APSI Alliance for Pathogen Surveillance Innovations – India

Wastewater Surveillance of pathogens contributing to antimicrobial resistance

Protocol Handbook 2024

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INDEX

1.0 Background

Antimicrobial resistance (AMR) occurs when microbes like bacteria, fungi and parasites, evolve to resist the drugs designed to kill them and as a result, illness persists in the patient's body, increasing the possibility of transmitting to others. Inappropriate use of antibiotics in any field like agriculture, medicine, aquaculture and food industry has resulted in the wide dissemination of resistance. AMR has been ranked among the top 10 global health threats by the world health organization (WHO). As per the report of Antimicrobial Resistance Collaborators, published in 2022, in the year 2019, 4.95 million deaths were linked to bacterial AMR worldwide, of which roughly 1.27 million deaths were specifically attributed to bacterial AMR. The six leading pathogens *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, were responsible for ~0.93 million deaths. It is estimated that by 2050, an annual death toll of 10 million will be attributed to AMR if appropriate policy measures are not taken and India could contribute to 20% of it. According to the World Bank Report, AMR could lead up to 7.5% decline in livestock by 2050. Under high AMR impact scenario, it has been estimated that world could lose over 3.8% of annual GDP by 2050. Thus, immediate attention and actions are necessary towards the geographical surveillance of AMR and identifying the classes of antibiotics which are at the highest risk of being innocuous.

Wastewater surveillance for AMR

The signatures for antimicrobial resistance are carried in the form of antimicrobial resistance genes (ARGs) in the pathogens either on their genomes or on mobile genetic elements. Wastewater surveillance for AMR involves detection of these ARGs in the wastewater. Wastewater samples are collected from the sewage treatment plants or open drains and are monitored for the signatures of ARGs. The samples are concentrated and used to extract the nucleic acids to detect either the pathogens or identify the antimicrobial resistance genes that are carried by these pathogens.

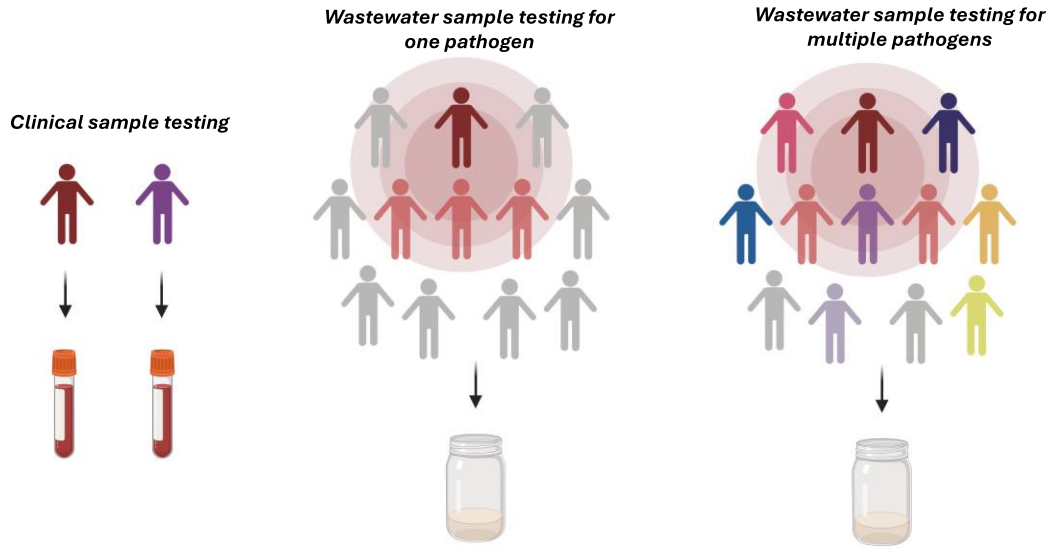

Figure 1: Wastewater surveillance as a complementary surveillance system for clinical surveillance of pathogens and associated traits

