Deliverable 2.3
WP2 Annual Report Y3
Workpackage 2

Responsible Partner: 36-INSA, 2-AGES

Contributing partners: 7-SZU, 14-UT, 23-UoS, 25-NUIG, 33-NVI

GENERAL INFORMATION

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Leader	Manuela Caniça (33-INSA)
Other contributors	Adriana Cabal (2-AGES)
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ANNUAL REPORT Y3 (WP2)

Description of action

The deliverable *D-JRP15-FED-AMR-WP2.3* (in the project proposal named as D-JRP15-FED-AMR-WP2.4) consists of a brief summary of WP2 activities performed in the first year (Y3: M25-M39) and was intended to be established jointly with tasks **WP2-T1, WP2-T2, WP2-T3.2, WP2-T4** and **WP2-T7,** some of which will continue in Years 4 and 5.

WP2 aims at determining the naturally occurring Antimicrobial Resistance genes ARG background load and the microbial biodiversity in the tested environmental compartments (see **Deliverable 2.1, Annex 1)** for more information.

Description of the deliverable

In task **WP2-T1** (entitled “Assemble list of sampling compartments and points. Determination of test areas representative for the European regions (North, West, East, South)”), tThe consortium members of WP2 contributed to compile the final list of sampling compartments and points, having been carried out according to the collectors from East (Czech Republic, Poland), West (Austria, Ireland and Great Britain), North (Estonia and Norway), and South (Portugal). The final list of compartments according to partners, the sample timeline by collectors, the sample distribution, transport and conservation of sampling by compartment were achieved and are already available to the participants via the OHEJP website and in the AGES site (https://fed-amr.ages.at). A unique identifier by compartment and time point was given to all samples planned to be collected in the frame of the FED-AMR project, ensuring a correct traceability of all samples from their collection until their processing and analysis at the laboratory. The description of the HOALs and main catchment areas within FED-AMR was elaborated. Participants of WP2 (2-AGES, 7-SZU, 14-UT, 23-UoS, 25-NUIG, 33-NVI, 36-INSA) provided input and advice according to their expertise and involvement in the sampling. The sampling list provided in this task supports harmonization of testing procedures and enhances comparability of the results obtained from those regions of Europe. The leader (36-INSA, Manuela Caniça) and deputy leader (2-AGES, Adriana Cabal) of this task provided preparatory and final work and the remaining participants took part, namely during the teleconferences made monthly by the project leader, with all members of the consortium. The end month was delayed to 30. This task is finished.

In task **WP2-T2** (entitled “Establish common protocol for sampling and data analyses to facilitate comparability of the results between European test areas (North, West, East, South) and local sampling locations”), the generation of common protocols supported the harmonization of testing procedures and will enhance the comparability of the results obtained from different regions of Europe. The task was finished later than planned (M33) due to the numerous compartments and procedures involved. 2-AGES, 7-SZU, 14-UT, 23-UoS, 25-NUIG, 33-NVI and 36-INSA were the partner institutes that participated in the execution of this task. First protocols for sample collection were made available by the leader (36-INSA) and the deputy leader (2-AGES). Therefore, all compartments have already their respective protocol(s) of sampling, which were uploaded and regularly updated on AGES FED-AMR internal website (https://fed-amr.ages.at), as well as in the OHEJP website, available for all FED-AMR partners at both. Culture and antimicrobial susceptibility testing protocols provided a harmonizing framework in the microbiology laboratory, for bacterial isolation and identification, as well as for antibiotic resistance determination in the strains obtained from the different collected samples, such as faeces (from pigs, wild animals and farmers), manure, soil, water, crops and feed. This procedure is applied to samples that may harbour bacterial strains of human, veterinary, zoonotic or environmental origin and aims at identifying six bacterial species of clinical relevance for humans (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella spp*, *Staphylococcus aureus* methicillin-resistant, *Enterococcus faecium* and *E. faecalis* vancomycin resistant). The protocol for DNA extractions (eDNA and total DNA) was finished for samples of all compartments (pig and wild animals feces, and feces from farmers, manure, soil, crops, river water, groundwater, wastewater and feed). The protocols for molecular and genomic analysis were finished (see tasks **WP2-T3**, **WP2-T3.2** and **WP2-T4**). All these protocols were part of the deliverable **D-JRP15-FED-AMR-WP2.1**. By now, the FED-AMR consortium has decided after voting to dispense with qPCR; the main reason is that in order to carry out the detection of resistance genes in extracellular DNA, the gene enrichment technique can target more than 8,000 ARGs per sample while a qPCR supports only few AMR markers. In addition, all the institutes are dedicating much time of their work in COVID diagnosis and therefore this new strategy will now provide both the consortium and the project not only with more data but also with faster results, which is actually quite relevant due to the time constraints we are facing (due to COVID). Moreover, the planned budget for 16S metagenomics and target enrichment was restricted, not covering the total amount of samples collected by the partners. Furthermore, the original project proposal included only 4 countries as collectors of samples, now there are additional 4 countries that also provide samples from individual compartments, as considered an asset. Therefore, the new expected budget for the analysis of these samples by 16S amplicon sequencing and AMR target enrichment has been adjusted. For additional information, see tasks **WP2-T3** and **WP2-T4**. This task was finished in M33.

