Deliverable

2.1 Matrix-dependent sample handling protocols

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Deliverable Description

D2.1 Matrix-dependent sample handling protocols

The overall aim of deliverable D2.1 was to collect and compare matrix-dependent sample handling protocols. Reasonable and standardized sample collection techniques, sample transport and storage conditions are of paramount importance as they can have an impact on the quality of information obtained during downstream applications such as next-generation sequencing. Sub-optimal sampling steps may significantly influence raw data quantity and quality, quality of processed data, sequence depth and the coverage of the obtained genomes. Additionally, the composition of microbial populations within a sample can significantly change not only during the sampling step itself but also during transport and further storage as some microbes are able to rapidly adapt to changes in their environment. Sub-optimal sampling conditions may also heavily influence nucleic acid stability. Especially RNA can rapidly degrade when cooling of samples is not guaranteed or in case the cold chain is disrupted during transport. All such adaptation or degradation events can finally influence the outcome of the sequencing results.

Thus, there is a need to identify optimized protocols for sample collection and transport procedures. Simple applications or actions such as keeping the cold chain or using stabilizing reagents or buffers can notably contribute to maintain a high quality of the respective sample. However, treatment or sample handling also depends on the matrix of the sample. For example clinical or animal samples (e.g. blood/tissue/serum) have to be usually transported, stored or handled in a different way when compared to e.g. feed or environmental samples. In order to find out which sample matrices and protocols for handling, transport and storage are available and used by COMPARE project partners, a survey on matrix-dependent sample handling protocols was conducted by the Technical University of Denmark (DTU) in collaboration with the Robert Koch Institute, Germany (RKI). All questions included in the survey were aimed to identify protocols and standard procedures in sample collection, sample handling, sample transport and sample storage, as well as the sample matrices, storage or stabilizing media or buffers and sample containers. All questions were designed in order to collect information on factors that might influence the outcome of whole genome sequencing.

The survey identified different matrix-dependent sample handling protocols used by different partners and thus enables COMPARE partners to conduct further sample stability experiments (e.g. long-term storage at different temperatures or in different buffers/reagents) using different sample matrices (e.g. solid/liquid/ bacteria or athropodes). Thus the outcome of the survey and of the experiments essentially contributes not only to other tasks within WP2 concerning dowstream applications (e.g. sample processing; sequencing and data anlyses) but also to other workpackages within the COMPARE project dealing with sample handling and processing during testing and validation steps (WP1-8 or also WP13). Results of the survey were compiled in an survey report and made available on the DTU Compare share site to all project partners

In conclusion, the results obtained during the survey valuably contribute to other WPs within the COMPARE project. The basis of further sequence stability experiments and downstream applications is laid and thus deliverable D2.1 was fullfilled.

Methods

The survey was developed using the online survey software surveymonkey (https://www.surveymonkey.com/) and sent to institutes and organisations within the COMPARE network. Participants (n=72) were invited to conduct the online survey within two weeks (from 07.09.2015 until 21.09.2015). The survey contained 23 questions (Table 1), including questions on general participant information and further questions asking for information on types of samples, types of sample matrices, pathogen domains relevant for sampling and content of available protocols for sample processing (collecting, handling, transport).

