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Microbiology of hospital wastewater

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4.1 Introduction

Wastewater includes any type (e.g., from agriculture, domestic means, industries, human excretion, commercial sectors, pharmaceuticals, healthcare units) of water which quality has been deteriorated under anthropogenic influence [1]. Hospital wastewater (HWW) is quite different from the wastewater discharged from other sources and is hazardous and infectious. It consists of wide range of several micro- and macropollutants, discharged from operation (surgery) rooms, wards, laboratories, laundry, polyclinics, research units, radiology, and medicine and nutrient solutions used in microbiology laboratories [2]. The micro- and macropollutants include radioactive isotopes, pharmaceuticals, stock cultures, heavy metals, media, pathogens, drugs, cotton particles, disinfectants, and chemical compounds [3]. The contraceptive-rich pharmaceuticals present in HWW were reported to be associated with effects of endocrine disruption, for instance, exposure to pharmaceutical waste containing estrogen or androgen caused sex reversals in fishes and thus, reproductive impairment [4]. The hospital effluent discharged directly into the ponds has caused eutrophication, which is evident by oxygen depletion, dense algal blooms, and death of aquatic animals [1]. The discharge of wastewater depends upon the capacity of hospital and generally water varying from 400 to 1200 L/day/bed is consumed by the hospitals [5].

The HWW with many microbes and emerging infectious particles such as prions, viroids, and toxins is hazardous for the environment, and ultimately human health. However, in many countries, HWW is directly discharged into sewage water without pretreatment, it then undergoes treatment along with the municipal wastewater but the treatment could not be sufficient to remove micropollutants from HWW [6]. Moreover, the pharmaceutical compounds present in HWW undergo biological transformation and form conjugate compounds, those toxicity could be even higher than the present metabolite [7]. Thus wastewater pretreatment methods designed for municipal wastewaters are not able to remove the pharmaceutical-related micropollutants or conjugate compounds, which are generally present in low concentration than other macropollutants (chemical oxygen demand, biological oxygen demand, phosphorus, and nitrogen) and thus, could not be removed using conventional wastewater treatment plant (WWTP) operations [8]. Moreover, the pathogens present

in HWW could cause microbial population imbalance in existing and operating municipal WWTP and metals or heavy metals present in the HWW can affect the biological processes like nutrient removal. The heavy metals are in fact nonbiodegradable as compared with other organic pollutants and thus move to other pollutant sources [9].

The HWW can act as an ideal growth medium for various pathogenic microbes including bacteria, viruses, fungi, and parasites. The wastewaters from hospitals also consist of several resistant bacteria and antibiotic residues as well, which could inhibit the growth of susceptible bacteria, thereby increasing the population of resistant bacteria in the receiving water. Resistant bacteria discharged into environment either act as vectors to carry a transmissible gene or as the reservoirs for antibiotic-resistance genes (ARGs) that could pose a threat to public health [10]. In addition, fungi which have capability to grow at faster rate and can spread their spores to external environment also pose a serious threat to environment and human health [11]. The absence of specific pretreatment technologies for HWW also increased the frequency of gastroenteric viruses in aquatic bodies [12]. The direct discharge of HWW into municipal wastewater containing disease-causing parasites has also increased the risk of skin infections and other harmful diseases in humans [13]. The specific and advanced technologies are required to be developed for HWW treatment to prevent the release of harmful contaminants into the environment. Therefore the microbial community analysis of HWW is utmost importance to assess the pollution load and to develop the specific treatment methods for protection of the environmental health. Thus authors explain about the various pathogens present in HWW, ARGs, and tools used to study the ARGs. Moreover, metagenomics, a recent approach used to study different microbial communities and gene-specific identification, are also explained in this chapter. In addition, horizontal gene transfer (HGT) approach, which can efficiently contribute to spreading of ARGs, is discussed briefly.

4.2 Microorganisms and pathogens originating from hospital wastewater

The HWW poses a serious threat to humans with respect to contagiousness and drastic spread of infective diseases in healthcare units, society, hospital employees, and environment [5]. Different solvents, pharmaceuticals, and radionuclides are used in hospitals for purpose of diagnostics, disinfection, and research. After the drug consumption, various drugs remain nonmetabolized or partially assimilate in the human body and thus excrete as such and end up into wastewater. The residual quantities of disinfectants used for treatment of skin microbial infection and to disinfect instruments and surfaces of hospitals, also end up in the HWW, resulting in an increase in the population of pathogenic microbes. The pathogenic microflora present in HWWs also come from medical devices, atmosphere, and water used in the hospital practice, and the pathogens are released mainly in the form of excreta of patients [14]. Therefore HWW consists of a mixture of pathogenic microbes including bacteria, fungi, yeasts, algae, viruses, protozoa, parasites, and bacteriophages. The effluent from hospitals is usually discharged and treated with domestic wastewaters without any prior

treatment [15]. The pathogens in the receiving water, if untreated, survive for a long time in soil or water and enter into the food chain causing infectious diseases and health risks to human beings [16].

4.2.1 Bacteria

Due to the economic reasons and lack of resources for analysis of actual pathogens, certain bacteria have been used as indicators for contamination of water since decades [17] (Box 4.1). However, it has to be considered that removal of coliforms, which is indicator of fecal contamination, could not be directly correlated with removal of viruses, pathogens, fungi, protozoa, or other bacteria from water (samples) [17]. In addition, the indicator bacteria such as *Escherichia coli* and *Clostridium perfringens* when compared with pathogenic bacteria, protozoa and viruses are more sensitive to inactivation through processes such as natural competition, wastewater treatment, and high temperature [18].

Traditional detection of pathogenic bacteria involves selective culture media and biochemical characterization methods. These techniques are inexpensive and simple; however, sampling error, time consumption (5–11 days), tedious, and monospecific detection (detection of only one type of pathogen) are the major limitations [17]. The enzyme-linked immuno sorbent assay (ELISA) is used for laboratory diagnostics of different pathogens. The polymerase chain reaction (PCR) has been adapted in many ways: nested PCR, multiplex PCR, real-time PCR, fluorescence, and digital PCR [19] (Box 4.2) for analysis of waterborne pathogens.

BOX 4.1 Fecal indicator bacteria

Criteria for selection:

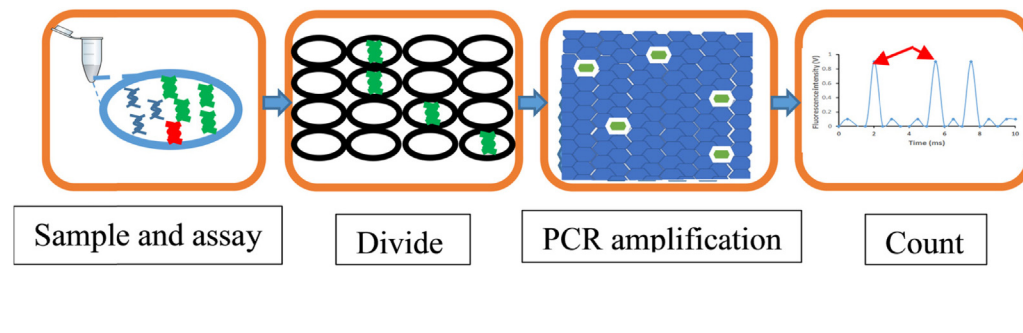
- Should be present only in contaminated water, not in uncontaminated ones
- Should not be able to grow and proliferate in water
- Should be present in intestinal tract of warm blooded animals
- Should have similar survival pattern as pathogens present outside the host
- Should be easily detectable
- Should be useful for all types of water
- Should be relatively cheap to use

Total coliforms are higher in number than any other pathogens. The subgroup fecal coliforms indicate fecal contamination of water and thus, indicate the presence of other pathogens as well, which are lower in number, hard to detect. However, lower number of pathogens are enough to cause morbidity and mortality to humans. They are detectable by inexpensive cultural methods and do not pose any health risk to laboratory workers. However, there are few limitations of fecal indicator bacteria, which have been discussed in the text.

BOX 4.2 Digital PCR (dPCR)

dPCR is a precise quantitative method to measure the nucleic acid. It requires similar assay reagents as used for standard PCR methods, but counts each and every target molecule in a digital format, which enables its wide range applications for high sensitive data analysis with limited sample, accurate next generation sequencing library quantification, improved copy number variation, and allele detection.

Principle: The sample is separated into large number of partitions, so that individual nucleic acid molecules are localized and separated within many separate regions. The different parts will consist of negative (0) or positive (1) reactions and nucleic acids will be counted on the basis of PCR end products (positive reactions). dPCR also varies from conventional PCR as it does not consider copy number based on number of amplification cycles, eliminating the need to rely on uncertain exponential data to quantify nucleic acid, providing precise and accurate quantification.



The molecular techniques are better than plating and biochemical method with advantages of comparatively shorter time and detection of specific pathogen [20]. However, there is still requirement for low cost, portable, and user-friendly diagnostic device such as disposable microfluidic chip method for detection of various pathogens altogether. Therefore the evolution of modern facilities and sophisticated biological tools is under development for accurate, faster, and specific determination of microbes. Several studies have identified the bacterial diversity from HWW (Table 4–1).

The research on HWW has revealed that the most predominant pathogenic bacteria found are of genus *Bacillus*, which count for 80%–90% with *Staphylococcus* and *Streptococcus* varying from 5% to 10% [37]. The wastewater coming from the laboratories and hospitals also consists of multiple microbes with drug resistance properties including *Acinetobacter*, *Enterococcus*, and *Pseudomonas* species. *Staphylococcus aureus* is the most common pathogenic gram-positive bacterium with high level of multidrug resistance (MDR). According to studies, the predominance of MDR bacteria varies from 0.58% to 40% depending on the size and origin of HWW [38]. This bacterium is mostly associated with nosocomial infections. The nosocomial infection is also called as hospital-acquired infection, which is acquired by patients in medical care units by various means including medical equipment,

Table 4–1 Major bacterial isolates found in the hospital environment from different countries.

Bacterial isolates	Methods of identification	Treatment	References
<i>Aeromonas</i> , <i>Kluyvera</i> , other Enterobacteriaceae	Matrix assisted laser desorption/ionization-time of flight mass spectroscopy and gene sequencing	Ciprofloxacin, cotrimoxazole, chloramphenicol, cephalosporins, fluoroquinolones, and aminoglycosides	[21]
Carbapenem-resistant Enterobacteriaceae	Microbiological methods and qPCR	Carbapenem combinations, polymyxins, fosfomycin, ceftazidime-avibactam	[22]
<i>E. coli</i> , <i>Salmonella</i> , <i>Shigella</i> , <i>S. aureus</i>	Microbiological and biochemical tests	Ioperamide, trimethoprim-sulfamethoxazole, cefotaxime, β -lactams, quinolones, macrolides, nafcillin, sulfa drugs, and vancomycin	[23]
<i>Enterococci</i>	Microbiological methods and PCR	Piperacillin-tazobactam, imipenem, meropenem, and ampicillin	[24]
ESBL-producing Enterobacteriaceae	Microbiological and 16S rRNA sequencing	Carbapenem, cephamycin, nitrofurantoin, clavulanic acid, and tazobactam	[25]
<i>Faecalibacterium</i> , <i>Aeromonas</i> , <i>Prevotella</i>	16S rRNA-based denaturing gradient gel electrophoresis analysis	Chloramphenicol, ciprofloxacin, cotrimoxazole, aminoglycosides, metronidazole, ureidopenicillins, carbapenems, clindamycin, and pavilion	[26]
<i>Klebsiella</i> , <i>Pseudomonas</i> , <i>Serratia</i>	Microbiological and molecular methods	Ampicillin/sulbactam, ceftazidime, cefepime, levofloxacin, norflaxacin, gentamycin, cefepime, aztreonam, and aminoglycoside with antipseudomonal β -lactum	[27]
KPC-producing <i>Aeromonas</i> spp., Enterobacteriaceae, and ESBL-producing <i>K. pneumonia</i> , <i>Enterobacter</i> , <i>E. coli</i>	Microbiological, PCR, and 16S rRNA sequencing	Chloramphenicol, ciprofloxacin, cotrimoxazole, cephalosporins, fluoroquinolones, ampicillin, ticarcillin/clavulanate, ertapenem, and merpenem	[21,28]
MDR <i>E. coli</i>	Microbiological and molecular methods	Isothiocyanates, trimethoprim, nitrofurantoin, and pivmecillinam	[29,30]
MDR <i>E. coli</i> , <i>S. aureus</i>	Biochemical finger printing and/or random amplification of polymorphic DNA (RAPD)-PCR	Natural isothiocyanates, cephalosporins, nafcillin, sulfa drugs, and vancomycin	[31]
	Biochemical tests, PCR, and sequencing		[32]

