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The Role and Implementation of Next-Generation Sequencing Technologies in the Coordinated Action Plan against Antimicrobial Resistance

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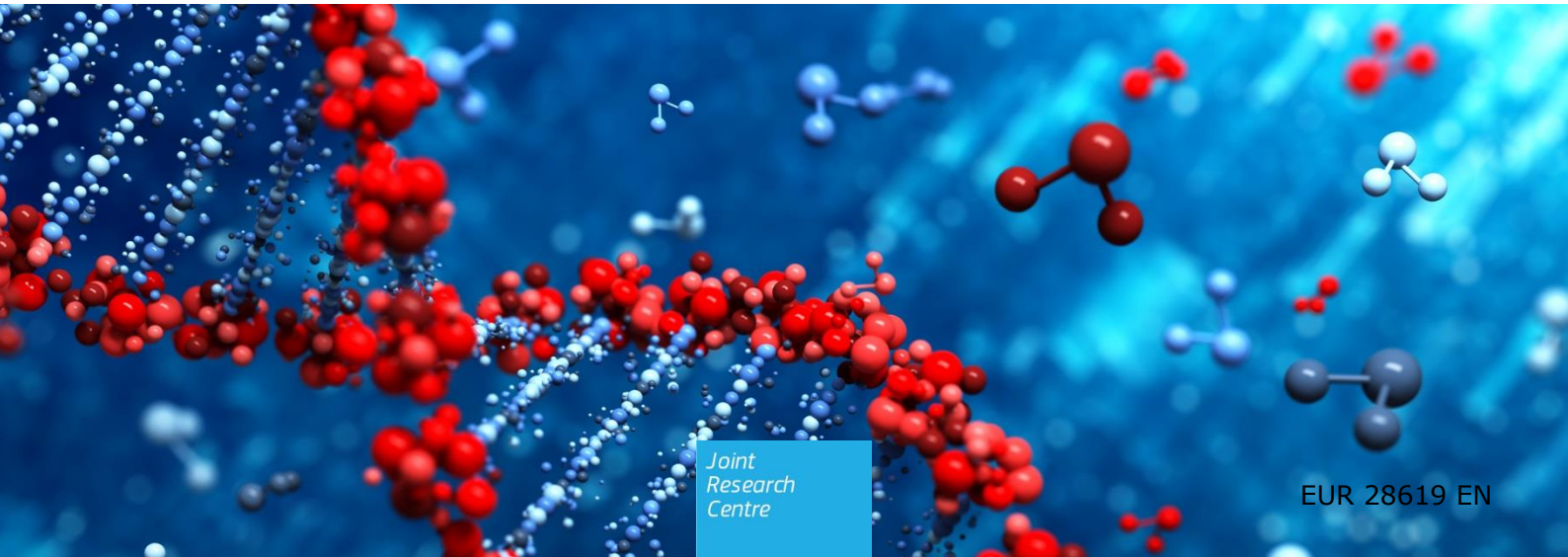

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Foreword

On the 21st and 22nd of March, a two-day workshop was organised by the Knowledge for Health and Consumer Safety Unit of the Joint Research Centre, on *the Role and Implementation of Next-Generation Sequencing Technologies in the European Action Plan against Antimicrobial Resistance.*

The workshop brought together 15 international experts representing the top expertise in the use of NGS to detect the genetic determinants of AMR in diverse fields: clinical, human health, animal health, food and environmental monitoring. Representatives from the JRC, EMBL-EBI and standardisation authorities were also present in the discussions.