TABLE 1: QUESTIONS INCLUDED IN THE SURVEY

Question #	Question content
1	Participant information (Contact name/Institute name/Country)
2	Which type(s) of samples do you handle?
3	Which type(s) of sample matrix do you handle (for animal, human and clinical samples)?
4	Do you have specific protocols for collecting, handling and transporting samples (food, animal, feed, human and/or clinical)?
5	Which domain is relevant for the sampling?
6	Overall purpose of the protocol
7	Is the type/brand of sample container specified in the protocol(s)
8	If the type/brand of sample container IS SPECIFIED in the protocol(s), would ANY medium for transportation be added to the container (e.g. RNA-later, PBS)?
9	If the type/brand of sample container IS SPECIFIED, would ANY ADDITIONAL substance be added to the transport medium (e.g. RNA-later, anticoagulant, or inhibitor)?
10	If the type/brand of sample container IS SPECIFIED, could any known substance MIGRATE from the container into the sample (e.g. inhibitor(s))?
11	If the type/brand of sample container IS NOT SPECIFIED, would ANY medium for transportation be added to the container (e.g. RNA-later, PBS)?
12	If the type/brand of sample container IS NOT SPECIFIED, would ANY ADDITIONAL substance be added to the transport medium (e.g. RNA-later, anticoagulant, or inhibitor)?
13	After collecting the sample, will a transport box be used for temporary storage (please select all that apply)
14	During transport of the sample, is there a request that the sample be kept at a certain temperature? (please select all that apply)
15	If the temperature of samples during transport is monitored, how is this done? (please select all that apply)
16	During transport of the sample, is there a request that the sample be kept at a certain atmosphere?
17	During transport of the sample, is there a request that the sample be kept a certain pH?
18	During transport of the sample, is there a request to maintain a certain time period?
19	Upon arrival of the sample to the laboratory, is there a request to process the sample (i.e. purify the DNA/RNA) within a certain time period?
20	Upon arrival of the sample to the laboratory, does the protocol describe a possibility of storing the sample within a certain time period (with the purpose of purifying the DNA/RNA at a later stage)?
21	Upon arrival of the sample to the laboratory, if the protocol describes a possibility of storing the sample, at which temperature should it be stored (with the purpose of purifying the DNA/RNA at a later stage)?
22	If storing the sample in a freezer, does the protocol describe the necessity to add a cryoprotective substance, e.g. glycerol?
23	Which type of sample matrix would you consider most relevant and suitable for the purpose of testing the effect of collecting, handling and transporting of samples for sequencing results (metagenomics analysis)?

The responses were collected as single or multiple options from multiple choices with additional free text (see Annex I) for remarks and comments. Information and data collected via survey monkey was compiled with tools implemented in surveymonkey.

Results

Respondents

From 72 invited COMPARE participants, 15 completed the survey (response rate 20.8%). Participants were registered from Germany (n=4), United Kingdom (n=2), The Netherlands (n=2), Italy (n=2), Denmark (n=2), France (n=1), Belgium (n=1) and Greece (n=1). The 15 participants represented 13 institutes or organizations, two institutes were represented by two participants each.

Sample, matrix and domain priorities

Information regarding types of samples which are handled was provided by all participants. Duplicate answers were given and total answers were n=39. Samples which are handled by institutes and organizations were mainly animal (n=11, 73.4%), clinical (n=10, 66.7 %), human (n=9, 60%) and environmental (n=7, 46.7%). Only 1 participant chose the option for food samples (n=1) and feed samples (n=1), (Table 2).

TABLE 2: QUESTION 2, TYPES OF SAMPLES

Which type(s) of samples do you handle?			
Answer Options	**Yes**	**No**	**Response Count**
We handle food samples	1		15
We handle animal samples	11		15
We handle feed samples	1		15
We handle human samples	9		15
We handle clinical samples	10		15
We handle environmental samples	7		15
		answered question	15
		skipped question	0

Respectively, also 78.5% of all participants (n=11) and 42.8% (n=6) considered animal samples and clinical samples most relevant for testing the effects of collecting, handling and transporting samples on sequencing results.

Respondents were also asked to name sample matrices which are handled (Question 3, Table 1). 13 participants gave multiple (n=64) answers and preferred tissue (n=11, 84.6%), blood (n=10, 76.9%), swabs (n=10, 76.9%) and feces (n=9, 69.2%) (Table 3). Additional samples were specified in the free text option (Annex I, A1).

TABLE 3: QUESTION 3, TYPES OF SAMPLE MATRICES

Which type(s) of sample matrix do you handle (for animal, human and clinical samples)? Select all that apply.		
Answer Options	**Response Percent**	**Response Count**
Tissue	84.6%	11
Blood	76.9%	10
Feces	69.2%	9
Urine	46.2%	6
BAL	46.2%	6
Liquor	46.2%	6
Swabs	76.9%	10
Other (please specify)		6
	answered question	13
	skipped question	2

Protocol availability and purpose

For all types of samples, except for food samples, specific protocols were indicated as available (Table 4).

TABLE 4: QUESTION 4, PROTOCOLS FOR SAMPLE PROCESSING

Do you have specific protocols for collecting, handling and transporting samples (food, animal, feed, human and/or clinical)?			
Answer Options	**Yes**	**No**	**Response Count**
For food samples	0		15
For animal samples	10		15
For feed samples	1		15
For human samples	4		15
For clinical samples	6		15
Comment			3
		answered question	15
		skipped question	0

Information regarding the domain, which is relevant for the sampling and sequencing outcome, was given by all participants. Virus domain was chosen by most of the participants (n=10, 66.7%), followed by bacteria (n=9, 60%). Four respondents (26.6%) and three respondents (20%) chose parasites and unknown domain as relevant for sample handling (Table 5).