(Continued)

Table 4–1 (Continued)

Bacterial isolates	Methods of identification	Treatment	References
Methicillin-resistant <i>S. aureus</i> , vancomycin-resistant <i>S. aureus</i>		Doxycycline, minocycline, trimethoprim, sulfamethoxazole, nitrofurantoin, linezolid daptomycin, and tigecycline	
MDR <i>E. coli</i> , <i>P. aeruginosa</i> , <i>Acinetobacter</i> , <i>K. pneumoniae</i>	Biochemical tests	Natural isothiocyanates, ampicillin, piperacillin/tazobactam, ceftazidime, cefapime, levofloxacin, meropenem, and ertapenem	[33]
<i>P. aeruginosa</i> , <i>Enterobacter cloacae</i> , <i>Klebsiella pneumoniae</i>	Microbiological methods and sequencing	Penicillin, cephalosporin, imipenem, meropenem, fluoroquinolones, and aminoglycosides	[34]
<i>Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Actinobacteria</i>	Microbiological, qPCR, and 16S rRNA sequencing	Azithromycin, doxycycline, carbapenem, tigecycline, ceftazidime, and penicillin G	[35]
<i>Streptococcus</i> , <i>Acromobacter</i> , Ruminococcaceae	16S rRNA sequencing, and qPCR	Meropenem, imipenem, trimethoprim- sulfamethoxazole, ticarcillin, and cephaosporins	[1]
Vancomycin-resistant <i>Enterococci</i>	Microbiological, biochemical, RAPD, and 16S rRNA sequencing	Chloramphenicol, doxycycline, nitrofurantoin, and ampicillin/sulbactam	[36]

bed-linens, or through infected patients [39]. The hospital-acquired infections resulted in increase of methicillin-resistant *S. aureus* (MRSA) isolates. The MRSA is associated with infections of skin and soft tissues and has been a global threat for human health. In the United States, 72,444 cases of MRSA infections were reported in 2014 [40].

E. coli and *Pseudomonas aeruginosa* are also commonly found in HWW along with other nosocomial pathogens including *Candida albicans*, *Klebsiella Proteus*, and *Enterobacter* species [37]. Most of the times, *E. coli* is harmless and is important part of human intestinal tract, but some of the strains are pathogenic to humans. The *E. coli* 0157:H7 is related to many outbreaks of water and food-borne illness and also responsible for 63,000 hemorrhagic colitis (bloody diarrhea) cases in the United States annually [41]. A study reported on HWW of Brazil also confirmed the presence of other bacterial species including *Citrobacter freundii*, *Klebsiella ornithinolytica*, *Proteus mirabilis*, *Pantoea agglomerans*, and *Serratia rubidacea* [28]. Many *C. freundii* infections have been reported in bloodstream such as septic shock, pneumonia, hypothermia, oliguria, and thrombocytopenia [42]. Another bacterium *P. mirabilis*, which is found in HWW, is responsible for urinary tract infections and is further accompanied by development of kidney stones [43]. *P. agglomerans* mostly affects plants but also causes opportunistic infections in immunocompromised individuals causing skin infections [44].

4.2.1.1 Fluctuations in pathogens load in hospital wastewater

An important prospect about fluctuations of pollution was discussed [44], according to which, the microbial load present in wastewater was directly correlated to several hospital activities. The higher microbial load was present in HWW during the day period as compared with evening and early morning. Another study [44] was also conducted on HWW collected during early hours (EH) and late hours (LH) of the day in Abia state, Nigeria. The microbiological analysis revealed the presence of higher total bacterial count (7.9×10^4 CFU/mL) at LH as compared with that (6.7×10^4 CFU/mL) at EH of the day. The similar results were also observed for total coliform count, which was also higher (2.2×10^3 CFU/mL) at LH as compared with that (1.8×10^3 CFU/mL) at EH due to the fact that hospital activities at night time were more restricted than day time. According to the recent study, the HWW samples were collected from three hospitals in different cities of Romania [35]. Three hospitals (H1, H2, and H3) were having different numbers of beds and inhabitants. The microbial community analysis results revealed that wastewater from H1 was dominant by 97.27% of the *Proteobacteria* phylum, while from H2 was dominant by 44.7% of *Proteobacteria* phyla followed by *Firmicutes* (33.68%), *Bacteroidetes* (16.4%), and *Actinobacter* (4.9%) and from H3 was dominant by 71.4% of *Proteobacteria*, *Bacteroidetes* (13.7%), and *Firmicutes* (13.1%) [35]. The similar taxonomic composition was also reported in various studies using wastewater collected from different hospital facilities, nonhospital medical facilities, and municipal treatment plant [26,45,46]. The dominant nature of *Proteobacteria* was correlated to their presence in human feces, long-term survival ability in wastewater, and exposure to several antibiotics present in HWW. Moreover, the dominant nature of *Firmicutes* could be correlated to their capacity to survive in extreme environmental conditions and high contaminant

levels [47]. In 2018 Buelow et al. [48] detected abundant bacterial taxa to be different from urban and suburban WWTP influents and comprised of Camphylobacteraceae, Aeromonadaceae, and Carnobacteriaceae. HWW also consisted of several different members of human gut microbiota such as of genus *Streptococcus* and family Ruminococcaceae due to the sampling location in close proximity of the hospital sanitation areas as compared with WWTP influent. These microbes are not well suited to survive in such complex environment, which results in progressively decreased levels of these bacteria in urban WWTP influent [48]. Another study compared the relative abundances of the most abundant bacterial orders in WWTP for 7 years and showed that the WWTP environment was dominated by phyla Actinobacteria, Bacteroidetes, and Proteobacteria, and the wastewater community was highly stable and unique to its environment [49].

Hospital sewage also contained high levels of anaerobic bacteria including genera such as *Bifidobacteriales*, *Clostridiales*, *Bacteroidales* that were likely to originate from the human gut [1]. The release of antibiotics from hospitals results in creating a selection pressure on bacteria and as a result, effluents from hospitals contain high numbers of resistant bacteria as well as antibiotic residues. Prevalence and spread of antibiotic-resistance bacteria (ARB) in the environment are a major problem worldwide due to improper antibiotic usage and lack of effective HWW management systems. Therefore ARB and ARGs are particularly studied among the hospital contaminants. For example, one study reported *E. coli* from hospital effluent to be multiresistant toward ampicillin, streptomycin, sulfamethoxazole, cephalosporin, ciprofloxacin, and tetracycline [29]. The hospital water environment includes potable water, faucets, wastewater drainage systems, and effluents can be the reservoir of nosocomial pathogens (ARB such as MDR Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas* species), thus, increasingly dominating the HWW microbiome [50]. Commonly found isolates such as *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae* in hospital effluents have shown carbapenem resistance and have disseminated around the globe [50].

Baricz et al. [51] reported antimicrobial effect of a crude bacterial extract of *Janthinobacterium lividum* against MDR bacteria of both clinical and environmental origin, for example, MRSA, methicillin susceptible *S. aureus*, *Enterococci*, and Enterobacteriaceae [51]. Such studies not only help to provide promising candidates for development of new antimicrobials but also to propose new or improved treatment technologies to reduce the burden of antimicrobial resistance (AMR) in environment.

4.2.2 Fungi

The microbiological analysis of several HWWs showed the prevalence of fungal species along with bacterial and coliform species. The fungi have simpler nutritional requirements and have higher capability to grow at lower water activity as compared with bacteria [52]. Moreover, the fungal populations can easily spread their spores to external environment, hence affecting the human beings directly [11,52]. This is the reason for its prevalence in hospital and clinical environment because if healthcare facilities are even considered free from fungus, but nature and environment factors such as temperature, moisture, and

nutrients could provide easy and favorable conditions for the extensive growth of fungal species in storage containers holding clinical waste. The fungal infections could range from moderate to fatal depending upon the infection site as well as immune system of the affecting individual [53]. The prevalence of invasive fungal species has been reported to increase since three decades, due to increase in number of immunocompromised patients. The moderate fungal infections include athlete's foot and ring worm infections (cutaneous infections) in immunocompetent patients and life-threatening infections include mucosal and systemic infections [53]. The fungal isolates, associated diseases, and treatment methods reported in various studies are presented in Table 4–2.

The study reported by Neely and Orloff [54] stated the prevalence of *Aspergillus* species in the hospital waste collected from the United States and even worse than that, this species was capable enough to survive for more than a month on the hospital waste. The longer survival of *Aspergillus* can result in reoccurrence of fungi and has higher chance to cause disease. Moreover, many spores have capacity to remain dormant under adverse conditions and then again develop into fungi, when conditions become favorable. The other fungal species were also detected but the survival capacity of *Mucor* (> 20 days), *Paecilomyces* (11 days), and *Fusarium* (10 days) species was lower than that of *Aspergillus* species. The survival efficiency of different fungal species is related to the presence of specific structure of spores. The spores of *Aspergillus* species are spiny and rough and thus capable enough to adhere to any type of waste and can survive longer time. However, spores of *Fusarium* species are smooth and thus have less capability to adhere and survive. The wastewater collected from Nigeria revealed the presence of total fungal count of 3.5×10^3 CFU/mL during the day time activities in the hospital [72]. The most prevalence fungal species in the HWW were *Aspergillus* (33.3%), followed by *Candida* (28.7%), *Cryptococcus* (14.9%), and *Penicillium* (14.2%) species. The fungal diversity (29 species) was reported to be higher for waste samples collected from hematology section of Malaysia hospital [72]. Moreover, gloves waste consisted of the highest number of fungal species (19 species) among all the different types of waste collected from hospital. The high fungal count of 7×10^5 CFU/mL was also reported in soil collected from hospital dumpsite, which was correlated with the presence of high organic material present in the hospital waste. The fungi are heterotrophic microorganism, which have capacity to consume organic compounds from the surrounding environment [72]. The common fungal species identified in hospital waste were *Penicillium rubrum*, *Penicillium viricadum*, *Trichothecium roseum*, *Rhizopus nigricans*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Microsporum canis*, and *Aspergillus niger*. Among them, *A. niger* was found to be dominant fungal species with 34.5% due to the fact that it released variety of enzyme such as amylases, pectinases, and proteases and had capacity to utilize number of organic compounds as a substrate [73]. *A. flavus* and *A. niger* can cause disease named as aspergillosis and are considered as pathogens for both humans and animals. They can cause either bronchopulmonary (coughing and wheezing) or invasive aspergillosis (affects the lungs and could spread to throughout the body) [74]. More than 200,000 cases and 30%–95% mortality rate have been reported for aspergillosis infections worldwide. The nonpathogenic black bread mold called *R.*

Table 4–2 Major fungal isolates found in the hospital environment from different parts of the world.