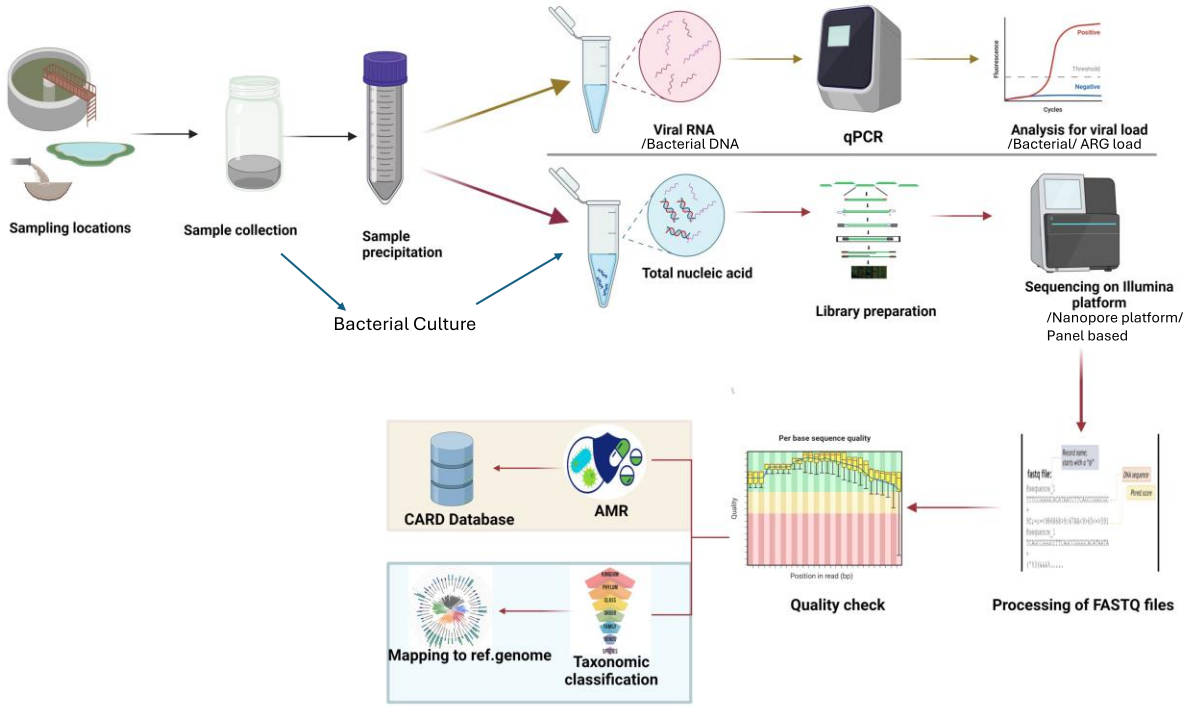

Figure 2: Schematic showing the overview of wastewater surveillance for AMR

Requirements:

Reagents and plasticware: Sample collection bottles, masks, gloves, bucket and rope, 0.1% hypochlorite, falcons, sample tubes, polyethylene glycol- 8000 (PEG; Sigma Aldrich; Cat No. P5413), Sodium chloride (NaCl; Sigma Aldrich; Cat no 33205), DNA/RNA Extraction and q-PCR Kits (details mentioned in subsequent sections)

Equipment: Centrifuge with slots for 50 ml tubes and 1.5ml tubes, water bath, vortex, nanodrop, RT-PCR machine.

1.1 Sample Collection

The person collecting the sample should wear gloves and, cover nose and mouth by a mask. They can wear a personal protective gown. Personnel must be aware of hazards that exist during sampling and the associated risks, safe working procedures, and emergency preparedness.

Collect around 500 ml to 1 litre of wastewater sample in sterile bottles by grab sampling method and transport to the laboratory immediately. If the duration of transport from the sampling site to the laboratory is longer than 2-3 hours, samples should be transported at 4°C, packed in ice packs. If the transport of samples involves intermediate handling steps by untrained personnel (for example transport of samples through courier or public transport), the samples should be inactivated at the site of sample collection by adding disinfectant (20 ml of 0.1% hypochlorite for 1000 ml collection volume). The bottle should be sealed in secondary sealing bags (multi-layered). The surface of the sample container should be wiped with disinfectant. Before packaging, the samples should be appropriately labelled with collection site name, sampling location co-ordinates, date and other relevant information. Precautions must be taken to avoid

leakage at any step. The sample should always be maintained at 2-8 °C immediately after collection, for transport and until processing.

