nereus COST ACTION ES1403

Deliverable of WG1

Deliverable 1

Protocol for assessing relative abundance of mobile ARGs in effluents and downstream environments based on experience being gathered through running project

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ACRONYMS

AR	Antibiotic Resistance
ARB	Antibiotic-Resistant Bacteria
ARB&ARGs	Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes
ARGs	Antibiotic Resistance Genes
qPCR	Quantitative real-time Polymerase Chain Reaction
MGEs	Mobile Genetic Elements
NGS	Next Generation Sequencing
TWW	Treated Wastewater
WWTPs	Wastewater Treatment Plants

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1. Introduction

Antibiotic resistance (AR) is considered to be one of the most significant human health risks of the 21st century. Although this phenomenon is strongly associated with hospitals and other clinical environments, there is a growing realization that it is also linked to anthropogenic activities such as animal husbandry and wastewater treatment that disseminate antibiotics, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) to natural environments, thereby contributing to the propagation of antibiotic resistance. The capacity of ARGs to be mobilized across phylogenetic and environmental barriers, thanks to their association with mobile genetic elements (MGEs), has led to their characterization as *"contaminants of emerging concern"*. This has facilitated a myriad of studies that have developed and applied culture-independent molecular techniques and especially quantitative real-time PCR (qPCR) to assess the diversity and relative abundance of ARGs in the environment at the molecular level. Although qPCR-based assessment of ARGs enables researchers to assess the abundance of ARGs on spatial and temporal levels or as a result of different treatments, a major drawback of the method is that: (i) due to differences in standard qPCR techniques applied, it is often not possible to compare results from different studies and therefore almost impossible to make conclusions regarding ARG dynamics of a more global level, and (ii) even after standardization of the methods, it still requires some apriorism regarding the gene to monitor.

The development of next generation sequencing (NGS)-based methods has revolutionized biology and enabled the field of metagenomics where whole microbial gene profiles can be acquired from natural environments without the need for culture-based methodologies, which do not allow screening of the vast majority of the non-cultivable bacteria (up to 99.9% in certain natural environments). These high throughput-screening molecular methods are increasingly applied to assess the diversity and distribution of bacterial populations and functional genes, including ARGs, in natural and engineered environments. Nonetheless, the large data sets generated in these analyses necessitate highly exhaustive bioinformatic tools in order to identify ARGs and their associated MGEs, and to compare their abundance and diversity in different samples.

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The extensive quantities of data generated by metagenomic analyses of environmental microbiomes necessitate supporting databases that can synchronize data and specifically pinpoint relevant ARGs and MGEs in these environments. In the past years, several such metagenomic datasets have emerged, including ones that specifically target ARGs and MGEs in the environment. Nonetheless, discussion among WG1 members highlighted the fact that, generally, these databases remain vastly underexploited, and often they are threatened with closure at the end of a grant period due to lack of support funding after years of implementation (for instance the insertion sequence “IS finder” database and its “IS saga” metagenome exploring module). WG1 members underscored that fact that there are almost no funding programs that support utilization and maintenance of existing data/databases and therefore the data is inevitably lost. The exploitation of existing metagenomic data for the identification of relevant/abundant ARGs and associated MGEs in many different receiving environments is possible as far as the means are provided. In-depth analyses of specialized databases to pinpoint co-linkage between ARGs and MGEs would be a means to specifically address the question of the dissemination risk of specific ARGs and identify the mobile platforms associated with the dissemination of individual ARGs. Therefore, there is an urgent need for funding agencies to open the possibility of exploitation of the data that are continuously being accumulated.

While culture-based methods are limited because they target very specific microbial communities within complex environmental microbiomes, they provide several advantages compared to molecular-based methods when evaluating antibiotic resistance dynamics in WWTPs and downstream environments, and therefore should still be applied to complement molecular and metagenomics analyses. First, characterization of MGEs and associated ARGs in targeted strains can provide vital information on ARGs transfer dynamics within these taxa. Additionally, specific fecal bacterial taxa (*i.e. Enterobacteriaceae*, *Enterococcus*) are often strongly associated with AR in clinical settings, and theretofore the culture-based approach is often more beneficial to specifically focus on AR within specific groups of higher epidemiological potential. Furthermore, certain taxa such as *Burkholderia* species and *Enterobacteriales,* have hyper-potential for acquisition and dissemination of functional genes and therefore these taxa, which represent “hubs” within gene transfer networks, may be much more indicative regarding the mobilization of ARGs than other environmental taxa (Kloesges et al., 2011). Finally,

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stakeholders (*i.e.* WWTP laboratories) generally do not have the resources and facilities for conducting molecular and metagenomics analyses and therefore, if optimized, the targeting of specific AR fecal indicators can be optimized to allow these stakeholders to ascertain the scope of AR in WWTPs using available methods.

