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Technical Report: Sampling and testing of Shiga toxin-producing *Escherichia coli* (STEC), Enterotoxigenic *Escherichia coli* (ETEC), *Cryptosporidium* and antimicrobial resistance (AMR) in *Salmonella* and *Campylobacter* spp. in the European Union

This is a public deliverable of One Health EJP Joint Research Project, *Integrative Action-2.2,* OH-HARMONY-CAP: One Health Harmonisation of Protocols for the Detection of Foodborne Pathogens and AMR Determinants.
https://onehealthejp.eu/jip-oh-harmony-cap/

OH-Harmony-Cap is a 2.5 year project which aims to collect information on current capabilities, capacities and interoperability at both the National Reference Laboratory (NRL) and the primary diagnostic level. The quantitative description of current and best practices and the development of harmonised protocols will identify and possibly close the gaps and support future studies of how best to detect and characterise foodborne pathogens across the One Health sectors. OH-Harmony-Cap Consortium comprises 15 One Health EJP partners.

1. Introduction

An efficient and effective public health surveillance system requires the harmonised application of the most appropriate sampling, detection, characterisation, data management and reporting procedures in the human and veterinary fields and in laboratories testing food, feed and environmental samples across the European Union (EU). This is essential in outbreak detection and investigation but also in the ongoing monitoring of foodborne and zoonotic pathogens. Despite improvements in recent years, differences in capability, capacity and communication practices have hampered the development of an integrative system. Moreover, the possibility to share harmonized data, such as from strain characterisation, is pivotal in a public health effective risk assessment perspective.

The main objectives of the One-Health-EJP-CAP Joint Integrative Project Harmony are: [1] to collect information on current capabilities, capacities and interoperability both at the National Reference Laboratory (NRL) and the primary diagnostic level, focusing on a set of microbial foodborne hazards; [2] to provide a quantitative description of the current and best practices; [3] to contribute to the development of harmonised protocols and [4] to identify potential research fields to detect and characterise food borne pathogens across the one health sectors. The target organisms included in the work are Shiga toxin-producing *Escherichia coli* (STEC), Enterotoxigenic *Escherichia coli* (ETEC), *Cryptosporidium* spp. and antimicrobial resistance (AMR) in *Salmonella* spp. and *Campylobacter* spp.

This document, the first in a series of three reports, covers ‘sampling and testing’ procedures for these targets in the EU. Future reports will cover ‘characterisation’ and ‘data management and harmonised reporting’. The report is divided in sections covering each of the target microorganisms with subsections on current practices, best practices, detection methods and the latest sampling technological developments. The information provided on current practices is based on the responses to a questionnaire completed by European public health, veterinary and food testing institutions and National Reference Laboratories reached via the European Union Reference Laboratory (EURL), the European Food Safety Authority (EFSA) Zoonoses networks, European Centre for Disease Prevention and Control (ECDC) Food and Waterborne diseases network, among others. 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 practice in the EU: Sampling, testing (detection & confirmation)

The STEC questionnaire was designed using the EU survey tool and comprised of 16 questions. There were 49 respondents to the questionnaire on current STEC sampling, testing, characterisation and data management in the EU (see appendix 1) and the results on 'sampling and testing' may be summarised as follows:

- 41 of these laboratories test for STEC
- the type of samples tested included human clinical (17), animal (13), food (24), feed (6), and environmental specimen (11) laboratories
- three laboratories always take their own samples, 11 indicated sometimes they sampled and 27 never do.
- of those that do not take samples, 15 are involved in designing the sampling plan, among which three use a statistically based method while the other 12 use a sampling plan recommended by a national, European or other authority
- the majority (all except 5) of laboratories were accredited to ISO/IEC 17025 or ISO 15189
- all laboratories except 5 used ISO/TS 13136 or equivalent PCR for testing. The remaining five laboratories indicated they used "Detection of toxin (EIA, FFCT, VCA or other)"
- in 2019 the number of samples tested in individual laboratories ranged from 0 to 15,000
- nine laboratories indicated they did not undertake confirmatory testing but all of these, except one, indicated they sent isolates to the national reference laboratory (NRL) for this purpose
- confirmatory methods used were based on enrichment and IMS and/or direct plating using a range of different media including SMAC, CT-SMAC, TBX, MacConkey, Enterohemolysin agar, Chrom-Agar STEC, Chrom-Agar O157, STEC Colorex, Gassner agar and ECC prisma
- the majority of these methods were accredited
- 11 of the laboratories used biochemical confirmatory testing although these varied considerably depending on the laboratory
- the majority of laboratories (35) also used conventional PCR and/or real time PCR accredited confirmatory methods
- 34 of the 41 laboratories stored isolates in their own laboratory and/or in the NRL

2.2 Sampling including best practice in designing statistically based sampling plans & most appropriate sampling methods

2.2.1 Human infections

Few countries/laboratories test all patients with diarrhoea for the presence of STEC, but the criteria used when deciding if a patient should be tested for this organism, the number of samples to be taken and tested, and the number of isolates to be typed varies between laboratories within a given country and between Member States (MS) (EFSA, 2020). Human cases of STEC infections are reported in accordance with Decision No 1082/2013 annually to the European Surveillance System (TESSy) held by the European Centre for Disease Prevention and Control (ECDC) database in accordance with the EU case definition for STEC/VTEC infections (Decision 2018/945/EU). However, metadata are frequently lacking.

2.2.2 Sampling in foodborne outbreaks

Monitoring of foodborne outbreaks by the MSs and in European Economic Area (EEA) countries and the annual reporting to EFSA is mandatory under Directive 2003/99/EC. Whereas reporting foodborne outbreak data is based on harmonised specifications, there is a lack of mandatory harmony in the National foodborne outbreak investigation systems. Thus, differences in the number and type of reported outbreaks may indicate differences in the sensitivity of the national surveillance systems for identifying and investigating foodborne outbreaks (EFSA 2020) hampering a comparison of the incidence of STEC infections in different MSs.

2.2.3 Sampling in food, animals and the environment

Monitoring and reporting of STEC along the food chain is mandatory under Directive 2003/99/EC, but not fully harmonized. Following an outbreak linked with sprouts in 2011, most Member States significantly increased the number of official control testings aiming to detect the presence of STEC in food placed on the market. These controls cover both imported and domestically produced foodstuffs (EFSA 2020). The only existing regulatory limit (microbiological criterion) for STEC in a food commodity such as sprout seeds, was set out in Regulation (EC) No 2073/2005. These products must comply with 'Absent in 25 grams' for STEC O157, O26, O111, O103, O145 and O104:H4 during their shelf-life.

