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Characterisation of Methods

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1. Introduction

A key objective of the OH-Harmony-CAP project was to collect information on current capabilities, capacities and interoperability at both the National Reference Laboratory (NRL) and the primary diagnostic level. A specific objective of WP3 was to examine current and best practice in the One Health sectors (public health, animal health and food/environment testing labs), identify existing knowledge gaps and propose new studies and/or methods to fill them in the areas of 'Sampling & testing', 'Characterisation' and 'Data management & harmonised reporting', specifically targetting Shiga toxin producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), *Cryptosporidium*, and AMR in *Salmonella* and *Campylobacter*.

This document, the second in a series of three such reports, covers 'Characterisation'. A previous report on 'sampling and testing' procedures has been published (https://onehealthejp.eu/jip-oh-harmony-cap/) and a future report will cover 'data management and harmonised reporting'. Our report is divided into sections covered each of the target organismsm with subsections on: [1] current routine strain characterisation in Europe; [2] gaps in strain characterisation that are inhibiting food safety regulation/policy in the EU; [3] recommendations on what typing methods, AMR and virulence gene testing, etc. should be undertaken as part of a future harmonised approach to strain characterisation, and [4] recommendations on how this harmonised strategy should be implemented.

The information provided on current practices is based on a questionnaire (see Appendices 1-4), completed by public health, animal health, food testing and national reference laboratories (EURL network of NRLs, EFSA Zoonoses network, ECDC Food and Waterborne Disease network, etc.) in the EU. The other sections were prepared by experts in the respective areas, with reference to the peer reviewed and other relevant technical literature.

2. Shiga toxin producing *Escherichia coli* (STEC)

2.1 Current routine strain characterisation in Europe

2.1.1 Responses to the questionnaire

This section summarizes the responses to the Shiga toxin producing *E. coli* (STEC) questionnaire used in WP3 of OH-Harmony-CAP regarding the characterisation of isolates. The design of the questionnaire and general information of the respondents has been previously described in Deliverable 3.1.2. In summary, 41 laboratories responded that they tested for STEC including human clinical (17), animal (13), food (24), feed (6) and environmental samples (11). A total of 33 (80.5 %) of the 41 laboratories stored isolates, either in their own laboratory and/or at the NRL (14 labs storing the strains at their own facilities, 6 responded that the strains are stored at the NRL level, and 13 stored them at both NRL and their own lab).

2.1.1.1 Biochemical testing

Eleven (27%) out of the 41 responding laboratories performed phenotypic characterisation using biochemical assays. One laboratory did not specify the biochemical tests performed. Out of the remaining 10 laboratories, where further information was available:

- 9 laboratories perform indole test for confirmation, alone (2) or together with additional phenotypic characterisation (7)
- 6 laboratories carry out tests to identify lactose fermentation and seven test for β-glucuronidase activity, in combination with other phenotypic testing
- 7 laboratories perform strains characterisation through additional phenotypic tests (e.g. ONPG, LDC, URE, Voges-Proskauer test and others usually included in the API20E test)
- 3 tested for the phenylalanine deaminase
- 2 laboratories carry out motility testing
- 4 test for enterohaemolytic activity.

2.1.1.2 Testing for somatic (O) and flagellar (H) antigens

Among the 41 laboratories responding, 25 (61%) perform tests for somatic (O) antigens. The methods employed include PCR-based testing, serological and whole genome sequenceing (WGS) based or a combinations of these methods. The distribution of the methods among the laboratories performing tests for identifying O antigens are shown in Figure 1. A total of 14 (34 %) perform the flagellar characterisation, H-typing, of STEC. For those who apply WGS based serotyping alone or in combination with other methods (10 laboratories), 3 laboratories used SeqSphere, 2 used Aries in combination with either Ridom or CGE, 2 used BioNumerics, either alone or in combination with CGE's SerotypeFinder, while 1 laboratory used an in-house pipeline in combination with the DTU/CGE's SerotypeFinder and another used an in-house pipeline, including the DTU/CGE SerotypeFinder.

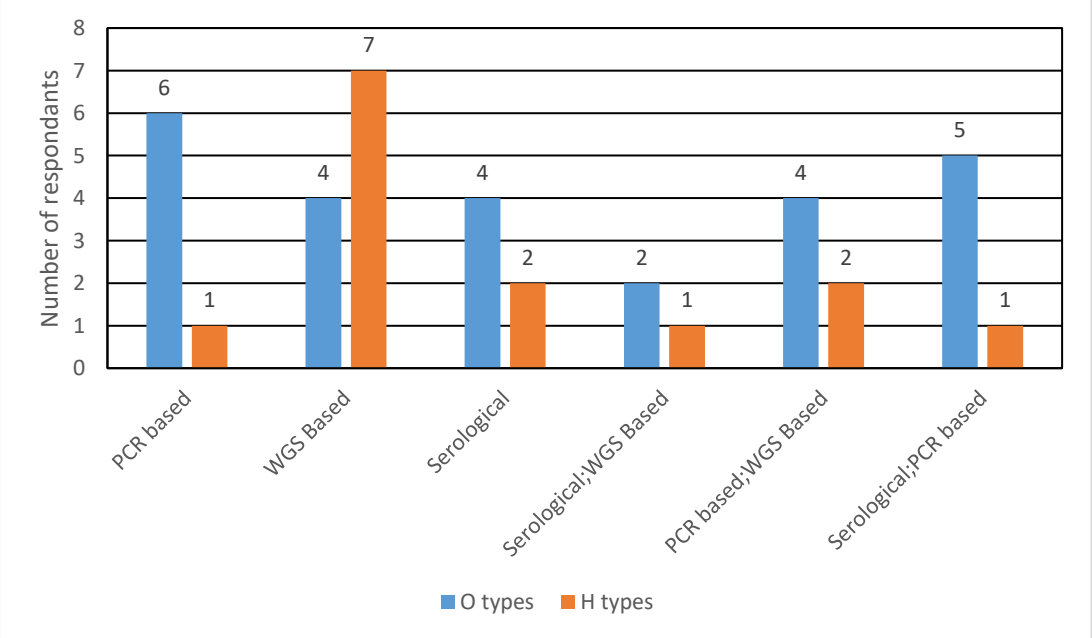

Figure 1. Distribution of methods used for somatic (O) antigen and flagella (H) antigen testing among the 25 and 14 laboratories performing the tests, respectively.

2.1.1.3 Virulence gene detection

A total of the 35 responding laboratories tested their isolates for STEC associated virulence genes (Figure 2). Most of the responding laboratories tested for the presence of the shiga toxin-encoding genes (*stx*1 n=34 and *stx*2 n=33) and the gene encoding the adhesin intimin (*eae* n=32). In total, 17 laboratories carried out *stx* subtyping, and 5 of these also performed *eae* subtyping. Seventeen of the laboratories which test for virulence genes also tested for the presence of *agg*R. From the responses to the questionnaire it is not possible to discriminate between laboratories using PCR or WGS for the detection of virulence genes. However, we would assume that both methods are in use, and that the majority of the responding laboratories applied PCR.

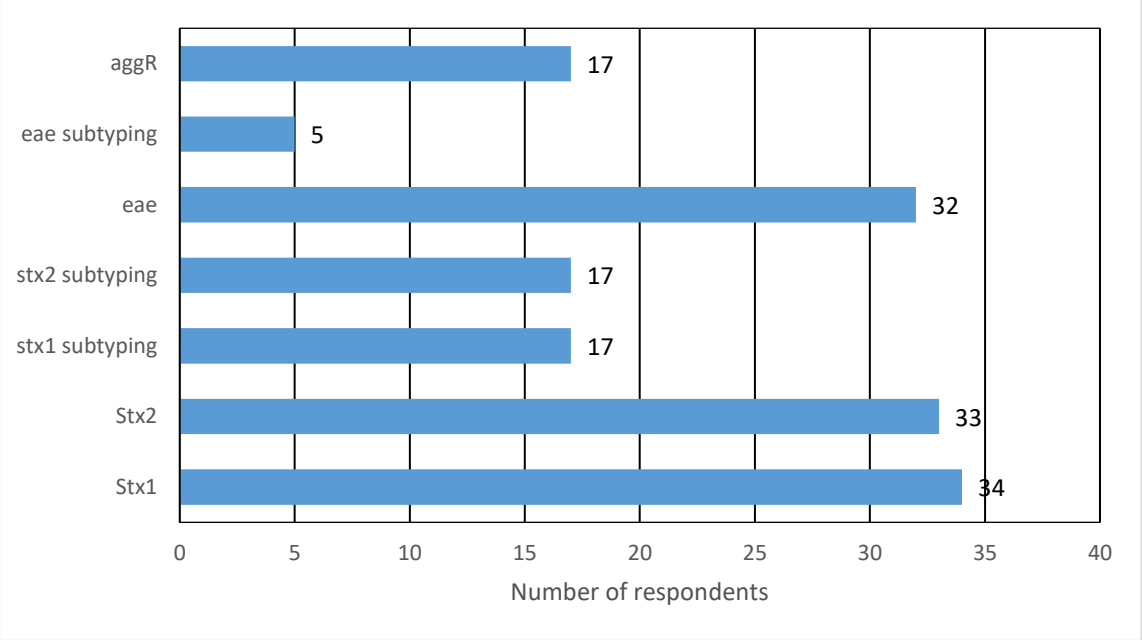

Figure 2. Number of laboratories testing for the different virulence factors.

2.1.1.4 Phylogenetic typing

In total, 17 (41%) laboratories reported that they performed genetic comparisons to determine the relatedness of isolates ("phylogenetic" typing). Typical methods used included WGS based, pulsed field gel electrophoresis (PFGE), multi locus sequence typing (MLST) and multiple locus variable number tandem repeat analysis (MLVA) or combinations of these (Figure 3).

Of the 15 labs that responded that they used WGS-based analyses:

- 9 used cgMLST (core genome MLST)
- 4 used Core genome (cg)/Whole genome (wg) MLST (whole genome MLST)
- 1 performed cgMLST in combination with SNP (single nucleotide polymorphism) analysis
- 1 used SNP analysis to determine the relatedness of STEC strains.

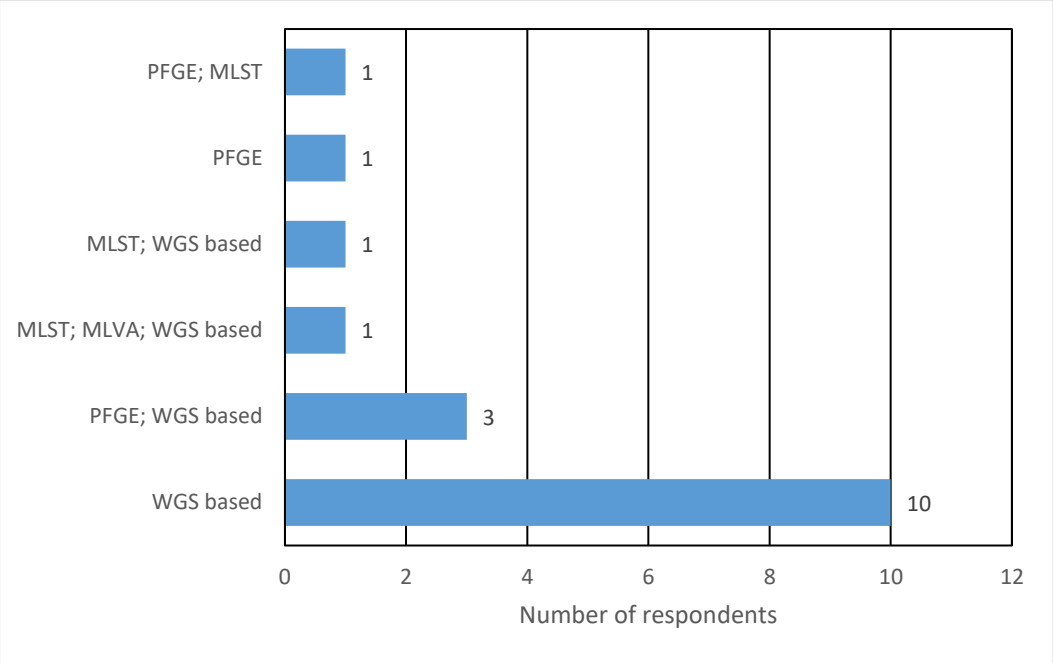

Figure 3. Number of laboratories performing phylogenetic analyses and the methods used.

2.1.1.5 AMR testing

A total of 13 of the 41 laboratories tested for AMR in STEC isolates. Common methods for testing, included genotypic methods, broth agar/agar dilution methods, disk diffusion and the E-test. The number of laboratories using the different methods or combinations is shown in Figure 4. Of the 3 laboratories that were only using genotypic methods, 2 used ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), while the third stated that they used an inhouse method and DTU virulence finder based (although it is not clear why this is being used for AMR purposes). The single lab that uses both broth/agar dilution and genotypic methods stated that they used PCR and "WGS NRL" database with no further information given.

