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Chemical Toxicities of Both Intestine and Environmental Water Caused Genetic Recombination in Bacteria with the Creation of MDR Genes and Drug Void

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Abstract

Civilization and Scientific Endeavour in Science, Engineering and Metal Industry followed by biochemistry revolution in health industry between 1650 - 1970 is great achievement but mega industries are polluting the water resources creating huge genetic mutants among the microbes. Intestinal bacteria, however experienced more toxicities due to consumption of huge antibiotics between year 1940 - 1980 creating XDR mutants whose genomes as well as plasmids have been analyzed here. Unresponsiveness to drugs has increased from bacteria to fungi and parasites suggesting we have created a life-threatening condition among the unseen microbes. We have classified many *mdr* genes and drug efflux genes which are heterogeneous. Beta-lactamases (blaTEM, blaOXA, blaCTX-M and blaNDM) are mostly diverged causing penicillin, cephalosporin and carbapenem drugs ineffective. Acetyltransferases (*aac, cat*), phosphotransferases (aph, strAB) and adenyltransferases (aad) were located in most plasmids. PenA, tetM binds drugs increasing drug MIC. Whereas gyrA, sul1/2/3 and blaOXA mutations have created more drug void. Sadly, large MDR conjugative plasmid have ~20 transposable elements and 10 recombination genes where new *mdr* gene creation may take only few days. Isolation of 300 *mdr* genes in the Human Microbiome Project registered the calamity of 21st Century science and civilization and inhibition of gut bacteria is thus a threat. We propose that, *mdr* genes creation is to protect human and animal from extinct. Other hypothesis is, unless toxin and virulence genes activated, *mdr* genes in bacteria are likely non-toxic to health. It also may be postulated that rapid increase of hormones, heavy metals and pesticides contamination in foods and too much antibiotics, psychoactive drugs and steroids use are likely caused different types of metabolic diseases and cancer in human irrespective of age, sex and locality.

Keywords: *Drugs Contamination; mdr Genes; Superbugs; Antibiotics Void; Gut Microbiome*

Introduction

Human race has been developed tremendously in last few decades due to advancement of science [1]. However, population increase, racial and political uncertainty and mass weapon production have created a society that is designed to save human but not humanity. In the past, we mostly utilised natural resources like fruits, animals, woods, fishes and caves or wood houses. In the past, population are greatly balanced by many diseases, flood, storm and malnutrition. Our mobility was limited due to river, hills, deserts and deadly jungles

with snakes and animals. So, there was a balance between human and nature where nature was dominated where chemical toxicities in water and air were low [2].

Civilization in Europe between 1700 - 1800 and their move into United States capturing whole world lead to the World War I and World War II followed by creation of the United Nations and World Health Organization to save this mankind [3,4]. Mega Industries are protected by Patent rights and likely rich countries are becoming more rich and poor countries are getting hard time to give scientific utilities to countrymen. India has only 40 crores people during independence and now 125 crores [5]. That means we need 125 crores computers and mobile phones and we have to purchase all from China or patent holder, United States. So, make in India programme has initiated where big industries are made. We need much oil, electricity and steel where mining activity has increased increasing pollution in water and air. Similarly, we need uranium for space research, steel and aluminium for rail road, buses and millions units of modern flats. All forests have gone (reduced 70% to 33% after independence) to increase agricultural land and housing to feed 700 millions people of this Earth and at least 230 mt food is required per year. As a result fertilizer and insecticide in agricultural land and effluent from mega industries are increasing in water creating severe pollution. Due to multi-resistance, we need hundred antibiotics and anticancer drugs and thereby, big pharmaceutical industries are made again polluting nearby rivers [6]. Big as well as 250 small countries are in danger and likely small countries suffer most to tackle pollution and disease epidemics. Heavy metals like lead, uranium, polonium, mercury and arsenic are in the drinking water of many Indian villages. PM2.5, PM5 and PM10 particulate contamination in air due to travel industry have increased ten times, carrying MDR bacteria and fungal spores in dust particles. Plant hormones and insecticides toxicities are maximum in vegetables. Heart problem, diabetes, sugar, arthritis, headache, and cancer have increased tremendously irrespective of age. Education time has increased ten years and so the marriage time leading to increase in polygamy with unauthorized sex workers which has caused high spread of gonorrhoea, syphilis, jaundice and genital disorder. Various toxicities are reported in the figure 1 causing genetic changes in the microorganisms who divide rapidly. Scientist claimed that all genetic changes to create multiple MDR genes happened to protect gut microbiota (biofilm) in the intestine from antibiotics and to continue vitamin synthesis required for > 30000 enzymatic reactions [7]. Now, we use 200000 tons of antibiotics per year and ng-µg quantity ampicillin and tetracycline drugs contamination per ml sea and river water were reported. Antibiotics void may cause tremendous calamity in coming decades with ~3% reduced world GDP, a statement recently given by DGs of UN and WHO whereas G-20 Leaders have issued AMR ACTION PLAN. Antibiotics affected 30,000 enzymatic reactions due to insufficient vitamins supply by microbes of the gut. We have addressed the toxicity index in bacteria by analyzing the genetic changes in plasmids as available in the database.

Materials and Methods

Ganga River water collection and drug selection

Water from Ganga River was collected at 8 am on Monday from Kolkata-700001 area. About 100 µl of water was spread onto 1.5% LB-Agar plates with different antibiotics concentration at 5 - 50 µg/ml. As imipenem resistant bacteria were present low (0.08 - 0.2 cfu/ml water), a modified method was followed. 2 ml 5x Luria-Burtoni media was added into 10 ml Ganga River water in presence of 2 - 5 µg/ml imipenem and was incubated 18 hrs to get imipenem resistant bacterial population. We also used meropenem to get meropenem resistant bacteria. Antibiotics were purchased from HiMedia and stored at 20 - 50 mg/ml stock solution at -20°C. Antibiotic paper discs were purchased from HiMedia according to CLSI standard. Antibiotic papers are: Amp-30 (ampicillin), Met-10 µg (methicillin), VA-10 µg (vancomycin), CAZ-30 µg (ceftazidime), AT-50 (aztreonam), Str-30 (streptomycin), COT-25 µg (Cotrimoxazole), LOM-15 µg (lomefloxacin), TGC-15 µg (tigecycline), AK-10 µg (Amikacin), LZ-10 µg (linezolid), and IMP-10 µg (imipenem). The antibiotic stock solutions are as follows: ciprofloxacin 50 mg/ml in water, ampicillin 50 mg/ml in water, streptomycin 50 mg/ml in water, tetracycline 20 mg/ml in ethanol, chloramphenicol 34 mg/ml in ethanol, cefotaxime 25 mg/ml in water.

