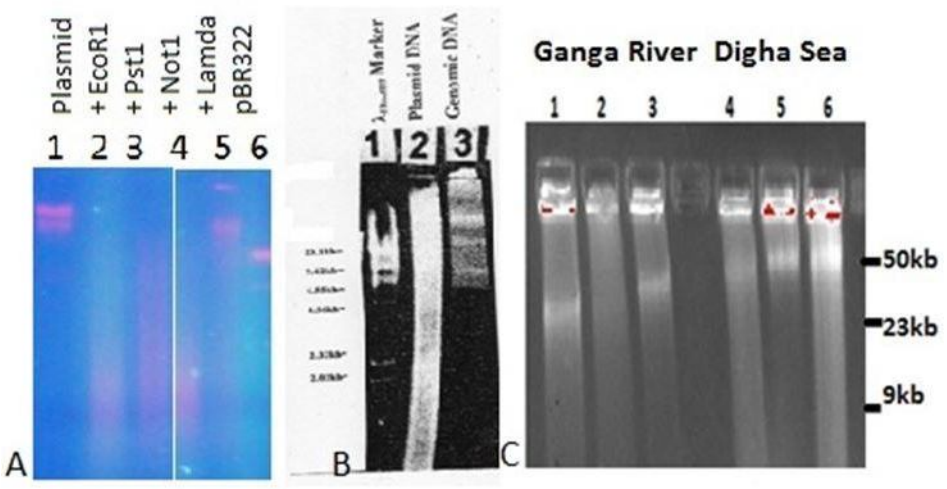

Figure 6. Molecular mechanism of MDR genes activation. To understand the nature of plasmids contaminated in MDR-bacteria, we performed two experiments here. 1st isolated plasmids were digested with many restriction enzymes (A) but no distinct band obtained. This suggested that heterogeneous plasmids were present. 2nd we did short term Pulse field Gel Electrophoresis to trap the small plasmids and contaminated plasmids in genomic DNA. We found (B) many heterogeneous plasmids again and indeed genomic DNA contained plasmids contamination. We detected (C) high molecular weight plasmids in superbugs of Kolkata Ganga River and Digha Sea water after long run of agarose gel at low voltage. Lanes may contains some contaminated chromosomal DNA or very large plasmids (>100 kb). Smear may contains low molecular weight plasmids termed as nano-chromosomes (Chakraborty, 2015).

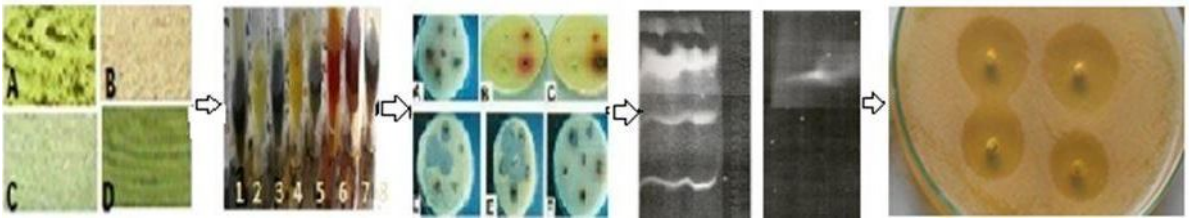

Figure 7. Flow diagram of isolation of MDR-Cure. Plants parts were cut into pieces with a heavy knife and grinded in Philip Mixi for two minutes (panel 1). 10 Gms plant parts extracted with 40 ml ethanol for overnight and concentrated (panel 2) and Kirby-Bauer agar hole assay were performed (panel 3). Active extracts were concentrated and ran a preparative TLC (panel 4) and active part was cut under UV shadow and extracted with ethanol and the potency of active chemicals were determined again by Kirby-Bauer agar hole assay (panel 5). Active component was further separated by TLC or chromatography and MASS, NMR, FTIR were performed for chemical and structure identification (data not shown).

bacterial infections (Chakraborty, 2015). Synthetic chemistry now produces new drugs every day and Genome Projects have helped to identify protein sequence of toxins and drug target enzymes (Xu et al., 2014). Recombinant DNA technology has helped to produce enough protein for crystallography and computer-aided graphics are giving virtual screening of inhibitors (Chakraborty, 2016a). Thus advent of antibiotics, anti-nucleoside analogues, steroids and psychoactive drugs advanced the modern medicine to cure many disease epidemic and we have forgotten our old tradition to cure the diseases using herbal drugs (Cowan, 1999; Cushnie and Lamb, 2005; Mckenna, 2013). However, emergence of drug resistant pathogens, high cost and toxicities have given an alternate choice for peoples to depend on natural remedies or herbal medicines again (Laxminarayan, 2014). Many modern widely used drugs, including strychnine, aspirin, taxol, quinine, topotecan, etoposide and artemisinin are of herbal origin (Sastri, 1956; Chakraborty et al., 1994). Two best known anti-malarials (quinine and artimisinin) have been purified from Indian and Chinese medicinal

plants, Cinchona (Rubiaceae) species and worm plant, suggesting the important of the present study combining traditional knowledge and using modern molecular techniques (King and Strynadka, 2013).