Fungal isolates	Diseases associated	Type	Drugs used for treatment	Country of study	References
<i>C. albicans</i>	Candidemia	Invasive and noninvasive	Azoles, polyenes, echinocandins, flucytosine	United States	[54–65]
<i>Aspergillus Terreus</i>	Chronic lung diseases, hematologic malignancies, renal and liver diseases, allergic bronchopulmonary aspergillosis, aspergillus bronchitis, tracheobronchitis invasive, disseminated aspergillosis, etc.	Invasive and noninvasive	Echinocandins, itraconazole, voriconazole, posaconazole		
<i>Candida tropicalis</i>	Bloodstream infections, candidemia	Invasive and noninvasive	Azoles		
<i>Candida Krusei</i>	Candidemia	Invasive and noninvasive	Voriconazole, Echinocandin, fluconazole, lipid formulation amphotericin		
<i>Candida parapsilosis</i>	Bloodstream, skin, soft-tissue infections	Invasive and noninvasive	Fluconazole, echinocandins		
<i>A. flavus</i>	Invasive aspergillosis	Invasive and noninvasive	Voriconazole, posaconazole, itraconazole, caspofungin, isavuconazole, amphotericin B		
<i>Aspergillus fumigatus</i>	Invasive aspergillosis, pneumonia, sinusitis, brain abscesses	Mostly invasive	Echinocandin-triazole, voriconazole, caspofungin		
<i>A. niger</i>	Aspergillosis	Mostly noninvasive	Voriconazole, posaconazole, liposomal amphotericin B, isavuconazole		
<i>Fusarium</i> sp.	Fusariosis, onychomycosis, cellulitis, erythematous, necrosis	Invasive and noninvasive	Amphotericin B and voriconazole		
<i>Mucor</i> sp.	Bloodstream infections, candidemia, invasive sinusitis, mucormycosis, skin infections	Invasive and noninvasive	Amphotericin B		

<i>Paecilomyces</i> sp.	Infections in lower respiratory tract, bone, blood (immunocompromised patients), pneumonia, endophthalmitis, sinusitis, peritonitis, soft-tissue infections	Invasive and noninvasive	Voriconazol, posaconazole, ravuconazole		
<i>Aspergillus</i>	Invasive aspergillosis	Invasive and noninvasive	Amphotericin B, pimaricin, Voriconazole, posaconazole, itraconazole, caspofungin, isavuconazole, etc.	Nigeria	[66–71]
<i>Candida</i>	Candidemia, urinary tract infections, disseminated candidiasis, endophthalmitis, endocarditis	Invasive and noninvasive	Caspofungin, micafungin, anidulafungin, fluconazole, voriconazole, liposomal amphotericin B		
<i>Cryptococcus</i>	Cryptococcosis	Invasive and noninvasive	Fluconazole, Itraconazole, Amphotericin B		
<i>Penicillium</i>	Invasive infections, fungemia, endophthalmitis, pulmonary infection, esophagitis, cutaneous infection (mostly immunocompromised patients)	Invasive and noninvasive	Amphotericin B, itraconazole, ketoconazole, flucytosine, voriconazole		
<i>Trichophyton rubrum</i>	Keroin, Majocchi's granuloma, mycetoma, deep dermatophytosis	Mostly noninvasive	Terbinafine, Bifonazole		
<i>Rhizopus</i>	Zygomycosis, abscess, gastrointestinal, allergic diseases	Invasive and noninvasive	Amphotericin B		

nigricans was also found in abundance with 27.5%. *M. canis* can cause infection in upper and dead layer of skin of domestic animals, including dogs and cats and later on the humans can also get affected by the infection [75]. Other fungal species including *T. roseum*, *P. viricadum*, and *P. rubrum* are nonpathogenic fungus, which can only affect the humans with weak immune system. The *Curvularia* species have also been reported to be present in hospital waste and can cause rare infections in the respiratory tract and cornea of human beings. *Curvularia lunata* is also responsible for eumycetoma disease caused in farmers of Chandigarh and Rajasthan. Most of the agricultural regions use azole fungicides (imidazole, metalaxyl, tebuconazole, and propiconazole). Therefore the farmers responded poorly to antifungal treatment and were referred for long-term therapies [76]. The few fungal species of *Fusarium* (1.5%) and *Trichoderma* (2.2%) have also been reported in hospital waste, but infection caused by these fungal species is difficult to cure [77].

Over 1.2 billion people worldwide are reported to be suffered from several fungal infections and 1.5–2 million people die due to fungal infections each year, which are far superior than malaria or tuberculosis death rate [76]. Antifungal drugs are mostly used for treatment of fungal infections as they target plasma membrane, sterol biosynthesis, DNA biosynthesis, and β -glucan biosynthesis of different fungi. However, since past decades, the increased use of antifungal drugs resulted in development of resistance, leading to increase in morbidity and mortality. The drug resistance could be due to several reasons such as alterations of the drug target site, increased efflux of drugs, and biofilm formation. Although several studies have reported, antifungal drugs resistance is not at the same level as resistance in bacteria against antibiotics. Other reasons of high mortality rate with fungal infections include availability of limited treatment options against invasive fungal diseases and less susceptibility of immunocompromised patients to antifungal drugs [78]. For instance, in the United States, until 2013, there were reports of 19,262 organ transplants, which resulted in increased susceptibility to fungal infections in immunocompromised patients [78]. In addition, adverse effects of antifungal drugs and lack of effective antifungal therapies are the other reasons, which necessities the development of novel treatment strategies and next-generations of antifungal drugs. The development of ultrahigh-throughput screening techniques could help in advent of novel antifungal drugs by providing rapid, effective, and economical drug screening.

4.2.3 Viruses

Presently, the viral infections are in forefront as compared with pathogenic diseases caused by bacteria, fungi, and parasites (Table 4–3). Moreover, the detection, analysis, characterization, and epidemiology of virus is entirely different from bacteria because the bacterial indicators used to represent water contamination cannot actually represent viral contamination.

It has been reported that each of the material present in the hospital waste can carry viruses, and thus, viruses are able to survive for 5–8 days [78].

The viral hepatitis is very common and leading disease. Moreover, human immunodeficiency virus infections, Hepatitis B, and Hepatitis C are among the deadly infectious diseases,

Table 4–3 List of human pathogenic viruses.

Virus	Type	Transmission	Disease
Aichi virus, Human astrovirus, Lordsdale virus, Norwalk virus, Sapporo virus, Southampton virus	[ssRNA(+)]	Fecal-oral	Gastroenteritis
Australian bat lyssavirus, Duvenhage virus, European bat lyssavirus, Lagos bat virus, Rabies virus	ssRNA(–)	Zoonosis, animal bite	Fatal encephalitis
Kunjin virus, Louping ill virus, St. Louis encephalitis virus, Tick-borne powassan virus	ssRNA(+)	Zoonosis, arthropod bite	Encephalitis
Borna disease virus	dsRNA	Zoonosis, arthropod bite	Encephalitis
Barmah forest virus, Chikungunya virus, Mayaro virus, O'nyong-nyong virus, Ross river virus, Sagiyama virus, Semliki forest virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus	ssRNA(+)	Zoonosis, arthropod bite	Fever, joint pain
Coxsackievirus	ssRNA(+)	Fecal-oral	Meningitis, myocarditis, paralysis
Crimen-congo hemorrhagic virus, Punta toro phlebovirus, Rift valley fever virus, Sandfly fever sicilian virus, Toscana virus, Uukuniemi virus	ssRNA(–)	Zoonosis, arthropod bite	Hemorrhagic fever
Dengue virus, Yellow fever virus, West Nile virus	ssRNA(+)	Zoonosis, arthropod bite	Hemorrhagic fever
Dhori virus	ssRNA(–)	Zoonosis, arthropod bite	Fever, encephalitis
Dugbe virus	ssRNA(–)	Zoonosis, arthropod bite	Thrombocytopenia
Ebolavirus	ssRNA(–)	Zoonosis, contact	Hemorrhagic fever
Echovirus	ssRNA(+)	Fecal-oral	Common cold
Encephalomyocarditis virus	ssRNA(+)	Zoonosis	Encephalitis
Epstein-Barr virus	ssRNA(+)	Contact, saliva	Mononucleosis
Hantaan virus	ssRNA(–)	Zoonosis, urine, saliva	Renal or respiratory syndrome
Hendra virus, Nipah virus	ssRNA(–)	Zoonosis, animal bite	Encephalitis
Hepatitis A virus	ssRNA(+)	Fecal-oral	Hepatitis
Hepatitis B virus	dsDNA-RT	Sexual contact, blood	Hepatitis
Hepatitis C virus	ssRNA(+)	Sexual contact, blood	Hepatitis
Hepatitis delta virus	ssRNA-RT	Sexual contact, blood	Hepatitis
Hepatitis E virus	dsDNA	Zoonosis, food	Hepatitis
Human adenovirus	dsDNA	Respiratory, fecal-oral	Respiratory
Human coronavirus, Human rhinovirus	ssRNA(+)	Respiratory	Respiratory
Human cytomegalovirus	ssRNA(+)	Contact, urine, saliva	Mononucleosis, pneumonia
Human enterovirus 68, 70	ssRNA(+)	Fecal-oral	Diarrhea, neurological disorder
Human herpesvirus 1, 2	dsDNA	Skin contact, saliva	Skin lesions
Human herpesvirus 6, 7	dsDNA	Respiratory, contact	Skin lesions

(Continued)

Table 4–3 (Continued)

Virus	Type	Transmission	Disease
Human herpesvirus 8	dsDNA	Skin contact, saliva	Skin lymphoma
Human immunodeficiency virus	ssRNA-RT	Sexual contact, blood	AIDS
Human papillomavirus 1, 2	dsDNA	Contact	Skin warts
Human papillomavirus 16, 18	dsDNA	Sexual contact	Genital warts, cervical cancer
Human SARS coronavirus, Mers coronavirus	ssRNA(+)	Zoonosis	Respiratory
Human T-lymphotropic virus	ssRNA-RT	Sexual contact, maternal-neonatal	Leukemia
Human torovirus	ssRNA(+)	Fecal-oral	Gastroenteritis
Influenza A virus	ssRNA(-)	Respiratory, animal contact	Flu
Influenza B, C virus	ssRNA(-)	Respiratory	Flu
JC polyomavirus	dsDNA	Fecal-oral, urine	Encephalitis
Lake Victoria marburgvirus, Lassa virus, Pichinde virus	ssRNA(-)	Zoonosis, fomite	Hemorrhagic fever
Langat virus	ssRNA(+)	Zoonosis, arthropod borne	Encephalitis
Lymphocite choriomeningitis virus, Machupo virus	ssRNA(-)	Zoonosis, fomite	Encephalitis
Measles virus	dsDNA	Respiratory	Fever, rash
Mengo encephalomyocarditis virus	ssRNA(+)	Zoonosis	Encephalitis
Markel cell polyomavirus	dsDNA	–	Markel cell carcinoma
Mokola virus	ssRNA(-)	Zoonosis, animal bite	Encephalitis
Molluscum contagiosum virus	dsDNA	Zoonosis	Encephalitis
Monkeypox virus, orf virus	dsDNA	Zoonosis, contact	Skin lesions
Mumps virus	ssRNA(-)	Respiratory, Saliva	Mumps
New York virus, Puumala virus, Seoul virus	ssRNA(-)	Zoonosis, urine, saliva	Hemorrhagic fever
Poliovirus	ssRNA(+)	Fecal-oral	Poliomyelitis
Rotavirus A, B, C	dSRNA	Fecal-oral	Gastroenteritis
Rubella virus	ssRNA(+)	Respiratory	Rubella
Sindbis virus	ssRNA(+)	Zoonosis, anthropol bite	Pagosta disease, fever, joint pain
Varicella-zoster virus	dsDNA	Respiratory contact	Varicella
Variola virus	dsDNA	Respiratory	Variola
Zika virus	ssRNA(+)	Zoonosis, anthropol bite	Fever, joint pain, rash

Source: From ViralZone: www.expasy.org/viralzone, SIB Swiss institute of Bioinformatics.

transmitted by direct contact of blood from infected to another. However, these are yet easy to prevent, once detected at early stages and obligatory precautions are followed by patients [5]. The samples collected from different WWTPs have been analyzed for detection and characterization, and even research has been focused on removal of viruses from wastewater [79]. However, in spite of all the health risks associated with viruses, very few research [5] has been conducted specifically for HWW, and thus, requires special attention due to the fact that, it is one of the main sources to spread the pathogenic and deadly viral diseases. The modern techniques of molecular biology including PCR assays offer several advantages such as specificity, sensitivity, and wider data analysis for easy and faster detection of viruses. The occurrence of Rotavirus A, norovirus genogroup I and II, human adenovirus (HAdV), and hepatitis were detected in samples collected from two different hospital WWTPs. The load of rotavirus and HAdV present in hospital WWTP was 2 log cycle higher as compared with that present in urban sewage WWTP [80].