1.2 Sample Heat Inactivation

Samples are heat inactivated at 60°C for 30 minutes in water bath in the laboratory. If the samples are inactivated by adding hypochlorite, this step is skipped.

1.3 Concentration and/ or Precipitation

50ml of inactivated sample is used for polyethylene glycol- 8000 (PEG) based precipitation. 4 gm PEG-8000 (Sigma Aldrich) and 0.9 gm NaCl (Sigma Aldrich, SRL or HiMedia) is added to the sample in a 50ml falcon tube. Samples are incubated for 2 hours or overnight at 4ºC after thorough mixing. They are then centrifuged at 10,000 rpm for 30 minutes at 4ºC. The supernatant is discarded, and the pellet is used further for DNA extraction. 3 sets of 50 ml samples are used for PEG precipitation. One set is used for further downstream processing and the remaining two sets are stored at -80°C.

Note: PEG NaCl should be added to the sample after the heat inactivation while samples are little warm. High temperature of the sewage sample helps to dissolve the Polyethylene Glycol and Sodium Chloride properly. Vortex the sample to dissolve the PEG and NaCl.
All the procedures should be done inside the a biosafety cabinet following appropriate biosafety measures.

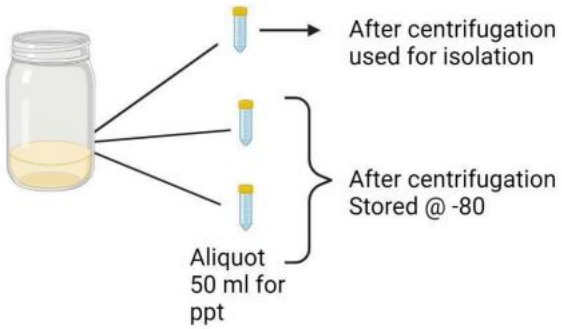

Figure 3: Schematic showing wastewater sample processing

Alternatively, filtration based method can also be used for sample concentration. Filter inlet samples through sterile fourfold muslin cloth with a pore size of 0.1 – 0.5 mm to remove large debris. After initial filtration, pass the samples through a 0.22 µm filter membrane using a vacuum filter assembly. Using sterile forceps, carefully roll the filter membranes to use it for DNA extraction after chopping into pieces and using bead beating method.

1.4 Nucleic acid extraction

PEG pellet from the 50 ml sample is used for nucleic acids extraction using Qiagen All Prep Power Viral DNA/RNA Extraction Kit (Qiagen; Catalogue No. 28000-50) following manufacturer's protocol. The nucleic acids are eluted in 100 ul elution buffer.

Important points before starting

Warm Solution PM1, provided in the kit, at 55°C for 10 min before use to dissolve precipitates. Use Solution PM1 while still warm. Shake to mix before using.

For RNA isolation, add β-mercaptoethanol (β-ME) to Solution PM1 to produce a mixture of Solution PM1/β-ME with a final β-ME concentration of 10 µl/ml. The mixture of Solution PM1/β-ME loses its effectiveness over time, so prepare a fresh batch each time you use the kit. You will need 600 µl of the Solution PM1/β-ME mixture per prep.

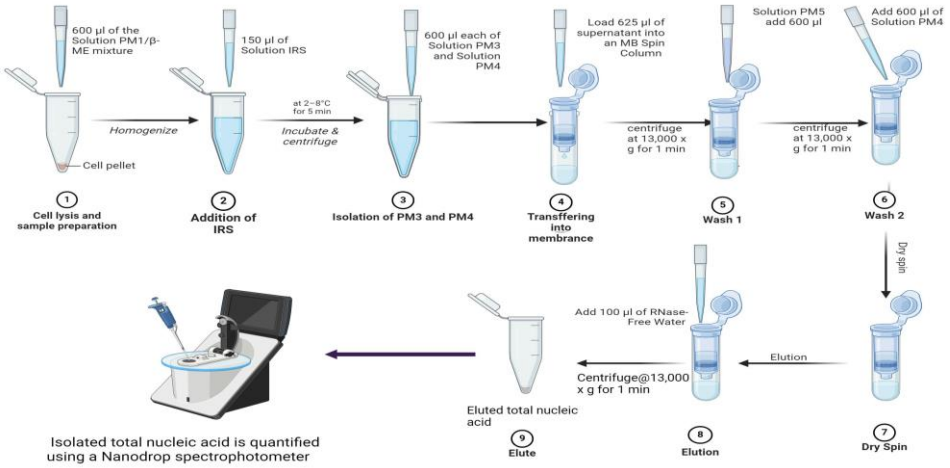

Figure 4: Pictorial representation of nucleic acids extraction

Procedure

All the reagents and buffers used in this section are provided in the Qiagen All Prep Power Viral DNA/RNA Extraction Kit.

