Review

Genomic Tools for the Impact Assessment of '*Hotspots*' for Early Warning of MDR Threats*

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INTRODUCTION

Antimicrobial resistance (AMR) has been a low-priority area of research in most developing countries even though it has much significant therapeutic value. The management of serious infections should include not only the treatment of patients but also the measures to ensure that microorganisms do not spread through hospital premises. Controlling the transfer of organisms among staff, patients, and the environment is important. Research related to antimicrobial use and resistance, regional variation, and intervention policies, according to the current health care conditions of every country, can be very challenging. Several antibiotics are currently available in global markets and are valuable therapeutics. The quality of antibiotics available has to fulfill pharmacopoeial requirements, and many of them have been subjected to microbial bioassays to calculate the potency[1-2]. Each microbial bioassay is based on measuring a zone of inhibition; the same strategy is used to detect antimicrobial resistance. Several studies have been completed on antimicrobial drug resistance by using the disk susceptibility method[3]; however, only limited data are available regarding the genomic detection of multi-drug resistance (MDR) related to microbial diversity. A study was also performed to assess the relationship between antimicrobial use and resistance in a hospital in Taiwan China using disk susceptibility testing on *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia, Pseudomonas aeruginosa*, *Serratia marcescens*, *Proteus* spp., *Escherichia coli*, *Acinetobacter* spp., *Enterobacter cloacae*, and other non-fermentative gram-negative bacilli that cause nosocomial infections[4-5]. Community-based studies have revealed a significant and diverse spectrum of resistance nationally, whereas there are only a limited number of hospital-based studies at both the regional and national levels.

Several culture-based and other (metagenomic) genomic tools are available to explore microbial diversity for the surveillance of MDR as an environmental threat. However, advances in genomic technology now offer a new approach for environmental monitoring and early assessment threats related to MDR. Sequencing and/or fingerprinting of microbial populations provide complete snapshots and the functional composition of microbial communities, unlike conventional analyses. Gene abundance, detected through qPCR or metagenomic/genomic markers, has been used for quantitative microbial risk assessment in fecal-contaminated recreational waters[6]. A similar approach for the detection of MDR and contamination in the environment could be applied for impact assessment. Hospitals with unacceptable environmental sanitization serve as primary '*Hotspots*' for antibiotic resistant microorganism, which contain genes that can be transferred to different niches.

Monitoring these MDR determinants is an uncommon and partially accepted practice. Furthermore, surveillance efforts and their details have not been linked to public health. The development and use of antibiotics has been among the most prominent of global health successes; however, owing to their overuse and misuse, selective pressure that drives bacterial genomic evolution has been enforced, which could lead to the development of multi-drug resistant bacteria. The release of selective pressure using appropriate antibiotics and proper sanitation within hospital premises could help to prevent MDR in microbial contamination[7]. Furthermore, improving the structure and function of public health care delivery systems and increasing the awareness of, MDR would help in controlling hospital-based infections. Genomic tools are highly important in the early identification of MDR-related threats. DNA sequencing, metagenomics, fingerprinting/

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genotyping, and probe hybridization tools can be used to assay entire microbial populations for MDR genes, which is not possible with conventional techniques[8-9]. In this review, we discuss the various genomic tools for exploring MDR contamination in environments and the early detection of threats. The emergence and spread of MDR at different '*Hotspot*' ecosystems is also discussed.

Problem Burden: Global and Indian Scenario

Antibiotic use has increased considerably in recent years, as this market experienced a sharp increase of 40% between 2005 and 2009[10]. Globally, it is predicted that 5%-10% of patients visiting hospitals develop an infection during their stay[11]. It was observed that methicillin-resistant *Staphylococcus aureus* (MRSA) alone infects approximately 94,000 people and kills almost 19,000 in the US each year[12]. Despite the problem of antimicrobial resistance, it is very difficult to obtain information regarding the diversity and distribution of MDR-associated microorganisms and genes, especially with respect to uncultivable bacteria. Inappropriate and excess use of antibiotics and the extraordinary genetic capacities of microorganisms are the main source of MDR development[13]. However, this issue has remained unknown due to the lack of routine nationwide surveillance and the unavailability of information in the public domain. Currently, such research and surveillance have become the main agenda of several global and national organizations[14]. The Indian Council of Medical Research (ICMR) as well as some private agencies has undertaken initiatives on a pilot scale for AMR surveillance. Likewise, The World Health Organization (WHO) performed an MDR surveillance study in a few cities in India using indicator microbes[15]. The UK Clinical Research Collaboration (UKCRC) is working on a '*Translational Infections Research Initiative*' with a £16.5 million partnership with another organization to perform research relevant to MDR and infection control, between 2008 and 2015. The Health Innovation Challenge Fund, and the Joint Department of Health and Welcome Trust Fund are also providing £2.8 million over 3 years to perform research using genomic tools that could help to improve local outbreak recognition and national surveillance. Currently, a number of agencies are funding research related to multidrug resistant microorganisms, which includes the UK-CRC '*Translational Infections Research Initiative*', the Health Innovation Challenge Fund, the NIHR Healthcare Associated Infection & AMR unit, and the Biotechnology and Biological Sciences Research Council (BBSRC), among others. Furthermore, fundamental research is also in progress through the Medical Research Council (MRC), Centre for Molecular Bacteriology and Infection, with a unique focus on the study of pathogenic bacteria, to understand the emergence and spread of MDR through genomic techniques[16].

In the absence of a monitoring organization on national and international levels, information on multi-AMR is unknown except for that regarding *Mycobacterium tuberculosis* and *Leishmania donovani*[17]. The infectious disease load in India is among the highest in the world; a recent report explained that improper and irrational use of antibiotics combined with poor sanitation are reasons for the easy transmission and acquisition of AMR. The most significant threat is posed by gram-negative microorganisms responsible for nosocomial sepsis in neonates and intensive care units (ICUs). Surprisingly, few members of *Acinetobacter* spp. have been observed to have high resistance to even the newly introduced tigecycline antibiotic. Gene mutation and gene transfer mechanisms are the major factors for dissemination of MDR-associated threats throughout environmental ecosystems. Currently, MDR has been observed in several environmental samples, and could cause a major threat to public health. Environmental samples were studied for the presence of the *NDM-1* (New Delhi metallo-*β-lactamase*-1) gene in seepage and drinking water samples in New Delhi. Twenty strains of bacteria were observed to contain the *NDM-1* gene in 51 of 171 seepage samples and 2 of 50 tap water samples[18]. Proliferation of multidrug-resistant *NDM* genes in a municipal sewage treatment plant in Northern China was also described. It was mentioned that river sediment did not contain *NDM-1* genes at detectable levels, whereas NDM-1-containing *Achromobacter* spp., isolated from wastewater treatment plants (WWTPs), were shown to transfer the gene to native wild *Comamonas* spp. Propagation of *NDM-1* genes through effluent and sludge of WWTPs highlights the need to better understand this issue to mitigate this transfer[19]. The important concern regarding the release of antimicrobials into the environment is the emergence and spread of AMR in various ecosystems. Information regarding the effects of antimicrobials on environmental bacteria is limited

and contradictory with reference to microbial resistance[20]. One published study states that urban WWTPs are reservoirs for antimicrobial resistant genes (AMRGs) and microbes that pose health risks to humans and animals[21-22]. Furthermore, extensive characterization of methicillin-resistant *Staphylococcus aureus* isolated from poultry food products in Germany showed that 37.2% of samples were positive[23]. Detection of the new *vga(E)* gene in methicillin-resistant *Staphylococcus aureus* isolated from poultry and cattle has also been previously reported[24]. Furthermore, the study of virulence factors and antimicrobial susceptibility in *S. aureus* using 360 fresh raw chicken samples has also been previously completed. Results showed that resistance to tetracycline was the most prevalent (97.56%), followed by methicillin (75.60%), sulfamethoxazol (31.70%), trimethoprim (31.70%), streptomycin (31.70%), gentamicin (29.26%), enrofloxacin (28.04%), ampicillin (26.82%), chloramphenicol (20.73%), and cephalothin (17.07%)[25].