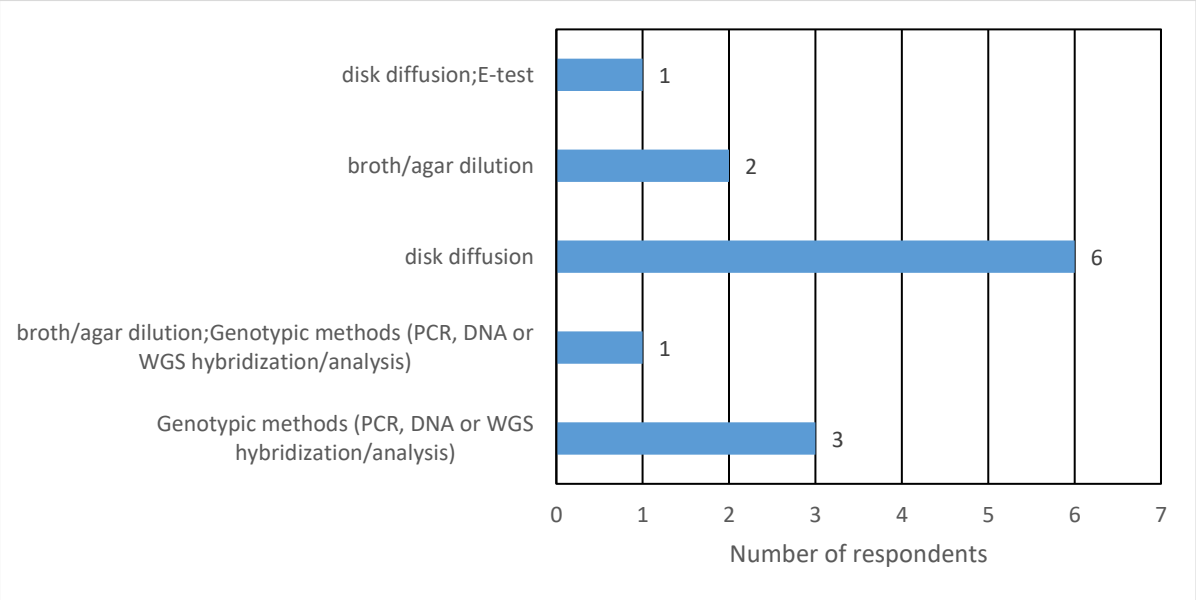

Figure 4. Distribution of methods used for AMR testing among the 13 responding laboratories.

2.1.2 Strain characterisation methods used in peer reviewed papers

The responses to the questionnaire confirm that WGS-based characterisation and typing methods were being increasingly used. However, other methods were also being applied. A literature search using PUBMED with the search string (Shiga toxin-producing *Escherichia coli*) AND (characterisation methods) AND (typing methods) was performed, with the criteria that the publications should be in English and published in the period from 2000 to 2021. The search returned 125 hits, where 2 were reviews. We also included 14 relevant papers identified from the search string (Shiga toxin-producing *Escherichia coli*) AND (characterisation methods). In addition, we looked at other sources, such as EURL for *E. coli,* EFSA, ECDC, OIE and FAO/WHO.

Two recent reports (FAO/WHO 2018, EFSA 2020) have described several methods including both phenotypical and molecular typing methods.Phenotypical methods included serotyping for identification of O- and H-antigens, sorbitol fermentation and the production of β-glucuronidase (limited to O157 serogroup), expression of enterohaemolysin and characterisation of STEC by looking at expression of Stx. Other phenotypical methods that have been used, are phage typing (Chinen et al 2009, Islam et al 2010). Serotyping for identification of O- and H-groups were still used, although it seems that the use of WGS for determining the serotypes of STEC was more and more common. More recently, MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectronomy) have been used for identification and differentiating of STEC. Christner et al (2017) used a novel data analysis tool after MALDI-TOF MS to identify isolates belonging to an outbreak, while Mclean et al (2018) reported that MALDI-TOF MS could differentiate *E. coli* O157:H7 from other STEC serogroups and distinguish *E. coli* O157:H7 from sorbitol fermenting *E. coli* O157. However, this method seems to have limited use.

Molecular typing methods are regularly being used, which was also confirmed by the respondents to the questionnaire. In the scientific literature the use of such methods are commonly reported. PCR-based genotyping for identification of O- and H-antigens and virulence gene profiling for *stx* genes and their subtypes, and other virulence genes, e.g. *eae, aggR, ehxA*, etc. are commonly used. Molecular methods for subtyping and fingerprinting such as pulse field gel electrophoresis (PFGE) and multilocus variable number tandem repeat (VNTR) analysis (MLVA) have been in use for several years. With the recent advancements and developments in sequencing technology, many laboratories have implemented WGS for typing and characterisation (FAO/WHO 2018, EFSA 2020). Single nucleotide polymorphisms (SNP) and core genome and/or whole genome multi locus sequence typing (cg and/or wg MLST)) are currently among the methods that are applied (EFSA 2020). MLST, using seven household genes have been applied, as described e.g. by Ziebell *et al.* (2008). Several other methods have also been used, including ERIC-PCR (enterobacterial repetitive intergenic consensus PCR) (Giammanco *et al*. 2020, Panutdaporn *et al*. 2004), RAPD-PCR (random amplified polymorphic DNA PCR) (Tutenel *et al.* 2003, Radu *et al.* 2001), RFLP (restriction fragment length polymorphism, electro typing, *P*-typing and ribotyping (Prager *et al* 2002). However, these methods have not, to the best of our knowledge, been in common use as they do not give the discriminatory information required. Several of these methods have been tested together with more commonly used methods, such as PFGE. Some have also only been

applied to specific serogroups/types. RFLP (restriction fragment length polymorphism) seems was used for discrimination of variants within a specific gene, such as in combination with PCR for genotyping of *fliC* (Beutin & Strauch, 2020).

AMR testing of STEC isolates is not useful as the use of antimicrobials in STEC infection therapy is controversial (Mor M and Ashkenazi S, 2014). In fact a few studies suggested that the use of antibiotics may increase the risk of developing haemolytic uraemic syndrome (HUS). Nonetheless, the AMR determination may be useful in the isolation of specific STEC strains, as was the case for the highly virulent Shigatoxin (Stx)–producing enteroaggregtaive E. coli (EAEC) O104:H4 strain which showed an extended spectrum beta-lactamase (ESBL) profile which could be exploited for its isolation (Sheutz et a.l 2011). Moreover, with the advent of WGS, the AMR profile can be easily predicted by analysing the sequences with tools, which may be also available as online resources, for the presence of AMR genes.

2.2 Gaps in strain characterisation that are inhibiting food safety regulation/policy in the EU

Molecular typing has developed rapidly in recent years. Many typing methods including PCR techniques and WGS, have become part of routine strain characterisation in many laboratories. Molecular typing provides essential tools for tracking microrganisms such as STEC in cases of disease and in the identification of vehicles of infection, as well as the capability to rapidly recognize outbreaks based on the ability to discriminate single isolated bacterial strains. According to the EU Reg. 2003/99 (Zoonosis Directive) STEC are priority microorganisms to be monitored in food and animals, nevertheless the current EU Food Legislation only includes microbiological criterion for STEC in sprouts ((Commission regulation No 209/2013: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32013R0209&from=ES), hindering the possibility of collecting data on the basis of a harmonised sampling strategy among EU member states (MSs). Regardless, since the last major STEC outbreak occurred in 2011, most MSs have significantly increased the number of official controls aiming at detecting the presence of STEC in food (EFSA 2020), leading to the possibility of having characterisation data for many STEC isolates.

The potential use of STEC strain characterisation for surveillance purposes, such as following the spread of strains and clones, early detection of international outbreaks, and prediction of epidemic potential of circulating strains, has been recently highlighted with a specific request form the EU Commission to EFSA on the creation of a database of typing data on STEC and other foodborne pathogens, in order to support a joint integrated analysis with a similar system already existing at ECDC (EFSA, 2014). At the time of the first creation of a EU repository of molecular features of STEC, the typing data indicated for STEC data collection included

- PFGE
- Serotyping
- Stx-coding genes and subtyping
- Additional virulence genes
- Phenotyping

Currently, WGS based typing is able to respond to several hazard characterisation questions all at once, representing a powerful tool for characterisation purposes. The widespread application of WGS is, however, hindered by the availability of the technology in all the laboratories carrying out the characterisation of STEC strains, as well as the lack of harmonisation for data analysis and concerns on the legal aspects associated with the collection of genomics data.

The characterisation of STEC strains is also essential for risk assessment, and recently several investigations have been carried out internationally (EFSA 2020, FAO/WHO 2018) to address the question of which STEC types are to be considered as potentially pathogenic to humans. Historically, the most common typing feature investigated was the serogroup, and a set of STEC serogroups most often isolated from HUS cases include O157, O26, O111, O103 and O145 (EFSA 2009). However, as other serogroups have also been associated with serious illness, serogroup is no longer a good indicator of pathogenicity (EFSA 2020). The pathogenicity assessment exercises recently carried out (FAO/WHO 2018 and EFSA 2020) highlight the importance of the characterization of subtyping of the *stx* genes. The determination of this feature is carried out by reference laboratories operating in the different sectors, and is part of the proficiency testing schemes carried out by the EURL for *E. coli* and by ECDC (https://www.iss.it/documents/5430402/0/Report_PT28_EN.pdf/c2cb7950-deb3-9853-6462-bd4a667eedd1?t=1615452108882; and https://www.ecdc.europa.eu/en/publications-data/ninth-external-quality-assessment-scheme-typing-shiga-toxin-producing-escherichia). Such a technique may not be routinely carried out by primary laboratories and official laboratories, representing an hindrance in the availability of data to inform food safety policy development. The lack of this typing data is also highlighted by the results reported by laboratories to which the survey was administered (17/35 carrying out this analysis) and by the data reported annually in the joint EFSA and ECDC zoonoses one health report (stx gene subtyping was only done for 6.1% of the food isolates, and even less for animal isolates, whereas more STEC isolates from human cases are *stx*-subtyped, 91.2% in 2019, EFSA and ECDC OH report 2020).

This gap is highlighted in the OH Report released in 2019 of ECDC and EFSA which highlights the importance of determining the virulence gene combinations (virulotypes) of the isolated STEC strains, emphasizing the *stx* gene subtyping, which would facilitate a more precise assessment of the risk connected with different STEC isolates (EFSA and ECDC OH report 2020 and EFSA BIOHAZ Panel, 2020).

2.3 Recommendations on what typing methods, AMR and virulence gene testing, etc. should be undertaken as part of a future harmonised approach to strain characterisation

Previously described methods of STEC characterisation were almost exclusively focusing on O157:H7, leaving a gap in the isolation and typing of other shiga toxin producing strains. Even considering the low sensitivity associated with culture-based methods, these persisted as the gold standard as they allow the selection of viable bacteria for typing (Parsons et al., 2016). Given the shortcomings of phenotypical agar-based methods, complementary molecular typing methods have been recommended. Classically employed STEC typing schemes are based on phenotypic serotyping (e.g: antisera agglutination assay)

combined with genotypic subtyping molecular methods (most usually PCR based methodologies. It is recommened that STEC characterisation includes serotype determination (include at least those serogroups with the highest epidemiologic relevance, as reported in the ECDC and EFSA OH Zoonosis Annual Reports), the presence of virulence genes, (i.e. virulotyping), *stx*-gene subtyping as well as testing for resistance genes for all clinical, animal, food and feed samples.

Considering the increasing number of typing methods available and the lack of consistency in nomenclature attribution, there is a growing need for a standardized approach. Method harmonisation is important not only from a clinical perspective, as toxin variations may confer different biological effects, but also from a public health surveillance perspective, to avoid generating data that cannot be compared between laboratories.

In an attempt to guide the subtyping of *stx*-encoding genes, a PCR protocol based in a multicentre study was developed (Scheutz et al., 2012). This could serve as a basis for a harmonised molecular typing technique that can easily be implemented in every country. New Stx genes subtypes have recently been reported, which are not targeted in the method decribed by Scheutz and colleagues: it would be important to understand their public health significance and subsequently develop new Stx subtyping methods including also the new subtypes.

Based on the EFSA report 2013 (Andreoletti et al., 2013), it is recommended that laboratories include the detection of the EAEC genes *aaiC* and *aggR* genes, as these are associated with enteroaggregative adhesion and therefore linked to a higher risk of developing severe disease. However, Boisen et al 2020 has recently suggested a molecular definition of EAEC comprising of *E. coli* strains harboring AggR and a complete aggregative adherence fimbriae AAF(I-V) or CS22 gene cluster. Therfore, we suggest that laboratories include the detection of *aggR* and not *aaiC*. Characterization of additional virulence genes should also include the entero-haemolysin-coding gene *ehly* and the genes encoding intestinal mucosa adhesion factors, intimin-encoded by the gene *eae* (Schmidt et al., 1995; Nataro et al., 1998), the Locus for Autoaggregation and adhesion (LAA) (Montero et al., 2017) and other adhesion factors.

When the typing is based on molecular techniques it is strongly recommended to use a combination of two or more methods, so a more sensitive and specific result is obtainable. Reports of false-positive STEC results associated with the use of a single detection method emphasize the disadvantages of depending on such an approach (CDC, 2001). Moreover it may not detect or identify “non-typical” emerging STEC strains.

Some of the discrepancies in strain characterisation between human, animal and food isolates were partially overcome with the publication of ISO/TS 13136:2012 (ISO, 2012), proposing a method to detect the major virulence genes of STEC, *stx* and *eae,* and the genes associated with the serogroups O157, O111, O26, O103, and O145. Even though this document facilitated the harmonisation of strain characterisation within the food sector, it does not cover *stx* subtyping which is also essential. This standard method is currently under revision by the CEN TC463 WG2 ad hoc group, and will include specifications on STEC strain subtyping and characterisation, further contributing to method harmonisation.

