Deliverable 2.1

Workpackage WP2-T1

Responsible Partners : ANSES and ISS

Contributing partners: ISS, RIVM, IP, IZSLER, IZSAM, ANSES, INRAe, WBVR, UCM, PIWET, DTU, SVA

GENERAL INFORMATION

European Joint Programme full title	**Promoting One Health in Europe through joint actions on foodborne zoonoses, antimicrobial resistance and emerging microbiological hazards**
European Joint Programme acronym	**One Health EJP**
Funding	**This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830.**
Grant Agreement	**Grant agreement n° 773830**
Start Date	**01/01/2018**
Duration	**60 Months**

DOCUMENT MANAGEMENT

JIP/JRP deliverable	**D.2.1**
Project Acronym	**CARE**
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Due month of the report	**M34**
Actual submission month	**M35**
Type *R: Document, report* *DEC: Websites, patent filings, videos, etc.; OTHER*	R **Save date**:
Dissemination level *PU: Public (default)* *CO: confidential, only for members of the consortium (including the Commission Services)*	PU
Dissemination *Author's suggestion to inform the following possible interested parties.*	OHEJP WP 1 ☒ OHEJP WP 2 ☐ OHEJP WP 3 ☐ OHEJP WP 4 ☐ OHEJP WP 5 ☐ OHEJP WP 6 ☐ OHEJP WP 7 ☐ Project Management Team ☒ Communication Team ☒ Scientific Steering Board ☐ National Stakeholders/Program Owners Committee ☐ EFSA ☐ ECDC ☐ Other international stakeholder(s): …… Social Media: ..... **Other recipient(s):** .....

WP2-TASK 1 : INVENTORY OF THE RESOURCES

INTRODUCTION

Authenticated reference materials (RMs) are necessary both for quality control in laboratories, like for day-to-day quality control, for validation of methods and as standard material in Proficiency Tests (PTs). Just a dozen biological reference standards is listed in the catalogue of EU certified RMs (https://crm.jrc.ec.europa.eu/). This is too few compared with more than 700 chemical items.

The CARE consortium aims to deliver an added value in setting and offering a large collection of well-characterized bacterial strains and genomes RMs with detailed information such as all the metadata that can be shared, virulence and antibiotic resistance properties, and gene contents for major zoonotic bacterial pathogens, including *Salmonella*, *Escherichia coli*, *Campylobacter*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Vibrio*, *Staphylococcus*, *Streptococcus* species in a One-Health perspective for trans-sectoral challenges.

OBJECTIVE OF WP2

To create **EUROpanelOH**, a reference database of bacterial strains and genomes for effective quality control analysis in food safety and public health protection across sectors.

The availability of RM for bacterial pathogens, including either reference isolates or genomic sequences, must be investigated among partners of CARE and OH-EJP by launching a dedicated survey. The survey includes the possibility to point at reference collections accessed by the partners through repositories made available by other organizations (e.g. online repositories, PulseNET, ECCO, WFCC, FAO ..). Besides, the aim consists of identifying the approach currently used by experts in the field to select appropriate RM, the localization where RM are stored, and the sector Animal / Food / Human from which RM have been isolated. This information will be used at the end to make a list of certified RMs for the bacterial pathogens considered of main interest in the field of OH-EJP. Attention will be paid to associate the reference isolates with a minimal mandatory set of metadata (e.g. source, year and country of isolation) and to include all kinds of analyses and characterization data obtained through validated standard methods. For antimicrobial resistance (AMR) genes, there is a need to have a single standardized challenge database that contains all validated antimicrobial resistance genes (ARG) classified by mechanisms. This will be used further as a gold reference data set to analyze RM genomes sequences. For all the listed and validated isolates, a link to the genomic entries eventually available in public repositories (NCBI, ENA or DDBJ) will be added. Similarly, other molecular characteristics (e.g. MALDI-TOF mass-spectra or toxin profiling) will be also considered when available. Finally, the location where the bacterial cultures are currently stored will be included in the document, in view of facilitating the work of WP3 which aims to make RM more easily accessible. A subset of well-characterized RM will constitute the first inputs for feeding WP3. **The D.2.1 is the shared database of resources.**