Origin of MDR Markers: Do We Really Know?

MDR genes are mostly present on plasmids and transferred within different bacterial species through various mechanisms. The issue became apparent when NDM-1 was first reported in 2009 and made front-page news in 2010. NDM-1 is an enzyme produced by the gene *blaNDM-1* and found in *Enterobacteriaceae* isolated from hospitals[26]. Furthermore, one study reported that the *NDM-1* gene had been detected in seepage water and drinking water[18], increasing its concern and focus worldwide. The first death due to an NDM-1-producing microorganism was reported in Belgium despite broad-spectrum colistin antibiotic treatment[27]. However, the exact origin of MDR in bacteria with respect to geographical region requires further scientific studies and discussion. One prestigious journal has also proposed changing the name NDM-1 to plasmid-encoding carbapenem-resistant metallo-*β*-*Lactamase* (PCM)[28].

In addition, several modified functional genes have been observed in an environment, which could be developed due to mutations resulting from the positive selective pressure of a particular compound or gene transfer mechanism. Functional genes are mainly responsible for particular activities observed in different geographical areas[29], and the same genes might be detected in other parts of the world. Thus, it is very difficult to authenticate the origin of specific microbial genes with respect to geographical area due to their extraordinary genetic capabilities.

'Hotspots' of MDR Contamination

Several '*Hotspots*' have been identified that play a significant role in the development, breeding, and spreading of MDR-associated bacteria in different ecosystems. Hospitals are the primary source for MDR development, and wastewater is a culprit for its breeding and spreading. Figure 1 depicts the flow of MDR and microorganisms within and outside the *'Hotspots'*. MDR travels through various '*Hotspots*' and enters the food web affecting public health. Tourists also play a key role in spreading MDR; however, the exact origin of MDR in prokaryotes, with reference to geographical area, is unknown. A few important '*Hotspots*' that play significant roles in the development, breeding, and spreading of MDR in various environmental niches are described in detail.

I 'Hotspot' of MDR: Hospital Environments as a Primary Source Various antimicrobials are commonly available and used in hospitals; these might considerably help in developing reservoirs of MDR. The primary '*hotspots*' generally are considered hospital-associated routes with significant numbers of AMR/MDR bacteria and gene pools[7]. Globally, nosocomial infections develop among 7%-12% of hospitalized patients with over 1.4 million people suffering from infections acquired in the hospital; this can be prevented through improved hygiene. Staphylococcus aureus and Pseudomonas aeruginosa are among the most common causes of hospital-acquired infections). A study was performed by the International Nosocomial Infection Control Consortium and observed that 46% of acquired infections were with Enterobacteriaceae and 27% with *Pseudomonas* spp.; this was followed by 6% *Acinetobacter* spp., 8% *Candida* spp., and 3% *S. aureus*[30]. The intensive and alarming spread of gene OXA-23 carbapenemase through *A. baumannii* in Egyptian intensive care units was previously investigated. The spread of such bacteria has critical health consequences and requires the use of harsh infection control actions[31]. Furthermore, nosocomial infections caused by carbapenem-resistant *Acinetobacter* spp. that were isolated from clinical samples from three different hospitals in western Algeria were studied using molecular epidemiology from 2008 to 2012. For clinical isolates recovered among the 113 isolates, 70.8% were found to be imipenem-resistant and a

blaOXA-23-like gene was detected in 50% of cases. However, a metallo-b-lactamase blaNDM-1-like gene was detected in 6.2% of isolates and a blaOXA-24-like gene in 21.2%[32].

The mechanisms that influence the development and transfer of MDR-associated genes and bacteria in complex environmental ecosystems are still poorly understood. However, several authors have suggested that hospital premises are the primary '*Hotspots*' for MDR-associated genes and microorganism, from where they can relocate to different environmental niches such as water, wastewater, rivers, marine ecosystems, and soil, among others[20,22,33]. In earlier studies, 547 cultures were isolated from Egyptian intensive care units. Among them *Klebsiella* spp. along with *A. baumannii* was the most prevalent species. Although *A. baumannii* represented only 10% of the total isolates, it exhibited the maximum rate of carbapenem resistance (74%). PCR results demonstrated that all antimicrobial resistant isolates carried both the *blaOXA-23* and *blaOXA-51* genes along with 85% class 1 integrase and only 2.5% metallo-beta-lactamase (*blaVIM*) genes. They also suggested that isolates from different hospitals were genetically linked to each other based on ERIC-PCR results[31]. Similarly, microbial cultures were isolated from culture plates containing meropenem spread with hospital sewage and screened for *blaNDM* genes by PCR[34]. A different *Acinetobacter* strain, from several locations of the hospital, was found to harbor *blaNDM-1*; this strain could also spread to various environmental niches[5]. These results indicate that the hospital sewage in China contains significant amounts of the *blaNDM-1* gene, suggesting that hospital sewage is an important but often overlooked reservoir of MDR-associated bacteria.

II 'Hotspot' of MDR: Wastewater Breeding System

Antibiotic resistant bacteria and genes are often observed in wastewater, allowing them to spread throughout the environment[35]. The discharge of untreated sewage and effluents or sludge from sewage treatment plants has been suggested to spread MDR determinants in natural aquatic systems, which could create significant public health issues[22]. The dumping of antimicrobials into the environment through sewer lines and/or premises without complete disposal is a common practice within hospitals in developing countries. The abundance of the ampicillin resistance gene (*ampC*) in 100 ng of total DNA was found to increase in wastewater samples receiving hospital effluents[36]. Earlier it was discussed that the detection of vancomycin and imipenem resistance genes is linked to hospital wastewater[36]. Moreover, multi-resistant *P. aeruginosa* was more frequently found in hospital wastewater, compared to domestic waste, indicating that hospitals are one of the '*Hotspots*' for breeding antibiotic resistant bacteria and genes[37]. Guo, et al., (2011)[38] also suggested that hospital wastewater represents an important source of multi-drug resistant genes (MDRGs) and the highly prevalent, antibiotic resistant *Enterobacteriaceae*.