Other typing methods are needed for public health purposes, outbreak investigation and source attribution. Phylogenetic typing techniques, such as PFGE and MLVA, when based on standardized

protocols allow data comparison across countries and multistate surveillance of emerging clones. Harmonization of these typing methods is partially assured in laboratories belonging to the PulseNet organisation as although PulseNetCDC considers WGS to be the current gold standard, protocols for MLVA of *E.coli* O157 are still available on their website (Anon, 2013). Thus laboratories are required to follow standardized genotyping methods, to have a shared nomenclature and share information in real-time. These standardize molecular typing protocols could serve as a template for interlaboratory harmonization of surveillance typing methods.

With the increasing availability and easier access to WGS technologies, it is recommended for use in STEC strain typing. Once the obstacles to the implementation of WGS technology are overcome, it provides a faster and more cost-effective typing method when compared to the forementioned traditional methods. *In silico* analysis enables laboratories to overcome some of the most frequent gaps associated with STEC serotyping and/or virulence genes. The reporting of “O group unidentifiable” strains and antisera cross reactions are, for the most part, overcome, as is the issue of identifying emerging serotypes or untypable *E.coli* strains, as all the information on the O and H antigen encoding genes can be retrieved from the WGS data (Abdalhamid, 2019; EFSA, 2020).

E. coli strains, and not just STEC, harbouring antimicrobial resistance determinants should also be routinely assessed as these are subjected to specific surveillance programs monitored by EFSA. These programs focus mainly on ESBL and AmpC enzymes producing strains, as this may confer resistant to antimicrobials deemed critically important for human medicine recommended by the WHO. It is recommended to change from traditional molecular methods of AMR characterization to WGS, as this technology allows a more in-depth scrutiny of mobile genetic elements, like transposons and plasmids, to better understand their dissemination, as well as facilitating the identification of specific mutations that confer AMR.

An effort should be made to implement WGS as a large-scale and broadly used typing method. The advantages associated with this technology are only attainable if a harmonized approach is employed, otherwise the same problems of data comparison and interpretation will arise. WGS implementation should be based on standardized and validated methods and some aspects surrounding its implementation, particular in middle and lower-income countries, need to be addressed. It is recommended that standardised databases are created, and an effort should be made to upload both sequence data and metadata in a harmonized way. The adoption of an “open access” policy to data sharing is also advised. Considering the multiple pipelines available (Parsons et al., 2016) for data analysis, it is also recommended to standardise the software used to analyse and interpret the sequence data. Many programmes for WGS analysis are freely available to use and implememtation of these could facilitate more wide-spread use. Developing training programs designed for professionals of human health, animal health and food safety is a stepping stone to implement a reliable and successful surveillance program based on this technology and should not be overlooked. It is important to note that the implementation of all the forementioned measures does not replace the development of better isolation methods from human, food, feed, animal, and environmental samples.

In conclusion, the genomic information provided by WGS has an enormous potential for rapid molecular characterisation and facilitates comparison with new or current datasets in the event on an outbreak. A

unified effort should be made to move from currently available methods, such as PFGE, to this technology. Compulsory data reporting of human, animal, food, and feed data to EFSA/ECDC should be required and every MS should follow the same case definition and outbreak investigation systems. It is of the utmost importance that an effort is made to implement the recommendations on WGS methods in all member states (MS), as only then is harmonisation achieved.

2.4 Recommendations on how this harmonised strategy should be implemented

The importance of STEC strain characterization, especially in terms of *stx*-genes subtyping, is highlighted in the recent opinion released by EFSA on the "Pathogenicity assessment of Shiga toxin-producing *Escherichia coli* (STEC) and the public health risk posed by contamination of food with STEC" (EFSA 2020), and resources should be invested to achieve the level of recommended strain typing.

There is currently a change in typing and characterization from phenotypic and molecular based approaches to molecular methods only, with emphasis on the use of WGS based methods. Although the costs associated with WGS based methods have been reduced considerably in the last few years, it is still relatively expensive and requires technical expertise. In order to implement harmonised WGS based methods, training and proficiency testing should be available.

However, in order to be able to use WGS based methods for typing and characterization, isolates are still needed. It is thus important to continue the development of improved isolation methods for STEC from all types of matrices, from clinical samples to food, feed, water and environmental samples. Currently it is crucial to have an isolate for characterization in outbreak situations and also for risk assessment, and isolation of STEC should be prioritised more than ever. If reporting becomes compulsory, a minimum requirement of information should be requested, including both metadata and data on isolates.

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3.0 Enterotoxigenic *Escherichia coli* (ETEC)

3.1 Current routine strain characterisation in Europe

This section summarizes the responses to the ETEC questionnaire organised in WP3 of OH Harmony-CAP regarding characterisation of isolates. The design of the questionnaire and general information of the respondents have previously been described in Deliverable 3.1.2. Briefly, 19 (47.5%) out of 40 laboratories responded that they tested for ETEC, and these covered all the matrices listed, including human clinical (13 lab.), animal (4 lab.), food (4 lab.), feed (1 lab.) and environmental (3 lab.) samples. Among the laboratories testing for ETEC, 78.9% (15/19) were accredited according to ISO/IEC 17025 or ISO 15189. The remaining 4 non-accredited laboratories were from the public health area. A total of 16 (84.2%) laboratories stored isolates (10 laboratories responded always and 6 responded sometimes), either on their own laboratory and/or at the National Reference Laboratory (NRL) (7 laboratories stored isolates at their own facilities, 3 responded that isolates are stored at NRL level, and 6 responded that the isolates are stored at both own laboratories and NRL).

3.1.1 Responses to the questionnaire

3.1.1.1 Laboratory testing for heat-stable (ST) and heat-labile (LT) enterotoxins

Fifteen out of the 19 laboratories tested their isolates for ETEC enterotoxins (ST and LT). They used a range of methods (Figure 5). A range of methods were utilised the most common of which was PCR that was used by 15 laboratories. Four respondents used immunological methods, one laboratory used a commercial kit and one laboratory used DNA hybridisation.

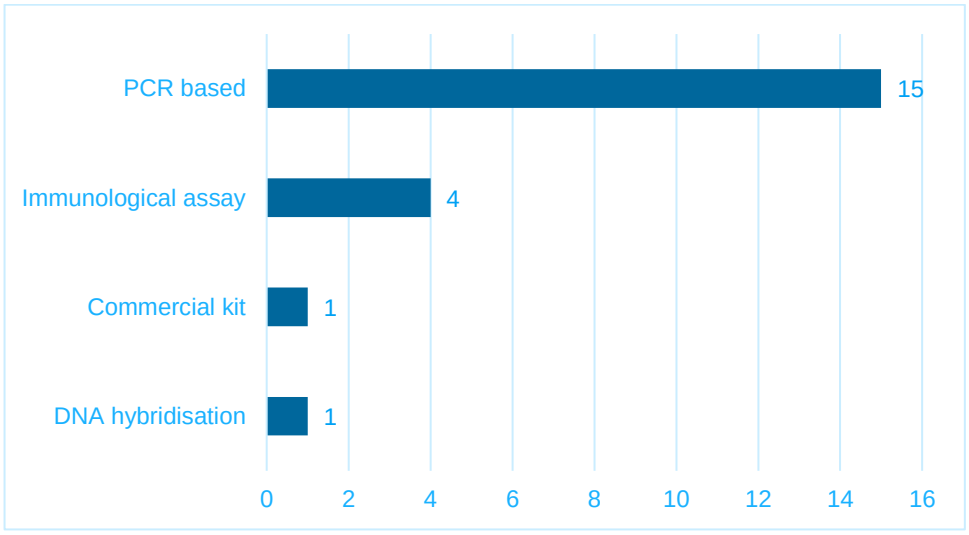

Figure 5. The methods used for detection of the toxins (ST and LT) among respondents.

3.1.1.1.1. Laboratories performing confirmatory tests

Eleven laboratories out of the 19 laboratories perform confirmatory testing and four are sub-contracting this service to other laboratories. One of the laboratories reported receiving samples from other laboratories for confirmatatory testing. Among the responding laboratories, 10have some accredited methods but only 6 have all the their confirmatory methods accredited. The non-accredited methods included; WGS, conventional PCR, real-time PCR, EURL Protocol, *"in-house"* real-time PCR on TAC, and serological testing. The most commonly used methods and media used to grow isolates was buffered peptone water enrichment, SMA, TBX, TSA, Chromogenic Agar, MacConkey, and SSI Enteric medium.

3.1.1.1.2 Biochemical testing

Three laboratories reported that they perform biochemical assays to characterise ETEC. These laboratories performed the following tests:

- All laboratories perform indole test for confirmation;
- 2 laboratories tested for beta-glucuronidase, Voges-Proskauer test, urease, methyl red, gas production, citrate, lactose, carbohydrate fermentation, ornithine decarboxylase, lysine decarboxylase;
- 1 laboratory tested for phenylalanine deaminase, hydrogen sulphide, and arginine decarboxylase;
- 2 laboratories test for enterohaemolytic activity;
- 1 laboratory carry out motility testing.

3.1.1.2 Testing for Somatic (O) and flagellar (H) antigens

Ten of the laboratories test for somatic (O) antigens and 6 test for flagella (H) antigens. Among these laboratories, 7 used serological methods to identify O antigens, 4 used PCR-based methods and 2 used *in silico* WGS serotyping (Figure 6). Four laboratories used serological methods for the detection of H antigens, 1 laboratory used PCR methods and 2 laboratories used *in silico* WGS serotyping. Laboratories using WGS to identify the antigenic sturcture of an isolate use the Aries, Ridom and CGE SerotypeFinder .

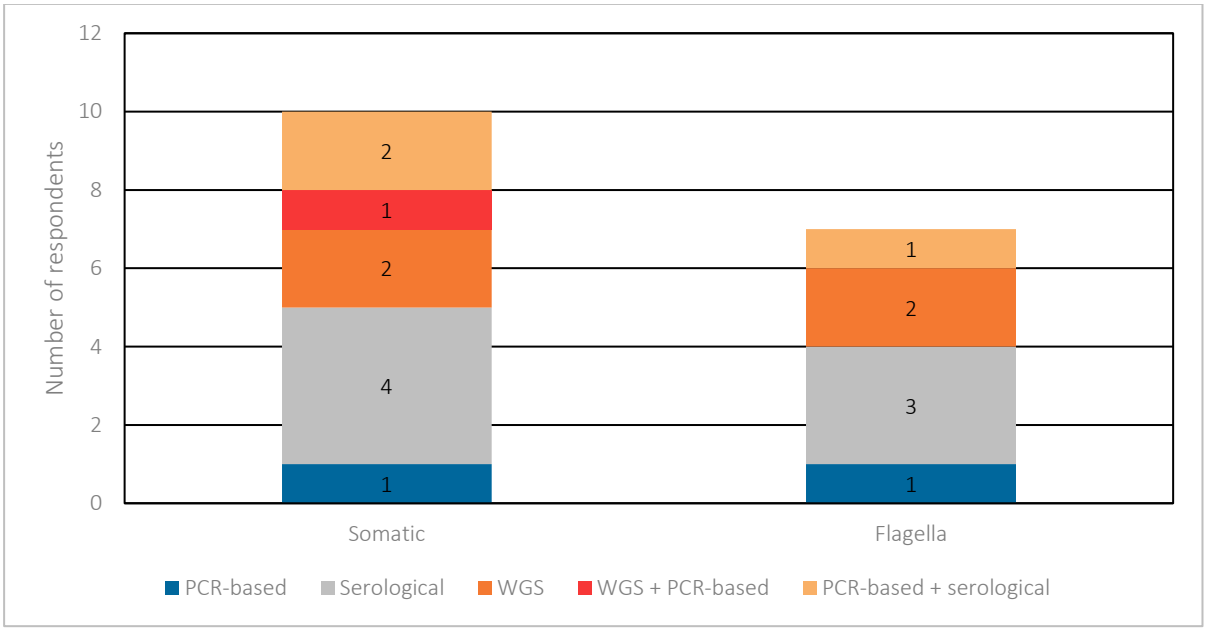

Figure 6. Methods used to determine the O and H antigens of ETEC among laboratories responding to questionaires.

3.1.1.3 Virulence gene detection

Characterisation of virulence factors in ETEC is carried out by 10 of the laboratories which test for ETEC. These include testing for the presence of toxin genes and colonization factors (fimbrial genes) using molecular methologies (Figure 7). Eight and 7 of the laboratories test for *elt* (heat labile toxin) and *est* (heat stable toxin), respectively. In addition, subtyping of toxin genes was carried out for STa (*estah* gene; human variant) by 6 respondants, for STa (*estap* gene; porcine variant) by 5 laboratories and STb (*estb* gene) by 4 laboratories. Two laboratories sub-typed LT genes (LT-1, A and/or B SU), and 1 laboratory tested for the *st2* gene.