Following a recommendation from EFSA to improve the monitoring of these pathogens in animal populations and foodstuffs (EFSA 2007), technical specifications for the monitoring and reporting of STEC in animals and food samples were published (EFSA, 2009), including a risk based sampling strategy for STEC, aimed at estimating the prevalence of STEC O157 contamination at slaughter, primarily on the hide of young cattle (3-24 months of age) and on sheep fleeces (between 4-12 months of age) (EFSA, 2009; Makela et al., 2012). Contamination rates on hides and fleeces provided a good estimate of STEC entering the slaughterhouses and gave an indication of the risk of carcass contamination. This document also provides general guidelines for carrying out specific surveys on the food categories that are most likely to be sources of STEC O157 and non-O157 infections in humans (EFSA, 2009; Makela et al., 2012).

2.2.4 Statistically based sampling plans

EFSA's technical specifications and reports provide guidance to MSs on the implementation of risk-based or random monitoring and the design of national surveys in animals and food (Makela et al.,

2012). It is preferable that monitoring targets all STEC types to better understand potential sources of human infections. Ready-to-eat (RTE) foods are of the highest sampling priority but food categories frequently involved in foodborne outbreaks should also be considered. Thus 'beef and products thereof', 'milk and dairy products', 'tap water including well water' and 'vegetables, fruits and products thereof' should be included in STEC monitoring and/or surveys.

Products should be sampled at retail as this is close to consumption, however additional stages along the food chain may also be considered, depending on the objective(s) of the testing. If undertaken at a National level, the National competent authority should be responsible for the design of the sampling plan. Sampling should be randomised but stratification criteria should be applied to ensure proportionate allocation of the samples to the target population. However, in outbreak investigation, suspect sampling should be applied. The decision on the primary sample size for each country will depend on the expected prevalence, the population size, the required confidence level and the desired accuracy. Where the statistical expertise to design is lacking, a minimum sample size of 384 may be used as it covers a broad range of prevalence-accuracy scenarios. Independent samples (epidemiological units) should be collected (i.e. only one sample per defined food category or subcategory should be collected in one place on one occasion). Only official staff or staff under the supervision of the competent authority should be responsible for sampling regardless of the place of sampling. Transportation of a sample must not affect the ability of the laboratory testing to subsequently detect or accurately estimate the concentration of STEC in that sample. In general, the samples should be transported under the same conditions as requested by legislation or as indicated by the food business operator. More details are available in the specific protocols to estimate the prevalence of STEC on bovine carcasses at slaughter, as well as in bovine meat at retail, where the above principles have been applied (Käsbohrer et al., 2010).

2.2.5 Sampling methods

In the absence of more specific rules for sampling animals and food, the relevant standards of the International Organisation for Standardisation (ISO) and the guidelines of the Codex Alimentarius should be used as reference methods. In Regulation (EC) 2073/2005, for example, more specific sampling methods for carcasses as well as food are described. Technical specifications published by EFSA give further guidance. For sprouted seeds, the most appropriate sampling methods are described in current EU legislation (Regulation (EC) 2073/2005, as amended by Regulation (EU) 209/2013). The general rules for sampling and testing require the preliminary testing of a representative sample of all batches of seeds and the sampling and testing of the sprouted seeds (where the probability of finding STEC is the highest) as well as the spent irrigation water at least once a month.

STEC may colonize the intestines of asymptomatic cattle and enter bulk tank milk via fecal contamination during milking, but these bacteria are rarely isolated from milk filters and milk. Thus, milk filters are a more suitable sampling target for monitoring STEC in milk (Jaakkonen et al., 2019, Artursson et al., 2018). Moreover, enhanced sensitivity can be achieved by using Cary Blair transport medium (Artursson et al., 2018)

Several methods are used to collect hide and carcass samples. The gauze or sponge swabbing method consists of sampling an area of the hide/carcass using a set direction and number of passes with a

cotton gauze or sponge moistened with buffer media to ensure capture of the bacteria and survival during transport. Sponge sampling is considered the standard method (USDA-FSIS, 2014, Stromberg et al. 2018).

Environmental sampling and testing has shown that STEC O157:H7 may persist in the farm environment for extended periods of time, suggesting that specific on-farm measures to reduce environmental prevalence and spread between groups of animals is required (Tamminen et al., 2019). Environmental sampling may be undertaken using gauze sampling socks, soaked with phosphate buffered saline (PBS) worn over plastic overshoes during walking around the pens together with a pooled fecal sample consisting of fresh faeces (around 1 cm^3 each) collected from 15-20 pick points on the floor or from the deep litter bedding (Widgren et al., 2015). Although a reliable method for identifying cattle herds with animals shedding STEC O157, this method may be less sensitive than testing rectal samples (Widgren et al., 2013).

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2.3 Detection including currently available detection methods & how these are applied as part of ISO or other peer reviewed methods

2.3.1 Introduction

The majority of STEC are indistinguishable from the ubiquitous commensal *E. coli*, except that they possess genes encoding the Shiga toxins (Stx). O157 was the first STEC serogroup identified and is responsible for the largest number of STEC infections worldwide, but the importance of several other serogroups as a cause of severe human disease have increasingly been recognized.

The common trait of all STEC strains is the ability to produce Stx, which is one of the most important virulence factors associated with human illness (Newell and La Ragione 2018). Therefore, a method

relying on this feature and not targeting individual serogroups represents a more effective strategy for diagnostic purposes. The presence of the *stx*-coding genes or the production of Stx may not be exploited directly for cultural purposes, but are usually the target of several methods for the detection of STEC. Methods include either the detection of the Stx itself in the test samples or the identification of the presence of bacterial strains possessing the genes encoding such toxins (*stx* genes). More details on the currently available methods for the detection of STEC are provided below.

2.3.2 Detection in humans

Several approaches and methodologies may be applied for the detection of STEC in the clinical laboratory. These include culture-based methods, immunological assays for STEC-specific antigens, and molecular tests targeting STEC-specific genes (Bryan et al 2015).

As for the cultural methods, it is usually impossible to use selective media to discriminate STEC from the commensal *E. coli*. However, certain STEC strains belonging to serogroup O157 are an exception to this, as they have peculiar metabolic characteristics (β-glucuronidase negative, inability to ferment sorbitol and resistance to Cefixime/Tellurite), which may be exploited with selective culture media. There have been several attempts to develop differential and selective media for STEC of other serogroups (Bryan et al 2015, Gill et al 2014). However, this has been hampered by the large variability in the biochemical characteristics of different strains and none of the proposed media are effective all of the time.