Isolation of plasmid DNA and genomic DNA

The plasmid DNA was isolated from overnight culture using Alkaline-Lysis Method as described in Sambrook., *et al* (1989). Simply, to bacterial pellet 0.1 ml solution-I was added and mixed well. Then 200 µl of cold Solution-II was added to make clear solution and then 150

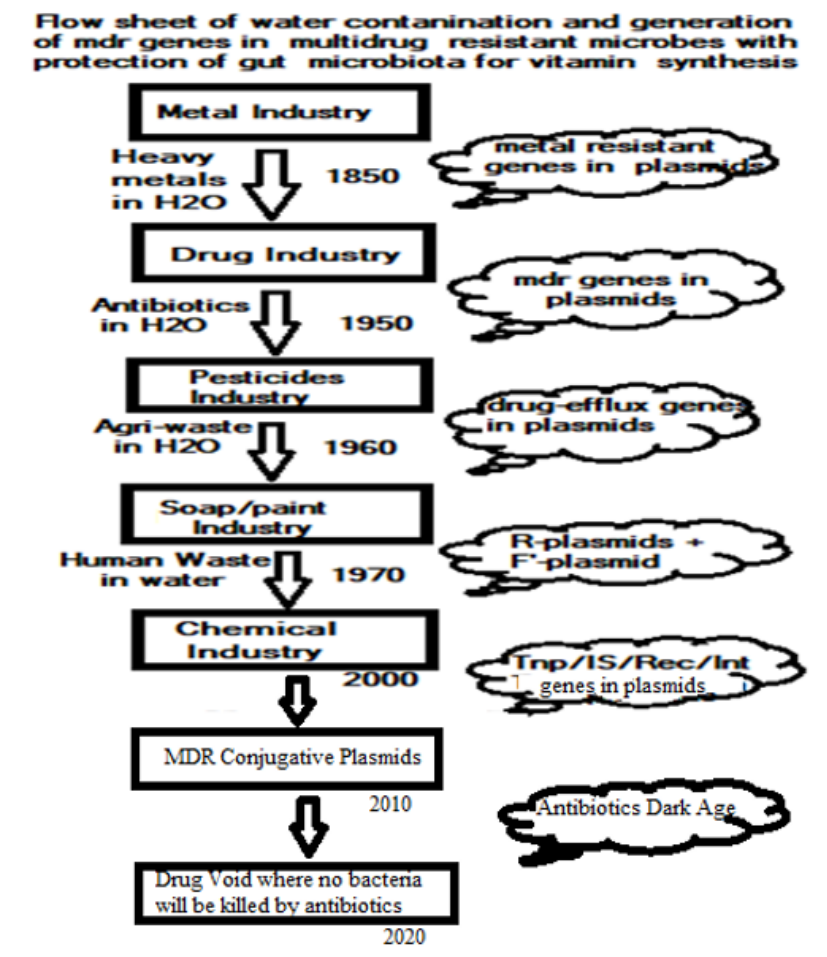

Figure 1: *Relation among the scientific development, population increase and industrial toxicities that cause genetic changes in microbes.*

µl cold of Solution-III was added and mixed well. After 10 minutes the solution containing huge whitish pellet of chromosomal DNA and cell debris were removed by centrifugation at 12000 rpm for 8 minutes. Then 1 ml ethanol added to supernatant (0.4 ml) and centrifuged at 12K rpm for 12 minutes. RNase treatment was done to remove RNA and plasmid DNA was dissolved in 30 µl TE solution.

Genomic DNA was isolated by Proteinase-K-SDS method from 1.5 ml overnight culture. After pronase digestion (10 µl of 10 mg/ml in water, 2 - 6 hrs incubation), the clear solution slowly extracted with 100 µl phenol-$CHCl_3$-isoamyl alcohol (25:24:1) and ethanol precipitated in presence of 25 µl 5M sodium chloride. Then centrifuged at 9000 rpm for 12 minutes and ethanol was added into upper aqueous solution and DNA recovered by centrifugation at 10000 rpm for 10 minutes. DNA pellet was dissolved in 50 µl TE buffer. 1 µl RNase A was added and incubated for 12 minutes at 37°C and extracted with phenol-$CHCl_3$-isomyl alcohol (25:24:1) again and was precipitated with 1/10 volume of 3M sodium acetate PH 5.2 and 2 volumes of 99.9% ethanol. The DNA pellet was finally dissolved in 40 µl TE buffer [9].

PCR and DNA sequencing

16S rDNA gene Sanger's di-deoxy sequencing was done by SciGenom Limited, Kerala, India). PCR amplification was carried out using 1.5 unit Taq DNA polymerase, 0.25mM dXTPs, 30 ng plasmid DNA template, 2 mM $MgCl_2$, for 30 cycles at 95°C/40" (denaturation)-50°C/45" (annealing)-72°C/2' (synthesis). The product was resolved on a 1% agarose gel in 1X TAE buffer at 60V for 3 hrs and visualized under UV light and photograph was taken [10]. The primers for 16S rRNA amplification and *mdr* genes are given below. NCBI BLAST analysis was performed for bacterial specific gene analysis (www.ncbi.nlm.nih.gov/blast) and data was submitted to GenBank [11,12].

Preparation of organic phyto-extract (MDR-Cure)

The barks of *Suregada multiflora* (Ban-Naranga), *Cassia fistula* (Bandor-Lathi) were collected on June-November 2017 - 2019 from medium sized tree at Midnapore district of West Bengal. Each 10 gms dried and grinded plant and spice parts (*Suregada multiflora*, *Shorea robusta*, *Cassia fistula*, *Trapa bispinosa* (peel), *Syzygium aromaticum*, *Cinnamomum zeynalicum*) was suspended in 40 ml ethanol and overnight extracts were mixed and concentrated 5 times (MDR-Cure) and 50 µl used for Kirby-Bauer agar hole assay [13]. Further, Preparative Thin Layer Chromatography (TLC) was performed using Methanol, water and Acetic acid (50:40:10) as mobile phase for 1 hr using 18 x 14 cm silica-gel plates. 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 performed at Bose Institute-Kolkata, IIT-Mandi and Indian Association for the Cultivation of Sciences-Kolkata.