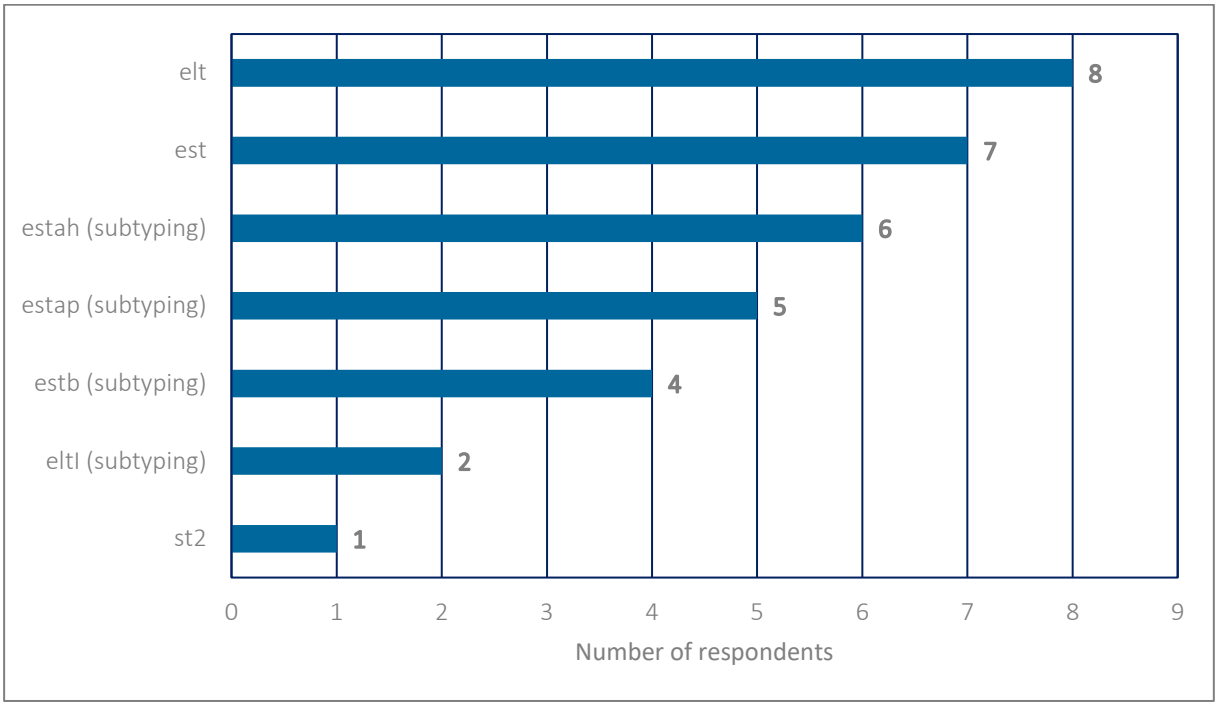

Figure 7. Number of laboratories that tested for each ETEC virulence factor.

Respondents were also asked which ETEC associated colonization factors were characterised by their laboratory. Two laboratories test for the presence of fimbrial genes, both testing for the presence of F4 (*fae*), F5 (*fan*), F6 (*fas*), F17 (*f*17a) and F41 (*fim*41a). One laboratory also tested for the presence of F18/F107 (*fed* gene). None of the laboratories tested for the presence of human colonisation factors (CFs).

3.1.1.4 Phylogenetic typing

Three (15.8%) out of the 19 laboratories reported thay performed genetic comparisons to determine the relatedness of isolates ("phylogenetic" typing). All three laboratories used WGS based cgMLST and 1 laboratory also used PFGE.

3.1.1.5 AMR testing

Seven (36.8%) of the respondant laboratories tested for AMR, by using broth/agar dilution (3 laboratories), disk diffusion (2 lab), disk diffusion/E-test (1 lab), and by using genotypic methods, namely PCR and "WGS NRL database" (1 lab).

3.1.2 Strain Characterisation methods used in peer reviewed papers

Based on data from the respondents to the questionnaire, it is clear that detection and characterization of ETEC is not widely performed. DNA-based methods are the most frequently used for ETEC characterization of enterotoxins and several colonization factos (CFs). Methodologies used a range from array technology to conventional PCR, real-time PCR (rt-PCR) and WGS. However, other methods like serotyping for identification of O- and H-groups are still in place.

The literature search to evaluate the methodology used for isolate characterisation was conducted on scientific publications available at PubMed. After an initial screening of all ETEC publications, a search string "(ETEC) AND (typing methods) AND (characterization methods)", limited to the time-period 2000-2021 and English language, returned 76 papers. According to the relevance for the purpose, 44 papers were excluded from further analysis, since they focus on detection instead of characterization/typing methods. Additionally, documents from a limited number of European and international organizations, including EURL *E. coli*, SSI/WHO Reference laboratories for enteric pathogens, ECDC, EFSA, FAO/WHO and OIE were also evaluated, but very limited information was found (only a protocol from EURL-VTEC 2013). Food safety agencies from outside Europe were excluded from the search.

Molecular methods were regularly used for the identification of O- and H-antigens, characterization and subtyping of genes encoding for ETEC enterotoxins (ST, LT, STa, STb, others) and/or associated CFs, either by conventional PCR, rt-PCR, or WGS (Nazarian *et al*. 2012, Alerasol *et al*. 2014). However, PCR reaction conditions, which includes different primers and probes targets, are highly variable among the studies, making it difficult to choose the best approach. There are also other methods, such as pulse field gel electrophoresis (PFGE), multilocus variable number of tandem repeat analysis (MLVA), multi-locus sequence typing (MLST), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism electro typing (RFLP), which are less frequently used, or used in combination with other methods ((Ramos et al., 2006, Robins-Browne et al., 2016, Yinghui et al., 2017, Michelacci et al., 2018).

Molecular typing has developed rapidly in recent years, and several DNA-based typing methods, like PCR and WGS, have become part of routine strain characterization, as molecular methods are replacing the phenotypic methods (Abraham et al., 2012, Iguchi et al., 2017, Kwon et al., 2017). Some laboratories had already implemented WGS for typing and ETEC characterization (Michelacci et al., 2018, Tang et al., 2019).

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As observed from the respondents of the questionnaire and the literature, antimicrobial susceptibility testing (AST) is very limited in ETEC isolates. As for the other *E. coli* pathotypes, AST is performed using standard broth/agar dilution, disk diffusion, and disk diffusion/E-test methods, according to Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, or genotyping (PCR and WGS) methods.

3.2 Gaps in strain characterisation that are inhibiting food safety regulation/policy in the EU

Common serotyping schemes, based on somatic (O) and flagellar (H) antigens, that are widely used to characterize other pathogenic *E.coli* can also be applied to ETEC but the same disadvantages, like antisera cross reactions and non-identification of certain serogroups, are to be expected. This typing

method is not suitable for a surveillance program, as it is unable to detect new and emerging serogroups that are assigned to the “untypable” category. In addition to O- and H typing, K typing of the acidic capsules has also been useful in the typing of ETEC. However, this is only done by one laboratory, the International *Escherichia* and *Klebsiella* Centre in Copenhagen.

Typical ETEC characterisation is based on PCR detection of heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). This typing method has a low discriminatory power as it can only classify strains as producers of one or both enterotoxins, or ST and LT alone. ETEC characterisation solely based on this approach leaves a major gap in the information retrieved from each strain, mainly on pathogenicity. Colonization factors (CFs) encoded by most strains play an important role when assesing the risk for disease in animals (primarily piglets and calves) and therefore in the development of clinical disease. CFs are also frequently included in strain characterization of isolates from animals and used in the development of vaccines. CF characterisation is almost non existing in the characterisation of human ETEC isolates. Serological typing, either by agglutination or immunoassay-based methods, is often considered the best method to distinguish between Colonization Factor Antigens (CFAs) (Vidal et al., 2019). The major limitation in applying this typing method as the standard methodology used in the EU is related to the limited access to antibody typing reagents, almost exclusively available to National Reference Laboratories and Reference Centres (Ghosal et al., 2007). Relying on a limited access typing method impairs ETEC surveillance, as data collation is restricted to a few institutes therefore preventing large scale data comparison. Another aspect hindering data harmonisation is the fact that the expression of CFAs is dependent on culture conditions (Chakraborty et al., 2001), which is a highly variable factor between laboratories.

In an attempt to close the gaps that arise from antibody-based methods, some PCR protocols have been proposed as a more reasonable alternative to the current practices without significantly compromising sensitivity or specificity (Ghosal et al., 2007, Sjöling et al., 2007, Vidal et al., 2009). The faster and more affordable nature of PCR-based methods are obvious advantages as far as large-scale application and standardization is concerned, it is however important to note that these methods only detect the presence of the CFs related genes and not their expression, leaving an information gap that would still need to be addressed by other methods (Ghosal et al., 2007).

Even though useful clinical information can be obtained from the characterization of virulence factors, it is not possible to determine strain relatedness as most toxin and virulence associated factors are encoded by genes located in plasmids and frequently flanked by insertion sequences. Evidence supporting this gap arise from studies showing that strains sharing the same serogroup or harbouring the same CFs are not clonally related (Pacheco et al., 1997, Pacheco et al., 2001). ETEC epidemiological investigations frequently use PFGE analysis for outbreak investigation and source attribution, as this is the typing method with the highest discriminatory power for phylogenetic assessment (Pacheco et al., 2001). Further research is required to provide guidelines on which virulence genes to include and which typing methods to employ. This will require a revision of the ETEC nomenclature and a comprehensive database including all the subtypes and variants of both the enterotoxins and the known CFs. This research should evaluate the role of minor putative CFs in the pathogenesis and assess if they should or should not be included in a harmonised typing method.

3.3 Recommendations on what typing methods, AMR and virulence gene testing, etc. should be undertaken as part of a future harmonised approach to strain characterisation

Gastrointestinal diseases are a global health problem and ETEC is the causative agent of a considerable number of cases, especially in low-income countries. Notably, the Global Enteric Multicenter Study (GEMS), found that most attributable cases of moderate-to-severe diarrhoea in sub-Saharan Africa and south Asia were due to four pathogens: rotavirus, *Cryptosporidium*, ETEC (ST-ETEC; with or without co-expression of heat-labile enterotoxin), and *Shigella* (Kotloff *et al* 2013). However, the real burden of ETEC infections may be underestimated since many countries do not perform ETEC screening and because this is not a notifiable disease. Vaccine development to reduce the burden of this disease is ongoing and a similar unified approach needs to be made towards the harmonisation of typing methods that will allow for the early detection of new trends in infections.

The detection of ST and LT toxins is clearly insufficient to characterize ETEC strains, as enterotoxins are only one of the virulence factors of this pathogen. Furthermore, the nomenclature of enterotoxins is sometimes confusing as different designations of the same toxin are in use. It will be a task for this project to revise and standardise the LT/ST nomenclature in collaboration with international experts in the ETEC field. Colonization factor (CFs) are vital for the pathogen to produce symptomatic infections and should therefore always be part of the strain typing protocol. Even though PCR-based methods have been developed for the detection of CFs encoding genes it is recommended that an assay capable of assessing these genes expression is included. Due to the limited access to CFs serotyping reagents, an approachable alternative could be the implementation of a reverse transcript-PCR (RT-PCR) that, being an RNA detection technique, is closely related to gene expression. However, this methodology is difficult to implement and needs further development, as some conflicting results with the serotyping arose in previous studies (Ghosal et al., 2007). The large-scale implementation of a PCR-based assay as the standard methodology is a good option but a protocol describing the reaction conditions, which genes to include and how to assess their expression is needed to make sure all laboratories produce data that can be shared and compared. Otherwise, the current gaps will not be overcome.

Whichever typing method is adopted as the standard for ETEC characterization needs to be based on a patronized culture protocol to avoid different CFAs expression influenced by culture condition (Chakraborty et al., 2001). The implementation of such a protocol cannot be neglected as it will influence all the downstream results.

Hybrid *E.coli* strains harbouring for example shiga toxin and enterotoxin genes or Shiga toxin and ExPEC associated genes are an emerging public health threat that cannot be overlooked and should be included in surveillance plans (Leonard et al., 2016; Bai et al., 2019; Brilhante et al., 2019). These hybrid strains are very often undetected in less well equipped laboratories, where WGS is not routinely performed and where the combination of virulence factors is not included in diagnostics guidelines nor in the surveillance network. The inclusion of more genetic determinants in ETEC typing is highly recommended and should be considered for all pathogenic *E.coli* as this will allow countries to have a better insight on hybrid strains epidemiology and the associated health burden (Bay et al., 2019).

As for most bacterial pathogens, antimicrobial susceptibility testing is based on Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. There is currently limited information on the antimicrobial practices of ETEC infections and of the associated antimicrobial resistance. A recent update on proposed antimicrobial susceptibility testing has been proposed by EFSA (2019). Based on this report it is recommended to include amikacin in the panel of antimicrobials tested, accompanied by the reduction of some of the dilution ranges of other antibiotics that constitute the current testing panel. This change was considered as there is increasing resistance rate to aminoglycosides, which are associated with carbapenemases *AmpC* or ESBL and fluoroquinolone resistance, mostly in Africa and in Asia (Fang et al., 2019).

With the increasing accessibility to WGS technology there is an opportunity to use this technology as the main method of strain typing. The application of this technology in ETEC typing could assist in the identification of new CFs and to evaluate their prevalence. This is valuable information not only to guide clinical decisions but also to advise on which virulence factors that should be included in the recommended surveillance protocols and in the choice of vaccines. Current DNA-based typing schemes for outbreak investigation and source attribution use PFGE analysis and, although it is considered a reliable approach to assess the phylogenetic relation between strains, it would benefit from the creation of a shared databases that would allow greater levels of data sharing (Sahl et al., 2017). A change from PFGE to WGS is expected to follow the global trend of moving to genome sequencing. It is however important to mention that all the advantages associated with the broader use of this technology will only be accomplished if an effort is also made to harmonise its implementation, otherwise similar gaps in interlaboratory data comparison are to be expected. It is recommended that standardised databases are created so sequence data and metadata can be uploaded in a unified way and that the same software is used to analyse and interpret the genomic data. A complete assessment of the core genome and of the accessory genome, which comprise the major virulence determinants, will give a more detailed insight into ETEC pathogenicity and epidemiology.

The adoption of policies that make data easily available is also important. Until recently only a small number of sequenced ETEC genomes were readily available and even less from strains isolated from animals. All the One Health sectors should contribute to a commonly shared database for data comparison. For a true harmonisation to be accomplished not only the detection and characterization methods need to be standardised but the isolation methods from human, food, feed, animal, and environmental samples should be revised and standardised.