Immunological-based methodologies are usually rapid and easy to use and provide indirect evidence of the presence of STEC by identifying the presence of Stx in stool samples or enrichment of the stools (Silva MA et al 2019). Several immunological assays, consisting of ELISA tests, for the detection of Stx in diagnostic biological samples have been developed, with some assays being commercially available. The detection of free Shiga toxin in stools can also be done by assessing the cytopathic effect exerted by Stx on Vero cells monolayers (OIE, 2018). The use of the Vero cell assay (VCA) requires expertise in cellular biology and in recognizing the Stx induced cytopathic effect, and requires approximately three days of analysis to obtain a result. Nonetheless, this assay is very sensitive and can detect the presence of the free faecal toxin after the bacterium has been cleared (e.g. an antibiotic treatment), supporting the diagnosis of STEC infection after the eradication of the STEC strain.

The molecular approach to detect the presence of Stx-coding genes is widely adopted. This determination can be done directly on stools or in the DNA extracted from stool enrichment or sub-cultures. Moreover, it is possible to simultaneously detect the presence of accessory virulence genes, such as those involved in colonization such as the intimin encoding *eae* or the enterohemolysin gene *ehxA*. In addition, the presence of specific serogroup-associated genes may also be determined. Nevertheless, only the isolation of STEC and the confirmation of the characteristics identified in the screening can give the definitive certainty of the concomitant presence in a single bacterial cell of all the targets present in the enrichment broth or in complex matrices, since Stx-coding genes can also be present in bacteriophages outside of bacterial cells. Moreover, the isolation in pure culture of the STEC strain is critical in terms of public health as it facilitates a full phenotypic and genotypic characterisation of the organism for epidemiologic purposes.

The molecular approach is usually based on the amplification of the virulence genes by PCR or real-time PCR, with the latter technique allowing lower detection limits. The same approach consisting in the detection of *stx* genes (and other virulence-associated genes) can be applied either in the biological sample (faecal or environmental sample) or on single *E. coli* colonies for their identification as STEC. Different PCR/real-time PCR reagents have been designed and deployed and are described in the scientific literature (Paton and Paton 1998, Perelle et al 2004, Nielsen and Andersen 2003) and/or available as commercial kits.

2.3.3 Detection of STEC in food

ISO 16654 is an international standard for food and feed published in 2001 targeting solely *E. coli* serogroup O157. This method was evaluated as being equivalent to the NMKL method N. 164, a standard procedure developed by the Nordic Committee for Food Analysis (NMKL, 2019). The ISO 16654:2001 standard describes the selective enrichment of test samples in modified Tryptone Soya Broth (mTSB) supplemented with 20 mg/l of novobiocin, followed by the immunomagnetic separation (IMS) with magnetic beads coated with antibodies against the lipopolysaccharide (LPS) antigen O157 and plating onto the differential and selective medium Cefixime/Tellurite -Sorbitol MacConkey agar (CT-SMAC) together with an alternative complementary solid medium for the isolation of *E. coli* O157. *E. coli* O157 bacterial cells captured by magnetic beads and then streaked on CT-SMAC appear as white colonies, due to the lack of sorbitol fermentation, a characteristic of most of *E. coli* O157 strains. As mentioned above, it has to be taken into account that sorbitol fermenting *E. coli* O157 have also been described and appear as cerise colonies on CT-SMAC and thus are similar in appearance to commensal *E. coli*. Moreover the method targets the O157 LPS and the need for further characterisation of the isolated strain, including the determination of pathogenetic features, is mentioned in the method, but the step for the confirmation of the *E. coli* O157 isolated strain as STEC (i.e. by determining the presence of *stx* genes) is not include in ISO 16654. The NMKL method currently reports an obligation to test the O157-isolate for the presence of *stx*-genes.

ISO TS 13136 was published in 2012 (ISO, 2012) with the aim of detecting STEC in food, and identifying the five main serogroups, namely O157, O26, O103, O111 and O145, which have been historically associated with the most severe STEC infections, including haemolytic uremic syndrome (HUS), and which are often referred to as ‘the top 5’. The approach of ISO TS 13136 is sequential and consists of a molecular-based screening of food enrichment cultures for the presence of *stx*, *eae* and the genes targeting the top five serogroups (only performed on *stx*+/*eae*+ enrichment cultures) and in the case of positivity to *stx* genes the attempt of isolation, as the standard method targets all STEC. The detection of the *eae* gene and serogroup-associated genes of the top 5 STEC serogroups facilitate isolation as, there are IMS-based procedures for these serogroups. However, this stage is hampered by the lack of selective and differential medium for growing and discriminating all STEC. Moreover, food samples may be heavily contaminated with background microflora and the only possibility to confirm the presence of STEC in the sample requires the testing of single colonies (50 colonies) for the presence of the Stx-coding genes.

STEC strain isolation is important as the typing of the isolate facilitates an assessment of risk to the consumer (EFSA, 2020) as well as facilitating the identification of sources and routes of transmission.
A real-time PCR based screening approach followed by isolation of STEC in food is part of an official Laboratory Guideline deployed by USDA for the detection of the top-7 STEC serogroups in USA in meat products (USDA 2019). Moreover, a method consisting of a similar approach for the detection of priority STEC strains (STEC strains possessing *eae* and belonging to serogroups O26, O45, O103, O111, O121, O145, or O157) in beef trim was developed and used in Canada since 2013 (Huszczynski et al 2013).

2.3.4 Detection of STEC in animals

Currently, there are no specific methods available to test animal faeces for STEC, but the same approach for food testing can be adapted to these samples. Detection of STEC *via* the presence of *stx* genes independently of serotype may be complicated by a high overall prevalence of STEC in specific animal categories (e.g. ruminants). The OIE terrestrial manual (OIE 2018) describes an approach for testing animal faeces or rectal swabs for the presence of *E. coli* O157 in agreement with ISO 16654:2001 standard (ISO, 2001) with a few modifications, including dilution of the faecal sample 1/10 in buffered peptone water (BPW) and incubating at 37°C for 6 h. This pre-enrichment is followed by the IMS as described in ISO 16654:2001.
The technical specification for the monitoring and reporting of STEC in animal and food samples issued by EFSA (EFSA, 2009) recommends to test for non-O157 STEC in agreement with the scheme reported in ISO TS 13136 international standard (ISO, 2012).

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2.4 Latest sampling/technological developments that should be incorporated into future sampling & testing activities

STEC are priority microorganisms to be monitored in food and animals in the EU as laid down in the Directive 2003/99/EC. Nevertheless, the current EU Food Legislation sets the only microbiological criterion for STEC in sprouts placed on the market, which shall be compliant with absence of STEC belonging to the top-5 serogroups plus O104:H4 in 25 grams. The reporting of STEC in the remaining food commodities as well as in animal samples are only generically described in Directive 2003/99/EC, leading to non-harmonized sampling strategies and plans in the different MSs and bias in the data reported. It is therefore essential that harmonized sampling plans for STEC are developed based on the risk assessment (EFSA, 2020). This is especially important as foodstuffs may be contaminated with low concentration of pathogens (e.g. seeds) or non-homogenously contaminated, making it crucial to define representative test portion sizes.