A long term Indian 135268 blood cultures MDR-study (2008 to 2014) recently had shown that MRSA *S. aureus* spread was 44% and carbapenem resistant *E. coli* was 11% but was highest among *Pseudomonas* sp. (49%), *Klebsiella pneumoniae* (56%) and *Acinetobacter* sp. (69%). Moreover, 98% *Salmonella typhi* was resistant to nalidixic acid now which was 20% when in 2008 (Gandra et al., 2016). Our genetic study and antibiogram surely extend such study confirming the accumulation of MDR genes in plasmids and chromosome. It is noteworthy to state that present conjugative mdr plasmids were so large (50 to 500 kb) that genomic DNA preparation might be contaminated with plasmids and cloning/sequencing of such big plasmids were also complicated task and needed huge funding (Doumith et al., 2012). So no such big MDR conjugative plasmids were sequenced in India but Indian Institute of Technology (IIT) had been initiated whole genome sequencing in India. Famous NDM-1 plasmids although were discovered in India, all credit for illuminating the nature of gene cassettes in NDM1 plasmids had gone to scientists abroad (Campos et al., 2015; Wang et al., 2015). We have detected large heterogenous plasmids in MDR bacteria of Ganga River at Kolkata and R-plasmids are absent (Chakraborty, 2014, 2016b).

It was postulated that MDR mechanisms would be similarly complicated with transcriptional activation and retroposon insertions as found in oncogenes, Human Ha-ras under retroviral promoter and gly to val 12th codon mutation greatly aggrevateed the carcinogenic potential of proto-Ha-ras which otherwise very mild carcinogenic (Chakraborty et al, 1991). Further, ID-retroposon insertions at the far upstream repressor sequences depressed the AvrII-AvrIII repressors with increased ras gene expression (Chakraborty, 2014). S174G mutation in blaKPC highly increased the Ki for amoxicillin-cavilinate showing clinical resistance (Chakraboorty, 2016b). Similarly, blaNDM-16 R264H mutation produced increased AMR as also L11F mutation in blaOXA-164 of *Acinetobacter baumannii* was more resistant to meropenem. PCR analysis of tetC and acrAB proton efflux genes suggested a very high copies could be present in *Escherichia coli* KT-1_mdr which was found resistant to many broad spectrum antibiotics like amikacin and linezolid. Further, we demonstrated many small DNAs with large conjugative plasmids in MDR-bacteria suggesting a view that integrons and miRNAs were constantly making the new genes in MDR-bacteria facilitating rapid insertions of mdr genes into F'-plasmid and chromosome similar to chromosome anuploidity in cancer cells (Chakraborty, 2015). In other words, the molecular mechanisms of AMR is very frightening as cancer is claiming many lives today although huge funding has been allotted in cancer research.

Antibiogram clearly suggested that highly resistant *Escherichia coli* were contaminated in Ganga River including Kolkata streets, rain water and Bay of Bengal (Digha Sea). Such bacteria indeed resistant to ampicillin, amoxicillin, cefotaxime, tetracycline, chloramphenicol, vancomycin, neomycin, azithromycin, sulphamethaxozole and streptomycin indicating huge MDR genes had been accumulated in individual bacteria with amplification and activation of *tet, mcr, acrAB, sul1/2t* as well as divergent *bla* genes and drug modifying genes like *cat, aac. aad* and *aph*. *bla*KPC gene was located in many *K. pneumonia* large conjugative plasmids (317kb, accession no. NC_022078; 251kb, accession no. NC_014312; 108kb, accession no. JX283456) as well as NDM1 gene was also located in conjugative plasmids (267kb, accession no. JN420336; 250kb, accession no. AP012055) (Table 3). Other *E. coli* plasmid pNDM_Dok01 and *K. pneumoniae* plasmid pKpANDM-1 have very similar to *Acinetobacter lwoffii* plasmid pNDM-BJ01 (accession nos. JQ001791 and JQ060896) with flanked IS*Aba125 elements and T4SS* genes (Hu et al., 2012; D' Costa et al., 2011; Campos et al., 2015; Manageiro et al., 2015). We have further demonstrated the amplification of such genes by PCR and sequencing is in progress. Indian ayurvedic medicines were initiated >5000 years ago as described in Charaka Samhita and Sasruta Samhita as well as Veda (Sastri, 1956). Thus our initiative to screen medicinal plants and spices were justified and we progressed a lot according to medicinal book, Chiranjib Bonoushashi. We have also initiated a Patenting approach in Kolkata Patent Office predicting such study would be needed huge funding particularly to identify the active principles to illuminate the drug target site (Sahoo et al., 2011; Drawz and Bonomo, 2010; Keating et al., 2011; Chakraborty, 2016b).