3.4 Recommendations on how this harmonised strategy should be implemented

The lack of well-defined and validated protocols should be be addressed as a priority to achieve a more efficient and harmonised strategy for ETEC detection, characterization and surveillance across laboratories of the different sectors.

Considering the global trend to move from phenotypical and molecular based methods to molecular methods only, based on WGS, it is essential to support this transition process, especially in closing the technological and human resource gap between countries. There should be a focus on implementing similar technologies and software, so the data can be shared and compared across the Europe and the

rest of the world. The large-scale implementation of these typing approaches requires qualified professionals to produce reliable results, and these is a need to provide training. Once guidelines are available, and are implemented, regular external quality assurance programmes should be carried out to evaluate the methods efficacy. However WGS is still relatively expensive, requires technical expertise and a harmonised method for ETEC isolation from human, animal and food samples is still required. It is estimated that the prevalence of ETEC infections is underestimated by non-notification. Mandatory notification should be adopted to compel countries to include this pathogen in their surveillance. Guidelines for data reporting is also essential including the provision of relevant metadata.

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4.0 *Cryptosporidium*

4.1 Current routine strain characterisation in Europe

The *Cryptosporidium* questionnaire was composed of 15 questions. Of the 54 respondents, 44 laboratories reported that they tested for Cryptosporidium, and 19 laboratories reported that they perform confirmatory testing for *Cryptosporidium*. Those laboratories performing confirmatory testing used different approaches: modified Ziehl-Neelsen microscopy (7), Auramine phenol microscopy (1), Immunofluorescence microscopy (2), Immunochromatographic rapid test (1), DNA detection by PCR (8) and IMS (1). The majority of those performing confirmatory testing (10/17) reported using accredited methods. For the question "If applicable, please indicate what characterisation is routinely performed (tick appropriate)", 17 laboratories reported they perform routine characterisation by 'PCR-based tests', and one of these also used WGS. A number of laboratories shared their protocols, including protocols for characterisation, with OH-Harmony-CAP for an evaluation and scoring exercise.

4.2 Gaps in strain characterisation that are inhibiting food safety regulation/policy in the EU

Cryptosporidiosis outbreaks in humans caused by food or water contaminated with *Cryptosproridium* spp. oocysts are detected relatively often in Europe. Altogether 11 outbreaks and 468 cases were reported in 2019 (EFSA and ECDC 2021). A zoonotic species, *Cryptosporidium parvum*, was implicated in eight outbreaks. No information on species was available for the remaining outbreaks. In addition to food and waterborne infections, zoonotic transmission from direct contact with infected animals as well as human-to-human transmission (including secondary cases) were reported. The transmission of *Cryptosporidium* spp. occurs mainly via faecal-oral route by accidental ingestion of oocysts, which are

shed in faeces of infected hosts and which are the infective, environmentally-resistant stage and the target diagnostic stage, as well as the stage that can contaminate food or water (EFSA BIOHAZ Panel, 2018).

Several *Cryptosporidium* species and genotypes can cause infections in humans and animals (Caccio and Chalmers 2016, Beser et al. 2015, Björkman et al. 2015, Santoro et al. 2019, Ryan et al. 2021). Species level identification is necessary to support the detection of an outbreak, and to inform whether zoonotic transmission could be the cause. To confirm whether the cluster is an outbreak with a point source, further molecular typing should be performed. The level of characterisation applied should be informative for epidemiological investigations and the detection and management of outbreaks (Chalmers and Caccio 2016).

Xiao et al. (1999) described primers for species determination of *Cryptosporidium* on the basis of 18S SSU rRNA gene amplification and sequencing. Further modification was published by Santin et al. (2004).

Nested-PCR/RFLP-method described by the EURLP for molecular detection of *Cryptosporidium* parasites at species level is based on the amplification of a fragment of the gene encoding for a structural protein of the oocyst wall (Cryptosporidium Oocyst Wall Prorein, COWP) (EURLP, Pedraza-Diaz et al. 2001, Spano et al. 1997). The nested PCR assay can detect the parasite in faecal samples containing <500 oocysts per gram, and based on the restriction patterns generated by the enzyme Rsa I, it is possible to distinguish the species *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, *C. andersoni*, and the horse genotype. The identification of *C. suis* and *C. ubiquitum* requires another restriction analysis using the enzyme Alu I.

For subtyping, Alves et al. (2003) described a protocol for *gp60*-based subtyping of e.g. *C. parvum* and *C. hominis*. For other species, approaches have been described, e.g. Stensvold et al. (2014, 2015) described protocols for *C. meleagridis* and *C. viatorum*. The discriminative ability of *gp60*-typing is limited, in particular if the result is a type that is common in the region (Cacciò et al. 2015; Santoro et al. 2019), and in case of mixed infections (Dettwiler et al. 2021). Typing based on *gp60* has been applied in One Health outbreak setting (Thomas-Lopez et al. 2020). Based on the questionnaire and available literature, WGS is not used routinely for *Cryptosporidium* typing at present. External Quality Control programmes exist for *Cryptosporidium* species level identification as well as a ring trial for species level determination and subtyping.

4.3 Recommendations on what typing methods, etc. should be undertaken as part of a future harmonised approach to strain characterisation

A scoring exercise performed in OH-Harmony-CAP on a convenience selection of submitted protocols did not clearly identify superior typing method(s) over others. The available approaches have their benefits and limitations, and selection of method depends on preferences of each laboratory and the purpose of the typing.

Key thing to harmonise is that species level identification should always be performed. For subtyping, *gp60* has been applied in One Health settings. To help in interpreting the results, baseline data on circulating types should be collected. Of mention is the possibility to use CryptoGenotyper software

which will aid in data interpretation and nomenclature (Yanta et al. 2021). Novel typing approaches are being developed (e.g. in One Health EJP project PARADISE), and future harmonisation plans should aim to implement new methodology, where possible.

4.4 Recommendations on how this harmonised strategy should be implemented

The currently used methods are based on published articles, however providing training could be beneficial. External Quality Control programmes and ring trials are encouraged. Uptake of novel methods that are being developed should be anticipated. Collaborations, in particular across laboratories of different sectors, are useful and can support gathering background information on circulation of the different *Cryptosporidium* species and subtypes.

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5.0 Antimicrobial resistance (AMR) testing in *Salmonella* and *Campylobacter*

5.1 Current routine strain characterisation in Europe

Forty-nine laboratories that responded to the questionnaires tested for antimicrobial resistance (AMR) in either *Salmonella* or *Campylobacter* or both. A breakdown of the samples tested for each bacterial genus is shown in Figure 8.

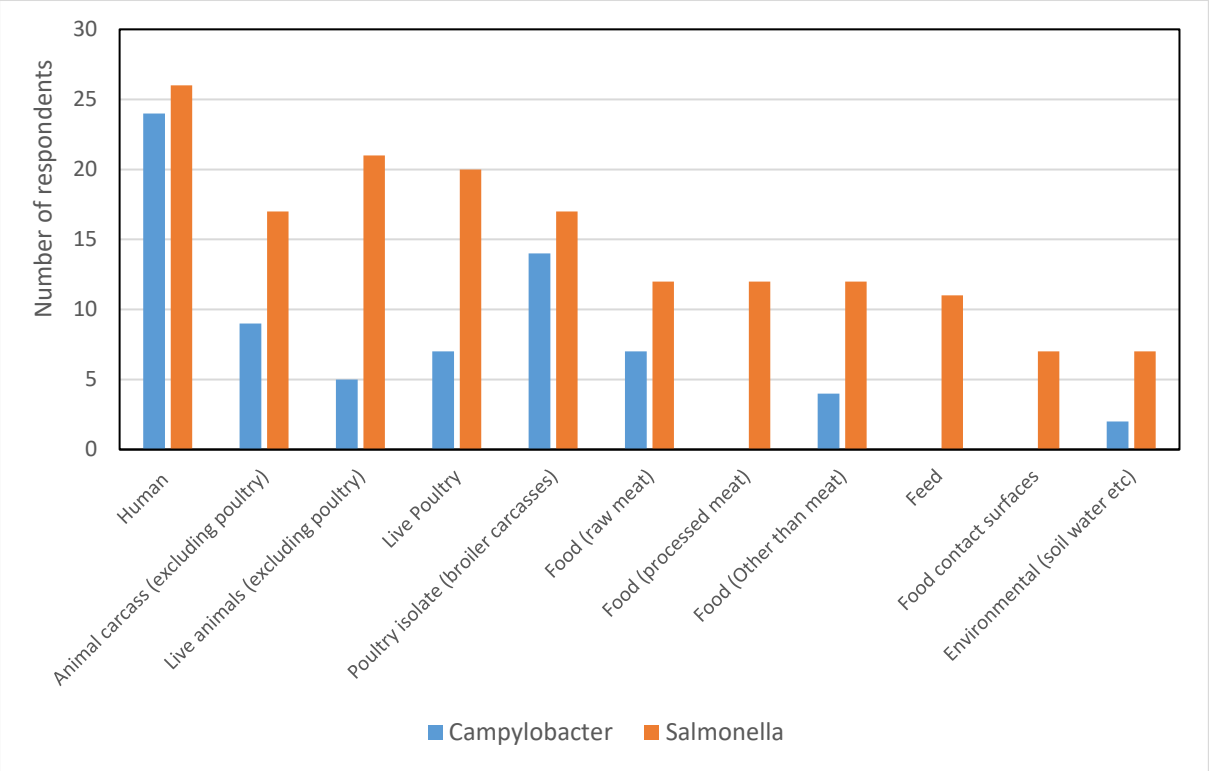

Figure 8. Number of respondents testing each sample type characterising *Salmonella* and *Campylobacter* for antimicrobial resistance.

5.1.1 Phenotypic characterisation

Phenotypic characterisation of AMR is carried out for *Salmonella* and *Campylobacter* using either; disk diffusion assays, MIC broth/agar dilution, Gradient strips (E-tests or similar), genotypic methods (PCR, DNA or WGS hybridization/analysis), and Vitek (Figure 9). The MIC methods were most often used for phenotypic characterisation of the AMR profiles of *Salmonella* and *Campylobacter*.

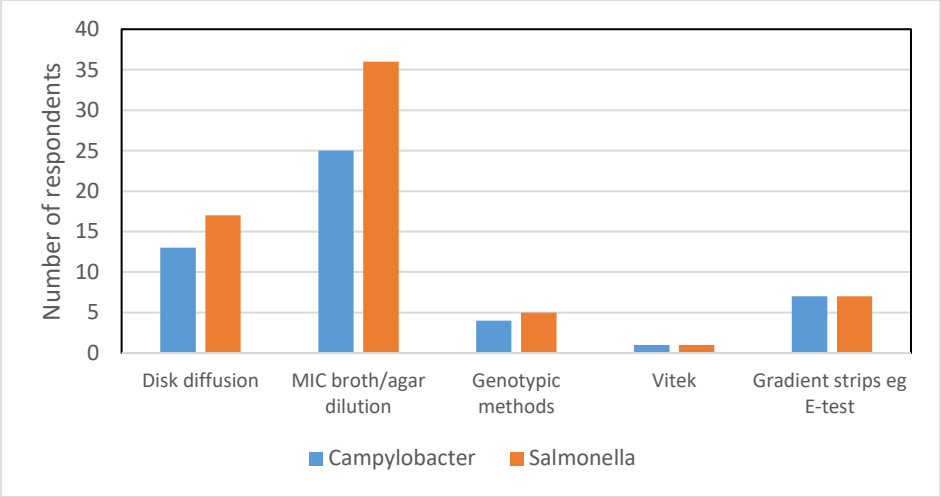

Figure 9. Number of respondents using each method for charcterisation of AMR in *Salmonella* and *Campylobacter* spp.

5.1.2 Genotypic characterisation

Six laboratories tested for specific AMR genes or point mutations, either by WGS or PCR/Sanger, most specifically for tetracycline resistance genes, fluoroquinolones-resistance associated point mutations, macrolides-resistance associated point mutations, aminoglycosides resistance genes, and 2 laboratories tested for all *in silico* resistance mechanisms. One laboratory also tested for macrolides-resistance associated point mutations and *in silico* by WGS. Although 8 laboratories answered that they do not test for specific AMR genes or point mutations, they do test for the above mentioned mutations.

5.2 Gaps in strain characterisation that are inhibiting food safety regulation/policy in the EU

Directive 2003/99/EC requires MSs to monitor and report comparable data on AMR of zoonotic agents isolated from food-producing animals and food (EC-European Commission, 2003). This directive is supplemented by the monitoring of AMR in human isolates conducted in accordance with Decision 1082/2013/EU (ECDC, 2016), and Commission Implementing Decision 2012/506/EU. This One Health approach to AMR requires intersectoral harmonisation at national and international levels, to allow comparison of data. Accordingly, several technical specifications for harmonised monitoring and reporting of antimicrobial resistance in *Salmonella* spp. and *Campylobacter* spp. have been issued since 2012. Nevertheless, comparison of AMR systems between countries and sectors still shows a lack of standardization and harmonisation (Mesa *et al.* 2020, https://zenodo.org/record/4381374#.YZPi2WDP2Uk). Indeed, AMR data can vary by sample type

(clinical/non-clinical), laboratory method (disk diffusion, broth microdilution (BMD), conventional PCR, real time PCR, WGS), antibiotic panel, data type, i.e. quantitative (minimum inhibitory concentration in mg/L or inhibition zone diameter in mm) vs qualitative data (susceptible-intermediate-resistant (SIR)), interpretative criteria, European Committee on Antimicrobial Susceptibility Testing (EUCAST), Clinical and Laboratory Standards Institute (CLSI) among others, and/or the evaluation criteria adopted (epidemiological or clinical).