4.2.3.1 Human adenovirus

The HAdV consists of double-stranded linear DNA (Fig. 4–1) and comes under category of nonenveloped viruses, which makes them resistant to heat, dry, and acidic conditions [78]. The adenovirus consists of outer capsid and inner core with several histone proteins. The elongated fiber proteins on the surface of the virus interact with receptor of host cell such as coxsackie and adenovirus receptor and MCP and start infection in the host cells. After

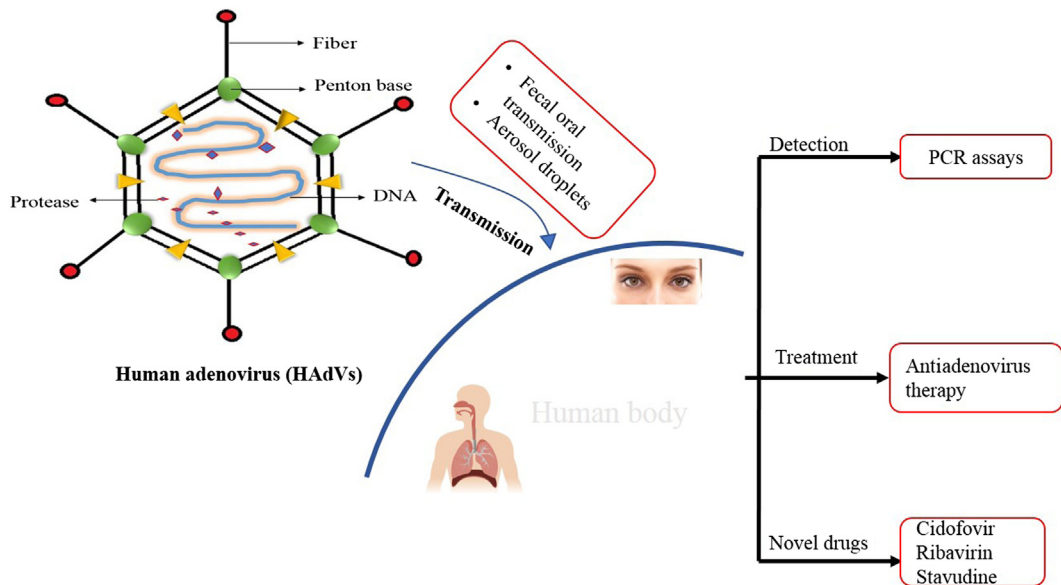


FIGURE 4–1 Human adenovirus structure, transmission, detection, and treatment.

interaction between virus fiber proteins and host cell receptors, uncoating of virus particles takes place resulting in dissolution of viral protein in the endosome. Ultimately, the translocation of adenovirus takes place with microtubules through cytoplasm toward the nucleus [81]. HAdV is mostly profound in patients with weak immune system and acute lower respiratory disease [82]. Seventy-nine types of HAdV have been reported [83], and out of them, HAdV 40/41 is mostly prevalent in aquatic systems such as sea water, sewage, rivers, surface water, and drinking water [12].

The HAdV is placed among the contaminant candidate list for drinking water by U.S. Environment Protection Agency and is a real factor of concern for the human health [84]. It can cause several diseases in humans including ocular infections, conjunctivitis, genitourinary infections, pharyngoconjunctival fever, hemorrhagic cystitis, exanthema, encephalitis, and pneumonia [85]. The HAdV is more frequently found in wastewater than any other enteric virus. The HAdV is reported to be transmitted through fecal-oral transmission, aerosol droplets, and contaminated materials. It can survive for the extended period of 3–8 weeks in the environment without host. Due to its stable and persistence nature in aquatic systems, it could also be considered as an indicator of fecal contamination as suggested by various studies [86,87].

The prevalence of HAdV species varies according to the hospital environment. According to Prado et al. [80], the HAdV 40/41 (species F) was among the most prevalence genotype found during molecular characterization of viruses, detected during analysis of samples collected from two different hospital WWTPs. The species F come from the hospitalized children reported with acute gastroenteritis disease. The other reported HAdV strains include species C and D, which are associated with conjunctivitis and respiratory tract infections [80]. The frequency of HAdV has also been tested for the samples (102 in number) collected throughout year from hospital WWTP located in Tunisia City, Tunisia and 64% samples were detected positive for HAdV and most prevalent species were species F (HAdV 40/41) [12]. In the similar way, the prevalence of HAdV was detected in samples collected from different regions of the world such as 92.1% in Poland, 76.9%–92.3% in Greece, 92% in Norway, and 100% in Brazil, while samples collected from Morocco (45.5%), Italy (21.6%), and Taiwan (27.3%) consisted of low prevalence of HAdV [78]. The high and low prevalence of HAdV reported in various countries could be collaborated by the fact that the circulation of virus varied according to the geographical regions and epidemiological community profile. However, whether it was high or low prevalence, still presence of HAdV questions the ability of WWTPs to remove the gastroenteric viruses and highlights the urgency for development of effective environmental control programs and innovative HWW treatment plants before discharging the HWW into the sewage system.

The detection of HAdV includes cell culture method, antigen detection, and PCR method. Serology is also used sometimes to detect adenovirus-specific antibodies. There is no effective treatment against HAdV, essential precautions should be taken to control infections, which include washing of hands, disinfection of instruments as well as application of infection control protocol in hospitals against adenovirus to prevent outbreaks [88].

4.2.3.2 Rotavirus

The rotavirus consists of 11 segmented double-stranded RNA and its surface is surrounded by three layers: the outer capsid, inner capsid, and central core. There are seven rotavirus groups that have been reported from A to G and studies have revealed that groups A, B, and C affect both humans and animals, while D to G are reported to cause infection mainly in animals. The dual (binary) classification system has been used for detailed classification of rotavirus on the basis of two outer capsid proteins; the glycoprotein VP7 and protein VP4 (Fig. 4–2), where the glycoprotein VP7 defines for the stereotype G and protein VP4 defines for stereotype P [89]. The viral attachment to the host cell surface is by glycoprotein VP7 or surface protein VP8. After interaction with host cell, calcium-dependent endocytosis takes place and thus, uncoating of the particle occurs, resulting in release of double layer of the viral particle into cytoplasm. Further, transcription and translation takes place in the cytoplasm and viral proteins are synthesized by cellular ribosomes. After assembly, virus particle is released from the infected cell through cell lysis, and infection is further spread into the host [90].

Rotavirus is associated with around 111 million cases of acute gastroenteritis in the newborn babies and children across the world, and 352,000–592,000 deaths have been reported for children with age of <5 years globally. After infection and replication, rotavirus is discharged into surface water, groundwater, drinking water, and wastewater, able to transmit effectively through water due to its stable and resistant nature under adverse environmental conditions [91]. The rotavirus can survive at ambient temperature of 30°C–35°C and can exist on inanimate objects for 60 days without host. The rotavirus is mostly prevalent in humans and 11%–42% of positive samples are associated with group A rotavirus [92,93].

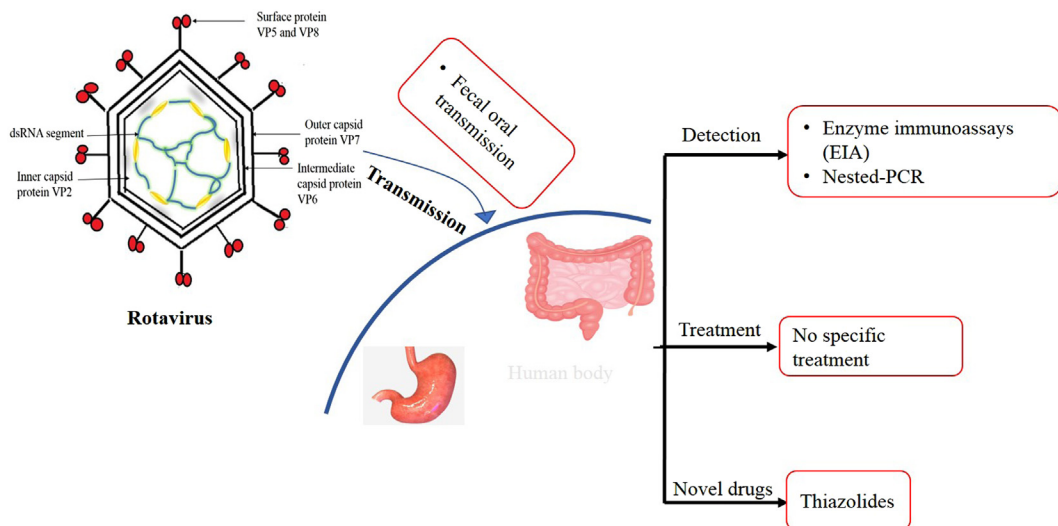


FIGURE 4–2 Rotavirus structure, transmission, detection, and treatment.

The detection methods of rotavirus include electron microscopy, ELISA, passive particle agglutination tests, polyacrylamide gel electrophoresis, and RT-PCR. The vaccination with safety and efficacy profile for rotavirus A has been developed and licensed in 2006 after the withdrawn of first ever vaccination, which was associated to cause intestinal intussusceptions in children [94].

The prevalence of rotavirus in different seasons (summer, autumn, and winter) was detected by collecting 60 different samples in the period of 9 months from HWW located in Shiraz, Iran. Enzyme immunoassays (EIAs) were performed and positive specimens were further investigated with nested-PCR by various primers. Moreover, the virus concentration method such as the pellet and two-phase is reported to enhance the concentration of virus by 50- to 100-fold before applying EIA. About 15 samples (25%) were found to be positive for rotavirus A and the highest prevalence was detected in autumn with frequency of 46.67% followed by 33.33% and 20% of prevalence in winter and spring, respectively [95]. Other studies also confirmed the prevalence of rotavirus A in cold weather as compared with other seasons [96]. The molecular characterization of rotavirus further revealed the predominance of G1 genotype during various clinical investigations followed by mixed genotype of G1G4, which is associated with frequent water contamination and further generation of novel strains by genetic reassortment. Moreover, various studies have reported the prevalence of rotavirus in treated wastewater as well, which is a real concern for public health [95]. The studies on rotavirus highlight the significance of environmental surveillance tools for detection of novel genotypes and to further analyze the distribution and treatment of the hazardous effects caused by rotavirus in human beings.

4.2.3.3 *Norovirus*

The norovirus belongs to the family Caliciviridae, and is reported to be nonenveloped and contains a positive sense, single-stranded RNA, which is organized into three open reading frames (ORFs). The three ORFs encode three different proteins; ORF1 is of size 5 kb and encodes nonstructural (NS) polyproteins, ORF2 is having 1.8 kb size and encodes structural capsid protein VP1, while ORF3 with size of 0.6 kb encodes a VP2 protein to maintain the stability of capsid protein [97]. The norovirus life cycle has been presented in Fig. 4–3. The interaction of norovirus with the host cell surface takes place through histoblood group antigens. After release of the VPg-linked RNA genome of the virus into cytoplasm of host cell, the viral RNA translation takes place. Once the translation of the ORF1 polyprotein is done, further co- and posttranslation processing occurs by NS6 protease of virus, which results in the release of NS proteins for the formation of replication complex. Thereafter the viral replication takes place by RNA-dependent RNA polymerase using de novo and VPg-dependent mechanism. Furthermore, the replicated genomes are translated and further packed into the capsid for assembly of virion and exit [98].