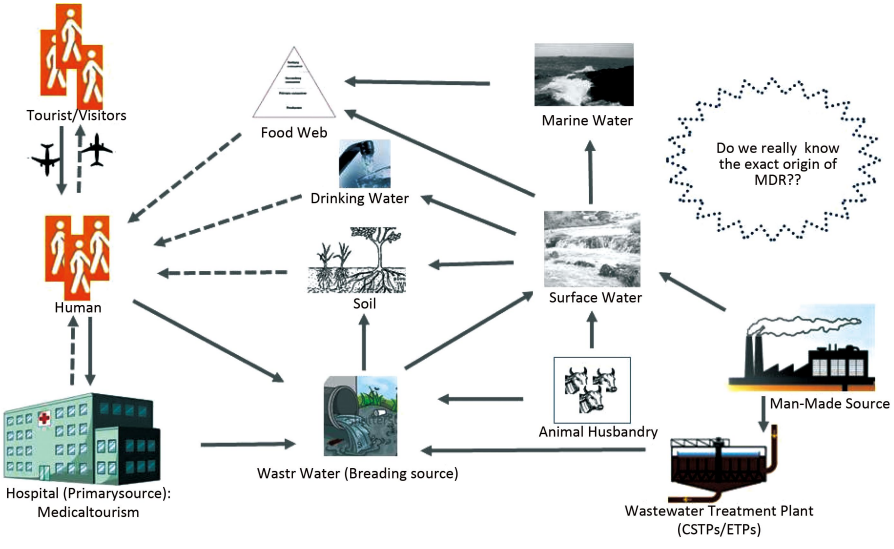

Figure 1. Flow of MDRGs and bugs pollution within and outside the hotspots.

WWTPs represent one niche that is believed to be a significant '*Hotspot*' for gene transfer, due to the high cell density maintained by nutrient-rich environmental conditions and the repeated pollution by both antibiotics and resistant bacterial populations[22]. Mobile elements such as plasmid-based resistance genes have been isolated and detected frequently in wastewater or activated sludge[22]; however, their direct participation in gene transfer remains to be established, due to the complex nature of the environment. Understanding the multiplication and spreading of these resistance genes through various wastewater treatment systems is critical to control the MDR threat. Several resistant bacteria and genes have been previously detected in wastewater systems[39]. *NDM-1* genes were more frequently observed in *Acinetobacter lwoffii* isolated from chickens in eastern China[40] and from tap water samples from New Delhi[18]. Recently, the *NDM-1* gene was noted in a hospital sewage sample[34]; however, there are limited or no significant data on the propagation of the *NDM-1* gene and its fate in environmental ecosystems[19]. The expansion of *NDM-1* genes in a municipal sewage treatment plant in Northern China has been previously reported[19]. Results suggested that a significant amount of the *NDM-1* gene was present in the effluent discharged from both WWTPs. In addition, *NDM-1* genes were observed at a much higher concentration in dewatered sludge, which has elevated the possibility of transfer into the indigenous microbial population. The biological treatment process provides an environment that is potentially appropriate for the spread of AMR, due to the continuous mixing of sub-inhibitory concentrations of antibiotics with the natural microbial populations[22].

MDR have been detected in several WWTPs in developed and developing countries, suggesting that it is a main source for the propagation of MDRGs and associated bacteria. Recently, two WWTPs in Morges and Visp, Switzerland were explored for the community-wide exchange of plasmid gene mobilization and selection[41]. The exchange of genetic material within the diverse and dense microbial populations of WWTPs was also previously demonstrated by plasmid replicon and contig analyses. Sentchilo, et al., (2013)[41] performed the sequencing through the combination of Sanger and 454 sequencing and assembled the subset of contigs from samples, from the WWTP in Morges, Switzerland, with a minimum of five-fold coverage consisting of 649 contigs covering 2,205,428 bp in total. Results indicated that the smaller replicon (M02_C11) was 1633 bp and encoded only a single *repA* plasmid replication protein, and the larger assembled replicon was 59,231 bp (M02_c11697). The increased sequence coverage of bacteriophage genomes indicates that they were highly represented in the WWTP. They also compared the metagenomic plasmid replicon analysis data from the two (Morges and Visp; Switzerland) WWTPs and suggested that the plasmid replicons were highly enriched in heavy metal and antibiotic resistance-associated proteins.

III 'Hotspot' of MDR: Gut Ecosystem Most human-associated microbes reside in the gut and consist of 800-1000 different bacterial species that are vital for life. However, approximately 80% of the gut microflora has not been cultured[42]. Many studies have demonstrated a diverse array of resistance genes in the human gut microflora, and have suggested that microbial communities change with exposure to antimicrobials[43]. The presence of resistance-encoding integrons in the gut flora of isolated individuals who are not exposed to antibiotic therapies was reported earlier[44]. Similarly, Sommer, et al., (2009)[43] studied fecal samples to examine the load and distribution of antibiotic resistance factors in gut microbial populations. DNA was extracted directly from the gut bacterial population of an individual who had not been given antibiotics for a year and was inserted into *E. coli* via expression vectors. The modified bacterial cells were cultured on antibiotic plates containing all thirteen drugs, and sequencing was performed to test for resistance factors. The results suggested that new resistance inserts were present in all samples and phylogenetic analysis showed that the most probable origin of antibiotic resistance factors was *Bacteroides* and Firmicutes, which dominate the gut microflora.

The human gut contains trillions of microorganisms comprising a broad range of bacterial species that influence public health. Gut microbial populations contain mostly bacteria and bacteriophages. It is known that bacteriophages can play a key role in human health by destroying pathogens in the same manner as antibiotics. It is expected that the human gut contains 10^{15} phages and thus could be one of the most densely populated ecological niches in nature[45]. It is the most diverse and abundant reservoir for phage communities, and varies depending on food habitat,

age, and health condition. A recent murine model of mimic revealed the human gut microbial population and temperate phage dynamic and observed the transient changes in the microbial community structure due to phage attacks. The extent of phage-microbial gene exchange was enhanced by environmental stress, and led to the emergence of antibiotic resistance in gut microbial populations[45]. The presence of prophages is assumed to give adaptive advantages to microbes such as an *Enterococcus faecalis* strain by enhancing amino acid availability with prophage induction. This allows the microbes to be more competitive in complex intestinal microbial communities and to maintain dominance over metabolically similar strains[45].

The gut microflora is often exposed to a variety of antibiotics both directly and indirectly. The human gut microflora warrants special attention due to its high microbial density and high accessibility[46], factors that could aid in promoting resistant bacteria. Xenobiotic-responsive genes across complex active gut microbial populations, drug metabolism, antibiotic resistance, and stress response pathways have been previously studied[46]. Results suggested that short-term exposure to antibiotics significantly affects the physiology, gene expression, and structure of the active gut microbial population, which ultimately alters host metabolic activity. Hence, attempts at personalized medicine should be considered due to variations in the active human gut microbial populations among different individuals, in the interest of better public health.

The gastrointestinal tract is continually exposed to several bacteria from the environment and often harbors antimicrobial drug resistance genes, which can be transferred to the native microbial population through horizontal gene transfer (HGT), which could enrich the AMR gene pool. The human gastrointestinal microflora is expected to be a '*Hotspot*' for the spread of antibiotic resistance[42-43]. The ampicillin resistant gene (*blaTEM*) has been detected in the human microflora by several authors[42-43]. Likewise, Sommer, et al., (2009)[43] also studied the AMR reservoir in the gut and the oral microflora of healthy humans. A variety of resistance genes were observed through culture-independent tools. In addition, most genes from the cultivable isolates were closely related to resistance genes from pathogenic isolates, showing an evolutionarily close relationship between clinical pathogens and resistance genes. Similarly, they identified a *blaTEM-1* gene in the gut microflora that has been detected in pathogenic strains of *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens, Neisseria meningitidis,* and *Haemophilus parainfluenzae* isolated around the world[43,47]. Furthermore, a study has suggested an increasing abundance of *blaCTX-M*, *qnrB*, and *qnrS* genes in the feces of healthy volunteers in the Netherlands immediately after returning from international travel. These findings contribute to the growing evidence that travelers might play key role in the spread of AMR[42]; however, the exact origin of AMR with respect to the geographical area needs to be discussed.