Regarding phenotypic methods of AMR surveillance, dilution methods, especially the broth microdilution (BMD), is the preferred testing method, and the only one that is accepted in testing of animals and food isolates. However, for human isolates, validated methods of gradient strip diffusion or disk diffusion according to EUCAST protocols, including interpretative criteria, are also accepted, and both methods show good to excellent correlation for *Salmonella* spp. and *Campylobacter* spp.. Additionally, reports on AMR should contain quantitative values rather than data on the 'susceptible', 'susceptible increased exposure', 'resistant' (SIR) categories to allow different thresholds for use in interpretation, either clinical breakpoints (CBP) or epidemiological cut-offs (ECOFF). This gap has however been overcome for various combinations of bacteria/antimicrobial substance, for which the ECOFFs equal the clinical breakpoints set for humans by EUCAST. The laboratory method itself can also be an issue. As an example, colistin, a key antimicrobial in human and animal health, listed as a first priority antimicrobial to be tested for human *Salmonella enterica*, diffuses poorly into the agar medium, and BMD is so far the only valid method in the technical guidance documents provided by the EUCAST and CLSI (CLSI, 2016; EUCAST, 2017). Therefore, disk diffusion results for colistin are not reliable (Ezadi et al., 2019). This technical issue impairs many public health laboratories, which perform disk diffusion only, to include this antibiotic in their surveillance program.

Molecular typing is an essential tool for surveillance purposes such as monitoring the spread of clones and strains, including AMR clones, early detection of outbreaks, and prediction of epidemic potential. These techniques can also help overcome the technical difficulties associated with phenotypic testing. As an example, a multiplex PCR protocol for detection of all currently known transferable colistin resistance genes (mcr-1 to mcr-5, and variants) in *Enterobacteriaceae* was developed for surveillance purposes (Rebelo et al., 2018).

The improvement in whole genome sequencing (WGS) and the availability of bioinformatics tools for real-time detection of AMR determinants is promoting the rapid and natural transition of phenotypic or conventional typing methods to routine WGS for public health surveillance. This technology will facilitate the detection of known resistance genes and resistance-associated mutations, the earlier detection of resistant strains (Köser et al., 2014; Ellington et al., 2017; Hendriksen et al., 2019) and allow an effective screen for novel and emerging antimicrobial determinants. There is however, a need for standardization of pipelines and databases as well as phenotypic predictions based on WGS data. Regarding this last point, data from literature showed that WGS analysis accurately predicts antimicrobial resistance phenotypes for *Campylobacter* spp., and for *Salmonella* spp. (Dahl et al., 2021; Zhao et al., 2015). For *Campylobacter* spp., a high correlation was found between phenotypic resistance and the presence of known resistance genes and/or point mutations, for macrolides, fluoroquinolones, tetracycline, and aminoglycosides (correlation above 98%, except streptomycin with a correlation of 92%). The same was

observed for *Salmonella* spp. Recently, Cooper et al, (2020) showed that antimicrobial resistance genes identification tools had ≥ 99% accuracy for predicting resistance to all the 15 antibiotics tested, except streptomycin (accuracy 94.6%). Since the resistance rate varies markedly between different *S. enteric* serotypes, it was important to ensure that WGS data analysis accurately determined *S. enterica* serotypes as well. Another issue is the definition of the ECOFFs which are based on available MIC and zone diameter distributions, and as more data is available, there might be a need to add or revise ECCOFs. This revision should consider the resistance mechanisms as well, in order to have a clear correspondence between phenotype and genotype. As an example, ECOFF for meropenem has been recently revised for all species, including *S. enterica* (August 2021) and the new criteria for non-wild type may not reflect the presence of resistance determinants (EU protocol for harmonised monitoring of AMR in human *Salmonella* and *Campylobacter* isolates ANNEX 1). Another issue concerns the emergence of *Campylobacter* spp. strains resistant to carbapenems (ertapenem and/or meropenem), reported in both and *C. jejuni* and *C. coli* (Lehours et al., 2018, Oleastro et al., 2019). Genomic analysis of these strains showed that the resistance mechanisms are complex, involving several chromosomal targets, such as the CmeABC operon, coding for a Resistance-Nodulation-Division efflux pump, and in the *porA* gene coding for the major outer membrane protein (Lehours et al., 2018). The complexity of carbapenem resistance mechanisms and the fact that it is still a rare event in *Campylobacter* spp. renders it difficult to correlate accurately the phenotype with the genotype, and more resistant isolates have to be studied at genomic level.

A cross-cutting issue regarding the application of WGS for surveillance purposes, including AMR, is the set-up of databases for molecular typing and data sharing across different countries and sectors. In addition, specifically for AMR, pipelines for genetic-based AMR prediction and molecular mechanisms of resistance identification must be harmonised. A considerable number of accessible tools are currently available for in silico prediction of AMR determinants, enough to support the transition to AMR tracking based on WGS data. However, the outputs obtained by different tools may not be fully comparable, and different input formats of the same data using the same tool (e.g., raw reads vs. assembled sequences, trimmed vs. non-trimmed reads; assemblies obtained by different software) can generate different results (Xavier et al., 2016). Therefore, a reliable genomic approach to assaying AMR gene content requires curated AMR databases that ensure a high concordance between phenotypes and genotypes. Particularly, the currently available tools are able to detect new variants of known AMR genes, but not to detect new AMR genes, nor can they predict that a certain gene or allele is expressed as a functional protein. Therefore, complementation by phenotypic tests is currently still necessary when searching for new AMR determinants. In addition, investment in machine learning schemes to identify novel resistance elements from genomic data and models for predicting MICs are required (Nguyen et al., 2019).

The first joint EFSA-ECDC molecular typing database was set up for both human and non-human isolates, and at the end of 2015, it was functional and limited to the collection of PFGE data of *Salmonella*, *Listeria monocytogenes* and Shiga toxin-producing *E. coli* isolates, and MLVA data for *Salmonella* Enteritidis and *Salmonella* Typhimurium isolates. Currently, it is possible to submit WGS data for the main foodborne pathogens, for human cases through ECDC, however, a centralized data analysis and real-time communication of the results to the data providers is not yet in place for most of

them. In addition, an integrated platform for the collection and analysis of WGS data for food and animal isolates is still not operational, and it is still unknown what data will be shared and how will be shared with the human sector.

From the data providers'/users' side, which are the public health and animal and food laboratories, there are also several constraints. Currently, the data collection system works based on the voluntary participation of the countries, the MSs in this case. This may pose a risk, since the data collected will only have added value if a significant number of MSs continuously and submit reliable and representative data in a timely manner.

Finally, one of the main gaps regarding the use of WGS for surveillance purposes is the inequality in access to WGS technology, especially when comparing the human sector, by far the most developed (ECDC, 2018), with the food, feed and animal sectors, as well as the discrepancies between countries, which still currently put them at different speeds. Therefore, investment must be made in order to democratize the access to this technology, with the support of EFSA and CDC and the collaboration of the EURLs.

5.3 Recommendations on what AMR testing should be undertaken as part of a future harmonised approach to strain characterisation and recommendations on how this harmonised strategy should be implemented

Harmonised monitoring and reporting of AMR from a public health perspective in *Salmonella* spp. and both *C. coli* and *C. jejuni* is currently taking place, as part of the monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. Considering recent trends in AMR, data collection needs and new scientific developments, EFSA has recently proposed several updates (EFSA, 2019). Regarding phenotypic testing, it was proposed that amikacin should complement the panel of antimicrobials for *Salmonella* and *E. coli* to improve detection of 16S RNA methyltransferases enzymes that confer resistance to all aminoglycosides except streptomycin, and which have been increasingly found in association with the carbapenemases AmpC or ESBL and fluoroquinolone resistance, mainly in Africa and in Asia (Naas et al., 2011; Wang et al., 2017; Fang et al., 2019). The inclusion of amikacin is accompanied by the reduction of some of the dilution ranges for other antibiotics of the current panel.

Regarding *Campylobacter*, it was proposed to remove nalidixic acid and streptomycin and the lowest concentration of gentamycin. In addition, it includes higher concentrations of erythromycin for better detection of high level resistance associated with the presence of the rRNA methylase *ermB* gene, and higher concentrations of ciprofloxacin in order to detect presumptively isolates harbouring mutations in the CmeABC operon, which might contribute to this high level of resistance (Yan et al., 2006; Florez-Cuadrado et al., 2016; Liu et al., 2017). Last resort options such as a carbapenem (ertapenem in this case) have also been included (EU protocol for harmonised monitoring of antimicrobial resistance in human Salmonella and Campylobacter isolates - Annexes August 2021), as it might be a therapeutic option against serious infections caused by MDR *Campylobacter*, especially *C. coli*, which are circulating in patients and in animals.

A future harmonised monitoring of AMR should provide data on the emerging resistances and mechanisms, of relevance, the emergence and spread of: [1] plasmid-mediated resistance to colistin; [2] MDR clones of *Salmonella* Infantis, with increased virulence and resistance to several antimicrobials mediated by mobile genetic elements (MGE); [3] *Salmonella* Kentucky with high-level ciprofloxacin resistance carrying plasmids coding for rare extended spectrum ß-lactamase- (ESBL) genes, and [4] high-level erythromycin resistant *Campylobacter* isolates harbouring *ermB* often present in MGE such as multidrug resistance islands.

In order to correctly address the complexity of emerging and novel resistance mechanisms, involving new MGE, efflux pumps, etc, and considering the dynamics of AMR, a trend towards the use of molecular methods, at the expense of phenotypic methods, is expected. This trend is in line with the Global Action Plan developed by the World Health Organization (WHO) to tackle AMR that highlights the need to strengthen the understanding of how resistance develops and spreads, and the underlying resistance mechanisms (WHO, 2015).

Classical molecular methods based on PCR, conventional, real-time and/or quantitative, are limited regarding the number of targets that can be addressed. In contrast, WGS provides the complete genomic sequence of a bacterium, a universal dataset from which, theoretically, any biological feature can be investigated, including the ability to detect AMR, and to track the evolution and spread of AMR bacteria in a hospital or the community. Additional traits of the bacterium such as virulence and strain type, including Multi Locus Sequence Typing (MLST), core-genome MLST, serotypes, etc, can also be obtained from WGS data, enabling enhanced detection and knowledge of the molecular epidemiology of resistant clones in a single assay. In the case of the foodborne pathogens, these complementary data can be extremely useful for source attribution and to understand the contribution of the use of antimicrobials in food-producing animals for the burden of AMR in humans.

Elucidation of the resistance mechanisms and their context is of the utmost importance for the effective control of AMR. As such, WGS data is capable of distinguishing between chromosomal point mutations in specific antibiotic targets or AMR genes located in the chromosome, which are vertically transmitted, and acquired resistance genes carried in transmissible plasmids or other MGE, which disseminate by horizontal gene transfer. Technically, full characterisation of MGE and their genomic context would require the use of long-read sequencing, as for example plasmids are difficult to reconstruct from WGS data using short-read sequencing only (Orlek et al., 2017; Berbers et al., 2020). However, there is still a high error rate associated with that technology, and accurate data can benefit from the combination of the long reads with the accuracy of short read sequencing, through the use of a hybrid assembly approach (Sović et al., 2016; George et al., 2017).

Considering the recent progresses in more rapid and affordable DNA sequencing technologies, including long-read sequencing, and the availability of increasingly robust and accessible bioinformatics workflows, the diagnostics and surveillance of the infectious agents is already changing, and will definitely change the paradigm of bacterial strain characterisation, including for surveillance purposes.

Currently, EFSA and ECDC are proposing to integrate WGS within the harmonised AMR monitoring, following a gradual and phased approach, that will lead to the full replacement of the phenotypic antimicrobial testing methods (ECDC, 2019; EFSA 2019). Particularly, ECDC Expert Opinion on WGS

for public health surveillance claimed that WGS-based typing would become the frontline microbial typing method for the investigation of multi-country outbreaks and disease and antimicrobial surveillance in the EU by 2020, at least for bacterial pathogens (ECDC, 2016). This assumes that all MSs should have WGS in place at human, animal and food testing laboratories in the future.

Recently, the European Commission, recognised that "Whole genome sequencing is a promising technique to replace conventional phenotypical testing in microbiology and is increasingly used worldwide. However, only a limited number of MSs are currently able to use WGS for AMR monitoring on a routine basis. It is therefore appropriate to authorise the use of WGS as an alternative to the conventional phenotypical techniques on a voluntary basis only, but to impose technical conditions on the WGS technique to ensure data comparability."

The future use of WGS for AMR surveillance will require that the laboratory passes EQA exercises before they form part of national surveillance systems to ensure the collection of high-quality, reliable data. Proficiency testing protocols for DNA extraction, library preparation, WGS, assembly, phylogenetic analysis and detection of AMR genes must also be established.

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Appendix 1: STEC questionnaire

Shiga toxin-producing *Escherichia coli* (STEC) Survey

Fields marked with * are mandatory.

OneHealth Harmony Shiga toxin-producing Escherichia coli (STEC) Survey

Dear Colleague,
We are undertaking this questionnaire to establish current practice in sampling, testing, isolate characterisation, data storage and reporting in our STEC testing laboratories covering human health, animal health and the food sectors throughout the EU/EEA. We will use this information to harmonise these activities and promote best practice across Europe thus ensuring everyone benefits from a testing system that serves our needs as European citizens. The deliverable will be a report on; [1] best practice in designing statistically based sampling plans; [2] the most appropriate sampling methods; [3] currently available detection methods; [4] how these are applied as part of ISO or other peer reviewed methods, and [5] the latest sampling/technological developments that should be incorporated into future sampling & testing activities. This will be disseminated, free of charge, to all participating laboratories.