Result and Discussion

Before 1600s we have no idea for microorganisms (virus, bacteria, fungus and parasites) that cause diseases and usually we blame ghosts, demons, Sun and Wind. But after the discovery of microscope by Anton Van Leeuwenhoek (1670) as he see bacteria in water and milk by his 200X single lens microscope. It about take 200 years to believe small living creature until further pioneering works by Edward Jenner (1790s), Lewis Pasteur (1860), David Koch (1880) and others. They proved that bacteria were the culprit of many diseases like TB, Cholera, Gonorrhoea and Typhoid as they purified the bacteria and injected into animal producing the diseases. Likely doctor's mind were busy to grow pure bacteria like *Vibrio cholerae* or *Staphylococcus aureus*, TB bacillus, *Salmonella typhi* and find different chemicals that kill it. Likely, 5% phenol as best disinfection as well as wine were found to reduce infections and death during surgery. Edward Jenner at that time (1789) kicked the concept of vaccination from his pioneering work to protect pox virus infection using live virus injection (Vaccination). Notably, discovery of antibiotics are in centre stage when Nobel Laureate Alexander Flaming discovered benzyl penicillin from slime mold *Penicillium notatum* in 1928. Penicillin inhibits bacterial cell wall peptidoglycan synthesis and thus a good drug as human have no cell wall and thus less toxic. Since then hundreds derivatives were made alone for penicillins (ampicillin, oxacillin, amoxicillin, cefotaxime, ceftriaxone, imipenem, meropenem and doripenem) for better drug usually called penicillinases resistant drugs. Dr. Selman Waksman developed over twenty antibiotics receiving Nobel Prize in Physiology or Medicine in 1952 as his streptomycin drug eradicated TB at that time. However, gradually (1930 - 1970) many potent β-lactamases like cefotaximases, oxacillinases, carbapenemases were appeared in bacterial conjugative plasmids and also have moved to chromosome islands [14,15]. We presented a snap shot of many MDR genes in table 1 as available in the GenBank Database.

Research suggested that huge oral antibiotics intake adversely killed 2 x 10^{12} diverse species of intestinal bacteria (gut microbiome) since 1940s creating an acute health hazard in human which is now balanced by probiotics (bifidobacteria) and vitamin-B complex capsules supplement. Scientists predicted that molecular signalling from small intestinal cells through interleukins like TGFβ, IL-10, IL-22 as well as signalling from bacteria synthesizing liposaccharides, vitamins, steroids, butyrate orchestrated to preserve symbiosis relation between human and bacteria [18]. Vitamins are converted into coenzymes (NAD+, THFA, FAD, Biotin, TPP, PALPO, B12 etc.) needed for every steps of > 40000 biochemical reactions in human and animal cells. Thus, MDR bacteria will be the resident of intestine favouring vitamin biosynthesis and immune-modulation needed for normal human metabolosome (See figure 2).

Name	Sequence of the primers	Tm	Size
P27F	5'-AGA GTT TGA TCC GAA CGC T-3'	62°C	1.4kb
P1392R	5'-TAC GGC TAC CTT GTT ACG ACT TCA-3'	65°C	
cmrF	5'-TTC GTT AGT CTG CCG TTG CT-3'	56°C	323bp
cmrR	5'-ATC GCT GGC AAA CAG GGT TA-3'	57°C	
tem-sF1U	5'-ATGATGAGCACYTTTAAAGT-3' Y=C/T	56°C	312bp
tem-sR1U	5'-TCATTCAGYTCCGKTTCCCA-3'Y=C/T; K=G/T	58°C	
tetF	5'-CTT CGC TAC TTG GAG CCA CT-3'	57°C	910bp
tetR	5'-GCA GAC AAG GTA TAG GGC GG-3'	57°C	
acrAB-F	5'-ATG CTC TCA GGC AGC TTA GCC-3'	59°C	.1kb
acrAB-R	5'-TGT CAC CAG CCA CTT ATC GCC-3'	59°C	
ctxF1U	5'-AACACMGCMGATAATTCACA-3' M=A/C	59°C	586bp
ctxR1U	5'-CCGCRATATCRTTGGTGGTG-3' R=A/G	61°C	

Table 1: *Primers used in this study (Chakraborty AK, 2015).*

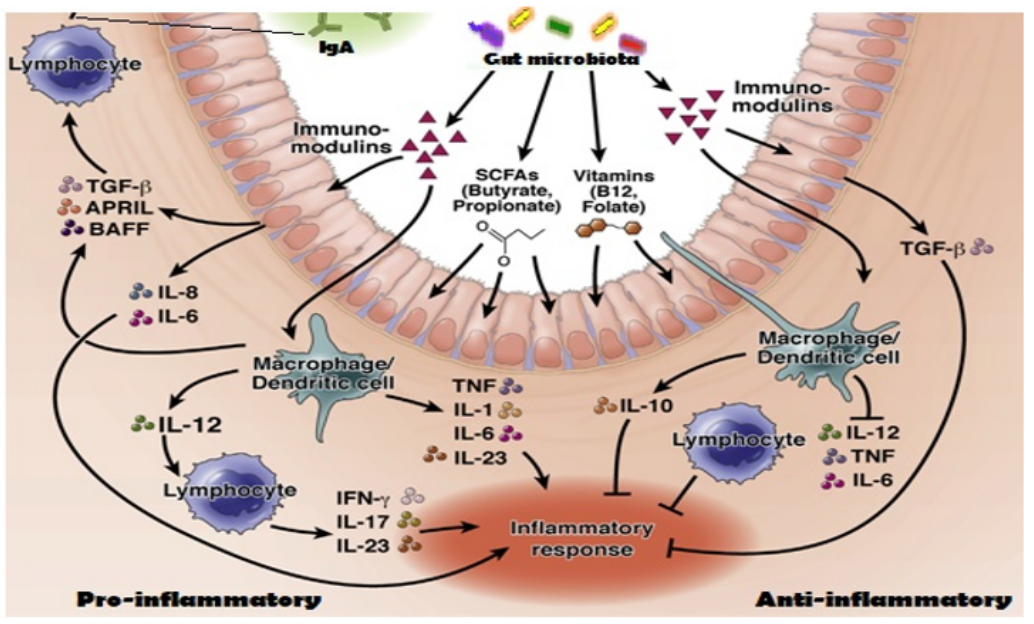

Figure 2: *Symbiotic relations between intestinal cells and gut bacteria.*

Gut symbiosis is very potential and without which many gene rearrangement are obvious. The major success came in 1965 when *amp* and *tet* genes were discovered in plasmid pBR322 which was made artificially from R-plasmids like pSC101 and pMB isolated from penicillin and tetracycline resistant bacteria. The *amp* gene codes for beta-lactamase enzyme which cleaves lactam A ring of benzyl-penicillin