WP2 Task 1 : Method

In order to achieve the WP2 objective, the first task was to make an inventory of the current use and existence of reference materials across OHEJP partner institutes, for selected, prioritized pathogens and antimicrobial resistance genes. Reference material (RM) for microbiological analyses on zoonotic

pathogens are available in the context of reference laboratories (e.g. EURL's). There is no EU level combining the reference collections linked to specific reference functions and sectors. Characterization of epidemic strains, antibiotic resistances, and virulence markers have progressed from culture-based methods to molecular fingerprints. Phenotypic and genomic data must be coupled for checking the authenticity of RM and promoting their use as controls. We will aim at providing insight to available and desirable RM, identifying sources of both strains and genomic data, including the field of antimicrobial resistance, provided by reference laboratories and other relevant sources such as online repositories and national collections.

Setting up a questionnaire :

During the kick-off meeting in Copenhagen with the WP2 participants, it was decided to build a questionnaire in order to collect the information of existing reference material available among CARE partners. The questionnaire was focused on zoonotic pathogenic species involved in three main sectors of interest: human, animal and food. Then, nine zoonotic pathogens species or genus were considered as main priority :
Salmonella, Listeria monocytogenes, Campylobacter, diarrheagenic *E. coli, Staphylococcus aureus, Streptococcus, Bacillus cereus, Vibrio, Yersinia enterocolitica*. Data regarding the candidate isolates was discussed and finally a list of minimal information was defined, and contained the following items:

- General data: Name of supplier institute, name of supplier lab and ID of reference material and contact person via e-mail
- Taxonomy: Genus, species, Maldi-TOF identification (if known)
- Sources: year and place (country) of isolation, sectors of isolation: 5 different sectors were defined: Human, Animal including environment of production, Food including environment of production, Feed and Natural environment
- Characterization data if WGS has been performed or not, if AMR by phenotypic or genotypic methods has been done or not, these kind of information will be helpful for identifying the status of the isolate further, especially for the need of additional characterization by WGS and AMR

After several exchanges and comments between partners, an excel template (see Annex 1) containing different sheets, one for each selected bacteria (nine sheets) with a total of 25 fields for metadata was sent to all participants on April 24th 2020 for completion. An additional tab called "instructions" explained partner how to fill the different fields and highlighted only those needed (called minimal information in red color on the excel table of Annex 1). Some other considerations and key-points were also transmitted jointly with the excel table in order to help partners for selecting their reference material for this project.

Data collection and purposes :

1) Some inclusion criteria were defined for RM to be included in the inventory :

- The RM will be strains that fall within the nine bacterial categories listed previously as prioritized pathogens for CARE
- They must be available for sharing between institutes all along the project (with an MTA through CARE consortium agreement and/or other manners such as the Nagoya protocol)
- As we are in OH project, sector of RM isolation must be at least mentioned, several sectors are identified: human / animal /food / feed / natural environment. The environment of food animal production will be included in "Animal" sector, the environment of food processing will be included in "Food" sector. More details regarding host, sources, animal species, food category can be added.
- Duplicate isolates were avoided, asking the partners to take care of excluding isolates sharing a same phenotypic/genotypic profile from a same source of isolation, whatever the year of isolation.
- Virulence markers mean only added remarkable or relevant markers and not the markers usually present in the species.

- Antimicrobial resistance data are split into two different spaces, one for phenotypic data and other one for genotypic data (AMR genes especially).

2) RM included in the inventory can be available for different purposes :

- Proficiency testing trials, CARE project through WP1 identifies three different PTs for detection of strain, characterization of strain, or outbreak investigation,
- Quality control and validation of already described methods or new innovative methods,
- Experimental tests, such as challenge tests or other experimental testing studies.

WP2 Task 1 : Results

The questionnaire was sent at the end of April 2020 during the critical sanitary situation in several countries, the deadline was on beginning of July but therefore, we extended the period for sending excel sheets until September 2020 as several partners were not able to fill the tables with all data.

Contribution from CARE partners

A total of 12 partner institutes contributed to the inventory of reference material. Most of them registered more than single pathogens, at least 2 to 3 different pathogens (IP, DTU, PIWET, IZSAM, IZSLER, SVA) to more than four to eight different pathogens (ANSES, UCM, ISS).