2. Objectives

The overall goal of **WG1** is to ***assess the epidemiological potential of ARGs released from WWTPs and propose conventional (culture-based or qPCR-based) and state-of-the-art molecular approaches that can be applied by research scientists and stakeholders to evaluate AR potential in effluents and effluent-impacted environments, including water bodies that receive effluent discharge and soils and crops that are irrigated with wastewater effluents.*** Four specific research objectives that support this goal are being pursued within the context of this WG:

(A) Propose standardized procedures used for ARB&ARGs detection and quantification in water and soil samples

(B) Identify the most prevalent and/or hazardous ARB&ARGs with ability to persist, spread and proliferate after wastewater disposal, including under wastewater reuse scenarios

(C) Assess the fate (whenever possible quantitatively) of ARB&ARGs discharged in treated wastewater and released in surface water or soils

(D) Identify the conditions favoring ARB&ARGs persistence or proliferation

These objectives will be fulfilled based on: (i) the input and experience of the WG members who are involved in several national and international project focusing on these questions; (ii) literature searches including reviews assembled by the NEREUS Blue Circle Society; and (iii) the joint activities of the WG1 discussions.

2.1 Deliverables

The following deliverables were defined for the second year:

D.1.1. *Protocol for assessing relative abundance of mobile ARGs in effluents and downstream environments based on experience being gathered through running project*"

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D.1.2. *List of the top 10 most prevalent and persistent, and the top 5 most hazardous ARB&ARGs in treated wastewater and surrounding environment, specifically focusing on antibiotic resistance genes associated with mobile genetic elements*

D.1.3. *Guidelines for the analysis of treated wastewater planned to be reused*

D.1.4. *List of ARB&ARGs to be taken into account for the risk evaluation for wastewater reuse*

3. Protocol for assessing relative abundance of mobile ARGs in effluents and downstream environments based on experience being gathered through running projects

This deliverable is based on the outputs of NORMAN network WG5, in which many NEREUS WG1 members participated, which focused on optimization and harmonization of quantitative PCR (qPCR) protocols for assessing the abundance of mobile ARGs. Other ongoing projects, such as the StARE details (Water JPI/0001/2013) and the ANSWER details (H2020-MSCA-ITN-2015/675530), are also assessing the relative abundance of ARGs in wastewater and in the surrounding environment, using the same protocols as NORMAN, as well as other approaches. This will support also an assessment of the degree of comparability of different methods. Although molecular-based methods can be more insightful, the use of culture-dependent approaches is still necessary, not only because targets are being measured and this can be a valuable complement to molecular methods, but also because culture-dependent methods are essential for a straightforward communication with key stakeholders. Hence, a global cefotaxime-resistant fecal coliform project was initiated within the framework of NEREUS WG1. To date, NORMAN network WG5 has surveyed the *sul1*, *qnrS, bla*$_{TEM}$, *bla*$_{CTX-M32}$, *vanA*, the class 1 integron integrase gene *int1* (a MGE recognized as a proxy for antibiotic resistance genes), and 16S rRNA (a proxy for total bacterial abundance). Within the StARE and ANSWER project, protocols have also been developed for *bla*$_{CTXM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{GES}$, *mecA* and *sul2* genes. A detailed overview of optimized qPCR protocols for quantifying ARGs and associated genes is summarized in Table 1.

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Table 1 - qPCR protocols based on analysis of the NORMAN network WG5.

Target gene	Primers	Primers sequence	qPCR product size	Conditions	Primers reference
16S rRNA	q_331F	TCCTACGGGAGGCAGCAGT	195 bp	95 °C - 10 min (1 cycle); 95 °C - 15 s, 60 °C - 1 min (45 cycles) Other: 1c	
	q_518R	ATTACCGCGGCTGCTGG			
bla$_{TEM}$	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	113 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (40 cycles) Other: 2a	Bibbal *et al*., 2007
	blaTEM-R	CTCAAGGATCTTACCGCTGTTG			
bla$_{CTX-M-32}$	CTX-M32-Fw	CGTCACGCTGTTGTTAGGAA	156 bp	95 ºC - 7 min (1 cycle); 95 ºC - 10 seg, 63 ºC - 30 seg (40 cycles); 95 ºC - 15 seg (1 cycle) Other: 3b	Szczepanowski et al., 2009
	CTX-M32-Rv	CGCTCATCAGCACGATAAAG			
sul1	sul1-FW	CGCACCGGAAACATCGCTGCAC	162 bp	95 ºC - 5 min (1 cycle); 95 ºC - 10 seg, 60 ºC - 30 seg (35 cycles) Other: 4b	Pei *et al*., 2006
	sul1-RV	TGAAGTTCCGCCGCAAGGCTCG			
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTG	118 bp		

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	qnrSrtR11	TGGCATTGTTGGAAACTT		95 ºC - 5 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles) Other: 2d	Marti and Balcázar, 2013
intI1	intILC5_fw	GATCGGTCGAATGCGTGT	196 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles) Other: 1c	(Barraud et al., 2010)
	intILC1_rv	GCCTTGATGTTACCCGAGAG			
*van*A	vanA3FP	CTGTGAGGTCGGTTGTGCG	65 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles) Other: 1c	Volkmann *et al*., 2004
	vanA3RP	TTTGGTCCACCTCGCCA			

For all protocols the constructed plasmid pNORM1 with sequences of all target genes was used. Conditions: 1) Power SYBR Green® PCR Master Mix; 2) SYBR® Select Master Mix; 3) DyNAmo ColorFlash SYBR Green Master Mix; 4) Fast SYBRTM Green Master Mix; a) 200 nM of primer; b) 300 nM of primer; c) 500 nM of primer; d) 600 nM of primer.

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