as well as ampicillin and amoxicillin whereas *tet* gene codes for drug efflux trans-membrane protein that kicks out drug from inside of the bacteria into outside increasing drug MIC. Scientists prepared semi-synthetic drugs that very active on penicillin resistant bacteria. But blaTEM, blaCTX-M-1, blaVIM, blaOXA-1-1, blaSHV could cleave amoxicillin, methicillin and oxacillin. Drug industry was alarmed as drug resistant pathogenesis was apparent in Europe, Asia and America and seems universal in their small plasmid and integron content. So, cephalosporin antibiotics were prepared and cefoxitin, ceftriaxone, cefotaxime seem very effective for few years between 1960 - 1980 but ESBL enzymes were appeared in plasmids which were found 20-90 kb plasmids containing multiple *mdr* genes like *blaCTX-M*-9 and *blaOXA-48, blaOXA-450* including *sul1/2, dhfr, catB3, aacC1, aacA1*, *tetA/C* and *strA/B* (See figure 3).

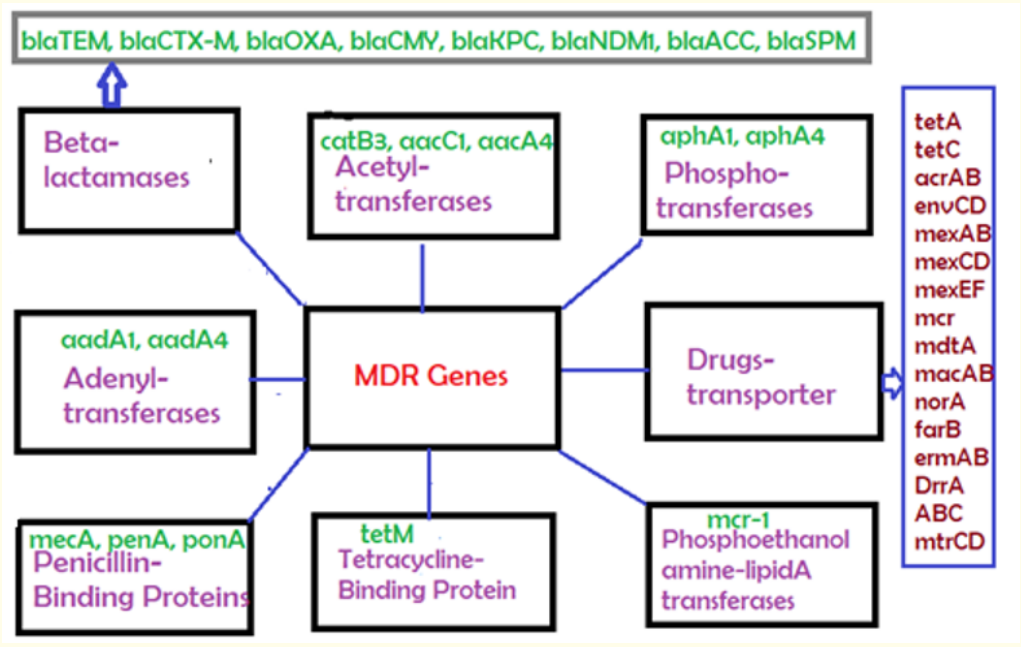

Figure 3: *Functional diversities of different mdr genes that cause antibiotics void.*

Interestingly, success of antibiotics still is continuing in poor countries where as Sweden, Germany, USA and Japan have banned to excessive use of antibiotics. Nevertheless, steroid antibiotics once in centre stage to clear bacterial infections but research indicated that steroids were very bad drug in excess due to activation of many regulatory genes following chromosomal unfolding. Thus, new drug discovery was slowed where as MDR bacterial population are increasing in water and air. XDR *Acinetobacter, Klebsiella, Staphylococcus, Mycobacterium* infections are steadily increasing in India.

Scientists discovered that due to over exposure of repeated antibiotics, drug resistance was increased but low molecular weight R-plasmids were decreased. Finer analysis however, indicated that F'-plasmid (62.5 kb) was combined with 6 - 12 *mdr* genes of R-plasmids making a very ideal *mdr* genes transmission machinery known as MDR Conjugative Plasmid (See figure 4). Sadly, Pubmed data analysis indicated that now > 95% of the isolated bacteria from human were drug resistant and major cases multidrug resistant bacteria have large plasmids with many *Tra* genes as well as many transposons (Tn5, Tn10) and recombination enzymes (TnpA, recA, ResA, Int) making an ideal place for new gene creation. Lastly, a new beta-lactamase blaNDM-1 was discovered in 2009 in New Delhi, India and such enzyme