Globally, thanks to the contribution of CARE partners, we have inventoried from all participant institutes a total of 2719 bacterial strains of the requested species, with the addition of 232 *E. coli* strains not explicitly described as diarrheagenic, one strain of *Listeria welshimeri*, one strain of *Listeria innocua*, two strains of *Bacillus mycoides* and 5 strains of *Yersinia pseudotuberculosis*. The 241 additional strains are indicated in brackets within the Table below. The overall number of candidate reference materials inventoried whatever the species and the sectors of isolation was thus of 2960 strains.

	Human	Animal&env.	Food&env.	Feed	Natural env.	Unknown	TOTAL
Salmonella	163	596	148	4	26	167	1104
L. monocytogenes	23	26	40 (+ *2 not monocyto.*)		14		103 (+ *2 not monocyto.*)
Campylobacter	100	342	71		46	2	561
Diarrheagenic *E. coli* (DEC)	17 (+ *1 not DEC*)	64 (+ *170 not DEC*)	2 (+ *42 not DEC*)			0 (+ *19 not DEC*)	83 (+ *232 not DEC*)
S. aureus	15	536	33		77	24	685
Streptococcus	10						10
B. cereus	6	6	12 (+ *2 B. mycoides*)				24 (+ *2 B. mycoides*)
Vibrio	4						4
Y. enterocolitica	21 (+*3 pseudotub*)	118	6		0 (+*2 pseudotub*)		145 (+*5 pseudotub*)
TOTAL	359 (+ 4)	1688 (+ 170)	314 (+ 44)	4	163 (+ 2)	193 (+19)	**2719 (+ 241)***

* In brackets : 232 *E. coli* strains not explicitly described as diarrheagenic, one strain of *L. welshimeri*, one strain of *L. innocua*, two strains of *B. mycoides* and five strains of *Y. pseudotuberculosis*.

Among overall listed isolates, the highest number of isolates was for *Salmonella* (1104) and to a lesser extent for *Campylobacter* (561), both are the main zoonotic pathogens, widely monitored in several countries in three main sectors (human, animal and food). Then, *Staphylococcus* (685) and *Listeria* (426) isolates also were well mentioned. Then collection for pathogenic (diarrheagenic) *E. coli* as well for *Yersinia enterocolitica* were around on the same level of isolates. A few of available reference materials was mentioned for *Bacillus cereus* (24 isolates), *Streptococcus* (10 isolates) and *Vibrio* (only 4 isolates). Therefore, a high variation in the number of isolates according to the species and to the level of monitoring for the different pathogens is highlighted through this RM inventory. The Animal sector gathers 57% of the total of declared RM. This is the sector where the pressure of monitoring is the highest; 359 isolates and 339 isolates were from the Human sector and the Food sector, respectively.

Detailed results by sectors

In Human sector, a total of 359 isolates was recorded, out of which near the half is represented by *Salmonella* (45%) followed by *Campylobacte*r (28%). Only, few isolates were declared for *Listeria*, *Yersinia enterocolitica*, diarrheagenic *E. coli*, *Staphylococcus*, Streptococci, *B. cereus* and *Vibrio* (order in decreased numbers of available isolates).

In Animal sector including environment of production, *Salmonella* and Staphylococci were the most registered isolates with 35% and 31% respectively of the total isolates (1686). Then, *Campylobacter* represents near 20% of declared isolates, *Yersinia enterocolitica* and diarrheagenic *E. coli* represent near 7% and 6% respectively of the total. Only a few *Listeria* (26) and *B. cereus* isolates (6) were declared.

In Food sector including environment of production, near half of the 339 declared isolates (42%) were from *Salmonella*, 21% from *Campylobacter*, 12% from *Listeria* and 11% from *Staphylococcus*. Six isolates were declared for *Yersinia enterocolitica*.

Natural environment recovered a total of 165 isolates, including *Staphylococcus* for half of them, *Campylobacter* for near 30%, *Salmonella* and *Listeria* for near 20% and 10% respectively.