The participating experts were:

Johan Bengtsson-Palme - University of Gothenburg, Sweden
Thomas Berendonk - Technische Universität Dresden, Germany
Burton Blais - Canadian Food Inspection Agency, Canada
Kok Gan Chan - University of Malaya, Malaysia
Teresa M. Coque - Hospital Universitario Ramón y Cajal (IRYCIS), Spain
Derrick Crook - University of Oxford, UK
Matthew Ellington - Public Health England, UK
Christoph Endrullat - German Institute for Standardization (DIN), Germany
Dirk Höper - Friedrich-Loeffler-Institut- Federal Research Institute for Animal Health, Germany
Ole Lund - Technical University of Denmark, Denmark
Jean Yves Madec - Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, France
Alan McNally - University of Birmingham
Thierry Naas - Hôpital de Bicêtre- Service de Bactériologie, France
Justin O'Grady - University of East Anglia, UK
Jessica Vamathevan- European Bioinformatics Institute (EMBL-EBI), UK

Chair:

Guy Van den Eede - Head of Unit Knowledge for Health and Consumer Safety, JRC.

Other participants from the Joint Research Centre:

Alexander Binder - Health in Society Unit, JRC
Alessia Bogni - Consumer Products Safety Unit, JRC
Dafni Kagkli - Fraud Detection & Prevention Unit, JRC
Teresa Lettieri - Water and Marine Resources Unit, JRC
Valentina Paracchini - Fraud Detection & Prevention Unit, JRC
Cristian Savini - Food & Feed Compliance Unit, JRC
Heinz Schimmel - Reference Materials Unit, JRC

Organising committee:

Alexandre Angers - Knowledge for Health and Consumer Safety Unit, JRC
Mauro Petrillo - Knowledge for Health and Consumer Safety Unit, JRC
Maddalena Querci - Knowledge for Health and Consumer Safety Unit, JRC
Alex Patak - Knowledge for Health and Consumer Safety Unit, JRC

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1 Introduction

1.1 Monitoring the rise and spread of Antimicrobial Resistance

Since their discovery, antimicrobials have played an essential role in the treatment of infections and have significantly improved the population's health. However, the rise of antimicrobial resistance (AMR, i.e. the ability of a microorganism to resist the action of an antimicrobial agent) now endangers the *status quo* of our healthcare system.

Evidence of antibiotic resistance is growing. For example, an alarming development was reported last year for one of the last-resort antibiotics, namely colistin, where a form of resistance was discovered that could be readily transferred to other bacteria (Liu et al., 2016). Researchers first discovered this resistance in China, quickly followed by findings – including by the JRC (Petrillo et al., 2016) - of similar resistance patterns in other countries, including in Europe. The spread of multiple-drug resistant bacteria already causes an estimated 25.000 deaths annually in Europe alone, a toll that is expected to increase[1].

For many years, the European Union, as well as other countries and international organisations, have been addressing the issue of the rise and spread of AMR. Their work includes the establishment of collaborative programs that raise public awareness and aim to align international actions in order to maximise their efficacy. These actions include the promotion of research and innovation to identify new antimicrobial compounds, the prevention of disease to minimise antimicrobial needs, and public awareness about their correct use.

These efforts depend, among other things, on the establishment of an efficient monitoring and surveillance scheme, implemented in a coordinated and international framework. This scheme is crucial for understanding the development and diffusion of resistance in order to provide relevant risk assessment data and evaluate the effectiveness of targeted interventions (see Box 1).

Box 1. AMR surveillance

The importance of an efficient framework for AMR surveillance and the efforts needed for its improvement have been stressed in the recent years.

For example, the WHO's 2014 Antimicrobial Resistance Global Report on Surveillance highlighted the existence of gaps in the methodology for integrated surveillance of resistance in human and foodborne pathogens and the need for the development of tools and standards for harmonized surveillance of AMR (World Health Organization, 2014).

In October 2015, a Declaration of the G7 Health Ministers stated that *"We consider that a lack of comparable data on the quantity and kind of use of antibiotics and the prevalence of AMR in the population results in an incomplete understanding of the antibiotic resistance situation. The availability of comparable international and national data is a pre-condition for targeted action within countries"* (http://www.g8.utoronto.ca/healthG8/2015-berlin.html)

Within the EU, data on surveillance of AMR in human health are compiled and released by the European Antimicrobial Resistance Surveillance Network (EARS-Net)[2] as part of ECDC surveillance. A "One Health" perspective is also encouraged, evaluating the impact of

[1] http://ec.europa.eu/dgs/health_food-safety/docs/amr_factsheet.pdf
[2] http://ecdc.europa.eu/en/healthtopics/antimicrobial-resistance-and-consumption/antimicrobial_resistance/EARS-Net/Pages/EARS-Net.aspx

antimicrobial resistance in humans, food-producing animals and food; in this optic, every year, EFSA and ECDC publish the European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food (see, for example, European Food Safety Authority and European Centre for Disease Prevention and Control, 2017).