Testing food samples for STEC already benefits from the use of established technologies such as real time PCR, which ensures the proper analysis of the samples and the appropriate level of sensitivity/specificity. Additional to this, there is substantial harmonisation in the methodologies adopted to test food for the presence of STEC, with more than 95% of the samples assayed yearly in EU and EEA countries tested with the approach depicted in the international standard ISO TS 13136:2012 (EFSA and ECDC, 2019). Nevertheless, recent PCR developments such as digital PCR which amplifies and detects individual molecules of target DNA, would facilitate the development of quantitative strategies to assess the real level of contamination of food and the infectious dose of the STEC triggering the disease. Both of these factors are crucial to understand the pathophysiology of infections in humans and to establish science-based measures to protect consumers' health.

Subtyping of STEC strains isolated from humans, food and animals is essential as it enables an assessment of the risk of severe infections (EFSA, 2020) and to trace the sources and routes of transmission. Additionally, determining strains' "signatures" facilitates the identification and monitoring, both temporally and geographically, as well as the diffusion and evolution of specific strains in order to develop control strategies (Newell and La Ragione, 2018). During the past few years, the field of DNA sequencing has taken massive steps forwards. Sequencing of entire genomes (whole genome sequencing, WGS) of foodborne bacteria can be easily achieved in the laboratory and analysis of this data allows a greater depth of characterisation. WGS provides high discriminatory power for foodborne outbreak investigation, source-attribution and hazard identification, potentially leading to a more targeted risk assessment. WGS is rapidly replacing the current phenotypic and genotypic reference methods, including serotyping, phage typing, pulsed-field gel electrophoresis (PFGE), multilocus variable-number tandem repeats analysis (MLVA) and multilocus sequence typing (MLST) (EFSA, 2019). Analysis of WGS data allows the identification of virulence factors and antibiotic resistance determinants, which would previously require a number of PCR tests.

The characterisation of STEC isolates is the key to the proactive pathogenicity assessment of STEC strains (EFSA, 2020) and WGS offers the ultimate analytical methodology. A number of freely available resources for WGS data analysis, both for bioinformatics savvy and naive laboratory personnel, make the use of WGS readily available. However, a major problem linked to the use of genomics data in outbreak investigation, and a priority intervention area, is the definition of thresholds for the level of variations between strains used with the different approaches for genome comparisons (SNPs, allele based MLST). No consensus has been achieved yet on suitable cut-offs for the identification of clusters, and these are likely to be dependent on context e.g. the population structure of the targeted STEC type.

Metagenomics is a culture-independent methodology with potential to contribute to either food testing or foodborne outbreaks detection/investigation. This approach has been applied for STEC detection in different matrices (Leonard et al., 2015; Gigliucci et al., 2018; Suttner et al., 2020), but there are still methodological constraints (e.g. the lack of harmonized methods, high costs, the low sensitivity in detecting certain *taxa* in the sample or the low amount of the target in the complex matrix investigated and limitations related to nucleic acid extraction protocols and library preparation strategies and bioinformatics pipelines, difficulty in assigning DNA sequence to the target isolate, etc.), which hinder

its development as a current detection method, and currently restricting this approach to research studies.

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3. Enterotoxigenic *Escherichia coli* (ETEC)

3.1 Current practice in the EU: Sampling, testing (detection & confirmation)

The ETEC questionnaire was designed using the EU survey tool and comprised of 15 questions.There were 40 respondents to the ETEC questionnaire (see appendix 2) and 19 of these laboratories test for ETEC. The main findings of the questionnaire may be summarised as follows:

- the type of samples tested included human clinical only (12 laboratories), animal only (3), food only (1), food and environmental (1), animal, food, feed and environmental (1) and human, food and environmental (1)
- two laboratories always take their own samples, four do so sometimes and 13 never do
- of the laboratories that do not take their own samples, two are involved in designing the sampling plan which is based on national, European or other authority recommendations
- 15 laboratories were accredited to ISO/IEC 17025 or ISO 15189
- 14 laboratories test for heat-stable (ST) and heat-labile (LT) enterotoxins
- 15 laboratories use a PCR based method, four use immunological assays, two also indicated they use culture based methods while one indicated that they use DNA hybridisation
- In 2019 the number of samples tested by individual labs ranged from 0 to 15,800
- 11 laboratories perform confirmatory tests while the other eight confirmed they did not undertake confirmatory testing but half of these send isolates to the NRL for confirmatory testing
- the confirmatory methods used are based on direct plating using a range of different media including SMAC, TBX, MacConkey, and Chrom-Agar
- In eight of these laboratories the testing and confirmatory methods were accredited
- three of the laboratories also use biochemical testing although these varied depending on the laboratory
- 17 of the laboratories used conventional PCR (10) and/or real time PCR (7) confirmatory methods and further three laboratories use WGS. Six laboratories indicated that the conformatory methods were accredited.
- 16 laboratories stored (always or sometimes) isolates in their own laboratory and/or in the NRL.

3.2 Sampling including best practice in designing statistically based sampling plans & most appropriate sampling methods

3.2.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infection is the most common type of colibacillosis of young animals (primarily pigs and calves), and it is a significant cause of diarrhoea among travellers and children in the developing world. ETEC adhere to receptors on the small intestinal epithelium by their adherence factors (fimbriae, pili or by afimbrial proteins) without inducing significant morphological changes. They secrete enterotoxins that reduce absorption and increase fluid and electrolyte secretion from the small intestinal epithelial cells. ETEC causes both human and animal disease but the

adherence factors are host-specific and these pathogens are therefore not considered as a zoonotic disease. Various heat stable (ST) and heat labile (LT) types have been described, but with different nomenclatures for identical genes. In this report, STa refers to both STh and STp with genes names *estah* and *estap*. *estIa* has been used for *estap,* and *st*, *sta2, sta3* and *sta4* has been used for *estah*. The gene encoding STb is *estb* in this report but has also been called *stb*. *eltI*Ah_1 encodes 28 different variants of the heat-labile enterotoxin B subunit and *eltI*Bh_1 encodes for 28 different variants of the heat-labile enterotoxin B subunit.