can cleave all penicillins, cephalosporins and carbapenems [14]. Further, many plasmids as well as chromosome of MDR bacteria have PenA, PonA and mecA proteins having strong binding capacity to penicillin drugs and thus inactivate penicillins as also the case of tetM protein which has strong affinity for tetracycline. StrA and StrB proteins phosphorylate streptomycin but more diverged enzymes like aphA1 and aphA4 types enzymes are abundant in plasmids and all phosphorylate drugs like streptomycin, amikacin, neomycin whereas ANT enzymes adenylate drugs to inactivate it (See table 2). Large number of acetyl transferases other than early discovered *cat* gene, were activated in MDR plasmids like *aacC1* and *aacA1* genes. Why so many isomeric *mdr* genes are generated to perform similar job? Yes, too many new drug derivatives were made and doctors hardly advised poor patients to perform drug sensitivity tests. In truth, no drug sensitive test is necessary in India to prescribe antibiotics. So, doctor first gave Amoxicillin+Cavilinate and then Tetracycline, then Cefotaxime and then finally Meropenem and Linezolid or Colistin. As a result, all intestinal bacteria became dead and no vitamin synthesis continued. In truth, human and animal cannot synthesis vitamins and during cooking at very high temperature and pressure all plant-derived vitamins are destroyed. In India, poor peoples hardly can eat fruits leading to crisis of vitamins. US Human Microbiome Project (HMP), European Metagenomics of the Human Intestinal Tract (MetaHIT) and Human Genome Project have demonstrated the beneficial functions of the normal gut flora (> 35000 species) on human health. Between 1940 - 1960, no vitamins were given to patients as well as no probiotics, leading to crisis in many patients for coenzymes (made from vitamins) needed in more than 30,000 enzymatic reactions for metabolism (sugar-fat-protein-nucleotides) and energy production (ATP synthesis at the ETC). Save your soul works for bacteria and also human cells are symbiotically controlled creating *mdr* genes many fold and it seems drug void will continue [7]. Rapid gene rearrangement have occurred as many DNA topoisomerases, recombinases, integrases, polymerases and ligases were assembled in MDR conjugative plasmids. Likely, such MDR bacteria have signalled due to secretion of many interleukins and cytokines by small intestinal cells that likely have facilitated new gene synthesizing machinery and MDR gene creation in bacteria (See table 2). Thus, both bacteria and human saved each other by inactivating antibiotics. All doctors became fool this way as mechanism of bio-film synthesis in the gut and its destruction by antibiotics, have not been included in the many medical text books. Antibiotics over exposure are a criminal offence in the Sweden, USA and Germany. Sadly, we see in the poor countries still we use ampicillin, tetracycline, ciprofloxacin, erythromycin and streptomycin increasing MDR TB, syphilis, fever, gonorrhoea, cholera, dysentery and typhoid. Essentially, we have also destroyed the utility of vaccination until we know the function of the ¼ of the unknown genes in large plasmids of MDR bacteria. This is very serious as, MDR plasmids are acquiring many unknown genes and such genes many form more *mdr* genes due to presence of transposons and mobile elements in most large MDR plasmids.

Klebsiella plasmid pNDM5-LDR contained blaNDM-5 which hydrolyses carbapenems and many conjugative *pil* genes were detected. *Acinetobacter baumannii* plasmid (accession no. KU549175) has Zeta-toxin, Hemolysin but also mph *mdr* gene and *thi* genes involved in vitamin B1 biosynthesis as well as few metal resistant genes. *Kluyvera ascorbata* strain WCH1410 plasmid pMCR_1410 contained deadly MCR-1 gene which inactivated the superbug drug colistin and was discovered in lately (accession no. KU922754) and also found in coliform bacteria (accession no.MF093645). *Vibrio cholerae* harbouring IncA/C2 plasmid (pMRV10) was reported with *blaCMY-2, aac*(*3)-IIa, blaCTX-M-2, blaTEM-1, floR, str*A/B*, sul1/2, dfrA1, tetA, mphA, mdr*-genes. Similarly, *Bacillus anthrus* plasmid pX01 (accession no. CM002399; 171kb) has toxin gene (protein id. AFL55645) and also in pBMB293 plasmid. *Mcr-1* gene has taken colistin drug from selves in 2016 and such MCR-1 gene were reported in *Escherichia coli* plasmids, pWJ1 (261kb), *pLV23529-MCR-3* (33 kb) *and* pKP37-BE (35 kb) (accession nos. KY924928, LT598652, KY964067). *Enterobacter cloacae* small plasmid pNDM-T1 has blaNDM-1 gene which also made large MDR conjugative plasmids with blaKPC gene (Table 3).

Bacillus thuringiensis plasmid pBMB293 (Accession no. CP007615, 294kb) has no *mdr* gene but genes for enterotoxins (protein id. AIM34697), dipterans toxin (protein id. AIM34741) and proteins for new *mdr* gene creation like DNA polymerase β, reverse transcriptase and DNA topoisomerase III. Nevertheless, without toxins and signalling growth regulatory genes, many *mdr* genes are harmless and rats injected with MDR bacteria stayed good for at least 3 months [13]. *E. coli* strain MCR1_NJ has medium sized plasmid with blaNDM-1 gene (accession no. KX447767) with many *vir* genes and *Tra* genes.