The norovirus is divided into seven genotypes, among them three genotypes GI, GII, and GIV are responsible for infection in humans, and GII with genotype 4 (GII.4) is a predominant infectious virus associated with 18% of the diarrheal diseases and gastroenteritis infections around the world. In addition to GII.4, various other non-GII.4 containing viral strains

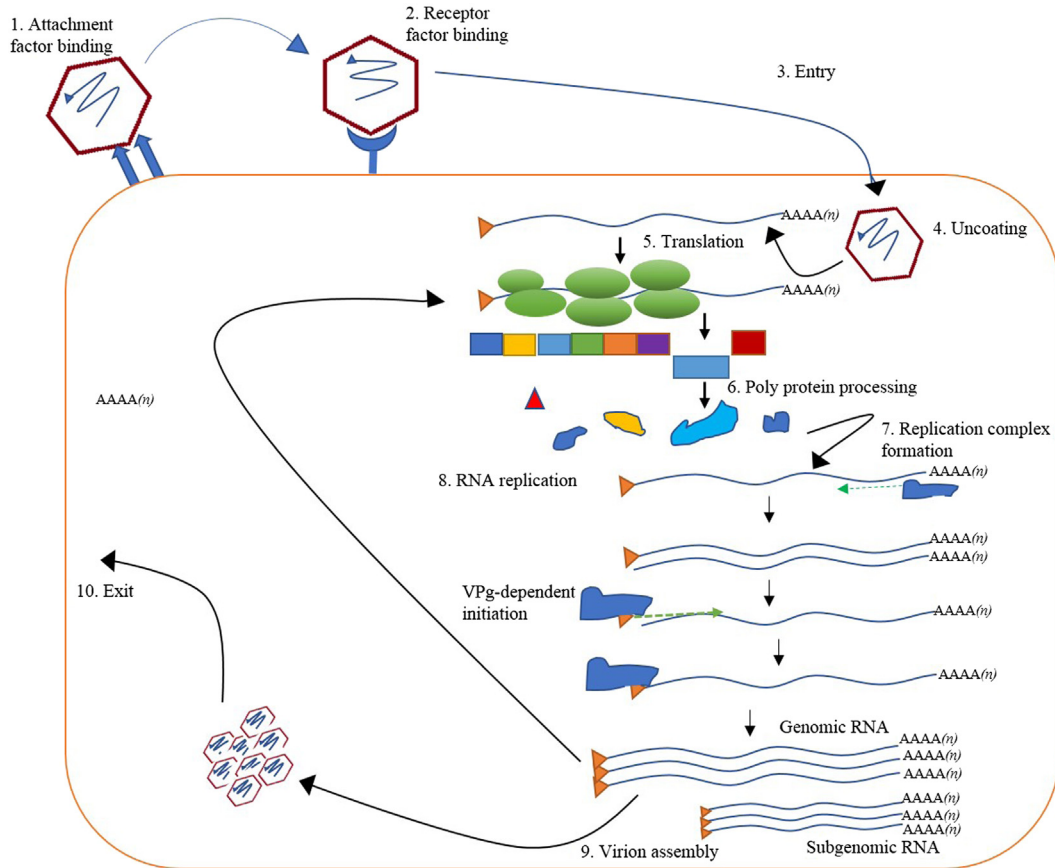


FIGURE 4-3 Life cycle of the norovirus.

are also responsible for gastroenteritis infections, for instance, the gastroenteritis emerged in Southeast Asia in 2014–15 was associated with G11.17 viral strain. Norovirus is associated with 677 million gastroenteritis cases and 210,000 deaths annually around the world [99]. The emission of waterborne norovirus is caused by contamination of water systems including drinking water, surface water, mineral water, and groundwater [100]. As the infectious individuals shed the virus into the water system, the samples collected from HWW are considered useful for study of norovirus epidemiology at population level [99]. The transmission of norovirus is through fecal–oral route from person to person or animal to person by contamination food or water intake. Depending on the surface temperature and conditions, the norovirus can survive for longer period outside the host. It has capacity to survive for weeks on the hard surface, until 12 days on contaminated fabrics and for years in the contaminated still water [101]. The norovirus infection in humans can be detected by real-time PCR, multiplex gastrointestinal platforms, EIAs, and genotyping as well.

The development of real-time PCR for detection of norovirus is considered as significant advancement with various environmental applications. The prevalence of norovirus was studied by Ibrahim et al. [100], where 102 samples were collected from 5 different basins of hospital WWTP located in Tunisia. The 65% of the samples were positive for norovirus GII followed by norovirus GI, which was detected in 1% of the samples collected from wastewater, while frequency of norovirus was lowered moving from one basin to another. However, there is requirement of tertiary wastewater treatment before recycling of water for bathing and other purposes. Different studies have revealed the prevalence of norovirus in different seasons, for instance, studies on HWW collected from Italy and Sweden have showed occurrence of norovirus GII in spring and summer seasons [102,103]. However, the study from China has revealed the prevalence of norovirus in winter conditions, which could be explained by variability in temperature during different seasons, immunity level of the host, humidity as well as socioeconomic conditions of different countries [104].

The emerging recombinant norovirus has been analyzed by illumine Miseq and Sanger sequencing technique by collecting different clinical and wastewater samples within Australia and New Zealand. The prevalence of pandemic variant (GII.Pe/GII.4) was found in Sydney during 2014 and 2015, while decline in pandemic virus was observed in 2016 with emergence of five new recombinant strains. These new viruses were held responsible for emergence of gastroenteritis outbreaks during November 2016 [99]. The use of new generation sequencing tools has advanced and reformed the genome sequencing of different pathogens present in wastewater.

The norovirus has been responsible for 210,000 deaths per year. In 2016 World Health Organization (WHO) identified norovirus as a priority disease for vaccine development. However, several vaccines are still in trials phase and preclinical stages and no licensed vaccine has been available for norovirus infection treatment. The cost effectiveness, target population, and public acceptance are the major considerations for vaccine development [105]. The public awareness about the norovirus and prevention measurement should be spread for protection of people from norovirus infection due to lack of treatment available.

4.2.3.4 Hepatitis A

Hepatitis A virus (HAV) is a nonenveloped virus, belongs to the Picornaviridae family, and has positive sense single-stranded RNA (Fig. 4–4). The RNA consists of only one ORF, which encodes a large polyprotein [106]. There are six genotypes of the virus reported and genotype I, II, and III are infectious to human population and are further subgrouped into A and B, while IV, V, and VI affects other primates than human beings [107]. There is a genetic nucleotide variation of 7.5% in each of the subgenotypes, which has been evaluated with PCR by VP1/VP2A junction present in 168 nucleotide fragment [108].

The interaction of the HAV with host cell involves endocytic pathway. The host cell receptor involved in interaction with virus is TIM-1 (HAVCR1) and it is reported to cycles between plasma membrane and lysosome of host cell with calthrin-mediated endocytosis. The replication, translation, and assembly of virus occurs inside the cytoplasm of host cell [108]. The genetic analysis of HAV could be correlated with the outbreaks of viral infections and transmission mode as well. The main transmission mode for the virus is fecal–oral route, which

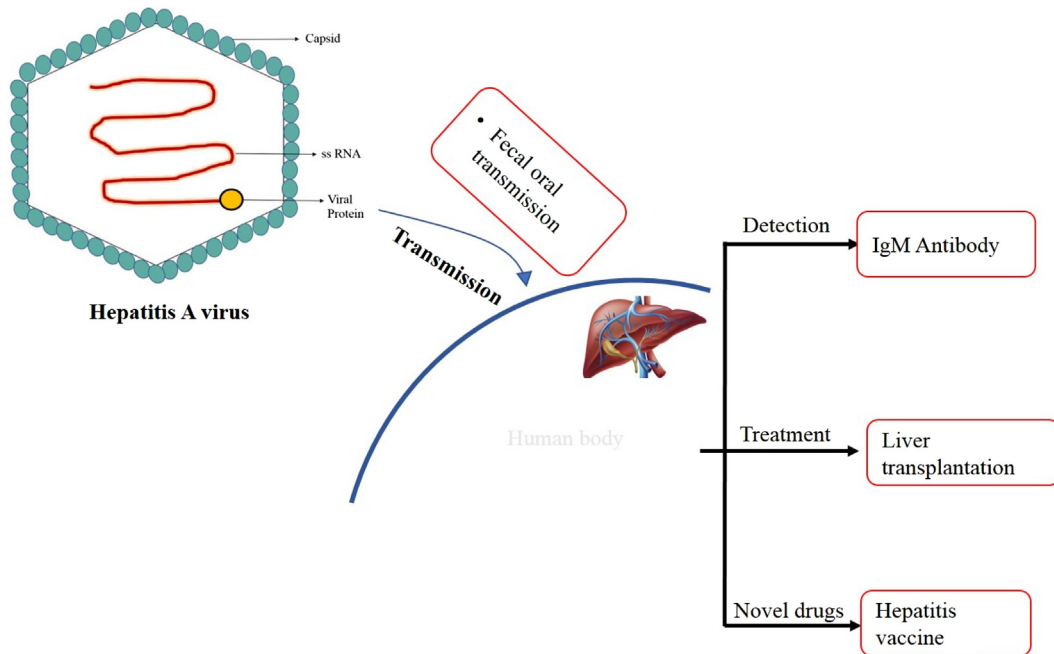


FIGURE 4–4 Hepatitis A virus structure, transmission, detection, and treatment.

could be directly transmitted through one human to another or indigestion of contaminated food as well [109]. The HAV has capacity to survive for months outside the host body, it can survive through freezing temperature but can be killed, when exposed to high temperature of $>80^{\circ}\text{C}$. The geographical distribution of the virus has been mainly present in India, Africa, Middle East, Central America, and South America. The HAV is considered as one of the most important food-borne pathogens and is a major cause of hepatitis in humans with reported cases of around 1.5 million globally. It has been estimated that half of the hepatitis cases are related to HAV. The wastewater coming from the hospital could be emerging source of HAV due to the fact that human excretion contains enormous amount of virus; moreover, virus is highly resistant to harsh environmental conditions and is able to survive for longer periods [110]. The HAV is also detected in rivers, raw or treated water, and dam water as well [111]. These viruses are a real cause of concern for human health; moreover, there are no strict regulations in existence related to monitoring of these viruses in environment and water resources [112]. The consumption of contaminated food has also been directly linked with outbreak of HAV at several places. For instance, there were 397 reported cases of HAV in Michigan between August 2016 to October 2017 with 15 fatalities and 320 hospitalization. The outbreak was linked to consumption of raw scallops served in sushi chain. In another case at Hawaii, 292 cases suffering with hepatitis A infections were reported between June and October 2016, which were linked to contaminated frozen strawberries [113].

The prevalence of HAV was detected in different clinical samples collected over a period of 2 years from Patras and Alexandroupolis located in Greece. The nested real-time PCR revealed the presence of 100% genotype-1 and particularly subgenotype A [114]. The predominance of genotype-1 and subgroup A has also been reported in wastewater samples collected from Brazil [80]. The prevalence of virus was also reported to be higher during the lower number of cases reported for hepatitis A, which could be directly correlated to the shedding of virus into feces. The detection of IgM antibodies and anti-HAV in blood is related to hepatitis A infection. The presence of anti-HAV in blood indicates infection or past vaccination. The HAV vaccination within 2 weeks of infection is the prevention method for HAV infection and includes two-dose series for individuals above age of 12 months. The vaccination includes inactivated virus and is safe for immunocompromised persons [113].

4.2.4 Parasites

The HWW is heavily loaded with pathogens and discharged directly into aquatic bodies could be directly evident by skin infections and intestinal parasites. Around the globe, millions of people are affected by deadly parasites infections, for instance, 2.8 million suffer from Giardiasis, 50 million from Amebic dysentery, 740 million affected by hookworm infections, 795 million detected with Trichuriasis, and 1.2 billion are infected with Ascariasis [13]. These parasites are mostly transmitted through fecal–oral route under poor hygienic conditions, contaminated water and food sources, and poor wastewater disposal practices. The parasites in their ineffective stages such as cysts, eggs, and oocysts survive under environmental adverse conditions and through many wastewater treatments processes due to the presence of protective outer layer. Therefore parasites have capability to survive in wastewater for extended time period in comparison to viruses and bacteria [115]. The samples collected from hospital sewage treatment plant located in Zaria, Nigeria contained several eggs, cysts, and oocytes of various parasites. About 1648 eggs, cysts, and oocysts were present per liter of wastewater. *Ascaris lumbricoides* was the common parasite found in HWW with 307 eggs per liter (18.67%) of wastewater followed by 287 eggs (17.42%) of *Taenia* spp., 253 eggs (15.35%) of *Schistosoma* spp., 176 eggs of *Toxocara* spp., 135 eggs (8.19%) of *Ancylostoma* spp., 130 eggs (7.89%) of *Cryptosporidium parvum*, and 58 eggs (3.52%) of *Giardia lamblia*. Moreover, 92 eggs (5.58%) of *Trichuris* and *Hymenolepis* spp. were also found in HWW. The cysts of *Entamoeba histolytica* and *A. lumbricoides* were found in many studies and remained viable for longer period of time in pond effluents that was further used to irrigate raw vegetables, thus, entering the food chain and then directly to humans [13]. *C. parvum* causes the disease Cryptosporidiasis and *G. lamblia* causes Giardiasis, both could be life threatening if found in persons with weak immune system. The children diarrhea in many cases is caused by the *Cryptosporidium* species [116]. *Taenia* species can cause cysticercosis in humans that can infect muscles, brain and can ultimately cause onset seizures in adults, while it can also cause bovine cysticercosis in cattle after consumption of contaminated water. The main symptoms of parasitic infection include nausea, vomiting, malabsorption, diarrhea, and stomach cramps [117]. Therefore the wastewater should be treated before discharge into water bodies otherwise they could be a great risk to public health.