Conclusion

AMR is a serious worldwide problem and we proved the similar situation in Ganga River water at Kolkata. We must act now to infuse huge funding from Government and also from Drug Companies for diagnostic and therapeutic research of MDR pathogens in India. It has been proposed by WHO to reduce the contamination of antibiotics, pesticides, detergents, paints, molecular biology chemicals and heavy metals in water (Basu et al., 2013). We must be aware, at what pressure 10-15 mdr genes were accumulated in a single conjugative plasmid and why all common bacteria are accepting such plasmids? Otherwise, we had created huge toxic chemicals contamination in water where all microbes were in a life threatening pressure that might be facilitated the combination of R-plasmids with F'-

Table 4. Chromosomal integration of MDR genes in MDR-bacteria.

Names of bacteria	Chromosome acc. no. / Size (bp)	mdr gene/ % similarity	Position in bacterial chromosome/ Size (bp)	Copy no.
Escherichia coli BW25113	CP009273/4631469	acrAB/100% envCD /100% cmr/ 99%	476132-482170/ 6038 3400624-3411591/ 11k 878844-880412/1568	One Three One
Klebsiella pneumoniae	NC_021232/5270770	emrD/99% acrAB/82%	29052-30607/955 4323257-4328383/5127	One Two
Stenotrophomonas maltophilia (MDR)	AM743169/4851126	mexAB/80% mexEF/79% aph/80%	4177460-4178947/1487 1879272-1880782/1510 2143348-2144475/1127	Two one
E. coli 0103:H2 (Toxin producing)	NC_013353/5449314	acrAB/100% envCD/98% cmr/100%	489265-495303/6038 4090536-4094991/4455 968010-969578/1568	One One One
Salmonella enterica	CP007557/4685859	acrAB/84% acrB/74% emrD/82%	508579-514081/5507 3444035-3447110/3087 3874357-3875522/1165	Two One
Bacillus thuringiensis	NC_005957/5237682	PBP/ n.d. MFS, ABC	2101873-2103330/1457 n.d.	One Many
Acinetobacter baumannii	CP001921/3949614	blaPER/99%	284625-285864/1239	One
A. baumannii AB030	CP009257/4335793	blaOXA-23/100%	3304402-3305175/833 4496984-4297817/833	Two
Acinetobacterbaumannii TCDC-AB0715 (MDR)	CP002522/4138388	bla_{OXA-23}/99% mexD/66% aadA1/A4/99%	2760564-2761566/1002 2169029-2169609/580 267616-266813/803 etc	One One Three
*Staphylococcus aureus,*MRSA	BX571856/2902619	norA/93%	781930-784247/2650	One
Bacillus subtilis *Citrobacter spp.*S-77	AP012496/4043042 NZ_DF830265	bmr/99% blaCMY-13/86%	2326257-2327658/1401 212590-216842/4252	One One
A. baumannii NCGM-237	AP013357/4021920	blaOXA-23/100%	3159738-3160571/833	One
S. aureusOCL, MRSA	CP000046/2934567	norA/94% Lactamase-B/ n.d.	775486-778125/2639 73402-74726/424	One One
Klebsiella pneumoniae	FO834906/5438894	acrAB/82% acrB/77%	1458887-1463969/5127 4240126-4240699/573	Three

GenBank database (www.ncbi.nlm.nih.gov/blast) was searched for MDR-bacterial chromosomes where one or more MDR genes were integrated from conjugative transposon-tagged MDR plasmids. Such finding was important as gene doses from chromosome and from MDR plasmids were additive and such bacteria were PDR or XDR suggesting no antibiotic will be able to kill such bacteria due to cumulative actions of beta-lactamases, drug modifying enzymes, drug efflux proteins and further target modifications like VanA, PBPs, rRNA and outer membrane porins.

plasmids. Such conjugative plasmids easily could donate the mdr genes into common bacteria came to close contact (Chakraborty, 2015). Further, MDR-bacteria increased the mdr gene doses by moving mdr genes into its chromosome (Table 4). The author hypothesised that plasmids used in molecular biology and recombinant technology experiments might also transfer the MDR genes (*amp, neo, tet*) to environmental bacteria but would be needed experimental evidences to support this view. MDR genes (Beta-lactamases and drug efflux proteins) were highly acquired by MDR bacteria in Kolkata as confirmed by blaTEM gene sequencing where 132 codon (alanine amino acid) had polymiphormism in two cases similar to amp gene of plasmid pBR322 (data not shown). Recently, we have detected imipenem resistant MDR bacteria in Ganga river water (0.2 to 3 cfu/ml of water) when 10 ml water was assayed in vitro liquid media insted 0.1 ml as previously used. It seems such bacterial plasmids lacks *cat* and *strA/B* type genes but *tet*. Overall, we need new inhibitors of MDR genes to keep old drugs active saving poor nations from AMR calamity and Indian herbal medicine (MDR-Cure) could be studied further using FTIR, NMR and MASS spectrometry to unravel the structure of active antibiotic principles. In truth, heterogeneous phyto-antibiotics, gene medicines (ribozyme, dicer, and miRNA), bacteriophage therapy and DNA nanotechnology are getting priority research to overcome MDR bacterial pathogenesis (Chakraborty et al., 2016). Nevertheless, we should stop taking the raw Ganga River water in religious occasions.

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