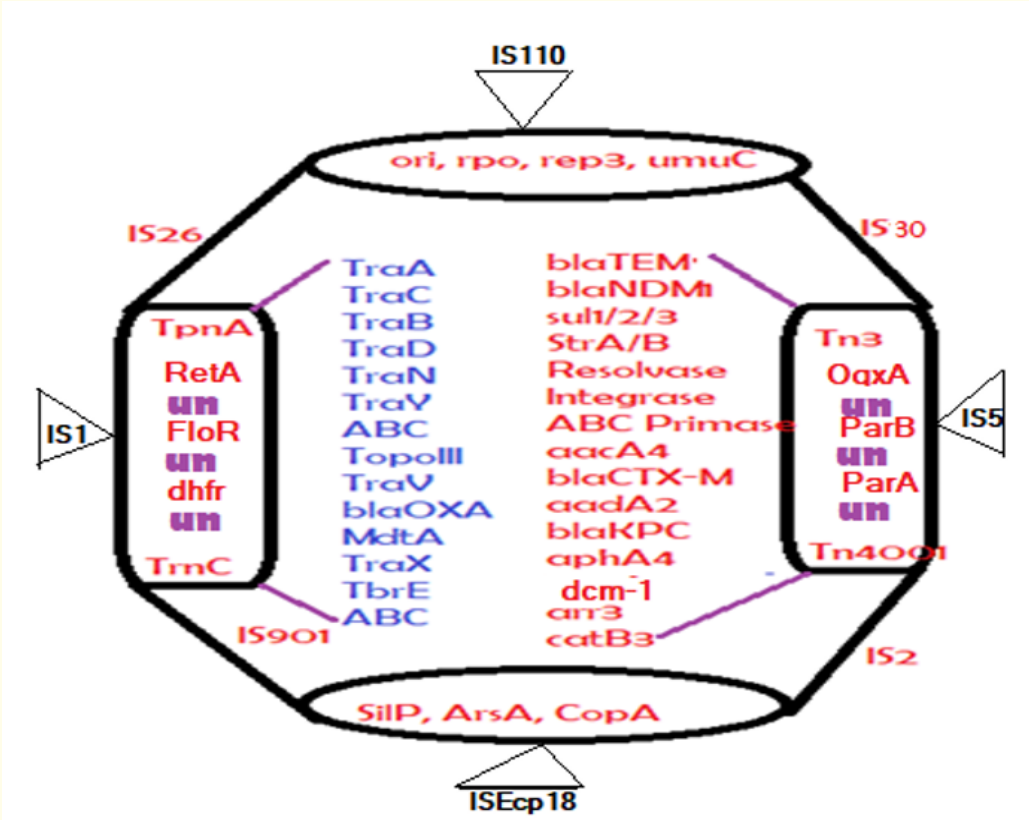

Figure 4: *A sketch of modern MDR conjugative plasmids that are efficient to donate mdr genes to bacteria and to make new mdr genes due to presence of many IS-elements, Tra conjugative genes and transposons.*

Genes	Subclass	Amino acids	Accession number	Protein Id	Function
bla	blaTEM	286 aa	J01749	AAB59737	Cleaves ampicillin
	blaCTX-M	291 aa	X92506	ABN09669	Cleaves cefotaxime
	blaSHV	286 aa	X98098	AAD37412	Cleaves amoxicillin
	blaKPC	293 aa	AF297554	AAG13410	Same + imipenem
	blaNDM1	270 aa	KC539430	AGC54622	Same + meropenem
	blaOXA1	276 aa	AF227505	AFG30109	Cleaves oxacillin
	blaIMP	246 aa	S71932	AAB30289	Cleaves imipenem
	blaVIM	266 aa	AJ291609	ABV21756	Same
	blaAMP-C	382 aa	AF124204	AAD28044	Cleaves cefoxitin
	blaSPM	276 aa	AY341249	AAR15341	Cleaves ceftriaxone

tet	tetA	399 aa	X75761	ALS39162	Kick out tetracycline
	tetA	424 aa	HM453327	AHC55487	Same
	tetB	401 aa	KP899806	AKJ20239	Same
	tetC	396 aa	KC590080	AGL61405	Same
	tetD	394 aa	AB089602	BAC67150	Same
	tetE	405 aa	JN315882	AEW70668	Same
str	strA	267 aa	M28829	AAA26443	Phosphorylates streptomycin
	strB	278 aa	LN555650	CED95339	same
cat	catB3	210 aa	EF516991,	ABP52023	Acetylates chloramphenicol
aac	aacA1	185 aa	AB061794	BAB72153	Acetylates amnoglycosides
	aacA4	152 aa	JN596279	AEZ05102	Same
	aac(3')	286 aa	M62833	AAA21890	Same
	aac(2')	210 aa	U72743	AAB41701	Same
aad	aadA1	263 aa	AF324464	AAK13440	Adenylates aminoglycoside
	aadA4	263 aa	AY138986	AAV34365	Same
	aadA5	262 aa	KT175895	ALO62079	Same
	aadA7	265 aa	DQ520937	ABG01709	Same
aph	aph(6')	266 aa	X01702	CAA25854	Phosphorylates aminoglycosides
	aph(4')	341 aa	V01499	CAA24743	Same
	aph(3')	264 aa	U32991	AAA85506	Same
	aph(2')	294 aa	NC_018107	WP_000155092	Same
RND	acrB	1027aa	M94249	WP_001132469	Kick out drugs
	mexB	1045aa	L11616	WP_023101049	Same
MFS	norA	388 aa	D90119	BAA14147	Quinolone efflux
	mdtE	385 aa	CP000247	ABG71588	Same
ABC	macB	664 aa	APBE01000048	EMU20415	Kick out macrolides
	DrrA	316 aa	HE971709	CCK28451	Same
SMR	Mmr	107 aa	LHCK01000002	KPU48951	Kick out dyes
	emrE	110 aa	Z11877	CAA77936	Same
EMR	emrA/B	~243aa	X03216, Y00116	CAA26964, CAA68299	Same
MCR	mcr-1	541aa	NG_050417	WP_049589868	Add phospho- ethanolamine
VanHAX	vanA	287aa	KR047792	AKE81063	Vancomycin Resistant

Table 2: *Major classification of different MDR genes in bacterial plasmids and chromosome with metal resistant, chemical resistant and drug resistant phenotypes.*

Accession number	Size (kb)	Beta-lactamases, drug transporters, metal resistant and antibiotic inactivating enzymes were found	GenBank Year	MDR Pathogens
NC_018107	353	aac3'-IId, aph2', terA/C/F, Sul1, ANT3''-Ia, dhfr, blaCTX-M-3, aacA4, blaSHV-12, aph3'', blaTEM	2017	*Klebsiella oxytoca*
CP022170	192	mexC, aac6'-1b, cmlA5, ANT3''-1a, dhfr, aac6'-1b-cr, OXA1, sul1, mphE 2', floR	2017	*Aeromonas salmonicida*
NC_022078	317	MFS, merBC, cat, sul1, aac3', cmr, tetA, tetG, ABC, blaKPC,blaCTX-M-24, blaVEB-3, aph3'-Ia, copB/C	2017	*Klebsiella pneumoniae*
KC543497	501	Ter2, OXA-10, MFS, TEM-8, ble, catB3, aac6'. IMP-9, neo	2016	*Pseudomonas aeruginosa*
LN555650	299	terA/C/F/W/Y, blaAmpC, sul1, arsB, silA, strA, dhfr, catA1, blaACC-1, aadA1, aacA4, blaVIM-1	2015	*Salmonella enterica*
CP019001	272	KPC, mphA, sul1, aac6'-II, cmlA, aph6'-1d, CMY-2, MFS, tetB, aac6'-1a	2015	*Escherichia coli*

Table 3: *Large number of mdr genes accumulation in MDR conjugative plasmids of superbugs.*

As depicted in figure 5A and 5B, *Escherichia coli* KC-1_mdr and KT-1_mdr are only imipenem susceptible other 7 antibiotics (ampicillin, vancomycin, ciprofloxacin, amikacin, tetracycline, azithromycin) have no effect and linezolid is partially susceptible. Later, we have also demonstrated the *tet, acr, amp, aac, cat* genes in many *mdr* plasmids isolated from Ganga River water as demonstrated in figure 6. But we need more work to confirm the rest of the genes and full length sequencing of MDR plasmid from such bacteria is demanding. In truth, it is very hard to get large quantity of pure plasmid from such bacteria due to multiple large plasmids are present with low copy number and such plasmids are heterogeneous [8]. Many *mdr* bacteria were detected in Ganga River water and such bacteria were resistant to frequently used old drugs like streptomycin, ciprofloxacin and tetracycline (Figure 6).