In these efforts, the definition of AMR refers to clinical or epidemiological cut-off (ECOFF) breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[3]. Their significant implementation across European laboratories is an essential component of the consistency required for clinical reporting of antimicrobial susceptibility results (Brown et al., 2015). These methods are "phenotypic", meaning that they rely on observing the extent to which live bacteria are affected by the antimicrobials.

1.2 A potential role for Next-Generation Sequencing

At the end of 2014, a report from the "The Review on Antimicrobial Resistance" stated that "advances in genetics, genomics and computer science will likely change the way that infections and new types of resistance are diagnosed, detected and reported worldwide, so that we can fight back faster when bacteria evolve to resist drugs".[4]

Indeed, the potential to predict antibiotics resistance of bacteria by determining the sequences of their genomes and the plasmids they host has long been discussed (see Box 2). With the advent of Next-Generation Sequencing (NGS) technologies, our modern capability to generate a wealth of nucleic acid sequence information - when coupled to the appropriate bioinformatics information systems - allows both a profiling of microorganisms (as single clones or as a community) and the detection of potential antimicrobial activities in a single experiment.

Box 2. Detection of AMR using DNA sequencing

An important aspect for the monitoring of antimicrobial resistance using sequencing-based methods (compared to phenotypic approaches) is the importance to understand and take into account the molecular mechanisms of these resistances. Several mechanisms have been described in the literature, and include:

1. The production of an enzyme that digests/metabolizes the antimicrobial.

2. The production of efflux pumps that remove the drug(s) from within the cell.

3. The modification, through mutations, of the intracellular target of the antimicrobial so that their interaction is lost.

4. The activation/up-regulation of alternate pathways that allow survival through the bypass of the pathway disrupted by the antimicrobial.

5. The down-regulation of the expression of the pores through which the drug enters the bacteria.

Mechanisms 1-3 generally involve modifications in the pathogen's DNA sequence and/or content through horizontal gene transfers or specific mutations in the endogenous genome sequence. As such, these modifications can most probably be efficiently detected by sequencing-based methods. Mechanisms 4 and 5, on the other hand, represent environmental adaptation through signal transduction pathways and their detection through non-phenotypic approaches will likely present a case-by-case challenge.

In the recent years, a wealth of scientific articles have been published, describing development and trials of NGS-based methods in the determination of AMR in various contexts, both for the clinic (see, for example, Bradley et al., 2015; Sherry et al., 2013; Votintseva et al., 2017), food (see, for example, Hasman et al., 2015), and the

[3] http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211.pdf

[4] "Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations", December 2014

environment (see, for example, (Bengtsson-Palme et al., 2014, 2016; Noyes et al., 2016).

In June 2016, in its conclusions on the next steps under a "One Health" approach to combat antimicrobial resistance, the Council of the European Union called upon the Commission and the Member States to "align surveillance on AMR in humans, food, animals and environment at EU level"[5].

This mention of environmental surveillance on AMR is a challenge with the currently available methods. Phenotypic interpretation of AMR for environmental bacteria is difficult, largely because there are no guidelines for resistance due to their lack of clinical relevance and difficulty (if not impossibility) in culturing them. Still, AMR in non-pathogenic environmental bacteria could be a crucial factor in the development of resistance pathogens due to the potential of horizontal gene transfer of resistance genes from non-pathogenic to pathogenic hosts.