IV 'Hotspot' of MDR: Manufactured Obvious Disasters Over the last two decades, increasing amounts of antimicrobials have been manufactured, used, and widely disseminated into the environment as a common practice. This provides constant selection and maintenance pressure on natural dynamic microbial populations in the environment. Obtaining an accurate measure of the quantity of antimicrobials manufactured is difficult, but it has been estimated that millions of tons of antibiotics have been released into the environment over the last decade[44]. The dumping of excessive amounts of ciprofloxacin into rivers by pharmaceutical manufacturers in Hyderabad (central India)[44] has been extremely horrific. Similar levels of pollution cannot be denied elsewhere in the world. Available antimicrobials in environmental niches can promote the development of resistance genes among natural microbial populations, which helps to develop superbugs. The term 'superbugs' refers to microbes with multiple enhanced mutations, endowing high levels of resistance to many antimicrobials. Resistant superbugs physiologically damage local native populations of flora and fauna and can lead to an imbalance in the natural functions of ecosystems.

Several human-associated activities aid in the development, breeding, and spreading of AMR in the environment. Animal husbandry and urban activities have a severe impact on the Jiulong river water in China[48]. A study of 16 water samples, collected and analyzed for various AMRGs, suggested that the swine industry and urban activities might be responsible for the high concentrations and relative abundance of AMRGs in river water. Similarly, the prevalence and mechanisms of lincosamide and macrolide resistance in 72 *Staphylococcus aureus* isolates from cows with clinical mastitis in China has been reported[49]. These isolates were confirmed to

have high levels of resistance to azithromycin (93.1%), erythromycin (93.1%), tylosin (40.3%), spiramycin (41.7%), clindamycin (36.1%), and tilmicosin (27.8%).

HGT: Emerging MDR in Various Environmental Niches

Plasmids have been documented to be a vital driver of DNA exchange and genetic change in prokaryotes due to their independent replication and frequent self-transfer. Plasmids can accumulate, distribute, and rearrange non-essential genes, and can offer an advantage for proliferation under selective pressure. WWTPs might be the largest open and uncontrolled microbial *'hotspot'* for HGT due to their high species diversity and density. Plasmids of microbial communities in WWTPs are expected to select for necessary functions, dependent on compositions and operating conditions[41]. Inappropriate use of antimicrobials promotes resistance among microorganisms; however, spreading and propagation is among the most dangerous and critical parameters. Reservoirs of antibiotic resistance can accumulate through the mutation of existing genes (vertical evolution), through the acquisition of new genes via HGT, and/or from direct uptake from the environment. Among them, HGT involves disseminating AMR within diverse microbial populations. Mutation-driven resistance mostly occurs during antibiotic treatment; HGT requires contact between recipient and donor microorganisms for the transfer of resistance. This process can occur via three main mechanisms including transformation, transduction, and conjugation, and is frequently observed in prokaryotes. This aids in the propagation of MDR in natural dynamic microbial populations and play a significant role in their evolution. Many antibiotic resistance genes are carried on plasmids, phages, transposons, or integrons that can act as vectors for transfer to other members of the same bacterial species, and/or to different genera or species.

MDR has emerged as a medical problem due to resistance genes being passed on horizontally via conjugation to other bacteria that are more pathogenic than the original plasmid host. The sharing of genes among bacteria through HGT takes place through mobile genetic elements; in a few cases, the presence of a small amount of antimicrobials in the environment is a critical signal that promotes gene transfer. HGT plays a major role in the development and spread of resistance among bacteria both on environment and community levels. Recent conjugation experiments with *Achromobacter* spp. isolated from native bacterial populations in Haihe River sediment and a WWTP were reported to confirm the potential propagation of *NDM-1* genes in these environments[19]. Resistance genes, mostly found on plasmids, are highly unstable throughout their life span, and are subject to a high frequency of HGT in dense microbial populations. Genetic elements exist in genomic islands (GI) of plasmid-free strains responsible for the survival of microbes with antimicrobials in environmental niches. Plasmid-free strains, harboring specific genes, play an important role in research due to their stability in natural conditions.

Resistance genes are often transferred as genomic islands on transmissible plasmids and act as vital sources for spreading resistance determinants. The spread of resistant bacteria from '*Hotspots*' to other environmental areas and subsequently to other geographical locations via different elements has put the public health at risk. However, the exact origin of such antimicrobial genes, with respect to geographical area, continues to be an issue of scientific debate. However, the identification of a *blaNDM-1*-carrying plasmid (pNDM-BJ01/pXBB1) and its variants suggest that a common plasmid has been transferred among diverse bacterial species at different locations[34]. The rapid and widespread dissemination of resistance determinants by HGT, and subsequently the selection for resistance, threatens to mitigate the efficacy of antibiotics. However, vigilant surveillance of emerging AMR in clinical areas, and subsequently in the environment, creates a powerful system to provide knowledge of such threats in advance.

Exploration of Genomic Information to Expose MDR Threats

Culture-based methods can take 24-48 h for rapidly growing bacteria such as *E. coli* or *Salmonella,* but several weeks for slow growing bacteria such as *Mycobacterium tuberculosis.* A tracking tool for MDRGs and bacteria from different '*Hotspots*' is illustrated in Figure 2. Several genetic tools for evaluating antimicrobial resistance are available and many of these are currently being used or will shortly become part of the standard testing procedure in

laboratories. Rapid analysis using appropriate genomic tools can be utilized for the surveillance of MDR-associated threats and could generate information for addressing issues raised by acquired resistance. The identification of imported resistance, carried by organisms, is also very critical. New molecular techniques for the detection of resistance, such as quantitative PCR (qPCR) or microarrays provide results within hours. Information on MDR-associated bacteria and genetic trends in environments can be exploited for the early forecasting of such threats. Sequencing of samples through metagenomic methods, followed by annotation and statistical analysis, provides complete information on diverse microbial populations along with their functional genes and metabolic pathways.

The conventional agar diffusion method is most commonly used for the detection of AMR in microbial populations, but it can be applied to only a limited number of microbes[50]. PCR-based approaches have yielded better insight into the molecular variability within microorganisms. PCR and subsequent amplicon analysis by gel electrophoresis are particularly helpful to identify MDR-encoded genes. The PCR product can be confirmed for desired nucleic acid targets through DNA band patterns after gel electrophoresis, probe hybridization assays, or sequencing. Among several genomic methods, sequence-based approaches are highly reproducible, which is not always the case for gel electrophoresis. A multiplex PCR method to identify the methicillin resistance (*mecA*) gene from isolated staphylococcal cultures has been reported[51]. For multiplex PCR, two different PCR products are generated simultaneously in the same reaction. In one reaction, the *mecA* gene was amplified and in the other reaction a nucleic acid sequence of the 16S rRNA gene, unique to *Staphylococcus*, was amplified[51]. Multiplex PCR assays are relatively simple to perform, are associated with low cost, and can be performed in 6-8 h. The multiplex *mecA* assay is more precise than other methods for detecting the *mecA* gene, particularly in coagulase-negative *Staphylococcus*.