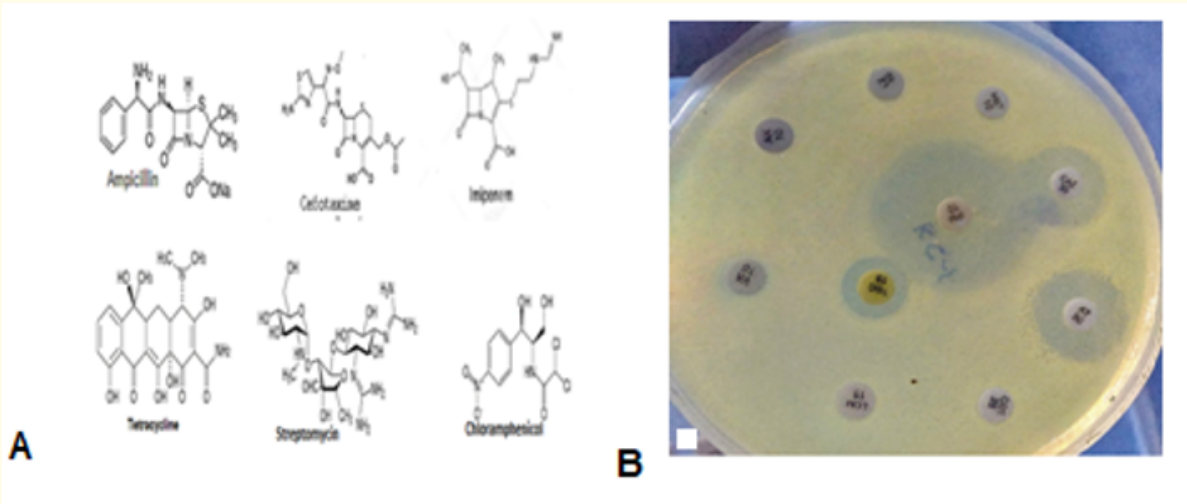

Figure 5: *Structures of useless antibiotics (A) and assay of multi-resistance using a Ganga River superbug (B).*

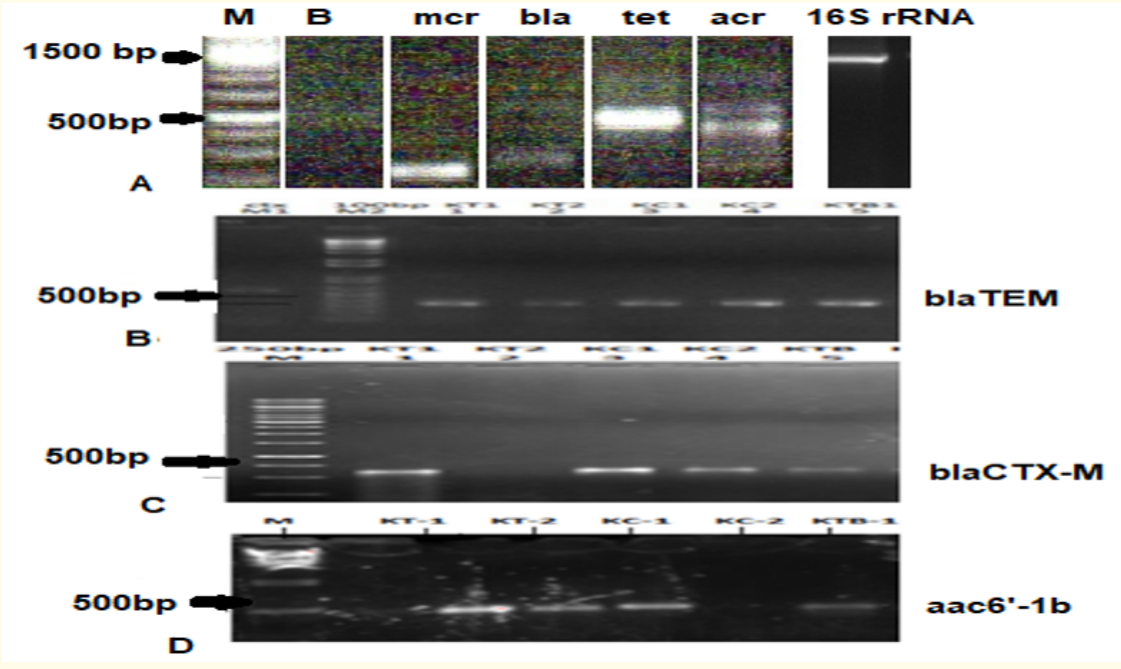

Figure 6: *SPCR detection and analysis of mdr genes in plasmids isolated Ganga River superbugs like Escherichia coli KT-1_mdr and KC-1_mdr strains. (A) superbug drug efflux genes assay, (B) blaTEM assay, (C) blaCTX-M assay and (D) aac6'-1b assay [13].*