1. Add 600 µl of the Solution PM1/β-ME mixture (see Important points before starting) to the 2 ml Collection Tube containing the pellet. Alternatively, you may add 600 µl of Solution PM1 and 6 µl of β-ME to the Collection Tube. Vortex the sample and the mix of Solution PM1/β-ME for 30 seconds. Incubate for 5 min at room temperature.
2. Add 150 µl of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min.
3. Centrifuge at 13,000 x g for 1 min. Avoid the pellet, and transfer the supernatant to a clean 2.2 ml Collection Tube (provided). Do not transfer more than 700 µl.
4. Add 600 µl each of Solution PM3 and Solution PM4. Vortex briefly to mix.
5. Load 625 µl of supernatant into an MB Spin Column and centrifuge at 13,000 x g for 1 min. Discard the flow-through and repeat until all the supernatant has been loaded onto the MB Spin Column. Note: A total of three loads is required for each sample processed.
6. Shake to mix Solution PM5 and add 600 µl to the MB Spin Column. Centrifuge at 13,000 x g for 1 min.
7. Discard flow-through. Add 600 µl of Solution PM4. Centrifuge at 13,000 x g for 1 min.
8. Discard flow-through and centrifuge at 13,000 x g for 2 min.
9. Place the MB Spin Column in a clean 2 ml Collection Tube.
10. Add 100 µl of RNase-Free Water (provided in the kit) to the center of the MB Spin Column membrane. Incubate for at least 1 min. Note: Eluting with 100 µl of RNase-Free Water will

maximize DNA/RNA yield. For more concentrated DNA/RNA, a minimum of 50 µl of RNase-Free Water can be used.
11. Centrifuge at 13,000 x g for 1 min. Discard the MB Spin Column. The DNA/RNA is now ready for downstream applications. It can be stored at –65 to –90°C for long term storage.

Following kits can also be used for nucleic acids extraction as per manufacturer's protocols

Sr No	Kit Name	Manufacturer
1	DNeasy Power Soil Pro Kit (Catalogue number: 47016)	Qiagen
2	DNeasy Power Water Kit (Catalogue number: 14900-100-NF)	Qiagen
3	Wizard Enviro TNA Kit (Catalogue number: A2991) (note-PEG-based concentration method is not required while using this kit)	Promega

The volume of wastewater used for concentration can be modified as per requirement.

Checking the quality of nucleic acids

Take nanodrop reading to know the yield and quality of nucleic acids. The 260/ 280 (general range: 1.8- 2) and 260/ 230 (general range: 2- 2.2) ratios are determined before further processing the samples for sequencing.
Load on 0.8% agarose gel to get an estimate of the quality and quantity of the nucleic acids.

1.5 Quantitative PCR based detection of antimicrobial resistance genes and pathogens:

The nucleic acids can be used for quantitative polymerase chain reaction (qPCR) based detection of antimicrobial resistance genes (ARGs) and pathogens using either TaqMan probes based method or SYBR based method.
The following TaqMan based qPCR kits can be used as per manufacturer's protocol for detection of ARGs in wastewater samples

Sr No	Kit Name	Manufacturer
1	Quantiplus® Antimicrobial Resistance Detection Kit -Carbapenamase Resistance (Cat No: QL-AMR-25)	Huwel Life Sciences, India
2	Hi-PCR® Carbapenemase Gene Quantification Probe PCR Kit (Cat No: MBPCR227-50R)	Himedia
3	Hi-PCR® Extended Spectrum Beta-Lactamases (ESBLs) Gene Quantification Probe PCR Kit (Cat No: MBPCR226-50R)	Himedia
4	Hi-PCR® Colistin Resistance Encoding Gene Quantification Probe PCR Kit (Cat No: MBPCR228-50R)	Himedia

5	Quantiplus® MDR-TB Fast Detection Kit (Cat No: QLF-MDR-TB-100: 100 rxns)	Huwel Life Sciences, India

TaqMan based qPCR kits for other ARG/ pathogen targets can also be used as per requirement.