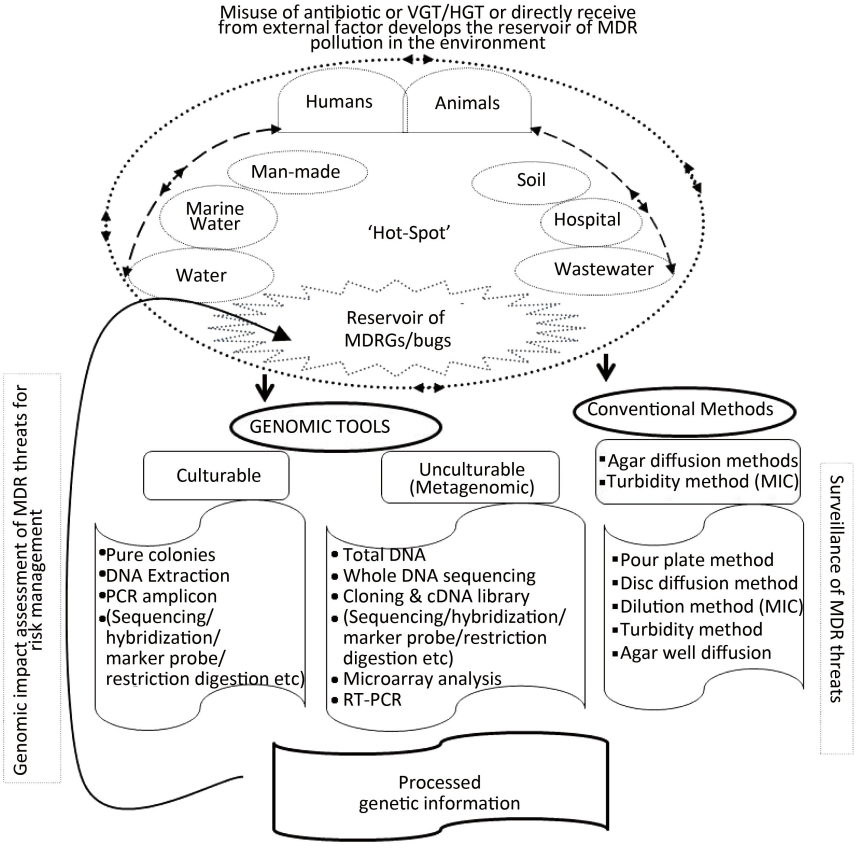

Figure 2. Tracking/analysis tools for MDRGs and hugs pollution in different.

Table 1. Specific Sequence of Primers for the Amplification of Antimicrobial Resistance Genes with its Amplicon Size

Antimicrobial Resistance Genes (AMGRs)	Primer	Amplicon Size (bp)	Ref
Beta-lactam genes			
blaTEM	GAGTATTCAACATTTTCGTACCAATGCTTAATCAGTGA	857	Rosengren et al. 2009.[54]
blaSHV	TCGCCTGTGTATTATCTCCCCGCAGATAAATCACCACAATG	768	Rosengren et al. 2009.[54]
Extended spectrum beta-lactam gene			
blaOXA-51	TAATGCTTTGATCGGCCTTG	353	Poirel et al. 2011.[55]
blaOXA-51	TGGATTGCACTTCATCTTGG		
*bla*OXA-58-like	AAGTATTGGGGCTTGTGCTG	599	Woodfored et al. 2006.[56]
*bla*OXA-58-like	CCCCTCTGCGCTCTACATAC		
blaIMP	TCGTTTGAAGAAGTTAACGG	568	Martin-Lozano et al. 2002.[57]
blaIMP	ATGTAAGTTTCAAGAGTGATGC		
blaSPM	AAAATCTGGGTAGGCAAAGG	271	Zong et al. 2008.[58]
blaSPM	ACATTATCCGCTCGAACACG		
blaOXA-24	CAAATGAGATTTTCAAATGGGATGG	123	Mesli et al. 2013.[32]
	TCCGTCTTGCAAGCTCTTGAT		
	OXA24-probe-FAM- GGTGAGGCAATGGCATTGTCAGCA		
Metallo-β-lactamase			
blaNDM-	ACTCGTCGCAAAGCCCAG	1013	Rasheed et al. 2013.[54]
	CTCATGTTTGAATTCGCCC		
blaNDM-	GGTTTGGCGATCTGGTTTTC	624	Zong et al. 2008.[58]
	CGGAATGGCTCATCACGATC		
NDM-1-533	CTCGCACCGAATGTCTGGC	532	Lou et al. 2014.[19]
	GCGGCGTAGTGCTCAGTGTC		
NDM-1-807	GGAATTGCCCAATATTATGC	806	Lou et al. 2014.[19]
	CGCAGCCCAATATTATGC		
NDM-1	Pre-NDM-A	984	Kaase et al. 2011.[5]
	CACCTCATGTTTGAATTCGCC		
NDM-2	Pre-NDM-B		
	CTCTGTCACATCGAAATCGC		
qPCR NDM 1	CGCCATCCCTGACGATCAAA(NDM-1-214a)		
	CTGAGCACCGCATTAGCCG(NDM-1-214s)		
Methicillin-resistant			
vga(B)	GAATAAGGCGCAAGGAATGA	601	FeBler et al. 2011.[23]
	TAGCTTGGCAAAAGCAACCT		
vga(E)	GAAATATGGGAAATAGAAGATGG	1685	Hauschild et al. 2012.[24]
	TAGATTTGGCAAGATCGAGC		
Aminoglycoside genes			
Aac(3)-IV	GTGTGCTGCTGGTCCACAGC	705	Rosengren et al. 2009.[54]
	AGTTGACCCAGGGCTGTCGC		
Aph (3')-*Ia* (*aphAl*)	ATGGGCTCGCGATAATGTC	700	Rosengren et al. 2009.[54]
	CTCACCGAGGCAGTTCCAT		
aacA-D	AATCCAAGAGCAATAAGGGC	227	Momtaz et al. 2013.[25]
	GCCACACTATCATAACCACTA		

Continued

Antimicrobial Resistance Genes (AMGRs)	Primer	Amplicon Size (bp)	Ref
Tetracycline genes			
tet (*A*)	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG	740	Rosengren et al. 2009.[54]
tet (*C*)	CCTTATCATGCCAGTCTTGC ACTGCCGTTTTTTCGCC	627	Rosengren et al. 2009.[54]
TetK	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360	Momtaz et al. 2013.[25]
Sulfonamide genes			
Sul1	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	547	Rosengren et al. 2009.[54]
Sul2	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	543	Rosengren et al. 2009.[54]
Streptomycin genes			
strA	CCTGGTGATAACGGCAATT CCAATCGCAGATAGAAGGC	546	Rosengren et al. 2009.[54]
strB	ATCGTCAAGGGATTGAAAC GGATCGTAGAACATATTGGC	509	Rosengren et al. 2009.[54]
Macrolide msrA	GGCACAATAAGAGTGTTTAAAGG AAGTTATATCATGAATAGATTGTCCTGTT	940	Momtaz et al. 2013.[25]