3.2.2 Human infections

Surveillance of human infection is focused either on the clinical outcome, which for ETEC usually is a short period of self-limiting watery diarrhoea, abdominal pain, fever, headache and, less frequently, vomiting, or on laboratory results only. Heterogeneity exists in the criteria used when deciding if a patient should be tested for ETEC infection, in the number of samples tested and isolates typed. Very few countries/laboratories test all patients with diarrhoea for the presence of ETEC, but the increased use of diagnostic tests on DNA extracts directly from faeces has lead to an increase in the number of stool specimens being tested for these bacteria. In spite of ETEC being primarily associated with travellers' diarrhoea, there have been multiple foodborne outbreaks in Denmark and elsewhere (Ethelberg et al., 2010; Pakalniskiene et al., 2009; Jain et al., 2008; Yoder et al., 2006). ETEC infection acquired in the EU/EEA countries are most often food or waterborne and in rare cases the suspicion of ETEC as a cause of a foodborne outbreak should be considered. In 2012, an ETEC outbreak in Norway was associated with imported chives added to scrambled eggs (MacDonald et al., 2015). In Denmark, extrapolation of the number of cases reported on the island of Funen in 2012, where all stool specimens were examined for ETEC, indicated an incidence of 24.3 per 100,000 inhabitants. It was estimated that an extra 1,071 human cases were overlooked because patients in the rest of the country were not included in the survey. Human cases of ETEC infections are not reported to the European Surveillance System (TESSy) held by ECDC. Except in relation to foodborne outbreaks, ETEC is not notifiable in the majority of the European/EEA countries and therefore ETEC cases are probably under-ascertained as this pathogen is not included in the routine panel of tests for gastrointestinal pathogens. ETEC may therefore be under-recognized as a cause of foodborne illness in the EU, and the most basic metadata are lacking. However, ETEC should be suspected as the cause of illness when stool cultures are negative for routine enteric pathogens. The median incubation period is 24–48 h, the diarrhoea-to-vomiting prevalence ratio is 12.5, and the median duration of illness is more than 60 hours (Beatty et al., 2004; Dalton et al., 1999).

3.2.3 Sampling in foodborne outbreaks

Monitoring of foodborne outbreaks by MSs and EEA countries and the annual reporting to EFSA is mandatory under Directive 2003/99/EC. Whereas reporting foodborne outbreak data is based on harmonised specifications, there is a lack of mandatory harmonisation of the national foodborne outbreak investigation systems. Differences in the number and type of reported outbreaks may indicate

differences in the sensitivity of the national surveillance systems in identifying and investigating foodborne outbreaks (EFSA 2020).

3.2.4 Sampling in food, animals and the environment

ETEC are the most common cause of *E. coli* diarrhoea in farm animals (Dubreuil et al., 2016) which cause a rapid onset of secretory diarrhoea leading to dehydration in animals. Lethal ETEC infections occur as a result of severe dehydration and electrolyte imbalance (Dubreuil JD et al., 2016) and are a major problem in pig herds.

Monitoring and reporting of ETEC along the food chain is arbitrary, scarse and in no way harmonized. To date there are no recommendations from European or National Authorities on how to improve the monitoring of animal populations or foodstuffs for ETEC although technical specifications for the monitoring and reporting of ETEC in animal and food samples have been published.

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3.3 Detection including currently available detection methods & how these are applied as part of ISO or other peer reviewed methods

3.3.1 Overview

There is no standard method for testing for ETEC in the EU. These bacteria cannot be phenotypically distinguished on isolation media (Mac Conkey, TBX, etc) from commensal *E. coli* or other *E. coli* pathotypes. Thus ETEC identification relies on the detection of either the toxins LT and ST, or the presence of the genes encoding such toxins. During the last 20 years, efforts to detect and quantify the amount of LT and ST toxins or their coding genes from ETEC have been made, including the development of serological, immunological, colorimetric, PCR and qPCR methods. Normally the methods for detection of the toxins are applied in clinical/ health laboratories on blood or stool specimens while the detection for the presence of toxins plasmid genes are used both in clinical and food laboratories.

3.3.2 Toxin detection methods

Several commercial kits for the detection of ETEC toxins are available. These comprise ST Toxin detection kits such as *E. coli* ST EIA (this Kit is designed to detect ST enterotoxin in culture filtration or supernatants by competitive enzyme immunoassay ST-coated microtitre well-strips) and *E. coli* ST-EIA (detects ST enterotoxin by EIA using microplates and allows visual detection. Although negative-positive boundaries require the use of spectrophotometers for microplates) The latter is intended for research only and not for use in diagnostics laboratories.

LT Toxin detection kits include the VET-RPLA TOXIN DETECTION KIT (for the detection of *Vibrio cholerae* enterotoxin (CT) and *Escherichia coli* heat-labile (LT) enterotoxin in culture fluid filtrates by reversed passive polystyrene latex particles agglutination using antiserum taken from rabbits, immunized with CT that will react with both CT and LT). The technique of reversed passive latex agglutination (RPLA) enables soluble antigen such as bacterial toxins to be detected in an agglutination assay. Another LT toxin detection kit is the VET-RPLA BACTERIAL TOXIN DETECTION KIT (detection by RPLA method using microplates (96 well, V-type). However, as *E. coli* heat-labile enterotoxins and *Vibrio cholerae* enterotoxins share the same antigen factor and both are detected using VET-RPLA, a different enrichment culture is required for each. This kit is intended for research only and not for use in diagnostics laboratories.

3.3.3 Molecular detection

The European Union Reference Laboratory (EURL) for *E. coli* issued a procedure describing a molecular methodology to screen food samples for the presence of ETEC by the detection of targets including the genes that encode the LT, STh and STp, human and porcine variants of heat stable toxin (EURL VTEC, 2013). The detection is performed by real time PCR in an enrichment broth, and where positive, the broth is streaked onto suitable plating media for isolation. Characteristic colonies are then tested for the presence of toxin genes by real time PCR. A similar approach of enrichment and real time PCR assay may be adopted for testing faecal samples from patients with diarrhoea.

3.3.4 Identification and quantification of virulence factors of ETEC by high-resolution melting curve quantitative PCR (HRM-qPCR)

This method uses five amplicons with melting temperatures (Tm) well separated by HRM-qPCR and that express five fimbriae by ETEC and simultaneously gives an identification and quantification of the five target genes. The area of amplicons under the melting peak correlated linearly to the proportion of the template in the calibration mixture. The genes encoding fimbriae and enterotoxins are quantified by HRM-qPCR and/or qPCR. This multiplex HRM-qPCR allows for accurate analysis of HRM curves and simultaneously distinguishes and quantify fimbriae genes in the faecal samples (Wang et al., 2017).

3.3.5 Detection of ETEC in water samples from an endemic area by real-time PCR

DNA extracted from water filters may be analyzed using real-time PCR (Lothigius et al., 2008). This assay with primers against enterotoxin genes *estA* (STh), *estB* (STp) and *eltB* (LT) was designed to be able to detect as few as three bacteria per PCR reaction. Gene copy numbers were estimated to be four (LT), two (STh) and one (STp) per bacteria. Thus this method allows for highly sensitive detection and quantification of ETEC in water samples and has better results than the Elisa method GM11.