In summary, in the framework of Antimicrobial Resistance detection and monitoring, NGS technologies have the potential to:

- Provide an harmonised link between the surveillance in the environment and in the other important aspects of the "One Health" approach (clinic, food and food-producing animals)
- Provide added value to the monitoring currently established in each of these fields individually.

For this reason, a Workshop was organised in order to discuss the potential impacts NGS technologies could have, specifically, on the current international action plans against AMR, as well as to understand the next steps for their development and implementation in this context.

[5] http://www.consilium.europa.eu/en/press/press-releases/2016/06/17-epsco-conclusions-antimicrobial-resistance/

2 Summary of the Workshop discussions

Structured in three distinct sessions, the workshop addressed:

1. The added value of NGS technologies in the AMR action plan
2. Technical considerations in the determination of AMR using NGS technologies
3. Challenges in the implementation of eventual NGS-based methods for AMR determination

Because of a large overlap in the discussions of sessions 2 and 3, they are reported as one in this document.

2.1 The added value of NGS technologies in the AMR action plan

Next-Generation Sequencing, also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern technologies that allow nucleic acids sequences to be generated much more quickly and cheaply than previously. Coupled with Bioinformatics, NGS has revolutionised the study of genomics and microbiology. Applied to the prediction of antimicrobial resistance of an unknown isolate or within an environmental sample, it could provide many significant advantages to the currently used methods, including:

It can contribute to clinical decision making.

In a clinical setting, sequencing-based approaches the potential to provide different levels of information that can guide treatment with the appropriate antimicrobials. These include a) whether the infection is bacterial or viral, b) the type of bacteria, allowing the development and use of narrow spectrum antimicrobials, c) the eventual presence of genetic determinant of resistance and d) the actual predicted susceptibility to antimicrobials of the isolate. Efforts are still necessary and ongoing, in particular for points c) and d).

The time to complete the analyses remains an important factor with most sequencing technologies. This is not a concern for infections with slow-growing bacteria, such as tuberculosis, for which the advantages of sequencing over culture-based assays makes no doubt (see Votintseva et al., 2017; Walker et al., 2015). For faster-growing pathogens, the possibility to fully replace phenotypic testing remains to be evaluated, despite high accuracy in reported prediction rates.

Advances in sequencing technologies also shorten of time between sample acquisition and AMR prediction. For example, studies showed that, in the treatment of patients with urinary tract infections, metagenomics analyses on nanopore sequencing data provided information to adapt antimicrobial treatment in time for the second dose, within 8 hours of the first (Schmidt et al., 2017).

It provides information (in addition to AMR predictions) that can help in understanding outbreaks and guide intervention.

NGS is, by its technical nature, a technology able to provide a "complete" set of data on the genetic material in the analysed sample. The same data can then be analysed, through separate bioinformatics processes, to answer different questions.

If established around NGS, the same diagnostics/monitoring framework can provide information on many crucial aspects in addition to the resistance determinants for AMR, such as epidemiological typing for outbreak investigation, organism species and virulence factors of clinical relevance (Eyre et al., 2012; Quick et al., 2015).

By storing WGS sequence data, it is possible to retroactively analyse when new information appear.

This "completeness" of information includes the possibility for future analyses which were not planned or known to be relevant at the time the samples were sequenced, such as the rise and spread of new AMR. This was seen with the recent discovery of the *mcr-1* gene in colistin resistance and its retrospective monitoring in established genome sequence databases (Falgenhauer et al., 2016; Hasman et al., 2015)

It provides mechanistic information about the resistance.

Unlike phenotypic tests that provide information only regarding resistance/susceptibility to antimicrobials, NGS can reveal the molecular basis for this resistance. This information can feed in monitoring schemes, helping to understand the events leading to acquisition of resistance. In addition, NGS can characterise novel resistance mechanisms when they arise, through sequencing of isolates that are phenotypically proven to be resistant. This is a remarkable added value if compared to other nucleic-acid based techniques such as the polymerase chain reaction (PCR).

It is a technology that has potential to link the different fields: clinics, environment, food and animals.