4.3 Antibiotic-resistance genes prevalence in environment

Antibiotics are one of the most successful and important drugs used in therapeutic applications and the indiscriminate use of these compounds has made their way into the environment. The overuse and misuse of antibiotics not only in human therapeutics but also in veterinary, agriculture, and aquaculture applications [118] has led to the emergence of ARGs and ARB compromising or decreasing the effect of antibiotic compounds as they are becoming resistant to multiple drugs thus, causing a major concern.

Some studies have shown that resistant bacteria can also be present where antibiotic concentrations are low, saying that subinhibitory concentrations can also promote resistance among bacteria as similar to concentrations found in some aquatic and soil environments [118]. Just as natural antibiotics have existed for billions of years, ARGs are also ancient [119]. There have been occurrences of ARGs in places where anthropogenic activities are minimal such as genes encoding for β -lactamase in a remote Alaskan soil suggesting the environment to be a reservoir of ARGs [120]. Antibiotic-resistant bacteria have been reported from Lechuguilla Cave, New Mexico which has been isolated for more than 4 million years including some strains which were resistant to over 14 different antibiotics [121] and in ancient permafrost samples where communities harbored resistance mechanisms minimum 5000 years ago [122]. Such studies improve our understanding of the prevalence of resistance genes in environments much prior to human use of antibiotics. Within past years, pieces of evidence have shown mobilization of these resistance genes from the environment into pathogenic bacteria causing health risks to humans and animals and also, demonstrating a link between environmental and clinical resistance [123].

Moreover, due to the introduction of antibiotics from various human activities, the environment has turned into a reactor of ARB and ARGs contributing to the evolution and spreading of resistant genes. The phenomenon of emergence and spread of ARGs has increased so intensely that 70% of all hospital-acquired infections show resistance toward at least one family of antibiotics. HWW represents an important source of ARGs and ARB and such effluents are highly hazardous due to its infectious and toxic features [78]. Traditionally, resistance was viewed as a healthcare problem but now nonclinical environment has also been reported as an important factor in the dissemination of resistance genes [124]. A wide range of antibiotics and ARGs are being released from the hospitals and urban wastewaters which are received by WWTPs [125]. The WWTP also serves as a hotspot in the emergence and spreading of ARGs and ARB in the ecosystem [126]. Even after the treatment, some of the antibiotics and ARGs are still not completely removed and being released into the receiving water bodies making aquatic ecosystems an ideal place for acquisition and spread of such genes. Commensal bacteria of humans and animals also constitute a reservoir of such resistance genes which can enter the environment through sewer systems or use of animal manure [127,128]. Other than WWTP effluents, ARGs can reach the soils, sediments, surface water and groundwater bodies including drinking water systems [129,130] by surface runoff or infiltration of water that has been used for agricultural purpose [118].

It has been estimated by WHO that ARBs are responsible for 25,000, 23,000, and 38,000 deaths per year in European Union, the United States, and Thailand, respectively [131]. There has been increasing resistance in many human pathogens such as *E. coli*, *A. baumannii*, *Enterococcus* sp., *Klebsiella pneumoniae*, *S. aureus*, *P. aeruginosa*, *Serratia marcescens*, *Citrobacter* sp., and other *Enterobacter* species, and there have been a great number of growing reports concerning the prevalence and dissemination of these pathogens into various environmental settings [132–134]. Knapp et al. [135] reported ARGs from different major classes of antibiotics tested from 1940 to 2008 had significantly increased since 1940, along with tetracycline ARGs around 15 times more abundant than in the 1970s [135]. The resistant gene can be specific to one antibiotic, for example, ciprofloxacin or a group of antibiotics such as β -lactams and as a result, hundreds of ARGs are being detected [136]. Genes resistant to antibiotics are commonly observed for aminoglycoside (*aac*, *aad*, *aph*), chloramphenicol (*cat*, *cmI*), sulfonamide (*sulI*, *sulII*, *sulIII*, *sulA*), trimethoprim (*dfrA*, *dfrB*), quinolone (*qnrA*, B, S), tetracycline (*tetA*-E, G, H, J, Y, Z, etc.), vancomycin (*vanA*, *vanB*), macrolide (*ermA*-C, E, F, T, V, X), β -lactam, and penicillin (*bla*, *mecA*, *penA*) antibiotics in different environments [124,136,137]. Infections caused by resistant strains are difficult to deal and have led to increasing rates of infections, higher hospital costs, and high rates of morbidity and mortality, for example, strains producing extended-spectrum β -lactamase (ESBL) are responsible for higher rates of mortality [138].

There is a widespread occurrence of ARGs originating from different settings that has contributed to a web of resistance among humans, animals, and the environment and, even after their treatment, they can be still present and can contribute to their spread in the environment. Furthermore, there still exists an unexplored pool of genes that may have the potential to be used as ARGs and may be passed to pathogenic bacteria. Various hypothesis have been given for the mechanisms of resistance genes to traverse WWTP and their influence by the treatment process [139] (Fig. 4–5). Thus antibiotic resistance development includes a broader impact on the environment and human health rather than just a local health issue and has to be addressed. The current risk assessment is inadequate and requires advanced biological risk assessment evaluations to detect the proliferation of ARGs and antibiotics. This includes minimizing the resistance emergence and spread in the environment and their transmission to humans. To achieve this, it is necessary to achieve goals such as defining resistance in environmental samples and standardizing testing in those samples which will further require establishment of more comprehensible databases to combine both environmental and clinical metadata. It would help to understand relationships between resistomes of different settings and would improve the risk assessment of ARGs and ARB to further develop control strategies [140].

4.4 Tools used to identify antibiotic-resistance genes

The need for screening of ARGs present in bacteria is important for the optimal antimicrobial therapy to treat infections in patients and this need is increasing with increasing resistance

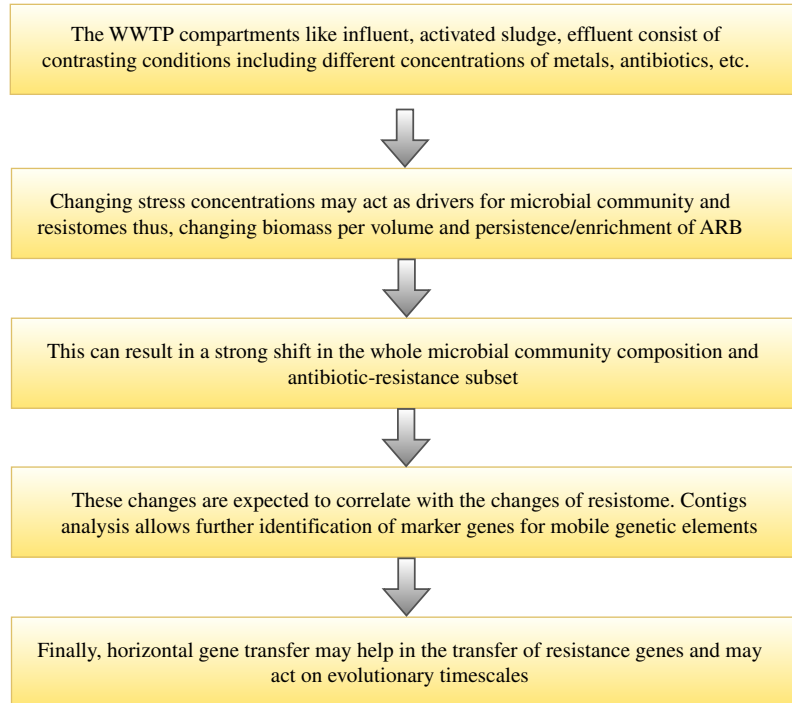


FIGURE 4–5 Hypothesis of the process that effects the microbial resistome during WWTP procedure.

among bacteria. Detection will also predict the spread of resistant organisms and genes throughout the environment. A diversity of detection methods is available for both phenotypic as well as genotypic determination of ARGs in isolates. Antibiotic resistance is a selectable phenotype and can be detected using growth inhibition assays using disc diffusion method, broth dilution, gradient strips, or other methods to determine the minimum inhibitory concentration (MIC) of antibiotics [141]. The MIC is calculated for each isolate and based on the results, isolate can either be susceptible or resistant to the antibiotic [142]. However, there remain certain problems associated with this method, such as gradation of resistance, time-consuming (can take several weeks for slow-growing bacteria such as *Mycobacterium tuberculosis*), and culture-dependent method, relate to only concentration of antibiotics (difficult to detect low-level resistance), and give no information about dissemination of resistance via mobile genetic elements (MGEs) [141,143]. To overcome these disadvantages, genotypic or molecular characterization methods such as PCR, hybridization techniques including microarray, and whole genome sequence (WGS) are used extensively to determine specific resistance genes and providing results within hours. Genotypic characterization has led to the identification of ARGs in fastidious bacteria which expresses few phenotypic features, for example, *Tropheryma whipplei*, that causes whipple's disease showed mutations in *gyrA* and *parC* gene owing to fluoroquinolones resistance [144].

The use of a plethora of PCRs such as standard, real-time, multiplex PCR to detect the presence of genes encoding resistance to aminoglycoside, chloramphenicol, macrolide, β -lactam, penicillin, trimethoprim, and tetracycline in bacteria is quite common [136,145,146]. Isothermal amplifications using loop-mediated isothermal amplification [147] PCRs are very rapid, performed at one constant temperature and have been developed to detect ESBLs genes and carbapenemases genes in bacteria but they cannot be used in multiplex to detect several genes simultaneously. Very recently, a paper-based chip which is integrated with LAMP and a switch molecule for fluorescent detection of ARGs has been developed to allow more convenient and efficient detection especially in resource-limited conditions [148]. Many other detecting and genotyping techniques based on the PCR have been developed to identify ARGs such as restriction fragment length polymorphism-PCR, mismatch amplification mutation assay-PCR, or PCR-single strand conformation polymorphism that have been used to detect *gyrA* mutations in quinolone-resistant isolates [149].

Schwartz et al. [150] used PCR technique to detect *vanA* (vancomycin-resistance gene), *mecA* (methicillin-resistant gene), and *ampC* (ampicillin-resistant gene) in wastewater, surface water, and drinking water biofilms [150]. Recently both PCR and RT-PCR have been used across Europe for detection of plasmid-mediated *mcr-1* genes harboring resistance against colistin [151–154] (Table 4–4). Multiplex RT-PCR has been used to identify Staphylococci from blood samples by targeting *mecA* and other species-specific genes [158]. Another example of using multiplex PCR is in the detection of prevalent ESBLs genes in *E. coli* that encoded mainly *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{OXA} [156]. It is also possible to devise and use multiplex PCR to detect 35 genetically diverse resistance genes for

Table 4–4 Commonly detected ARGs in various water environments.

Antibiotic class	ARGs	Source	Detection method	References
Sulfonamides	<i>sul(I)</i> , <i>sul</i> [6], <i>sul(III)</i> , <i>sulA</i>	WWTP effluent	PCR	[136,155]
Vancomycin, methicillin, ampicillin	<i>vanA</i> , <i>mecA</i> , <i>ampC</i>	Wastewater, surface water, and drinking water biofilms	PCR	[150]
Colistin	<i>mcr-1</i>	Pigs, wastewater	RT-PCR	[151]
Macrolides	<i>ermF</i> , <i>ermB</i> , <i>ermA</i>	WWTP effluent	PCR	[155]
β -lactams	<i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{VEB} , <i>bla</i> _{VIM} , <i>ampC</i>	WWTP effluent, drinking water, and sewage water	Multiplex PCR	[155,156]
Tetracycline, sulfonamides	<i>Tet39</i> , <i>sul</i> [6]	Wastewater, fish farms	PCR-southern blot assays	[157]
Tetracycline	<i>tetA</i> , <i>tetB</i> , <i>tetC</i> , <i>tetD</i> , <i>tetE</i> , <i>tetG</i> , <i>tetH</i> , <i>tetJ</i> , <i>tetY</i> , <i>tetZ</i>	Wastewater from hospital, farms, untreated sewage, effluent of WWTP	PCR, microarray	[136]
Chloramphenicol	<i>cmIA</i> ₁ , <i>cmIA</i> ₅ , <i>catB</i> ₂ , <i>catB</i> ₃ , <i>catI</i> , <i>catII</i> , <i>catIII</i>	Activated sludge, sewage water, natural water	PCR, molecular hybridization	[136]

tetracycline thus, making it one of the most widely used techniques [159]. However, these methods have low-throughput, sometimes gives false results, mostly ignore potential reservoirs of resistance that are nonculturable bacteria, and depend on primers that leave less room for discovery of novel genes.