For SYBR based method, QuantiTect SYBR Green PCR kit (Cat No: 204143, Qiagen) can be used as per manufacturer's protocols, using the following primers

Gene	**Forward Primer (5′-3′)**	**Reverse Primer (5′-3′)**	**Reference**
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	(Chen et al., 2017)
IMP 01	AACACGGTTTGGTGGTTCTTGTA	GCGCTCCACAAACCAATTG	(Chen et al., 2017)
NDM-1	ATTAGCCGCTGCATTGAT	CATGTCGAGATAGGAAGTG	(Chen et al., 2017)
KPC	CAG CTC ATT CAA GGG CTT TC	GGC GGC GTT ATC ACT GTA TT	(Nasri et al., 2017)
VIM	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	(Wang et al., 2014)
OXA 51	TAA TGC TTT GAT CGG CCT TG	TGG ATT GCA CTT CAT CTT GG	(Hubeny et al., 2022)
OXA 23	GAT CGG ATT GGA GAA CCA GA	ATT TCT GAC CGC ATT TCC AT	(Li et al., 2019)
OXA 48	AGG CAC GTA TGA GCA AGA TG	TGG CTT GTT TGA CAA TAC GC	(Nasri et al., 2017)
OXA 58	AAG TAT TGG GGC TTG TGC TG	CCC CTC TGC GCT CTA CAT AC	(Li et al., 2019)
SHV-01	TCCCATGATGAGCACCTTTAAA	TTCGTCACCGGCATCCA	(Wang et al., 2014)
CTX-M-01	GGAGGCGTGACGGCTTTT	TTCAGTGCGATCCAGACGAA	(Wang et al., 2014)
TEM	AGCATCTTACGGATGGCATGA	TCCTCCGATCGTTGTCAGAAGT	(Chen et al., 2017)
MCR-1	GGGCCTGCGTATTTTAAGCG	CATAGGCATTGCTGTGCGTC	(Hembach et al., 2017)

* can be customized for any gene, if primer sequence is known

Procedure

- Before use, all PCR components should be completely thawed on ice (4°C).
- Perform the amplification reactions in a clean area, preferably in a biosafety cabinet.
- Use of aerosol barrier pipette tips is recommended to reduce contamination risks from extraneous DNA templates.
- For detection of each AMR genes always set reaction for 16s rRNA gene as an internal control.
- Always set up the reaction in triplicates

a) Protocol for PCR Master Mix Preparation

- Perform PCR reactions for each DNA sample as per the following table:

Components	Volume
QuantiTect SYBR Green PCR kit	5 µL
Forward Primer (10 µM)	0.8 µL
Reverse Primer (10 µM)	0.8 µL
Molecular Biology Grade Water for PCR	2.4 µL

Template DNA (5 ng) *	5 µL
Total volume	10 µL

*Metagenomic or environmental DNA extracted from water/sediment extracted using any kit/protocol can be used

- Centrifuge the tube briefly at 6000 rpm for about 10 seconds. Place the tubes in Real-time PCR machine and set the recommended PCR program (mentioned below). Interpret the data from the amplification plot (observe the Ct values).

b) Recommended PCR program

Condition	Temperature	Time	No of cycles
Pre-incubation	95°C	5 minutes	-
Denaturation	95°C	15 seconds	45
Annealing	60°C (depends on the Tm of the primer pair)	1 minute	
Polymerization	72°C	20 seconds	
Melt curve	95°C	5 minutes	-
	65°C	1 minutes	-

c) Interpretation of the results

- Consider the sample positive for the respective AMR genes when at least 2 out of 3 replicates show similar Ct values and similar pattern of melt curves, and amplification curves.
- Amplification of internal control along with the target AMR gene for wastewater samples represents that the sample is positive for bacteria.
- Amplification of only the 16S rRNA gene but not the target AMR gene shows that the bacteria present in that sample doesn't have resistance genes.
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1.6 References:

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1.7 Acknowledgements:

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