Standardised methods for AMR monitoring that are specifically applicable to the environment have never been developed; culture-dependent methods established for clinical samples can't readily be applied to environmental samples (Berendonk et al., 2015) since the numbers of isolates necessary for the tests endpoints are different (set of species/prevailing species), most environment bacteria are not recovered in culture conditions and established criteria are not applicable (the main objective of these tests being to identify likelihood of therapeutic failure). In addition, AMR in non-pathogenic environmental bacteria (for which there are no guidelines) is relevant due to the possibility of horizontal gene transfer.

In order to achieve, as described by the Council of the European Union, a coordinated surveillance of AMR in humans, food, animals and environment at the EU level, new technologies are needed on which to base the framework. For this, NGS is a strong candidate, as extensive work is currently being done with this technology in all the fields.

Data accumulation allows better understanding and improvement of the system.

The information available from a set of whole genome sequences grows as the amount of available information increases. Building a monitoring framework on NGS will thus allow continuous self-improvement of the whole framework.

2.2 Technical considerations and challenges in the implementation of NGS-based methods for AMR determination

Recently, numerous articles, reports and guidelines, both in the scientific literature and regulatory frameworks, have been published assessing and promoting the use of NGS for pathogen detection in the clinic, environment and food chain. Although these documents often include AMR determination in their discussion, it is becoming clear that the use of NGS for AMR has its own - and not always overlapping - set of technical and implementation challenges.

The workshop brought together experts having approached the specific problem of using NGS for detecting the genetic determinants of AMR in the different areas. From these discussions, it became apparent that:

Each field has its own set of technical challenges and realities.

Despite many studies reporting the use of a common technology, NGS, for AMR determination, it is important to note that there exist very specific sets of requirements, difficulties and gaps that will need to be addressed on a field-by-field basis. Outside of a simple "monitoring" framework, additional needs can include, for example, proper risk

assessment (food, environment) and correct prediction of therapeutic success (human and veterinary medicine).

On a technical basis, considerations of varying relevance include whether the detected AMR gene is expressed or not, the presence of the resistance in the chromosome or in a plasmid, understanding interaction of bacteria harbouring the resistance with other bacteria, the limit of detection of the whole methodology (including the sample preparation), etc.

There are points of contact between the different fields, where the "problems" to solve are similar.

For all these differences, a "core" problem emerged that is common to all fields, which is the need to correctly and reliably identify the known genomic determinants of AMR from a set of NGS reads produced from the whole genome sequencing of a sample/isolate.

Different approaches for this have been and are being developed and used. There would be a lot to gain in involving, in future activities, scientists and experts active in this type of work from the different fields in order to share experience and identify best practices. Common challenges in this aspect include:

- **Identify a "best practice" bioinformatics strategy and implementation.** Define what approach to use (shotgun metagenomics, short/long read technologies, assembly or single reads analysis, …); minimal metadata requirements; how to determine "true" outcome to which the results should be compared when evaluating a strategy; minimum recommended sequencing depth; etc.
- **What reference database to be used in the analysis**. A lot of resources exist, such as ResFinder, CARD and SRST2 (see Xavier et al., 2016), which are often complemented with *in house* databases. The scope and quality of the reference database affects the outcome as the AMR screen will only return genetic determinants you are looking for. A recent review from a EUCAST subcommittee identified the establishment of a sustainable reference database as one of the main recommendations towards the use of NGS for bacterial antimicrobial susceptibility testing (Ellington et al., 2017).
- **A harmonised way to record and share information.** Sharing schemes exist and produce benefits, in particular collaborations between national reference centres. However, the situation varies between countries, and the existing collaborations rely on *ad hoc* networks. In this aspect, the COMPARE project, an EU project (funded by Horizon 2020) whose aim is to speed up the detection of and response to disease outbreaks among humans and animals through the use of NGS, is expected to play an important role.
- **Appropriate quality controls/reference materials/harmonisation.** There is currently no comprehensive quality management approach in NGS present which includes necessary requirements for proper documentation, containing standardised information about identified AMR resistance genes, for example. Several NGS standardization efforts have been done by American work groups, authorities and societies, thus the majority of standardisation efforts has been taken place in US. In addition, Illumina Inc., an US-based sequencing company, strives towards the establishment of internal developed standards due to the current held position as the market leader in NSG. However, due to the strong presence and importance of European bodies like CEN (European Committee for Standardization), the standardisation of NGS will become inspired and lifted up on the EU level in the future. There exists a high demand for NGS standardisation in clinical diagnostics, including AMR detection in a clinical setting. However, the same standards which will be established there, will not be necessarily applicable or reasonable in other application like food chain or environment. Hence, there are two options possible. First, the development of one common standard for all