Molecular hybridization is one of the oldest molecular techniques that have been used to detect the presence of specific ARGs and meanwhile several improvements have been done on probe designs and synthesis. Southern hybridization demonstrated *tet* and class-I integrons can be cotransferred from isolates present in soil to *E. coli* and/or *Pseudomonas putida* [160]. PCR-Southern blot assays were used frequently and reported tetracycline (*tet39*) and sulfonamide (*sulII*) resistance genes in *Acinetobacter* species from fish farms in Thailand [157]. Probes can be labeled with a variety of reporters including radioactive and nonradiolabeled systems. One nonradiolabeled method is fluorescence in situ hybridization that has been used for rapid detection of macrolide, clarithromycin, linezolid-resistances [136].

High-throughput DNA microarray is another technique that has been successfully used to detect ARGs in the test organism in comparison to a reference strain and works with high speed and high delicacy. In this technique, probes specific to the particular gene are spotted on a solid substrate (e.g., glass slide). DNA is labeled and hybridized, and the specific target-probe duplexes are detected. Comparison of genomic diversity in a large number of test isolates can be done for which WGS is not available [145]. It enables detection of a large number of single genes, mutations, MGEs and also can characterize strain at the molecular level [161]. It has been used for antimicrobial genes detection in a diverse range of bacteria [162]. Glass-based microarray has been developed for the detection of 17 tetracycline-resistance genes and one β -lactamase gene in multiple bacteria that used microarray probes c. 550-base pair PCR products [134]. Perreten et al. [163] developed a rapid and efficient screening of gram-positive bacteria using microarray for the presence of 90 ARGs which was further improved by additional oligonucleotides for detecting 117 ARGs [163,164]. Similarly, Alere microarrays have been developed capable of detecting 75 clinically relevant antibiotics and can be used in routine diagnostics laboratories [165]. Some of its disadvantages include, low detection limit, high cost (due to use of platforms with probes of short oligonucleotides or PCR products as well as fluorescent dyes) and problems in dealing with complex samples [166,167].

Just like PCR and microarray, WGS is another potential method to detect genes and mutations conferring antibiotic resistance. The main advantage is its ability to use and detect different targets simultaneously and to subtype-specific gene variants. One can possibly add new targets to the database for analysis and can perform rapid in silico reanalysis of sequenced isolates. The presence of ARGs within the WGS data has to be determined for which, comprehensive databases containing relevant target DNA is required along with the use of appropriate bioinformatics methods for obtaining information about the WGS data.

Some of the databases that have been used for detection of ARGs in curated WGS data are ResFinder, which is a web server and uses BLAST for identification of ARGs [168], comprehensive antibiotic-resistance database that uses BLAST and resistance gene identifier as

two analysis options [169], antibiotic-resistance gene-annotation that gives user an opportunity to use local BLAST in Bio-Edit software and analysis can be done without web interface [170], and antibiotic-resistance gene database that is a manually curated database unifying publicly available information on ARGs such as resistance profile, ontology, mechanism of action, and much more [171]. Other knowledge resources of AMR include antibiotic-resistance genes online, collection of antimicrobial peptides, database of antimicrobial activity and structure of peptides, antimicrobial peptide database, and BacMet which differ from each other on the basis of the knowledge on molecular level of ARGs they reflect and the scope of resistance mechanism they cover [172].

However, one major drawback of using molecular techniques for ARG detection is that new emerging resistances against some antibiotics may be overlooked as observed for *ndm-1* gene encoding resistance to carbapenem antibiotics which was first isolated using phenotypic methods and then characterized on the basis of genotype [141]. Thus, within the number of both phenotypic and genotypic methods, there are limitations involved with each one and one can perform a combination of these screening methods to monitor resistance. Thus the use of all these techniques helps in predicting the resistance genes, setting up control measures in hospital infections, adapt to a specific therapy and using them routine detection tools [173].

4.5 Metagenomics of hospital wastewater

The molecular analysis tools such as PCR, quantitative PCR, and 16s rRNA analysis have provided the in-depth knowledge about microbial communities present in wastewater since decades [174]. However, requirement of gene-specific primers, and species-specific approach used by these tools limit their activity for detection of certain targeted microbes providing incomplete information about microbial communities present in wastewater [175]. Recently, metagenomics analysis has been introduced, which overcomes the limitations related with conventional molecular analysis tools and is able to generate hundreds to thousands of sequences, providing complete profile of microbial communities present in unknown samples, thus high abundance of potential microbes are detected [176]. The metagenomics analysis consists of four steps; genetic material isolation followed by genetic material cloning, construction of library, and the analysis of genetic material from the metagenomics library.

Metagenomics along with Search Engine for AMR [177] can analyze the unknown samples collected from environment and can provide full-length ARG data. The HWW has been reported to have two overexpressed β -lactam-resistance genes (*bla*_{GES} and *bla*_{OXA}) as compared with the water collected from other aquatic bodies, which could be correlated with antibiotic usage over the time in hospitals and discharge of the residues of antibiotics in the wastewater [176]. The HWW that is derived from clinical speciality ward is determined to be major spot for the AMR. The wastewater derived from different wards of the hospital and the final effluent of hospital was tested and compared for resistance genes. The wastewater has a high abundance of class A β -lactamase resistant genes (i.e., *bla*_{KPC}, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}) and

the wastewater from clinical ward also consists of high level of *bla*_{KPC-2} genes (142,200 x/Gb), encoding for carbapenem resistance. Moreover, there was presence of assembled scaffolds of IncQ and IncF plasmids having quinolone-resistance genes (*qnrS1*, *qnrS2*) and the class A β -lactamase gene (*bla*_{TEM-1}) in wastewater, which further helps in proliferation of AMR [178].

Metagenomic functional selection has been integrated with deep metagenomic sequencing to trace validated ARGs in different environments. Studies have identified WWTP resistome consisting of novel ARGs thus, throwing the limelight on WWTP reservoir of uncharacterized resistance genes using these selections. Using such metagenomic methods relates the number of clinically relevant resistant genes to overall functional resistant genes and thus, helps in improving the risk classification in environments [49].

Metagenomics strategy also revealed the association of isolated *P. aeruginosa* from HWW with AMR frequency. The isolated strain was selected as a bio-indicator, due to its ability to survive and colonize in harsh environments and it was used to assess its viability, antibiotic susceptibility, and diversity in HWW. The samples collected during different treatment steps of HWW revealed that each treatment step was able to decrease the bacterial population by four logarithm cycle; however, the antibiotic resistance profile did not decrease at each treatment level, while, on the contrary, there was increase in AMR microbes and genes as well. The microorganism was able to remain survived in the spore form during different treatment steps and again transform into vegetative form when released into surviving environment [179]. The healthcare clinics and hospitals, where antibiotic use is more common among the major contributor to disseminate the pathogenic microorganism into the environment. In addition, shotgun metagenomics analysis of HWW collected from Turkey revealed the presence of more than 100 antibiotic resistance determinants and most common genes belongs to aminoglycosides and β -lactamases. The prediction of high resistome diversity in HWW raises an alarm for health concern of human population.

4.6 Horizontal gene transfer

An important step in coping with the serious problem of emergence and dissemination of ARGs is to understand the pathways for resistance gene spread. The ability of bacteria to respond to selective pressures and new environments can be explained by the acquisition of new genes by cells using horizontal transfer methods. Studies have shown around 75% of the genes in each genome have been transferred by HGT during evolution [180]. Many resistance genes are located on MGEs such as plasmids, transposons also known as jumping genes, and integrons that are capable of capturing and expressing gene cassettes [181], which are responsible for antibiotic resistance that functions as vectors for their dissemination [182]. Horizontal acquisitions might be neutral or have deleterious effects in the chromosome or confer a selective advantage to the host. Other than mutations, HGT has been one of the methods which are most responsible for the dissemination of ARGs among different bacterial species. It has caused the spread of antibiotic resistance from commensal and

environmental bacteria to pathogenic species. The ARGs transfer by HGT exists much back before the use of antibiotics by humans, for example, OXA β -lactamase genes that confer resistance against β -lactam antibiotics mobilized from chromosomes to plasmid millions of years ago [183]. But the rate of the resistance gene transfer has increased tremendously due to selective pressure caused by antibiotic use by humans in the last few decades.

Bacteria employ three mechanisms for HGT, that is, conjugation, transformation, and transduction (Fig. 4–6). Conjugation is said to have the greatest influence over the spread of ARGs. Conjugation requires a physical contact between two cells via pili or adhesions to transfer the genetic material. This mechanism has been found in bacterial cells with an exception of *Agrobacterium* species that uses HGT in plants. The conjugative machinery encoded by the genes on plasmids or by integrative conjugative elements on chromosomes facilitates this process. ARGs are mostly associated with conjugative elements like plasmids and transposons, and the transfer of these elements is most likely to occur by conjugation

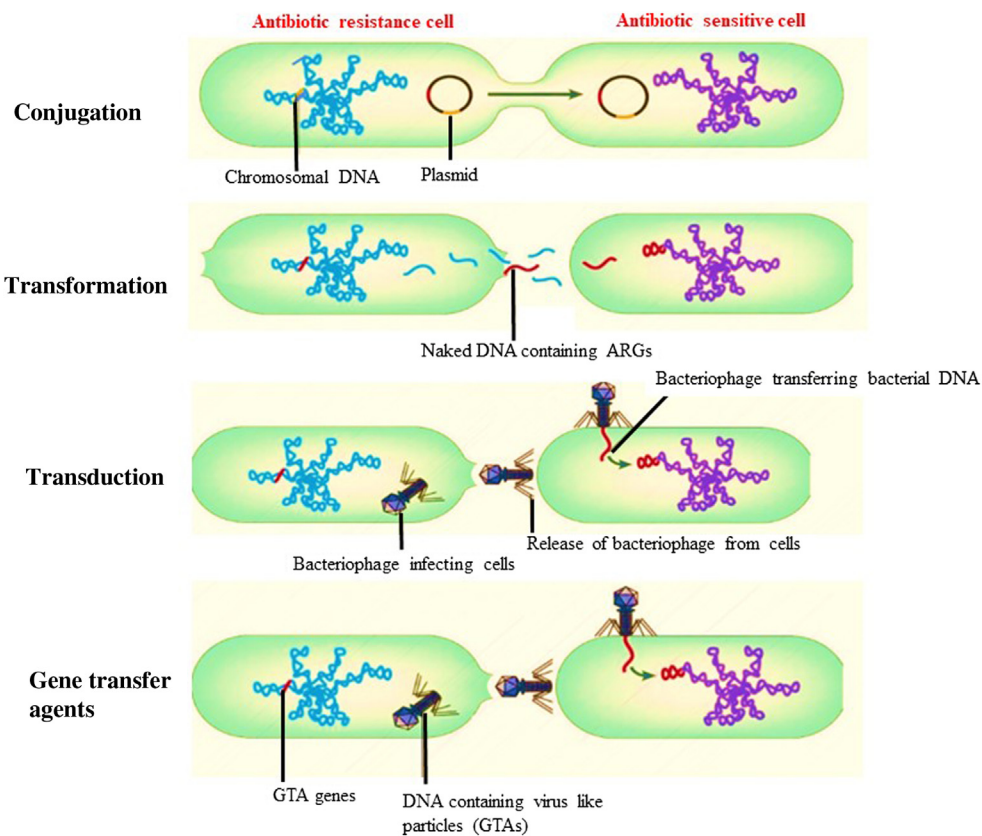


FIGURE 4–6 Different mechanisms of horizontal gene transfer shown by bacterial cell for transferring ARGs. Modified from E.Y. Furuya, F.D. Lowy, *Antimicrobial-resistant bacteria in the community setting*, *Nat. Rev. Microbiol.* 4 (1) (2006) 36 [184].

because it provides a more efficient way to enter the recipient cell and gives better protection in the environment [119]. Conjugation can be of various types such as (1) transfer of a self-transmissible conjugative plasmid, for example, RP4 plasmid of *E. coli*. (2) Mobilization of nonself-transmissible plasmids by the action of conjugative plasmid, for example, IncQ plasmid RSF1010. (3) Cointegration of two different circular plasmids to fuse to become one. (4) Conjugated transposons which may facilitate mobilization of plasmids or cointegration. The MGEs conferring ARGs transfer have been found in bacteria ranging from soil, water ecosystems to food, and human pathogens [185]. The transfer of the conjugative elements between bacteria over distant phylogeny indicates the emergence of multiresistance between different reservoirs [186]. *Bla*_{CTX-M} ESBL genes have been disseminated to various plasmids within Enterobacteriaceae and other pathogens and can be found in various geographical locations [187]. The conjugation of plasmids has caused dissemination of ARGs worldwide that encode resistance to β -lactamases, carbapenemases, quinolones, aminoglycosides, colistin, sulfonamide, tetracyclines, and other classes of drugs. Conjugation has been also reported between bacteria and eukaryotic cells and is observed in various environments such as soil, aquatic, marine sediment, wastewater, and activated sludge [124].