The use of restriction fragment length polymorphism (RFLP) analysis, to differentiate genes encoding vancomycin resistance determinants has been reported[52]. This tool is particularly convenient for evaluating vancomycin resistance related to the *vanC* gene group, as this type of resistance is very difficult to identify by conventional antimicrobial susceptibility methods. However, studies must be performed on the identification of *vanB* and *vanC-2* through RFLP analysis, as major DNA sequence variation among these genes has been reported[52]. In addition to these limitations, conserved DNA sequences can be present with vancomycin resistance genes, and hence, additional vancomycin resistance gene could be amplified in the same PCR reaction. Due to this reason, RFLP analysis can be used to differentiate amplicons of vancomycin resistance genes, provided that differential restriction sites are available in the associated genes. In addition, amplified ribosomal DNA restriction analysis (ARDRA) has been used previously to compare the prevalence of AMR profiles of clinical isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. A total of 1381 laboratory isolates were analyzed using 12 different antibiotics to assay the associated antibacterial susceptibility profiles; rates of resistance were found to be 68% for amikacin, 75% for ceftazidime, 68% for meropenem, 67% for ampicillin-sulbactam, 73% for ciprofloxacin, 71% for levofloxacin, 75% for cefepime, 0% for colistin, 75% for gentamicin, 74% for trimethoprim-sulfamethoxazole, 65% for imipenem, and 78% for piperacillin-tazobactam[53]. The amplification of antimicrobial resistance genes using specific primers has been performed previously by several authors as described in Table 1[54-59]. These specific primer sequences could be used further for the detection of various AMR-associated genes.

Sequencing is a more reliable and reproducible method to collect information on AMR in complex environments. Advances in sequencing technologies and bioinformatic tools have given additional importance to this area. Next generation sequencing is affordable ($0.10/Mb) and increases the sequencing depths and read lengths. Furthermore, the availability of bioinformatic analysis tools provides a platform for scientists to access and address regulatory queries. Simultaneously, decreased sequencing costs and increased sequencing depths have also allowed the exploration of more environmental samples, leading to comprehensive profiling of resistant microbes. Metagenomic techniques have emerged over the last few years as vital tools to explore the enormous reservoir of unexplored microbes. Moreover, conventional regulatory monitoring through

culture-based approaches can be extremely time consuming, a fact that has perpetuated the development of simple and adequate alternative techniques for the routine monitoring of resistant bacteria. Furthermore, culture-based techniques can only explore a small fraction of the total microbial population, and require the desired antibiotics as positive controls, which could again contribute to environmental contamination[9,60]. Expertise and initial capital costs for instruments are the major requirements for metagenomic analysis. Furthermore, metagenomic-based approaches have been extended to detect the genetic elements involved in resistance, and to enhance the understanding of microbial resistance patterns and associated gene abundance in dynamic and complex environments.

Insights into Antimicrobial Resistance through Metagenomic Approaches

Metagenomic technology provides in-depth analysis of microbial populations and access to data on the complete diversity of a microbial community, which is not possible through cultivation using petri dishes[61-62]. This approach is also helpful to assess genetic potential and to identify diverse microbial populations, as well as to identify genetic variations responsible for AMR. Metagenomics comprises culture-independent analysis tools using total microbial DNA, and employing approaches based on either expression profiling or sequencing. These can be helpful to detect MDR in different ecosystems and to increase the understanding of such sources. Similarly, AMR determinants carried on bacterial plasmids, viruses, or chromosomes within activated sludge have been identified and studied. Additionally, antimicrobial resistant microbes have been widely identified in water, wastewater, rivers, soil, marine, and agricultural environments[11,63]. An article has been published on the metagenomic screening of bleomycin resistance genes from activated sludge that is used to treat industrial wastewater containing phenolic compounds. In that study, two novel genes were identified[64]. The same approach was used earlier for the identification of novel antibiotic resistance genes including those conferring resistance to β-lactams, tetracycline, aminoglycosides, and bleomycin[64-65]. Furthermore, another study identified a novel tetracycline resistance determinant, *tet37*, through constructing metagenomic clone libraries analysis of the human oral cavity microbiota[65]. Functional metagenomics was also applied to screen AMR-encoding genes from the oral microbiota of 60 adult humans[65]; in addition, tetracycline and amoxicillin resistant clones were identified in each library. Similarly, an investigation showed the presence of different tetracycline and erythromycin resistance genes in human oral and fecal samples through metagenomic investigations in six different European countries[66]. Furthermore, eleven AMRGs including *tet*A, *tet*G, *aacC1*, *strA*, *ermB*, *cmlA5*, *vanA*, *dfrA1*, *sulll*, *blaTEM*, and *blaOXA-1* genes were detected and quantified by real-time PCR in river water[6,48]. Over the next few years, the application of metagenomics will expand to almost every field of life sciences, and especially to the identification of MDR in different environments[11].

Functional metagenomics has been employed to detect genes encoding proteins that inactivate antimicrobials for cultivable communities[67]. This approach only targets known functions and cannot be used easily for broad-spectrum screening. This method ignores potential resistance reservoirs with a higher number of uncultivable microbes, wherein primers are required, and therefore cannot be used for the detection of novel genes. Schmieder and Edwards (2012)[11] have recently functionally verified AMR through metagenomic approaches. Next-generation sequencing has also provided a different direction, wherein a small fraction of DNA is used without the need for cloning. This represents an important alternative to rRNA sequencing for evaluating complex microbial populations in environmental and clinical samples. Next-generation sequencing can also assess the diversity of uncultivable microbial populations, as well as changes in the community that result from antibiotic treatments. Previously, metagenomic sequences, representing resistance genes, were identified using reference databases and screening for particular motifs in the encoded protein sequences[7,31]. It was observed that these motifs and sequences were related to those previously detected in pathogenic bacteria isolated from hospitalized patients with different infections.

Ma, et al., (2014)[68] also applied a newly developed metagenomic method to investigate the diversity and abundance of resistance genes and mobile genetic elements in activated sludge, river water, aquaculture, sediments, anaerobic digestion sludge, and biofilm. Results indicated that the freshwater fishpond sediment had the highest abundance (196 pm), and that anaerobic digestion

sludge possessed the highest diversity (133 subtypes) of ARGs in the studied samples. Also reported was that rifampin resistance genes were detected in all diverse samples and consistently accounted for 26.9%-38.6% of the total annotated ARG sequences. Recent advances in the field of metagenomics offer new visions into the life of uncultured microbes, and could yield applications that result in a better understanding of microbial flora and MDR in '*Hotspot*' areas. However, since very few studies have been performed to explore of MDR gene pools using metagenomic approaches, further studies are required in this area.

Molecular Markers for MDR Detection

In the last decade, biological science has moved in exciting new directions and genomic science is emerging as an important discipline. Biomarkers yield biological outputs (genetic/phenotypic) to monitor processes. Genomic markers can be used to evaluate the relationships between species either by direct comparison or through phylogenetic analysis[29] and for the identification of resistance genes in various niches[69]. The most suitable approach requires synthesizing biomarkers to target reference MDR genes. Several antimicrobial resistance genes can be placed on mobile genetic elements; however, plasmid-mediated transfer has been a focus due to its medical and practical significance. Previously, labeled, specific target genetic fragments were used as biomarkers, and the reactions followed the same basic principles of hybridization[70].