possible applications or second, the primary development of NGS standards in a clinical setting, which could become translated into other applications like environment or food chain later on. The decision for one of these options will rely on standardisation bodies and the involved experts. Efforts in this direction should take into account specificities of the field such as fast obsolescence of systems and technologies (e.g. 454 and SOLiD), possibly by focusing on setting up quality metrics (mainly addressing, in a first instance, NGS performance characteristics) and best practices rather than restrictive prescriptions (Endrullat et al., 2016; Lambert et al., 2017).

Efforts will also be needed in the optimisation and, possibly, harmonisation of the early steps of the workflow - such as a sample preparation, DNA extraction and, if needed, target enrichment - as these are expected to have a strong impact on the results.

3 Conclusions

Apart from the specific conclusions linked to the different topics, one of the major recognised impacts of the workshop was that the experts involved in the different fields appreciated the opportunity to meet and interact with experts of other fields of applications.

In the monitoring of AMR in general and in the implementation of NGS technologies in particular, each field has its own set of technical challenges, requirements and realities. However, it emerged that there are points of contacts between them, where the "problems" to solve are similar.

In this optic, one of the major requests from the participants was for the JRC to follow up this type of discussion in a cross-discipline setup, in order to identify, discuss and possibly harmonise the elements which are common to the different fields.

For some fields, such as human and animal pathogen monitoring (including antibiotics resistance), some countries, such as France and the UK, reported ongoing collaborations with evident benefits for public health and for the management of outbreaks. It was suggested that understanding (mapping) the situation in the different countries, with a possible expanded sharing and coordination effort, would be greatly beneficial.

It was highlighted that the legislation in the different areas do not always take into accounts the importance of AMR monitoring and its translation into intervention, in particular in the environment. A need was identified to better understand the abundance and processes of AMR spread and evolution in the environment (and its relation to environmental and human health).

For all the effort involved, the experts emphasised the potential benefits of using NGS for AMR determination.

A list of potential follow-up activities has been suggested, as summarised in the Annexes.

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List of abbreviations and definitions

AMR	Antimicrobial Resistance
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EUCAST	European Committee on Antimicrobial Susceptibility Testing
NGS	Next-Generation Sequencing

Annexes

Annex 1. Proposed follow-up: Mapping the evidence on the risk related to AMR between the environment, food producing animals, the food chain and human health.

Rationale: An important element in correctly designing and implementing a surveillance scheme for AMR spread that would span humans, food, animals and the environment is to understand the risk associated with the spread of the genetic determinants of AMR between the different frameworks.

Proposal: An expert group should be set up to review the available evidence (see for example Ohidul and Tianlin; Williams-Nguyen et al., 2016), assess the demonstrated and potential risks and identify existing gaps in the current understanding of the impact of antimicrobial resistance spreading between humans, food, animals and the environment. A report would be produced and presented to the appropriate policy makers.

Possible impacts: Mapping this evidence and understanding when there is sufficient demonstrated risk to justify intervention (and understanding, if not, whether to apply the precautionary principle) will help the development of proportional AMR monitoring schemes, in particular for the environment which is currently not well developed nor encouraged. It could also lead to the establishment/strengthening of official networks between the existing clinic, food and veterinary frameworks in the different Member States. It may also affect existing risk assessment regulatory frameworks for environmental policies (e.g. the Water Framework Directive 2000/60/EC and the Committee for Medicinal Products for Human Use), that currently evaluate safe level of antimicrobials in the environment based on their toxicity and do not take into account the emergence of AMR, which is expected to occur at lower concentrations (see Bengtsson-Palme and Larsson, 2016).