The second mechanism is transformation, in which the cells take up the naked DNA from the environment. For transformation to take place, there are certain prerequisites like release and persistence of extracellular DNA in the environment, the recipient cells must be competent, and the DNA translocated must be stabilized by integration into the genome or by recircularizing into self-replicating plasmids [188]. The process takes place in two steps: first, the DNA substrate is transferred from surface to the cytoplasmic membrane which is mediated by type II secretion system, type IV secretion system, and type IV pili; and second, is the transport of DNA across the cytoplasmic membrane using cytoplasmic membrane channels [189]. With an exception of *Neisseria gonorrhoeae* that is a naturally transformable bacteria which responds to environmental conditions [188], most species develop competence under autoinducers, peptides, or stressful conditions, for example, aminoglycoside and fluoroquinolone antibiotics sublethal concentrations induce competence in *Streptococcus pneumoniae* and *Legionella pneumophila* [190]. Mao et al. [191] developed a method to extract extracellular and intracellular DNA from water and sediments. They obtained a higher concentration of extracellular DNA than intracellular DNA in sediments from a river basin that serves as a major ARG reservoir that could be transferred to indigenous bacteria through natural transformation [191]. Also, it has been shown in pneumococcal genome that the conjugated transposon disseminates via transformation in addition to conjugation [192].

Transduction is the process by which DNA is transferred with the help of bacteriophages. Bacteriophages can transfer DNA sequences like chromosomal DNA, MGEs such as plasmids, transposons and genomic islands that are advantageous to their bacterial hosts as well as serves in improving the survival of bacteriophages [193]. Transduction can be generalized where random host DNA is incorporated during cell lysis and specialized, where the prophage excises itself from the host genome and also incorporates flanking host DNAs [194]. ARGs transfer by transduction has also been reported in various bacteria, for example, β -lactamase gene transfers by P1-like bacteriophages in *E. coli* [195], erythromycin resistance

transfer by phage between *Clostridium difficile* strains [196], tetracycline and gentamycin resistance between enterococci [197], or transfer of resistance plasmids in MSRA [198]. Bacteriophages can have broad host range, that is, between different species or among different taxonomic groups [197] thus, indicating dissemination of ARGs via transduction in the environment, a common mechanism.

Other mechanisms such as gene transfer agents (GTAs) and cell fusion have also been described. GTAs are delivery systems that carry random pieces of host genome in capsids and get integrated into the host chromosome. The amount of DNA that GTA contain is insufficient to encode their proteins counterparts, unable to self-propagate thus, they do not necessarily carry GTA-encoding genes that is an important distinction from transduction mechanism [199]. The first GTA discovered was in *Rhodopseudomonas capsulate* called as RcGTA and they were able to transfer antibiotic resistance to bacteria by a mechanism similar to transduction which does not require cells contact and was resistant to DNases [200]. Apart from the evidence of GTA shown in various members of Rhodobacterales, for example, *Ruegeria pomeroyi* which contain a complete RcGTA-like cluster of these genes, *Roseovarius nubinhibens* and *Ruegeria mobilis* also showed rates of transfer of antibiotic resistance around 10^6 -fold higher than rates of transformation and transduction when added to microbial communities [199]. McDaniel et al. [201] reported GTA frequencies to be much higher, around a million times higher the frequency of transformation and transduction measured previously in the marine environment [201]. There are several advantages of GTAs over other mechanisms such as protection of DNA from environmental factors, transfer not constrained to cell–cell contact, and they contain random DNA from host rather than most of the bacteriophage DNA as observed in transduction. Another mechanism, cell fusion has been observed in *Haloferax* and *Sulfolobus* species [202,203]. Symmetric and bidirectional cell fusion has been observed in *Haloferax volcanii*. Studies have shown interspecies gene exchange in halophilic archaea where cells fuse forming a diploid state containing two different chromosomes of parental cells thus, facilitating genetic exchange and recombination followed by separation of hybrid parental cells [202]. Although new mechanisms are continuously being developed by bacteria and identification, mapping of these resistance mechanisms provides us with knowledge of sources of resistance and helps in designing new antimicrobial drugs.

4.7 Emerging infectious particles

4.7.1 Prions

The prions are infectious protein particles present in brain, which are responsible for degenerative neurological disorders. In humans, the PrNP gene present on chromosome 20 produces the PrP protein and prions results in transformation of PrP protein into abnormal disease causing isoform [204]. These pathogenic prions cause the functional disruption of neural cells resulting in cell death and leading to memory problems, trouble with movement and personality changes as well. The prions can result in many infectious diseases such as Creutzfeldt-jakob disease (CJD), variably protease-sensitive prionopathy, Gerstmann-Sträussler-Scheinker

disease, scrapie, variant CJD, fatal insomnia, and kuru. These transmissible disease impacts number of mammalian species, including sheep and goats, cattle, deer, moose, elk, and humans. The scrapie and CJD are of particular concern because they can be transmitted horizontally and they remain infectious in the environment for very long time [205]. For instance, the scrapie infection was persistent even after burial in soil for 3 years. The CJD disease is most common and deadly human prion disease and can cause infection in three ways: sporadic, genetic, and acquired. Most of the times (85%–90%), CJD occurs sporadically and approximately 1–1.5 million people are affected annually, because there is no cure available for CJD and other prion diseases [206]. The recently developed methods for detection of prion infectivity include protein misfolding cycling amplification, quake-induced conversion, and quantitative tandem mass spectrometric techniques [207]. However, the study of prions is highly challenging due to the fact that the high-resolution structure of prion isotherm is still to be determined and change in structure directly affect infectivity and pathology of disease.

Prions are likely to enter through live, infected hosts, and can be shed through saliva, urine, feces, mucus, and blood; and they enter in wastewater through hospital effluents, research facilities, homes, slaughterhouses, and mortuaries [208]. The WWTPs are not able to treat prions, for instance, after entering into municipal WWTP, the prions bind to sewage sludge, survive through anaerobic digestion and are further present in treated biosolids. These biosolids are further used for land application, which results in their introduction into environment. Thereafter they can be swept by wind and contaminated water with prions could further migrate into lakes, rivers, and oceans and thus, directly affecting the humans, aquatic life, and animals as well [209].

4.7.2 Viroids

Viroids are small (~ 250 – 400 nucleotides), single stranded, circular RNA particles, which are distinguished from viruses by smaller size, lack of capsid and also they do not encode any protein. They have capacity to reproduce and are mostly known to cause infection in plants; however, hepatitis δ , which cause infections in humans, has many similar properties with viroids and could be related to viroids [210]. A total of 29 viroids species has been identified, which are grouped into Avsunviroidae and Pospiviroidae. The viroids are mainly infectious for plants such as tomatoes, cucumber, avocados, potatoes, apples, coconut palms, and chrysanthemums and are responsible for million dollars' loss in agriculture revenue each year. The most commonly reported viroid plant diseases caused by viroid include apple fruit crinkle, tomato chloric dwarf, and chrysanthemum chlorotic mottle [211]. The yellowish curled leaves of plants are due to pairing of viroid RNA with messenger RNA of plants resulting in interference of proper translation. Some of the viroids cause devastating effects, for instance, Coconut cadong–cadong viroid has caused lethal effects in coconut palms in Philippines, resulting in loss of 40 million coconut palm [212]. Other reported effects of viroids include epinasty, rugosity, necrosis, chlorosis, stem shortening, color alteration of fruits, flowering, and ripening delays. The microarrays are identified as a significant tool for detection of many viroids simultaneously and even has ability to detect emerging or established viroid in new host.

4.7.3 Toxins

Potent protein toxins such as tetanus and botulinum toxins, anthrax toxin, epsilon-toxin, and enterotoxin can cause some most significant diseases in humans and animals. These protein toxins are most common virulence factors encoded by plasmids in *Clostridium* and *Bacillus* species, for example, tetanus toxin plasmid and conjugative toxin plasmid of *C. perfringens*. Extracellular vesicles (EVs) are also infectious particles said to be produced by all types of microorganisms. These EVs can contain nucleic acids, toxins, lipoproteins, adhesins, nutrient scavenging factors, and enzymes that can play major role in pathogenicity in HWW. Such vesicles from Gram-positive bacteria, mycobacteria, and fungi contain virulence factors that can elicit host immune responses. For example, *Cryptococcus neoformans* EVs have glucuroxylomannan (capsular polysaccharide) which is a virulence factor [212]. EVs from Gram-negative bacteria originate from outer membrane and are called outer-membrane vesicles which have been associated with cytotoxicity, virulence, invasion of host cells, and antibiotic resistance proteins. In addition to these particles, certain factors and conditions may further increase the virulence of EVs, for example, a study indicated the role of iron-limiting conditions in the virulence of mycobacterium EVs. These emerging infectious particles are not easily removed by conventional treatment technologies. Thus there is a requirement of more novel methods and techniques to deal with such infectious particles. Therefore these particles have to be readily analyzed for their presence and have to be studied for their removal from HWW due to their potential negative effects on human and animal life.

4.8 Conclusions and perspectives

The recent years have witnessed the emphasis toward management of HWW and various studies focused on the microbial communities present in wastewater have increased immensely. The pathogenic microbes present in HWW have affected the human health since decades and antibiotic resistance microbes are also increasing significantly with time. The development of resistance toward antibiotics has been observed worldwide and has challenged both public and animal health. The use and release of various antibiotic agents in different settings have not only led to the prevalence of ARGs in the environment but also spread and emergence of resistant bacteria. This has caused increased resistance in human pathogens and thus, making infections caused by them difficult to deal with, leading to higher mortality rates. However, the surveillance of its spread and prevalence in environment is limited and has to be expanded more due to the broad impact of antibiotic resistance on human health. Various culture-dependent and independent techniques have allowed characterization and exploration of ARGs and ARB, which have increased our understanding toward the evolutionary pathways for their dissemination within any community. Further, metagenomics tools have advanced the research toward analysis of complete microbial profile and increased the knowledge toward microbial abundance present in HWW. However, further planning and implementation of strategies, policies, and experimental approaches have to be done by collaboration of scientific community and public authorities to limit the

use of antibiotics, detection of microbial communities (resistant and/or sensitive) from wastewaters, and mapping resistance mechanisms to clearly understand the role of MGEs and extracellular DNA in evolutionary process.

Abbreviations

AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic-resistance genes
CCCVd	Coconut cadong- cadong viroid
CJD	Creutzfeldt-jakob disease
dPCR	Digital polymerase chain reaction
EH	Early hours
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immuno sorbent assay
ESBL	Extended-spectrum β -lactamase
EV	Extracellular vesicles
GTA	Gene transfer agents
HAdV	Human adenovirus
HGT	Horizontal gene transfer
HWW	Hospital wastewater
LH	Late hours
MCP	Membrane cofactor protein
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
ORF	Open reading frame
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RAPD	Random Amplification of Polymorphic DNA
ssRNA	Single-stranded ribonucleic acid
WGS	Whole genome sequence
WHO	World Health Organization
WWTP	Wastewater treatment plant

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