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3.4 Latest sampling/technological developments that should be incorporated into future sampling & testing activities

3.4.1 Introduction

ETEC is not a priority microorganism to be monitored in humans, food and animals in the EU. The current EU Food Legislation has no microbiological criteria for ETEC in food. The reporting of ETEC is, at best, limited to reports related to foodborne outbreaks. There are no harmonized sampling strategies or sampling plans in the different Member States (MS). Thu,s data on ETEC infections in the EU is lacking.

The application of WGS in the surveillance of STEC, which is a high priority organism, has led to the discovery of so-called hybrid or cross-over pathotypes referred to as STEC-ETEC (Nyholm et al., 2015; Leonard et al., 2016; Oh et al., 2017; Bai et al., 2019). This acquisition and combination of virulence genes from STEC and ETEC is poorly understood. A more systematic surveillance of and harmonized sampling plans for ETEC, their genes and their origin in relation STEC is needed in order to investigate where these genes are coming from and how they are exchanged between strains.

Foodstuffs may be contaminated with low concentrations of ETEC (e.g. RTE vegetables, salads and green toppings) or non-homogenously contaminated. These food items are often added to other heat treated foods such as sandwiches, scrambled eggs, fish or meat and thus it is important to define representative test portion sizes and serving conditions.

Testing food samples for ETEC already benefits from the use of established technologies such as toxin detection kits (EIAs or VET-RPLA) and various molecular detection methodologies such as the real time PCR or HRM-qPCR assays, which ensure the proper analysis of the samples and the appropriate level of sensitivity/specificity. However, there is a substantial lack of harmonisation in the methodologies adopted for testing for the presence of ETEC in animals or humans.

The majority of the technological developments for future sampling & testing activities mentioned above in section 2.4 for STEC also applies for ETEC. However, metagenomics may also be used differently to study the gut microbial metabolism in weanling pigs (Wang W *et al.* 2019), with the purpose of reducing the load of ETEC. Feed fermentation with *L. reuteri* has been shown to reduce the level of colonization of weaning piglets with strain ECL13795 (O149, virotype STb:LT:EAST1:F4) ETEC (Yang Y *et al.* 2015). Thus, feed fermentation supplied concentrations of reuteran may specifically contribute to the effect on colonisation of ETEC, but this has not been studied on a larger scale.

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4. *Cryptosporidium* spp.

4.1 Current practice in the EU: Sampling, testing (detection & confirmation)

The *Cryptosporidium* questionnaire was designed using the EU survey tool and comprised of 15 questions.There were 54 respondents to the *Cryptosporidium* questionnaire (see appendix 3) and 44 of these laboratories reported they tested for this organism. The data obtained may be summarised as follows:

- The type of samples tested by the laboratories, were as followed: 24 human samples only, seven animal samples only, and five environmental (soil, water, etc.) samples only; regarding combined samples, four laboratories tested for human/animal samples, two human/animal/food/environmental (soil, water, etc.) samples, one human/animal/environmental (soil, water, etc.) and one animal/food/environmental (soil, water, etc.) samples
- Eleven laboratories always take their own samples, with nine indicating they do so sometimes and 24 never
- Of those who never take samples, 13 are at least involved in designing the sampling plan (number, types of samples, etc.); among these, only one uses a statistically based method, but also a sampling plan recommended by national, EU or other authority and other (targeted sampling), nine use a sampling plan recommended by a national, EU or other authority; three laboratories use other sampling plans, including if clinically indicated or, when needed, advice may be given to veterinary practices on collection of faeces samples for diagnostic testing, appropriate sample, host animal etc.
- All the 44 laboratories, except six, were accredited to ISO/IEC 17025 or ISO 15189
- Regarding testing method, five use modified Ziehl-Neelsen microscopy only, 15 use this technique coupled with another technique (such as Antigen detection by enzyme immuno assay, DNA detection by PCR, immunochromatographic rapid tests, immunofluorescence microscopy), five laboratories reported DNA detection by PCR. Eight laboratories use immunochromatographic rapid tests only, four immunofluorescence microscopy only. Only one laboratory uses auramine phenol microscopy only, and 3 laboratories use other single techniques, such as carbol fuchsin coloring, immuno magnetic separation or direct microscopy of feces samples
- In 2019 the number of samples tested ranged from 7 to 16,348
- 19/44 laboratories indicated that they perform confirmatory tests
- 25/44 labs indicated that they did not undertake confirmatory testing while two of these indicated that they sent the samplse to the National Reference Laboratory for this purpose.
- DNA detection by PCR (8) and modified Ziehl-Neelsen microscopy (7) were the most used confirmatory method
- 10/19 of the confirmatory methods were accredited; for eight of the eight laboratories that use PCR, the method is not accredited
- 25 of the 44 laboratories stored (always or sometimes) isolates in their own laboratory and/or the National Reference Laboratory
- 18/44 laboratories refer to perform routine characterisationby PCR-based tests, including one by WGS
- 31 laboratories testing human samples indicated they also receive some or all of the following metadata: date of sampling, date of receipt in the laboratory, specimen, age, gender, geographic origin, drug therapy, medical conditions, outbreak investigation
- 15 laboratories testing animal samples typically come with information about the date of sampling, date of receipt in the laboratory, animal species, type of sample and geographic origin

- The great majority of laboratories (40/44) indicated that they had a specific system for data management, although two of these laboratories indicated they do not store information about the sample
- 40 laboratories store data electronically although six also use a paper format
- 21/44 laboratories reported to national authorities, four laboratories reported to regional authorities, while 20/44 do not report to a regional nor national authority. Only 1 laboratory reported to EFSA.

4.2 Sampling including best practice in designing statistically based sampling plans & most appropriate sampling methods

The best practice in designing statistically based sampling plans and the most appropriate sampling methods for *Cryptosporidium* spp. are largely similar to those for other pathogens, and important for obtaining robust, useful and comparable data (Directive 2003/99/EC; EFSA and ECDC 2019). In comparison with sampling for bacterial pathogens, there are some particular considerations when sampling for the detection of *Cryptosporidium* spp.

Designing sampling plans for detection of *Cryptosporidium* spp. is highly dependent on the context, which includes clinical diagnostic situations, epidemiological studies, monitoring, and outbreak investigations. Sample collectors are very diverse and include veterinarians, animal owners, private individuals, parents or guardians, local authorities, researchers and representatives of retail, catering, industry and suppliers. The objectives of the sampling include estimating the incidence or prevalence, confirming or ruling out suspected clinical diagnosis at individual or at farm/group level, and investigating an outbreak. Cryptosporidiosis outbreaks reported in literature include foodborne outbreaks, waterborne outbreaks as well as outbreaks linked to other settings (Robertson and Chalmers, 2013; Kinross et al., 2015; EFSA BIOHAZ Panel, 2018; Ryan et al., 2018; Thomas-Lopez et al., 2020; Zahedi and Ryan, 2020). The priority for medical doctors and veterinarians is the correct diagnosis so that an appropriate treatment can be pursued. *Cryptosporidium* infection is a relevant differential diagnosis for humans and animals with suggestive gastrointestinal signs, as well as in food and waterborne outbreaks of gastroenteritis.