The development of specific genetic markers to detect the abundance of resistance is a critical area of research[69]. Genetic markers have been used in various genomic techniques; among them, the most frequently used and rapid method is RFLP, which is visualized by Southern hybridization using different probes[29,52]. In addition to the DNA sequencing of an entire genome, several other types of tools are useful as acceptable genomic markers for the identification of resistant microbes. Genomic techniques such as single-nucleotide polymorphisms (SNPs), fluorescence in-situ hybridization (FISH), single-strand conformation polymorphism (SSCP), PCR-enzyme linked immunosorbant assays (ELISA), repetitive element palindromic sequence (rep-PCR), microarray analysis, and T-RFLP, among others are available, and can be used for the detection of *AMR*-associated genes in complex microbial populations. The effect of removing antibiotic-associated growth promoters or using alternatives to antibiotics, decreases the abundance of AMR-associated genes or the prevalence of pathogens in bacterial communities associated with poultry litter treated with streptogramin, which was demonstrated using T-RFLP with high throughput sequencing[71]. Similarly, a DNA microarray was also used for the detection of AMR-associated genes among Staphylococcal clinical isolates using specific biomarkers[72]. Furthermore, Di-Cesare, et al., (2012)[73] assessed natural microbial populations for MDR using a rapid and quantitative RT-PCR assay. All of the previously described techniques aid in predicting the exact scenario of AMR in various ecosystems.

The majority of AMR investigations has focused on pathogenic bacteria in clinical settings, despite widespread AMR in the environment[74]. The presence of such targets in these systems might facilitate their propagation in natural microbial communities. The dissemination of these bacteria and genes to the environment is mainly through mobile genetic elements, which could be further transferred to other microbes through HGT. Natural environments including soil, freshwater, wastewater, and marine ecosystems have been shown to harbor an abundance of AMR-associated genes, which could originate from hospital environments[11,44,48,75].

Etiological Agents of MDR

Most studies depend on the assumption that diverse species reside in different niches with specific abiotic environmental parameters. Small changes in abiotic parameters reflect the community structure of the ecosystem. Hospitals represent a significant niche for several microbial pathogens that are associated with epidemics of human disease, and MDR-associated genes can be present in these microbes due to various factors. Serious nosocomial infections caused by *Acinetobacter baumannii*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Burkholderia cepacia*, *E. coli*, *Serratia* spp., *Citrobacter freundii*, *Enterobacter* spp., *Enterococcus faecium*, *Proteus mirabilis*, *Haemophilus influenzae*, *Clostridium difficile*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* spp., *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae* are well known[44,76]. These hospital-associated etiological agents are a major reservoir for MDR. Several researchers have studied AMR-associated genes with respect to etiological agents and how they disseminate in

environments. With the increasing application of interactive genomic tools, it is easy to identify MDR determinants with a significant focus on both the direct and indirect functions of these genes.

Genetic tools are more reliable and rapid for tracing the epidemiological spread of AMR in the environment. Besides phylogenetic DNA markers, antibiotic resistance and virulence genes could also be identified simultaneously[22]. Amplicon band patterns are often used to determine the shifting of microbial communities, with time, in response to various factors including drug drug resistance. Several researchers have studied the value of PCR-SSCP tools for the identification of mutations in extended-spectrum β-lactamase-encoding genes in the Enterobacteriaceae family, and in genes from *Mycobacterium tuberculosis* that are associated with isoniazid, rifampin, ethambutol, and fluoroquinolone resistance[77-78].

MDR in Enterobacteriaceae, due to carbapenemase, has been an increasing important issue and could lead to dangerous handling limitations. Carbapenem resistance, caused mainly by carbapenemase production with the expression of extended-spectrum beta-lactamase (ESBL) or AMPC[17], is currently a critical and important issue. The reliable detection of carbapenemase genes and associated microbes is necessary for identifying outbreaks in the environment. However, carbapenemase detection in Enterobacteriaceae has become a challenge since carbapenemase-producing *Klebsiella* pneumonia have low carbapenem minimum inhibitory concentrations. Phenotypic tests including the modified Hodge test for carbapenemase detection are helpful, but show low sensitivity[79] and specificity for NDM, and therefore demonstrate the need for molecular methods. Multiplex PCR assays for carbapenemase genes have been described by several authors. Kaase, et al., (2012)[51] also investigated 132 clinical Enterobacteriaceae strains from Germany for MDR by multiplex PCR, identifying dominant carbapenemase-encoding genes including *NDM*, *VIM, KPC*, and *OXA-48*. In addition, all strains were compared for the presence of carbapenemases using the modified Hodge test[80] and a multiplex PCR assay, and it was observed that the sensitivity for the detection of *KPC-*, *NDM-*, *OXA-48-*, and VIM-encoding genes was 100% for the PCR assay. Recently, genomic and proteomic patterns of MDR in *Acinetobacter baumannii* at hospitals were also analyzed through bioinformatic tools[81]. Many etiological agents harboring MDRGs have been detected in environments, but continuous surveillance data is not available; such data could help to plan for and control threats.

Hospitals are a significant source of AMR-associated bacteria and genes that can travel to various environmental niches. The major resistant pathogens in hospital-acquired infections are vancomycin resistant *Enterococcus* (VRE), methicillin resistant *Staphylococcus aureus* (MRSA), and ESBL-producing *Klebsiella pneumoniae and E. coli*; in addition to these, there are also pan-drug resistant gram-negative bacteria including *Pseudomonas aeruginosa* and *Acinetobacter baumannii, Stenotrophomonas maltophilia and Burkholderia cepacia*. Similarly, the genera *Aeromonas* and *Acinetobacter* have also been evaluated for MDR, with specific emphasis on resistance to aminoglycosides, beta-lactams, carbapenems, and fluoroquinolones, suggesting that these genera might contribute to the spread, occurrence, and persistence of AMR-associated genes in environments[82].

Several etiological agents harboring MDR-associated genes have been identified in various niches; however, it is very difficult to routinely identify the entire MDR-associated population. Hence, it is better to exploit frequently observed dominant etiological agents that have MDR-associated genes and use them as indicators to routinely test for outbreaks in environmental niches. The World Health Organization has performed an MDR surveillance study in which *E. coli* was used as an indicator organism for high levels of resistance, especially for pathogenic isolates[15]. Results indicated that the rates of resistance were higher for antibiotics associated with prolonged use, and that resistance rates to newer antibiotics such as fluoroquinolones were particularly high in developing countries[15]. The identification of resistance patterns using only a few indicator microbes, through genomic tools, is possible, and such information regarding antimicrobial threats can be used, while conceptually framing a risk management strategy. Environmental audits and risk management of MDR through gene flow information, generated using different genomic tools, is described in Figure 3. Different genomic tools are available for the detection of emerging MDR in environments, and these tools could be help produce significant information. These gene flow informations are

useful for early warning of MDR threats in '*hotspots*', which are the primary source of MDR development and spread. Emerging threats could be control by taking appropriate actions at primary '*hotspots*', to ensure that these agents do not spread to other ecosystems.

Quantitative Assessment of MDR-associated Threats in Various 'Hotspots'

Current genomic approaches offer significant tools to quantify the proper structure of microbial populations[41]. Previously, qPCR was used to track resistance genes and reveal their spread within environments[6,36,48]. The extraction of DNA from whole communities, followed by a qPCR approach with specific primers, is valuable for the detection and quantification of MDR-associated targets including non-cultivable fractions of microbial populations[41]. Available extensive data of bacterial gene pools can be used to design suitable primers and probes for qPCR[48]. Metagenome and transcriptome analyses, using next generation sequencing, create databanks of reference genomes to compare gene sequences and gene activities. The amplification of target genes with known primers can then be performed through qPCR, which can help to quantitate MDR threats.