Annex 2. Proposed follow-up: Harmonisation efforts in the common aspects regarding the use of NGS in the detection of AMR common to environment, food producing animals, the food chain and human health.

Rationale: In view of the identified points of contacts between the "problems" faced when using NGS to identify genetic determinants of AMR whatever the context (clinic, food control, environmental monitoring…), there would be added value in continuing discussions across the different frameworks.

Proposal: Follow-up discussions should be organised, with the appropriate format to be determined (workshops, working groups …) in view of harmonising, as much as possible and useful, the common steps and resources. This could involve:

- Mapping existing networks in different areas (AMR/metagenomics for human health, animal health, food monitoring and environmental monitoring, AMR sequence databases, …). When missing, such a network could be initiated. (E.g. application of metagenomics for human health)
- Invite representatives from these networks (in particular, bioinformaticians) to identify and discuss the elements which are truly common between the different frameworks (to confirm/complement those identified during the present workshop).
- Identify the best forum to proceed with efforts to identify best practices, quality aspects, reference databases, etc.

Possible impacts: With the current impetus for the eventual establishment of an efficient and useful AMR monitoring framework that combines all the different aspects of the "One Health" initiative and the environment, it will be invaluable that the elements that can be harmonised are harmonised.

Annex 3: Workshop agenda

JRC WORKSHOP: THE ROLE AND IMPLEMENTATION OF NEXT-GENERATION SEQUENCING TECHNOLOGIES IN THE COORDINATED ACTION PLAN AGAINST ANTIMICROBIAL RESISTANCE.

JRC Ispra, 21-22 March, 2017

1st day: 21 March 2017

9:30 - 10:00 Arrival and coffee

10:00 - 10:30 Welcome and opening remarks

Setting the scene: background, format & aim of the workshop (Guy Van den Eede, JRC)

Session 1: Added value of NGS technologies in the AMR action plan

10:30 - 12:30 Invited presentations (15 min each), followed by round table discussions
Introductory presentations by:
Dr Kok Gan Chan, *University of Malaya, Malaysia*
Dr Thierry Naas, *Hôpital de Bicêtre- Service de Bactériologie, France*
Dr Justin O'Grady, *University of East Anglia, UK*

12:30 - 14:00 **LUNCH BREAK**

Session 2: Technical considerations

14:00 -15:00 Invited presentations (15 min each), followed by round table discussions
Introductory presentations by:
Dr Thomas Berendonk, *Technische Universität Dresden, Germany*
Dr Derrick Crook, *University of Oxford, UK*
Dr Dirk Höper, *Friedrich-Loeffler-Institut- Federal Research Institute for Animal Health, Germany*
Dr Ole Lund, *Technical University of Denmark, Denmark*

15:00-15:30 coffee break

15:30 -17:30 Continuation of discussions

17:30 **End of day 1**

20:00 **WORKSHOP DINNER (Villa Borghi)**

2nd day: 22 March 2017

Session 3: Implementation

9:30 - 10:45 Invited presentations (15 min each), followed by round table discussions.
Introductory presentations by:
Dr Burton Blais, *Canadian Food Inspection Agency, Canada*
Dr Christoph Endrullat, *German Institute for Standardization (DIN), Germany*
Dr Jessica Vamathevan, *European Bioinformatics Institute (EMBL-EBI), UK*

10:45-11:15 coffee break

11:15 -12:30 Continuation of discussions

12:30-13:30 **LUNCH BREAK**

Session 4: Conclusions and recommendations

13:30 -15:00 Summary of the conclusions from the different sessions. Identification of the next steps and follow-up activities

15:00 **End of Workshop**

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