Statistically based sampling plans require background information on the occurrence or prevalence and on the population sampled. The available data on these are often limited (Cacciò and Chalmers, 2016; Plutzer et al., 2018). For diagnostic use as well as for epidemiological studies, a single faecal sample per individual is usually sufficient, although the number of oocysts per gram faeces can vary greatly. For sampling animals such as calves, their age is relevant (Santoro et al., 2019). Moreover for epidemiological studies, clustering should be taken into account as animals on a specific farm are likely to have been subject to similarly exposed. For sampling water sources, spatial and temporal variability should be considered (Burnet et al., 2014). Due to a long incubation period, apparent relatively low awareness, and because most infections are self-limiting, it may be challenging to ensure that relevant samples are obtained, for example, in foodborne cryptosporidiosis outbreaks. Sampling biases and the

potential for imprecision due to the limited number of samples tested needs to be acknowledged and taken into consideration in the interpretation of the results.

The transmission of *Cryptosporidium* spp. occurs mainly via the faecal-oral route by accidental ingestion of the oocysts, which are shed in faeces of the infected hosts and which are the infective, environmental resistant stage and the target diagnostic stage. Apart from faeces (humans, animals), the other most relevant sample types collected are water (drinking water, wastewater and recreational water), foodstuffs (fruit, vegetables and juice consumed raw), and environmental samples (soil). Sample material needs to fit the aims of the investigation and the methods used for the detection of the parasite.

Cryptosporidium spp. oocysts do not multiply in the samples collected. They are resistant against environmental factors, but temperature and duration of sample storage and transport may be important for some analyses. Formalin should not be used for the fixation of samples if molecular typing is planned. Freezing may influence the success of some methods. It should be noted that the infective dose to humans is low and precautions need to be taken during sampling and sample handling to avoid occupational exposure.

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4.3 Detection including currently available detection methods & how these are applied as part of ISO or other peer reviewed methods

The genus *Cryptosporidium* comprises more than 20 species, including the morphologically indistinguishable *C. hominis* and zoonotic *C. parvum*, which together account for most human cases. The oocyst is the infective transmission stage for all hosts, and the target diagnostic stage.

Whereas for human and animal diagnostic purposes the samples are faecal samples, in outbreak investigations the typical sample material includes food matrices and water. Using methods and approaches that have been validated for the specific sample material is important. As oocyst load can be very low in the sample (especially in environmental samples), the detection protocols often include an oocyst recovery (isolation and concentration) step that, depending on the matrix, might include, for example, immunomagnetic separation or flotation. As the infective dose is low and these organisms do not multiply in the sample material, samples such as lettuce or water need to be substantially concentrated (sometimes from many liters to µl) to enable detection. No *in vitro* systems are available for diagnostic purposes.

The detection methods used are methods based on direct detection and include either microscopy or nucleic acid amplification (Chalmers et al., 2020). It is important to highlight that specific adjustments of the detection method are often necessary depending on the different sample types and matrices in which *Cryptosporidium* oocysts could be present (Berrouch et al., 2020; Ligda et al., 2019). However, the same methods can and have been used in One Health approaches, for example when testing faecal

samples from animals and humans (Thomas-Lopez et al., 2020). In addition to detection, species-level identification and typing are usually necessary for source tracing and in outbreak investigations (Zahedi and Ryan, 2020).

Traditional microscopic diagnosis relies on modified acid-fast-stained faecal concentrates (Ziehl-Neelsen staining), auramine-phenol staining and/or antigen detection by direct fluorescent-antibody or immunochromatographic assays.

Molecular detection methods are routinely used (Verweij and Stensvold, 2014). As a vast number of *Cryptosporidium* species may infect different hosts, a genus-specific PCR assay is a practical and relevant diagnostic approach in routine clinical laboratories. The extracted DNA can be stored for further characterisation, including genotyping (Zahedi and Ryan, 2020). The main targets for diagnostic PCRs typically include the SSU rRNA gene, the *Cryptosporidium* oocyst wall protein (COWP) gene, or the DnaJ-like protein gene. While partial SSU rRNA gene sequences are available for all species of *Cryptosporidium* known to infect humans, only a fraction of these species are currently represented by complete SSU rDNA sequences in GenBank.

To date, two international standards are available for the detection of *Cryptosporidium*, both based on microscopy. ISO 18744:2016 specifies a method that is applicable for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts on fresh leafy green vegetables and berry fruits, and ISO 15553:2006 is applicable for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in water.

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4.4 Latest sampling/technological developments that should be incorporated into future sampling & testing activities

Improvements in *Cryptosporidium* detection have been proposed for various types of diagnostic techniques routinely used, including microscopy, immunoassays, and DNA-based methods. The multiple matrices (e.g. food, feces, and environmental matrices) routinely tested for *Cryptosporidium* have specific limitations when it comes to detection, making the recovery of *Cryptosporidium* oocysts important. Since only certain *Cryptosporidium* species and subtypes are infective to both humans and animals, typing of *Cryptosporidium* is also essential in epidemiolocal studies and source attribution/tracking.

Oocyst recovery (isolation and concentration) is a crucial initial step for any successful detection technique applied to *Cryptosporidium*. Different matrices as well as different recovery procedures may result in inhibition in molecular assays (e.g. PCR). No standardized recovery technique is available for all matrices, although there are standardized and validated ISO methods for leafy green vegetables and berry fruits (ISO 18744:2016) and for water (ISO 15553:2006). Standardizing oocysts recovery and DNA extraction from multiple matrices would enable comparative prevalence studies. The EFSA project IMPACT (Standardizing molecular detection methods to IMprove risk assessment capacity for foodborne protozoan PArasites, using *Cryptosporidium* in ready-to-eat salad as a model) aims to develop a Standardized Operating Protocol (SOP) for oocyst recovery, DNA extraction, and qPCR-based detection of *Cryptosporidium* spp in leafy salads. The One Health EJP project PARADISE (Parasite Detection, Isolation and Evaluation) aims to develop and test novel enrichment strategies to overcome the limitations of current technologies. In this project, pre-DNA enrichment approaches will include aptamers and nanobodies, whereas for post-DNA enrichment, an approach using hybridization probes will be followed.

ELISA of copro-antigens could be used as a substitute for the detection of *Cryptosporidium* by microscopy in setups where case load is high and staining and microscopy expertise is lacking (Ghoshal,

Jain, Dey and Ranjan, 2018). Detection techniques using antibodies like these have some disadvantages, including potential low sensitivity and high costs.