In task **WP2-T3** (entitled "Assess microbial and ARG diversity with NGS in the selected test environments (metagenomics). Compare microbial and ARG diversity between ecosystems and over ecosystem boundaries. Characterization of cultivable environmental bacteria on complete nutrient and minimal media.") the FED-AMR consortium decided to introduce changes due to the situation caused by the COVID pandemic and the tight budget available for genomic analysis, as also indicated in task JRP15-WP2-T2. First, it was agreed by all WP2 participants to modify the aims of the culture protocol to be able to identify in all collected samples six bacterial species of clinical relevance for humans (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella spp*, *Staphylococcus aureus* methicillin-resistant, *Enterococcus faecium* and *E. faecalis* vancomycin resistant). This unanimous decision was taken to favour the identification of human, animal and environmental bacteria that may be resistant to one or more critically important antimicrobials, and that can have equal or similar genetic traits than human pathogenic bacteria. Antimicrobial resistance is now being evaluated through diverse antimicrobial susceptibility test depending on the availability of each test in the participant countries and includes determination of the minimum inhibitory concentration (MICs) by broth microdilutuion, E-test and/or disc diffusion. Secondly, microbial diversity was agreed to be evaluated and compared by detecting the entire 16S region (V1-V9) through 16S metagenomics, which is more sensitive than 16S amplicon sequencing. Third, we agreed on the evaluation and comparison of ARGs using a novel methodology based on gene capture probes (see task **WP2-T2** and subtask **WP2-T3.2**). This novel methodology allows us to dispense with both shotgun metagenomics (sub-task **WP2-T3.1**) and qPCR (task **WP2-T4**). As main advantage, target enrichment will analyze the presence or absence of several thousands ARGs, in contrast to qPCR, which only detect few of them. Moreover, we will avoid performance bias, since all samples will be analysed by the same persons and the same devices. In addition, quantitative data will be inferred from the number or reads that cover each detected ARG. Hence, we will invest tangibly more of the currently unspent budget in this novel methodology and therefore we decided to divert additional funds to target enrichment and 16S metagenomics. This task is ongoing.

Subtask **WP2-T3.2** (entitled "Gene enrichment with gene capture probes") was delayed to M31. As explained before, this subtask was recently re-evaluated regarding the *pros* and *cons* of achieving a better detection of ARGs in samples with complex bacterial communities and of different exDNA sources (e.g. soil), which needed to be well defined. To do so, we performed a pilot study involving four Austrian wastewater samples, which were processed between AGES and an external company and that helped on the above-mentioned decision-making. Briefly, the methodology included exDNA and total DNA detection through conventional shotgun metagenomics and the novel target enrichment assay in the collected samples. Results showed a better performance of the latter in regards of ARG detection. The outcome of this pilot study was shared with the FED-AMR consortium, which voted in favour of this methodology as a replacement of qPCR and conventional shotgun metagenomics. The first batch of DNAs from 2-AGES was analysed through target enrichment and 16S metagenomics in the month M36 and the remaining countries will send their DNA samples to the external company in Austria in year 4, as AGES did. This task is ongoing.

In task **WP2-T4** we aimed to "Quantifying clinically relevant ARGs in the tested compartments by qPCR or qPCR arrays". However, as explained above, the detection of ARG through qPCRs was eliminated from the project. The Scientific Supervisory Board (SSB) contributed to this decision-making process. Nevertheless, ARGs quantification will be performed, as it will be inferred from the number or reads that cover each detected ARG.

In task **WP2-T7** (entitled "Isolate and assess quantity, diversity and stability of free extracellular ARG encoding DNA in the tested environments. Sequence comparisons"). WP2 participants have extracted the exDNA and total DNA from all samples immediately after their collection by following the available DNA extraction protocol. The sampling campaign will finish in Spring 2021. As stated before, the target enrichment and 16S metagenomics assays are starting in M36 for the first batch of DNA samples.

E. coli, *K. pneumoniae*, *Salmonella spp*, *S. aureus* methicillin-resistant, *E. faecium* and *E. faecalis* vancomycin resistant strains have been retrieved from the collected samples. Antimicrobial susceptibility testing and Whole Genome sequencing is being performed in some institutes in a selection of strains, where the characterization of the ARGs harboured by those strains is also being investigated. The start and end of this task was delayed to M34 (year 3) and M50 (year 5), respectively.

Associated deliverables

D-JRP15-FED-AMR-WP1.2
Unified sampling and experimental protocols (according to Task **WP1.1**)
D-JRP15-FED-AMR-WP2.1
List of sampling compartments, points and European test areas and harmonized protocols in alignment with EFFORT project protocols available in data repository (Tasks **WP2.1**, **WP2.2**)
D-JRP15-FED-AMR-WP2.2
Preliminary data collection on ARG prevalence and ARG background load in the compartments analysed so far (task **WP2.4**)

Confidentiality of the document

The current deliverable is public.