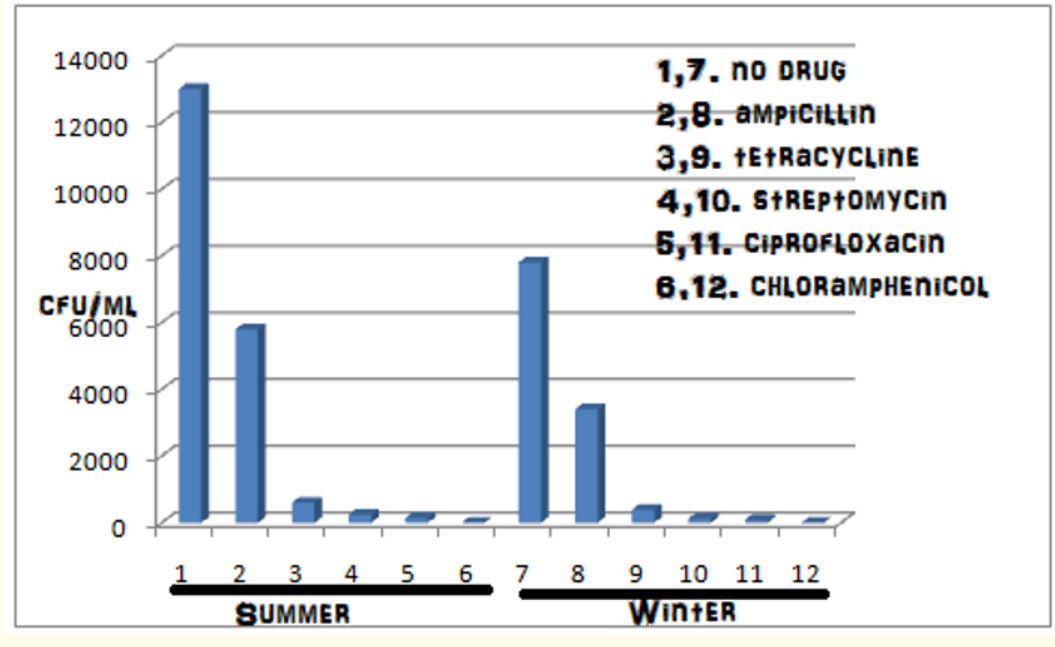

Figure 7: *Drug resistant bacterial load in Ganga River water during summer and winter.*

Conclusion

In India, Ganga River (2525KM) from the hills to the Bay of Bengal carry municipal sewage from large urban centres, trade effluents from industries and polluting waste from several other non-point sources resulting in its pollution [13]. The Ganga basin lies between East longitudes 73°02' - 89°05' and North latitudes of 21°06' - 31°21', covering an area of 1,086,000 sq km with many big cities located. Indian First Ganga Action Plan have reduced polluted waste in 25 class-I cities of India. The pollution level in the holy Ganga at Kumbh (Sangam) was estimated as 7.4 mg/L BOD during Kumbh Mela at the historical city of Allahabad on January 14, 2013. In truth, the bathing water BOD limit is 3 mg/L indicating Ganga River water is not suitable for bath but millions foreign visitors take bath in that occasion. The major contributors of pollution are tanneries, distilleries, paper mills and sugar mills. The Government announced the "Mission Clean Ganga" project on 31st December, 2009 with the objective that by 2020, no municipal sewage and industrial waste would be released in the river without treatment, with the total budget of around Rs.150,000 million rupees [18,19]. So more mega industries and more pollution and how many plasmids have to be made bacteria to live in so polluted water! Drug void and AMR is the problem of world as small or big, poor or rich, MDR spores are in air particulates (PM10) and will drop with rain everywhere during monsoon and winter. We found 40% of sea, river and rain water bacteria are ampicillin and amoxicillin resistant and studies indicated that AMR is progressively increasing (Figure 7). Eventually, due to surface water shortage, more and more people will move to Ganga River link and obviously during 2050, Ganga will be more polluted with more deadly bacteria like blaKPC2 and blaNDM1 *Escherichia coli*. We have assayed the village ponds water and found the drug-resistant bacteria every pond and proved rain water carry MDR bacteria.

We use million tons antibiotics per year by 7000 million peoples and many un-used drugs are thrown into sea and river sites contaminating water with drugs (ng-µg label). It was reported that blaAmp-C gene is induced by low concentration of penicillin drugs and tetO induction is a well known method of expression vector design [14]. In my opinion, expression of proteins in bacteria use many *mdr* genes as selectable marker may not be good practice. Much pollution for making and using cars, televisions, chemicals, paints, detergents and steel. Gut bacteria were terribly intoxicated and thus creation of MDR genes and antibiotics void are our dark side of science. Director General of WHO and UNDP and also Pharmaceutical Scientists are sure that 3 - 4% GDP reduction and poverty may be eminent between 2040 - 2050 if we could not able to discover alternate to antibiotics. In truth, 1940 - 2019 or 80 years we did too much nonsense use of antibiotics [8]. Gut bacteria synthesize 20 vitamins (nicotine, thiamine, folate, biotin, riboflavin etc) and other complex signalling molecules (LPS) and also nutrients (butyrate) for intestinal cells those are in tight symbiotic control synthesizing interleukins and cytokines (Figure 2). Save your soul works and *mdr* genes are created in plasmids and integrons. But that is not all, in India, Africa, Latin America; we still prescribe ampicillin and tetracycline to patients as well chickens, pigs, and animals, and also into agricultural land [20,21]. We absolutely need combined education in school for mandatory mathematics, life science techniques, civic laws and physiology. We see phage therapy, enzybiotics, nano-technology, gene-therapy and gene medicines are emerging as profitable pharmaceuticals but question remains as faithful remedies for the control of superbugs. MDR genes are an example of man made genocide but it is not bacteria, all life forms are mutated [10]. We need more funding and faithful research to know the calamity. We are using heterogeneous phyto-antibiotics to kill MDR bacteria and in my opinion poor countries should try such ancient therapy as depicted in Sanskrit books of India like Charaka Samhita and Atharva Veda [22,23]. How many peoples of these Earth can get newly antibody-drugs against cancer which cost $1000 - 2000 per dose? Bacteria are in symbiosis with plants who secrete anti-metabolites against bacteria. Thus, heterogeneous phyto-antibiotics from Indian medicinal plants may be ideal drug against superbugs [24]. *mdr* genes in expression plasmids must be regulated otherwise recombinant microbes will be contaminated in the environment. China Coronavirus outbreaks may be similar one as postulated in the Facebook and Newspaper. Creation of Oncogenic Harvery Sarcoma Retrovirus (HaSV) and Rous Sarcoma Retrovirus (RSV) with *ras* and *src* proto-oncogenes has given us similar threat due to overwhelming use of carcinogens in rat, mouse and chicken [25]. Thus, Recombinant DNA Technology should not be in the curriculum of Under Graduate Science students who do not know ampicillin resistant gene (*amp, bla*) are serious threat if escape into environment. I also see every drug resistant gene is available from companies as expression vectors and I am sure their escape into environment will be happening during bacterial transformation and isolation of plasmids for molecular examination [26]. Thus, we are increasing AMR and world leaders must act now.

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