Thank you for your cooperation,

Nadia Boisen and Flemming Scheutz
OneHealth-Harmony Coordinators
Declan Bolton
Work package leader

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* Does your laboratory test for STEC?
  - ○ Yes
  - ○ No

Sampling

* 1. What sample type(s) do you test?

- [ ] Human samples
- [ ] Animal samples
- [ ] Food samples
- [ ] Feed samples
- [ ] Food contact surfaces
- [ ] Environmental (soil, water, etc.) samples

* 2. Do you take your own samples? (tick as appropriate)
  - [ ] Always
  - [ ] Sometimes
  - [ ] Never

* 3. If 'Never' is your laboratory at least involved in designing the sampling plan (number, types of samples, etc.)?
  - ( ) Yes
  - ( ) No

* 4. If 'Yes' which best describes your sampling plan?
  - [ ] Statistically based
  - [ ] Recommended by national, EU or other authority
  - [ ] Other

Please specify:

Testing (detection)

* 5. Is your laboratory accredited according to ISO/IEC 17025 or ISO 15189?
  - ( ) Yes
  - ( ) No

* 6. which testing method does your laboratory use (tick as appropriate)
  - [ ] ISO/TS 13136
  - [ ] or equivalent PCR
  - [ ] DNA hybridisation
  - [ ] Detection of toxin (EIA, FFCT, VCA or other)
  - [ ] National standard

If, 'National standard', please specify:

* 7. What was the total number (negatives and positives) of samples tested in 2019?

Testing (confirmation)

8. Do you perform confirmatory tests?

- Yes
- No

Do you send samples/isolates to the National Reference Laboratory for this purpose?

- Yes
- No

Please complete the following section on CONFIRMATORY methods used.

- Direct plating
- Enrichment and IMS
- No plating

Media used

Accredited

- Yes
- No

* Biochemical tests (tick as appropriate)

- Yes
- No

Biochemical tests

	Yes	No
Arginine decarboxylase		
Lysine decarboxylase		
Ornithine decarboxylase		
Carbohydrate fermentation tests		
Lactose		
Citrate		
Gas production		
Hydrogen sulphide		

Indole	○	○
Methyl red	○	○
Motility	○	○
Phenylalanine deaminase	○	○
Urease	○	○
Voges-Proskauer tests	○	○
Enterohaemolytic activity	○	○
β-glucuronidase	○	○

DNA-based (tick as appropriate)

- ☐ Conventional PCR
- ☐ Real time PCR
- ☐ Array
- ☐ WGS

Reference (e.g PMCID, PMID, or DOI)

Are all the confirmatory method(s) accredited?

- ○ Yes (if all methods are accredited)
- ○ No

Which methods are not accredited?

* 9. Do you store isolates

- ○ Yes, always
- ○ Yes, sometimes
- ○ Never

Please indicate where the isolates are stored

- ☐ Own Laboratory
- ☐ National Reference Laboratory
- ☐ European Union Reference Laboratory
- ☐ Other

Characterisation

10. Please indicate what characterisation is routinely performed

O:H Serotype

* Somatic (O) antigen
  - ○ Yes
  - ○ No

Method used
- ☐ Serological
- ☐ PCR based
- ☐ WGS Based

Which software

* Flagella (H) antigen
  - ○ Yes
  - ○ No

Method used
- ☐ Serological
- ☐ PCR based
- ☐ WGS Based

Which software

* Virulence genes
  - ○ Yes
  - ○ No

Genes tested for:
- ☐ Stx1
- ☐ Stx2
- ☐ *stx1* subtyping
- ☐ *stx2* subtyping
- ☐ *eae*
- ☐ *eae* subtyping
- ☐ *aggR*

* Genetic comparison to determine relatedness of isolates ("phylogenetic" typing)
  - ○ Yes
  - ○ No

Method used

- [ ] pulsed-field gel electrophoresis (PFGE)
- [ ] multilocus sequence typing (MLST)
- [ ] multiple-locus variable-number of tandem repeat analysis (MLVA)
- [ ] WGS based

Please specify; cg/wgMLST, SNP-based or other

* Antimicrobial resistance testing

- ( ) Yes
- ( ) No

Method used

- [ ] broth/agar dilution
- [ ] disk diffusion
- [ ] E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)

Please specify the methods and database used:

Data/Data Management

* 11. Please indicate what metadata you receive with human clinical sample(s):

- [ ] Date of sampling
- [ ] Date of receipt in the laboratory
- [ ] Specimen
- [ ] Age
- [ ] Gender
- [ ] Geographic origin
- [ ] Drug therapy
- [ ] Medical conditions
- [ ] Outbreak investigation
- [ ] Not relevant

* 12. Please indicate what metadata you receive with animal sample(s):

- [ ] Date of sampling
- [ ] Date of receipt in the laboratory
- [ ] Animal species
- [ ] Type of sample
- [ ] Geographic origin
- [ ] Not relevant

* 13. Do you have a specific system for data management/storing data?

- Yes
- No

* 14. Do you store information about the sample?

- Yes
- No

If so, in which way;

- Paper format
- Electronic format

* 15. Do you report the results to a regional or national competent authority regularly?

- Regional: Yes
- Regional: No
- National: Yes
- National: No

* 16. Do you report testing results to ECDC or EFSA

- ECDC
- EFSA
- No

Contact information

* Name of Institution

* Contact person

* Email address

Final comments

Is there anything else you wish to add to the information you have provided?

Was there any question(s) that was not fully appropriate to your laboratory? and important information may be lost as a result of this. Please provide details.

Acknowledgement

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Appendix 2: ETEC questionnaire

Enterotoxigenic *Escherichia coli* (ETEC) Survey

Fields marked with * are mandatory.

OneHealth Harmony Enterotoxigenic Escherichia coli (ETEC) Survey

Dear Colleague,
We are undertaking this questionnaire to establish current practice in sampling, testing, isolate characterisation, data storage and reporting in our ETEC testing laboratories covering human health, animal health and the food sectors throughout the EU/EEA. We will use this information to harmonise these activities and promote best practice across Europe thus ensuring everyone benefits from a testing system that serves our needs as European citizens. The deliverable will be a report on; [1] best practice in designing statistically based sampling plans; [2] the most appropriate sampling methods; [3] currently available detection methods; [4] how these are applied as part of ISO or other peer reviewed methods, and [5] the latest sampling/technological developments that should be incorporated into future sampling & testing activities. This will be disseminated, free of charge, to all participating laboratories.

Thank you for your cooperation,

Nadia Boisen and Flemming Scheutz
OneHealth-Harmony Coordinators
Declan Bolton
Work package leader

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* Does your laboratory test for ETEC
  - ○ Yes
  - ○ No

Sampling

* 1. What sample type(s) do you test? (tick appropriate)

- [ ] Human samples
- [ ] Animal samples
- [ ] Food samples
- [ ] Feed samples
- [ ] Food contact surfaces
- [ ] Environmental (soil, water, etc.) samples

* 2. Do you take your own samples? (tick as appropriate)
  - ( ) Always
  - ( ) Sometimes
  - ( ) Never

* If 'Never' is your laboratory involved in designing the sampling plan (number, types of samples, etc.)?
  - ( ) Yes
  - ( ) No

* If 'Yes' which best describes your sampling plan?
  - [ ] Statistically based
  - [ ] Recommended by national, EU or other authority
  - [ ] Other

* Please specify:

Testing (detection)

* 3. Is your laboratory accredited according to ISO/IEC 17025 or ISO 15189?
  - ( ) Yes
  - ( ) No

* 4. Does your laboratory test for heat-stable (ST) and heat-labile (LT) enterotoxins?
  - ( ) Yes
  - ( ) No

* 5. which testing method does your laboratory use (tick as appropriate)
  - [ ] PCR based
  - [ ] Enzyme-linked immunosorbant assay
  - [ ] DNA hybridisation
  - [ ] Immunological assays (eg. latex agglutination)
  - [ ] Commercial kit
  - [ ] Other

* If, 'Other', please specify:

• Reference (e.g PMCID, PMID, or DOI)

• 6. What was the total number (negatives and positives) of samples tested in 2019?

Testing (confirmation)

• 7. Do you perform confirmatory tests?

- Yes
- No

• Do you send samples/isolates to the National Reference Laboratory for this purpose?

- Yes
- No

• Please complete the following section on CONFIRMATORY methods used.

- Direct plating
- Enrichment and IMS
- No plating

Media used

• Accredited

- Yes
- No

• Biochemical tests (tick as appropriate)

- Yes
- No

Biochemical tests

	Yes	No
Arginine decarboxylase		
Lysine decarboxylase		

Ornithine decarboxylase		
Carbohydrate fermentation tests		
Lactose		
Citrate		
Gas production		
Hydrogen sulphide		
Indole		
Methyl red		
Motility		
Phenylalanine deaminase		
Urease		
Voges-Proskauer tests		
Enterohaemolytic activity		
β-glucuronidase		

* DNA-based (tick as appropriate)
  - Conventional PCR
  - Real time PCR
  - Array
  - WGS

* Reference (e.g PMCID, PMID, or DOI)

* Are all the confirmatory method(s) accredited?
  - Yes (if all methods are accredited)
  - No

* Which methods are not accredited?

* 8. Do you store isolates
  - Yes, always
  - Yes, sometimes
  - Never

* Please indicate where the isolates are stored

- [ ] Own Laboratory
- [ ] National Reference Laboratory
- [ ] European Union Reference Laboratory
- [ ] Other

Characterisation

9. Please indicate what characterisation is routinely performed

O:H Serotype

* Somatic (O) antigen

- ( ) Yes
- ( ) No

* Method used

- [ ] Serological
- [ ] PCR based
- [ ] WGS Based

If, 'WGS', please specify software

* Flagella (H) antigen

- ( ) Yes
- ( ) No

* Method used

- [ ] Serological
- [ ] PCR based
- [ ] WGS Based

Which software

* Virulence genes

- ( ) Yes
- ( ) No

* Genes tested for (tick appropriate):

- [ ] *elt* (LT)
- [ ] *elt* subtype: (LT-I, A and/or B SU)

- [ ] *eltII* subtype: (LT-II, A and/or B SU)
- [ ] *est* (ST)
- [ ] *est* subtypes: *estap* (STa porcine variant)
- [ ] *est* subtypes: *estah* (STa human variant)
- [ ] *est* subtypes: *estb* (STb variant)
- [ ] *clyA*
- [ ] *eatA*
- [ ] *tia*
- [ ] *tibA* and/or *C*
- [ ] *tleA*
- [ ] *etpA*
- [ ] *astA*
- [ ] *lecA*
- [ ] *stl2*
- [ ] Other

* Please specify

* Colonization factors (tick appropriate)
- [ ] CFA/I
- [ ] CFA/II
- [ ] CFA/III
- [ ] CFA/IV
- [ ] *fae* (F4)
- [ ] *fan* (F5)
- [ ] *fas* (F6)
- [ ] *f17a* (F17)
- [ ] *fim41A* (F41)
- [ ] *fed* (F18 or F107)
- [ ] Minor colonization factors (CS)
- [ ] Not relevant

* Please specify the number (e.g CS1 and/or CS2, and/or CS3, up to CS30):

* Genetic comparison to determine relatedness of isolates ("phylogenetic" typing)
- ( ) Yes
- ( ) No

* Method used
- [ ] pulsed-field gel electrophoresis (PFGE)
- [ ] multilocus sequence typing (MLST)
- [ ] multiple-locus variable-number of tandem repeat analysis (MLVA)

- [ ] WGS based

* Please specify: cg/wgMLST, SNP-based or other

* Antimicrobial resistance testing
  - ( ) Yes
  - ( ) No

* Method used
  - [ ] broth/agar dilution
  - [ ] disk diffusion
  - [ ] E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)

* Please specify the methods and database used:

Data/Data Management

* 10. Please indicate what metadata you receive with human clinical sample(s) (tick appropriate):
  - [ ] Date of sampling
  - [ ] Date of receipt in the laboratory
  - [ ] Specimen
  - [ ] Age
  - [ ] Gender
  - [ ] Geographic origin
  - [ ] Drug therapy
  - [ ] Medical conditions
  - [ ] Outbreak investigation
  - [ ] Not relevant

* 11. Please indicate what metadata you receive with animal sample(s) (tick appropriate):
  - [ ] Date of sampling
  - [ ] Date of receipt in the laboratory
  - [ ] Animal species
  - [ ] Type of sample
  - [ ] Geographic origin
  - [ ] Not relevant

* 12. Do you have a specific system for data management/storing data?
  - ( ) Yes
  - ( ) No

* 13. Do you store information about the sample?

- Yes
- No

* If so, in which way;

- Paper format
- Electronic format

* 14. Do you report the results to a regional or national competent authority regularly?

- Regional: Yes
- Regional: No
- National: Yes
- National: No

* 15. Do you report testing results to ECDC or EFSA

- ECDC
- EFSA
- No

Contact information

* Name of institution

* Contact person

* Email address

Final comments

Is there anything else you wish to add to the information you have provided?

Was there any question(s) that was not fully appropriate to your laboratory? and important information may be lost as a result of this. Please provide details.

Acknowledgement

The Harmony project is part of the European Joint Programme One Health EJP. This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement Number 773830.

Appendix 3: Cryptosporidium questionnaire

Cryptosporidium spp. Survey

Fields marked with * are mandatory.