TABLE 5: QUESTION 5, DOMAINS RELEVANT FOR SAMPLING

Which domain is relevant for the sampling? (please select all that apply)		
Answer Options	**Response Percent**	**Response Count**
Bacteria	60.0%	9
Virus	66.7%	10
Parasites	26.7%	4
Unknown	20.0%	3
Comment		0
answered question		15
skipped question		0

The overall purpose of the protocol was provided as open text by 13 participants. Main answers given were concerning general quality and stability in diagnostics, detection and isolation of bacterial and viral DNA/RNA and animal/clinical sampling (Table 6).

TABLE 6: QUESTION 6, OVERALL PURPOSE OF THE PROTOCOL

Open-Ended Response
Detection of food borne bacterial pathogens
Defining the handling workflow for diagnostic samples; assure quality.
Protocol is designed to allow virus isolation and isolation of nucleic acids (RNA and DNA)
We essentially receive biological samples for diagnostic purposes (so, no active collection). We have rules for handling and storage of samples that potentially contain pathogens. We also organize ring trials and proficiency tests for which we have specific guidelines.
Protocols for report cases are stipulated to standardize diagnosis and conform to accreditation standards. Other submissions (e.g. testing-to-exclude) or specialist enquiries can vary according to the situation. Protocols are refined to give an accurate result, but only changed once the modifications have been shown to give an improvement.
Quick and sterile collection of post mortem samples from diseased animal species for pathogen detection
Clinical samples from animals and humans are gathered for clinical viral diagnostic purposes, viral metagenomics, and virus tissue culture propagation.
To minimize time from sampling to processing, and to maintain the temperature cold-chain.
- environmental study - outbreak investigation
To study the composition, species diversity; interactions among organisms and the evolution of organisms
To collect the caecum contents of broilers avoiding contaminations
To have good quality starting material for WGS (and virus isolation)
Stability

Sample container/ Media

The type/brand of container is specified in the protocols of 5 participants (38.5%). Additional remarks are given in Annex I, A2. From these specified containers, only 2 respondents chose additional medium (Tissue-Tek and RNA-later) to be added to the container. Also 2 respondents chose additional substances (RNA-later) to be added to the container. Only one participant considered inhibitors from the container to possibly influence the sample (Annex I, A3-4).

From the participants which indicated the container not to be specified in the protocol (n=8), 5 respondents named the medium to be added to the container, including PBW, PBS, Hanks salts and virus transport medium. Additional substances are given to the container by 5 respondents and specified as lysis buffer, antibiotics and anticoagulant (Annex I, A6-7).

Transport

After collecting the sample, 7 of 15 respondents do not use a specific transport box. Polystyrene boxes are used by 5 participants, 3 respondents use no transport box at all. 8 of 15 participants use a cooler brick during transport (Table 8). Most respondents agreed that the sample has to be kept refrigerated during transport (n=8, 57.1%) or even deeply frozen at -80°C or on dry ice (Annex I, A8). 78.6% (n=11) of all participants do not monitor the temperature during transport, and all respondents (100%, n=14) do not provide a certain atmosphere or pH during transport.

TABLE 8: QUESTION 13, BOXES FOR TRANSPORT

After collecting the sample, will a transport box be used for temporary storage (please select all that apply)		
Answer Options	**Response Percent**	**Response Count**
No specific transport box (excl. cooler brick)	21.4%	3
No specific transport box (incl. cooler brick)	28.6%	4
Polystyrene box (excl. cooler brick)	7.1%	1
Polystyrene box (incl. cooler brick)	28.6%	4
No transport box	21.4%	3
Comments		3
	answered question	14
	skipped question	1

Sample arrival and storage

Most respondents (57.1%, n=8) do not aim to process the sample within a certain time period, but have the possibility of storing the sample within a certain time period (64.3%, n=9). Storage conditions were specified mainly to be at -80°C (50%, n=7) and 4°C (35.7%, n=5), 85.7% of all respondents (n=12) agreed that no cryoprotective substance be added to the sample.