Previously, qPCR was used to determine the prevalence of eleven ARGs using a pyrosequencer, which was compared to the concentration of antibiotics in WWTPs[83]. AMRGs such as *bla*CTX-*M*, bla*TEM*, *qnrS, erm*(*B*), bla*SHV*, *tet*(*O*), *sul*(*I*), *tet*(*W*), and *sul*(*II*), were quantified in all analyzed sediment and biofilm samples. A significant increase in the relative abundance of ARGs in biofilm samples was observed in this study. Additionally, the detection and estimation of bacterial genera in each wastewater system was tested for MDR gene pools using qPCR. Results also indicated that the WWTP discharge was contributing to the spread of ARGs into the environment, which might affect the native bacterial populations of the recipient river[83]. The genus *Exiguobacterium* dominated the *OTUs* from the biofilms of river samples, appeared in the sediment, and was recently characterized as a carrier of AMR-encoding genes including those conferring resistance to beta-lactams and sulphonamides[84]. The identification and quantification of such dangerous MDR-associated threats within the environment will help at a regional and national level to identify important microbes for the early forecasting of MDR-related threats.

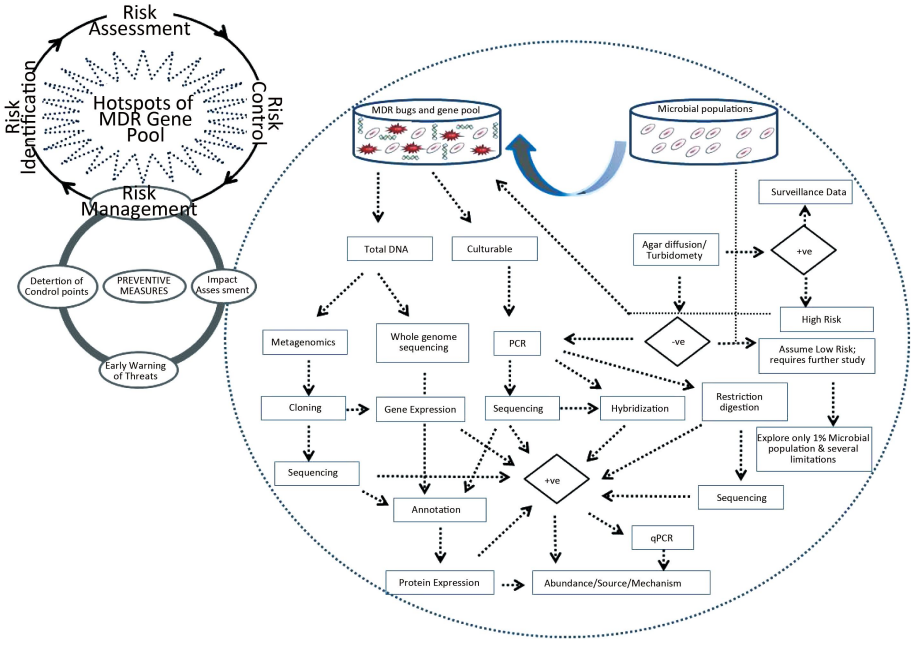

Figure 3. Environmental audit and risk management of MDR pollution with early warning through gene flow informations generated using different genomic tools.

Impact Assessment for Capturing Imperceptive Gene Flow Information

Over the next few years, whole genome and target gene sequencing, markers, probes, DNA fingerprinting, and other genomic technologies will move from research laboratories to widespread use[85]. This will enable the rapid identification of threats with specific reference to MDR. However, current genomic tools are also available for rapidly identifying target genes in dynamic microbial populations. This has provided tools for the reliable assessment of MDR-related threats. The AMR surveillance network (EARS-Net) and the US National Antimicrobial Resistance Monitoring System have studied the use of antibiotics and antibiotic resistant isolates. This has given global magnitude to emerging MDR research.

In the current scenario, many MDR-associated microbes, with relation to several antimicrobials, have been reported. However, studies on changes in the level of resistance to antibiotics such as carbapenem (often being the last option for infections treatment) are crucial for preventive measures. The identification of MDR scenarios in environments with the consideration of entire microbial populations is very time consuming and costly. However, routine surveillance of the more frequently obtained indicator bacterial species will mitigate this problem. Generally, the trend must be tracked by looking at changes in the number of combinations of resistance-associated genes rather than a single gene. In the UK, a five-year AMR strategy for 2013-2018[16] was initiated to identify indicators of etiological agents/microbes with reference to antibiotic resistance and susceptibility. Similarly, frequently observed MDRGs have to be taken into account concerning their growth trends and subsequently need to be monitored using appropriate genomic tools. The data generated through surveillance should be processed using bioinformatics and hits that are obtained can then be used for implementing policies for preventive measures.

MDR-associated environmental pollution continues to intensify. Because of this, a global strategy, along with stable research infrastructure, is urgently needed to develop new antibiotics and track their emerging resistance, as well as implement preventive plans. Due to increased information regarding MDR-associated microorganisms and gene pools, it is now possible to identify the early warning signs of threats, which allows for preparation in a proactive manner[17,35,69,85]. There is a critical need for continued surveillance of antimicrobial use and corresponding resistance at various levels. The sharing of local, regional, national, and global information relating to MDR-related pollution is needed to trigger appropriate preventive measures, which include early warnings, and to limit the spread of AMR. Simultaneously, attempts should be made to optimize the use of antibiotics with suitable treatment guidelines and education for professionals and common individuals.

CONCLUSIONS

AMR is a critical global issue; better understanding of the mechanisms through which resistance evolves, breeds, and spreads is required. The demonstration of MDR in a complex ecosystem remains a difficult task; however, it is essential for adequate risk identification, assessment, and management. In this respect, specific genomic tools have valuable potential for identifying the early warning signs of MDR-associated threats, through modeling and pinpointing the effect of various parameters in the ecosystem. The human gut ecosystem contains trillions of microorganisms that influence public health by metabolizing xenobiotics including antimicrobials. Short-term exposure to antibiotics significantly affects the physiology, gene expression, and structure of the gut microbial population, which could eventually alter host metabolic activity. The gut environment is also decided by the genetic diversity of host, represented by SNPs characteristics. Therefore, overall gut physiology is determined by the interaction between genotype-specific factors of SNPs of the host with gut microbial population, suggesting that personalized medicine is required to address the issue of MDR-associated threats.

Additionally, disinfection processes and advanced treatment are considered possible tools to control the spread of MDRGs and associated bacteria in the environment. However, more systematic studies on the effect of conventional (e.g., UV radiation and chlorination) and novel/alternative disinfection processes for the inactivation of MDR-associated microbes, as well as the capacity to control resistance in environmental ecosystems, are strongly recommended. Similarly, the information on MDR-associated microbes related to sampling sources and sites, major characteristics, and phylogenetic lineages represents a valuable tool to

better understand antibiotic resistance and control measures. The local and worldwide surveillance of highly resistant indicator microbial populations is crucial for the exploration of the prevalence, fate, and transmission of MDR-associated threats in different ecosystems. There is an urgent need to develop and strengthen antimicrobial policies, a national plan for control, and standard treatment guidelines for MDR in different ecosystems. Surveillance information, education, and communication, along with existing public health issues are also important aspects for assessing MDR-related threats.

Moreover, many significant questions have not yet been addressed with reference to the exact origin of MDR at specific locations. We believe that there are unidentified cellular mechanisms that allow for genetic rearrangement under selective environmental pressure that are independent of geographical ecological niches. However to address such important cellular events, further scientific debate and exploration are required.

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