OneHealth Harmony Cryptosporidium spp. Survey

Dear Colleague,
We are undertaking this questionnaire survey focusing on Cryptosporidium spp. to establish current practice in sampling, testing, isolate characterisation, data storage and reporting in laboratories covering human health, animal health and the food sectors throughout the EU/EEA that are performing testing for these parasites. We will use this information to harmonise these activities and promote best practice across Europe, aiming to ensure everyone benefits from a testing system that serves our needs as European citizens. We will summarize the results and report on; [1] best practice in designing sampling plans; [2] the most appropriate sampling methods; [3] currently available detection methods; [4] how these are applied as part of ISO or other peer reviewed methods, and [5] the latest sampling/technological developments that should be incorporated into future sampling and testing activities. The report will be disseminated, free of charge, to all participating laboratories.
Thank you for your cooperation,

Nadia Boisen and Flemming Scheutz
OneHealth-Harmony Coordinators
Declan Bolton
Work package leader

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* Does your laboratory test for *Cryptosporidium* spp.?
- ○ Yes
- ○ No

Sampling

* 1. What sample type(s) do you test? (tick appropriate)
- ☐ Human samples

- [ ] Animal samples
- [ ] Food samples
- [ ] Environmental (soil, water, etc.) samples

* 2. Do you take your own samples? (tick as appropriate)
  - ( ) Always
  - ( ) Sometimes
  - ( ) Never

* If 'Never' is your laboratory at least involved in designing the sampling plan (number, types of samples, etc.)?
  - ( ) Yes
  - ( ) No

* If 'Yes' which best describes your sampling plan?
  - [ ] Statistically based
  - [ ] Recommended by national, EU or other authority
  - [ ] Other

* Please specify:

Testing (detection)

* 3. Is your laboratory accredited according to ISO/IEC 17025 or ISO 15189?
  - ( ) Yes
  - ( ) No

* 4. which testing method does your laboratory use (tick as appropriate)
  - [ ] Modified Ziehl-Neelsen microscopy
  - [ ] Auramine phenol microscopy
  - [ ] Immunofluorescence microscopy
  - [ ] DNA detection by PCR
  - [ ] Antigen detection by enzyme immuno assay
  - [ ] Immunochromatographic rapid tests
  - [ ] ISO 18744 (only for food samples)
  - [ ] ISO 55553 (only for water samples)
  - [ ] Other

* If, 'Other', please specify:

* Reference (e.g PMCID, PMID, or DOI)

• 5. What was the total number (negatives and positives) of samples tested in 2019?

Testing (confirmation)

• 6. Do you perform confirmatory tests?

- Yes
- No

• Do you send samples/isolates to the National Reference Laboratory for this purpose?

- Yes
- No

• Please complete the following section on CONFIRMATORY methods used (tick appropriate).

- Modified Ziehl-Neelsen microscopy
- Auramine phenol microscopy
- Immunofluorescence microscopy
- DNA detection by PCR
- Antigen detection by enzyme immuno assay
- Antigen detection by immunofluorescence
- Immunochromatographic rapid tests
- Other

• If, 'Other' please specify

• Are all the confirmatory method(s) accredited?

- Yes (if all methods are accredited)
- No

• Which methods are not accredited?

• 7. Do you store isolates

- Yes, always
- Yes, sometimes
- Never

Please indicate where the isolates are stored

- [ ] Own Laboratory
- [ ] National Reference Laboratory
- [ ] European Union Reference Laboratory
- [ ] Other

If, 'Other' please specify

Characterisation

* 9. If applicable, please indicate what characterisation is routinely performed (tick appropriate)
  - [ ] PCR-based tests
  - [ ] WGS
  - [ ] Other

* Reference (e.g PMCID, PMID, or DOI)

* If, 'Other', please specify software

Data/Data Management

* 10. Please indicate what metadata you receive with human clinical sample(s) (tick appropriate):
  - [ ] Date of sampling
  - [ ] Date of receipt in the laboratory
  - [ ] Specimen
  - [ ] Age
  - [ ] Gender
  - [ ] Geographic origin
  - [ ] Drug therapy
  - [ ] Medical conditions
  - [ ] Outbreak investigation
  - [ ] Not relevant

* 11. Please indicate what metadata you receive with animal sample(s) (tick appropriate):
  - [ ] Date of sampling
  - [ ] Date of receipt in the laboratory
  - [ ] Animal species
  - [ ] Type of sample
  - [ ] Geographic origin
  - [ ] Not relevant

* 12. Do you have a specific system for data management/storing data?
- Yes
- No

* 13. Do you store information about the sample?
- Yes
- No

* If 'Yes', in which way:
- Paper format
- Electronic format

* 14. Do you report the results to a regional or national competent authority regularly?
- Regional: Yes
- Regional: No
- National: Yes
- National: No

* 15. Do you report testing results to ECDC or EFSA
- ECDC
- EFSA
- No

Contact information

* Name of institution

* Contact person

* Email address

Final comments

Is there anything else you wish to add to the information you have provided?

Was there any question(s) that was not fully appropriate to your laboratory? and important information may be lost as a result of this. Please provide details.

Acknowledgement

The Harmony project is part of the European Joint Programme One Health EJP. This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement Number 773830.

Apenddix 4: AMR in *Salmonella* and *Campylobacter*

AMR testing in Salmonella & Campylobacter Survey

Fields marked with * are mandatory.

OH-HARMONY
CAP

OneHealth Harmony AMR testing in Salmonella & Campylobacter Survey

Dear Colleague,
We are undertaking this questionnaire to establish current practices in AMR testing in *Salmonella* spp. & *Campylobacter* spp. in the EU/EEA. We will use this information to harmonise these activities and promote best practice across Europe thus ensuring everyone benefits from a testing system that serves our needs as European citizens. The information gathered will inform part of a technical report covering characterisation methods used for foodborne pathogens used in European laboratories. This will be disseminated to all participating laboratories.

Thank you for your cooperation,

Nadia Boisen and Flemming Scheutz
OneHealth-Harmony Coordinators
Declan Bolton
Work package leader

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* 1. Please tick if you AMR test *Salmonella* and/or *Campylobacter*?

- ☐ *Salmonella*
- ☐ *Campylobacter*
- ☐ Neither

Salmonella spp.
Sources of *Salmonella* isolates

* 2. What type of *Salmonella* isolates do you test for AMR (tick appropriate)?

- [ ] Human isolates
- [ ] Animal (excluding poultry) isolates –live animals
- [ ] Animal (excluding poultry) isolates –carcass
- [ ] Poultry isolates – live birds
- [ ] Poultry isolates – broiler carcasses
- [ ] Food (raw meat) isolates
- [ ] Food (processed meat) isolates
- [ ] Food (other than meat) isolates
- [ ] Feed isolates
- [ ] Food contact surface isolates
- [ ] Environmental (soil, water, etc.) isolates

* 3. Do you know/record the serotypes of the strains tested?
  - ( ) Yes
  - ( ) No

AMR testing

* 4. What methods do you use for AMR testing human isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
  - [ ] Other

* If, 'Other', please specify:

* 4. What methods do you use for AMR testing animal (live) isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
  - [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing animal (carcass) isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)

- [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing poultry isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
  - [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing food (raw meat) isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
  - [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing food (processed meat) isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
  - [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing food (other than meat) isolates?
  - [ ] MIC broth/agar dilution
  - [ ] Disk diffusion
  - [ ] Gradient strips, e.g E-test
  - [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
  - [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing feed isolates?

- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing food contact surface isolates?

- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 4. What methods do you use for AMR testing environmental isolates?

- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 5. Is your laboratory accredited?

- ( ) Yes
- ( ) No

If, 'Yes', to what standard?

* 6. What was the total number of *Salmonella* isolates tested for AMR in 2019?

* 7. Do you test for the presence of specific AMR genes or point mutations?

- Yes
- No

* If, 'Yes', which testing method does your laboratory use (tick as appropriate)

- Conventional PCR
- Conventional PCR and Sanger sequencing
- Real time PCR
- DNA array
- WGS
- PCR in combination with WGS

* 8. What specific AMR genes or point mutations do you routinely test for?

- pAmpC
- ESBL
- Carbapenemase
- mcr (colistin genes)
- qnr (flouroqinolone genes)
- Other

* If, 'Other', please specify

* 9. Do you store *Salmonella* isolates after AMR testing?

- Always
- Sometimes
- Never

* If, 'Always' or 'Sometimes'. please indicate where the isolates are stored

- Own Laboratory
- National Reference Laboratory
- Other

Data/Data Management

* 10. Please indicate what metadata you receive with human clinical *Salmonella* isolates that are tested for AMR (tick appropriate)

- Date of sampling
- Date of receipt in the laboratory
- Specimen

- [ ] Age
- [ ] Gender
- [ ] Geographic origin
- [ ] Drug therapy
- [ ] Medical conditions
- [ ] Outbreak investigation
- [ ] Not relevant

* 11. Please indicate what metadata you receive with animal sample(s) (tick appropriate)
  - [ ] Date of sampling
  - [ ] Date of receipt in the laboratory
  - [ ] Animal species
  - [ ] Type of sample
  - [ ] Geographic origin
  - [ ] Not relevant

* 12. Do you have a specific system for data management/storing data?
  - ( ) Yes
  - ( ) No

* 13. Do you store information about the sample?
  - ( ) Yes
  - ( ) No

If 'Yes', in which way?
- [ ] Paper format
- [ ] Electronic format

* 14. Do you report the *Salmonella* AMR results to a regional or national competent authority regularly?
  - [ ] Regional: Yes
  - [ ] Regional: No
  - [ ] National: Yes
  - [ ] National: No

* 15. Do you report the *Salmonella* AMR testing results to ECDC or EFSA?
  - [ ] ECDC
  - [ ] EFSA
  - [ ] No

Campylobacter jejuni and *Campylobacter coli*

Sources of Campylobacter isolates

* 1. What type of *Campylobacter* isolates do you test for AMR (tick appropriate)?
  - [ ] Human isolates
  - [ ] Animal (excluding poultry) isolates –live animals

- [ ] Animal (excluding poultry) isolates –carcass
- [ ] Poultry isolates – live birds
- [ ] Poultry isolates – broiler carcasses
- [ ] Food (raw meat) isolates
- [ ] Food (other than meat) isolates
- [ ] Environmental (soil, water, etc.) isolates

* 2. Do you know/record the species of the strains tested
- ( ) Yes
- ( ) No

* 3. What methods do you use for AMR testing human isolates?
- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 3. What methods do you use for AMR testing animal isolates?
- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 3. What methods do you use for AMR testing poultry isolates?
- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 3. What methods do you use for AMR testing food isolates?

- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 3. What methods do you use for AMR testing environmental isolates?
- [ ] MIC broth/agar dilution
- [ ] Disk diffusion
- [ ] Gradient strips, e.g E-test
- [ ] Genotypic methods (PCR, DNA or WGS hybridization/analysis)
- [ ] Other

* If, 'Other, please specify:

* 4. is your laboratory accredited?
- ( ) Yes
- ( ) No

If, 'Yes, to what standard?

* 5. What was the total number of *Campylobacter* isolates tested for AMR in 2019?

* 6. Do you test for the presence of specific AMR genes or point mutations?
- ( ) Yes
- ( ) No

* If, 'Yes', which testing method does your laboratory use (tick as appropriate)
- [ ] Conventional PCR
- [ ] Conventional PCR and Sanger sequencing
- [ ] Real time PCR
- [ ] DNA array
- [ ] WGS
- [ ] PCR in combination with WGS

* 7. What specific AMR genes or point mutations do you routinely test for?

- [ ] Tetracycline resistance gene
- [ ] Fluoroquinolones-resistance associated point mutations
- [ ] Macrolides-resistance associated point mutations
- [ ] Aminoglycosides resistance genes
- [ ] Other

* If, 'Other', please specify

* 8. Do you store *Campylobacter* isolates after AMR testing?

- [ ] Always
- [ ] Sometimes
- [ ] Never

* If, 'Always' or 'Sometimes', please indicate where the isolates are stored

- [ ] Own Laboratory
- [ ] National Reference Laboratory
- [ ] Other

Data/Data Management

* 9. Please indicate what metadata you receive with human clinical *Campylobacter* isolates that are tested for AMR (tick appropriate)

- [ ] Date of sampling
- [ ] Date of receipt in the laboratory
- [ ] Specimen
- [ ] Age
- [ ] Gender
- [ ] Geographic origin
- [ ] Drug therapy
- [ ] Medical conditions
- [ ] Outbreak investigation
- [ ] Not relevant

* 10. Please indicate what metadata you receive with animal sample(s) (tick appropriate)

- [ ] Date of sampling
- [ ] Date of receipt in the laboratory
- [ ] Animal species
- [ ] Type of sample
- [ ] Geographic origin
- [ ] Not relevant

* 11. Do you have a specific system for data management/storing data?

- Yes
- No

* 12. Do you store information about the sample?
  - Yes
  - No

If 'Yes', in which way?
- Paper format
- Electronic format

* 13. Do you report the *Campylobacter* AMR results to a regional or national competent authority regularly?
- Regional: Yes
- Regional: No
- National: Yes
- National: No

* 14. Do you report the *Campylobacter* AMR testing results to ECDC or EFSA?
- ECDC
- EFSA
- No

Contact information

* Name of institution

* Contact person

* Email address

Final comments

Is there anything else you wish to add to the information you have provided?

Was there any question(s) that was not fully appropriate to your laboratory? and important information may be lost as a result of this. Please provide details.

Acknowledgement
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