Only four isolates of *Salmonella* were reported from feed source. Two-hundred and twelve isolates were reported without any information on the sector, most of them (167) correspond to *Salmonella* isolates. Such results will be discussed with partners in order to obtain the information of sector at least and to have a statement regarding inclusion or not of such reference material.

Associated data provided

The database of resources (DELIVERABLE 2.1) was analyzed for the content of information given by the partners. There were only eight isolates (4 *Salmonella* isolates, 2 *Campylobacter* isolates and 2 *E. coli* isolates) from which no metadata are available due to confidentiality agreement. Such isolates could be used as EQAS materials for dedicated purposes. Moreover, countries of sampling were unknown or not mentioned for 91 strains. That corresponds to less than 0.03% of the total number of strains.

WGS data are already available for some strains and species. This is not enough however to create the sub-database for antimicrobial resistances as initially planned. Indeed, WGS data are available for 66% *Listeria* strains, 43% *Campylobacter* strains, 83% *Bacillus* strains, 25% *Salmonella* strains, 24% *E. coli* strains and 15% *Yersinia* strains. Only four strains were fully WGS characterized for *Staphylococcus* and none for *Vibrio* and *Streptococcus*. The AMR sub-database (DELIVERABLE 2.2) is postponed. It will be later on set up by using a unique bioinformatics tool (RESFINDER) onto the WGS data available and those that will be determined during the next phases of the workpackage.

Here are below the detailed results of the WGS information we got at this time :

	Number of strains	**WGS "YES"**	WGS "NO"	**% WGS "YES"**
Salmonella	1104	**277**	825	**25%**
Listeria monocytogenes	103 (+2 not monocyto.)	**70**	33 (+2 not monocyto.)	**68%**
Campylobacter	561	**243**	318	**43%**
E. coli	83 (+232 not DEC)	**20 (+2 not DEC)**	63 (+230 not DEC)	**24%**
Staphylococcus aureus	685	**4**	681	**0.6%**
Streptococcus	10	**0**	10	**-**
B. cereus	24 (+2 B. mycoides)	**20 (+2 B. mycoides)**	4	**83%**
Vibrio	4	**0**	4	**-**
Yersinia enterocolitica	145 (+5 pseudotub)	**22**	128	**15%**

Next steps required

Based on the inventory of which and how are available RMs currently used by CARE partners, a gap analysis will be conducted to address the group's needs. This will determine the gap-in-knowledge for associated metadata, the genomics sequences and possibly the mass spectrometry profiles of included strains; these complement of information will be investigated in dedicated tasks. Moreover, the gap analysis will investigate on the need to look for new RMs, especially to cover all the antibiotic resistance mechanisms, but also to cover under-represented serogroups or virulotypes which could be of interest. At the end, the reference collection (designated as « EUROpanelOH of RMs ») will be representative of what EU needs as controls for managing zoonotic infections (or food-borne zoonoses). A special attention will be paid for *Vibrio* for which only four strains were inventoried. An effort will therefore be engaged during the next phase to collect more *Vibrio* isolates.

CONCLUSION

Setting up a EUROpanelOH of RMs (strains and genomes) will result from a common effort made by different European expert and reference laboratories to define a set of minimal inclusion criteria for strain integration. The establishment of such a database would provide an effective tool for accurate and fast consultation, for the benefit of laboratories involved in microbiological analysis. This enables the easiest use of RMs for quality control and method validation to detect and characterize foodborne pathogens or to perform antimicrobial resistance testing. In a One-Health perspective, this would facilitate the development, the validation, the accreditation and finally the spread of diagnostic and typing methods for foodborne pathogens. In addition, this set of RMs may connect WGS data with phenotypical traits including mass spectrometry profiles, growth characteristics, antibiotic susceptibilities and origins of the strains. Except for the source of sampling that will be mandatory, it is important to note that not every entry will have in the first round of integration all the associated information available. It will be one of the major purposes of CARE to establish a trustable link between the phenotypic traits of the strains and their genomes at the final round of integration. The genome sequence information is becoming increasingly critical since the diagnostic technologies in the field of microbiological analyses have begun to migrate away from more traditional culture-based formats and there is still a need of consolidated datasets of reference materials for benchmarking novel analytical tools.