Summary and Conclusions

This report presents findings from the survey on sample handling and transport, which was designed to form the basis of the sequence stability experiments within WP2. In general, the majority of participants considered animal samples (78.5%) and clinical samples (42.8%) most relevant for testing the effects of collecting, handling and transporting samples on whole genome sequencing results with preferences for tissue (84.6%), blood (76.9%), swabs (76.9%) and feces (69.2%). Main domains relevant for sampling were specified as viruses (66.7%) and bacteria (60%). Factors considered relevant for whole genome sequencing outcome were mainly temperature during transport and storage as well as time between sampling and processing of the sample.

The survey identified different matrix-dependent sample handling protocols used by different partners and thus enables COMPARE partners to conduct further sample stability experiments (e.g. long-term storage at different temperatures or in different buffers/reagents) using different sample matrices (e.g. solid/liquid/ bacterial or athropodes) to optimize sequence outcomes concerning the two main domains viruses and bacteria. In accordance with the results of the survey, e.g. an experimental assessment of sample handling conditions is planned that will focus on the two factors temperature and time. Two different sample

matrices (feces and sewage) will be assesed and spiked with microorganisms from different phyla. The samples will be stored at four relevant temperatures (-80˚C, -20˚C, +5˚C, +22˚C) and examined after three incubation times (0h, 12h, 72h) representing the following realistic situations: immediate sample processing, sample storage over night, sample storage over a weekend. DNA will be extracted and sequenced and the metagenomic sequencing data analyzed.

In conclusion the outcome of the survey and of the experiments essentially contribute not only to other tasks concerning dowstream applications (e.g. sample processing; sequencing and data anlyses) within WP2 but also to other workpackages within the COMPARE project dealing with sample handling and processing during testing and validation steps.

Annex I: Additional text answers and comments

A1. Question 3, Sample matrices, answers specified as "other"

cell culture supernatant
Eggs (allantoic fluid), feathers, semen,
Nasopharyngeal aspirates and any other liquid clinical material that we occasionally process
Shellfish
pure culture
Bacterial strains

A2. Question 7, Type of specified container

Swab
Not in the protocols. However, IATA and ADR rules are applied and the contingency plans contain recommendations. It has to be understood that the samples are often shipped for diagnosis in general. Sample transportation is usually discussed prior to dispatch.
Polystyrene box
Necropsy number and type of tissue / fluid collected
Different containers are used for the different clinical/environmental/animal samples. Also, for sample device we use different material. For instance, swab (flocked), COPAN.
type yes, 25 ml sterile tubes, brand it is not specified
according to protocol

A3. Question 8, Medium if container is specified

PBW - Buffered Peptone Water
In case of snap-frozen material Tissue-Tek® O.C.T™ compound is added
Depending on the samples we work with/without medium. If we use a medium it might also vary from sample to sample.
depending on matrix and sample RNA-later for RNA detection

A4. Question 9, Additional substances if container is specified

depending on matrix and sample RNA-later for RNA detection

A5. Question 10, Migration of substances if the container is specified

possible inhibitors from the plastic container

A6. Question11, Medium if container is not specified

PBW - Buffered Peptone Water
Depends. PBS is an option that is frequently used. In some cases lysis buffers are an option. Discussed prior to dispatch.
Brand not specified,specified requirements Type of swab (no wooden shaft) Most samples are sent dry. Packaging must conform to IATA standards for Biohazardous material
virus transport medium for swabs: Minimum essential medium with Hanks salts containing 0.5% Lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 mg/ml streptomycin, 100 U/ml polymyxin B sulfate, and 250 mg/ml gentamycin
Depending on the samples we work with/without medium. If we use a medium it might also vary from sample to sample.
depending on matrix and sample RNA-later for RNA detection

A7. Question 12, Additional substances if container is not specified

Depends. Lysis buffers are an option. Discussed prior to dispatch.
Antibiotics and/or anti-mycotic and cell culture medium
Anticoagulant is specified for blood samples (EDTA, Heparin)
What is the difference between this and 11.
anticoagulant for blood samples, PBS or SP medium for swabs

A8. Question 14, Temperature during transport

Usually cooled (4-5°C) or on dry ice (depends on the sample.for e.g. metagenomic analyses or RNA viruses dry ice is preferred to keep the RNA quality as good as possible)
Field samples do not have temperature criteria stipulated during transport. Laboratory or research samples are transported as the operator deems appropriate
dry ice; without temperature monitoring
As cool as possible preferentially at -80 degrees Celsius
sometimes samples are frozen