Recently developed immunoassays for clinical stool samples such as ImmunoCard STAT and QUIK CHEK have proven to be a more reliable test concerning sensitivity compared to ELISA and are simple and rapid techniques. However, these techniques are not able to discriminate between species and can be less sensitive compared to PCR (Adeyemo, Singh, Reddy and Stenström, 2018). Another technique in development are biosensors, which are comprised of specific DNA or proteins immobilized on a transducer. The transducer converts targets binding to the specific DNA or proteins into a measurable signal (electrochemical, optical, mechanical etc.). This technique can analyze small sample volumes, reducing reagent consumption costs and energy consumption, is portable, and has a short assay time. Promising examples of this technique are the aptasensors developed for detection of *Cryptosporidium* spp. on fresh produce and in water (Iqbal et al, 2015, 2019).

(q)PCR is routinely used for the detection of *Cryptosporidium*. Currently, different genes are used as PCR targets by different laboratories, but ongoing and future studies using new genomic data acquired by sequencing may find new gene targets (Cunha, Peralta and Peralta, 2019). In general, PCR is more sensitive compared to microscopy and able to differentiate more or less to species level or beyond, based on the target loci. PCR can be problematic in the case of infections with multiple *Cryptosporidium* spp. as often only one type will be detected (Ahmed and Karanis, 2018). This problem can be overcome by using multiplex PCR or metagenomic approaches. New developments to improve the sensitivity of PCR include loop mediated isothermal amplification (LAMP), digital droplet PCR (ddPCR) and high-resolution melting PCR (HRM-qPCR) (Adeyemo et al., 2018, Lamien-Meda et al., 2020, Yang, Paparini, Monis and Ryan, 2014).

Sequencing techniques are fast becoming routine diagnostic techniques in many laboratories. Whole genome sequencing (WGS) followed by comparative genomics allow for a more sensitive detection of subtypes which will increase our understanding of *Cryptosporidium* population genetics and epidemiology (Fan et al., 2019). Studies show that sequencing can be directly carried out from faecal samples without the need of recovery of *Cryptosporidium* oocysts or DNA, using immune based separation techniques (Andersson et al., 2015; Hadfield et al., 2015). The expansion of databases with complete *Cryptosporidium* genome sequences of all species and subtypes is required for the improvement of sequencing techniques. These databases are being used to search for new biomarkers by detecting Variable Number Tandem Repeat regions in the *Cryptosporidium* genome (Morris et al., 2019, Pérez-Cordón et al., 2016). The previously mentioned One Health-EJP PARADISE project will also use metagenomics as an untargeted approach for the detection of foodborne protozoa and helminths in different matrices. This will include both *in silico* analyses of available metagenomes against a reference genome database that PARADISE will establish, and experimental work to test the applicability of shotgun and amplicon-based next-generation sequencing for the detection of foodborne parasites in food matrices.

16S/18S profiling and other metagenomic approaches targeting pathogens across the classical silos (bacteria, viruses, parasites, fungi) will likely be more and more routinely applied. It is important that reference sequence databases are exhaustive, covering all the *Cryptosporidium* species relevant to clinical and environmental microbiology, including the specific needs of a One Health setting.

Finally, testing viability of *Cryptosporidium* oocysts is important to improve predictions on human health risks, for example in recreational and drinking water. To test the infectivity of oocysts, currently *in vivo* animal experiments or *in vitro* studies using HCT-8 or Caco-2 cell lines are mostly used (Rousseau et al., 2018), but these are not applicable to routine use. Multiple innovative methods to determine the viability of *Cryptosporidium* oocysts have been published as a proof-of-concept including techniques using metabolomics (Beale et al., 2013), microfluidic impendance cytometry (McGrath et al, 2017), measuring excystation of oocysts (Paziewska-Harris et al., 2016), using the vital dye propidium monoazide (PMA) (Vande Burgt et al., 2018) or measuring heat shock protein mRNA (Garcés-Sanchez et al., 2013). However, none of these techniques is currently ready for routine use.

For all alternative and novel diagnostics it is important to validate the assays so as to determine the detection limit, specificity, sensitivity, repeatability and negative and positive predictive values. A number of ISO standards on microbiological detection should guide these validations (e.g. ISO 22174, ISO 20837, ISO 7218 and ISO 16140). Depending on the purpose of the test (diagnostics, surveillance, etc.) the most applicable technique should be chosen.

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5. AMR in *Salmonella* and *Campylobacter* spp. in the European Union

5.1 Current practice in the EU: Sampling, testing (detection & confirmation), characterisation and Data management

The AMR questionnaire for *Salmonella* and *Campylobacter* was designed using the EU survey tool and comprised of 15 questions for *Salmonella* and 14 questions for *Campylobacter*. There were 56 respondents to the *Salmonella* and *Campylobacter* AMR survey. Of these 49 indicated that they test for AMR including 34 in both bacteria, 12 in *Salmonella* and 3 in *Campylobacter*. The main findings may be summarised as follows;

AMR for Salmonella (n=46):

- The laboratories test mostly for human samples only (23 laboratories), 20 animal samples (including poultry) , food (13), feed (10), and environmental (7)
- 44 laboratories serotype *Salmonella* isolates
- AMR testing methods used for human isolates include disk diffusion, broth dilution, agar dilution, gradient strips (eg. the E-test) and genotypic methods (PCR, WGS)
- Similar methods are also used for AMR testing in animal, poultry, meat, other food and environmental isolates
- 35 laboratories indicated that they are accredited for AMR testing to ISO 17025: 2017, ISO 20776-1:2019, ISO 17925, ISO 15189, ISO 15189:2013 and the performance standard CLSI Vet 01-A4 2013, chapter 11, TREK
- In 2019 the number of Salmonella isolates tested in a given laboratory ranged from 0 to 5350 with the majority of laboratories (28) testing less than 200 and 23 testing less than 100 isolates
- 19 laboratories test for specific AMR genes using conventional PCR, real time PCR and/or WGS methods
- Regarding the specific AMR genes or point mutation routinely tested, 27 test for at least two of pAmpC/ESBL/carbapenemase/colistin genes/fluoroquinolone genes, and 3 test for carbapenemases only; 1 laboratory uses WGS targeting all AMR genes and most laboratories undertake these molecular tests to confirm phenotype results; 16 laboratories do not test for specific AMR genes or point mutation
- 32 laboratories always stored the isolates, 6 sometimes and 8 never
- These isolates are stored in their own laboratories only (14), in the National Reference Laboratory only (6) or both (18)

- 26 laboratories testing human samples indicated they also receive some or all of the following metadata: date of sampling, date of receipt in the laboratory, specimen, age; gender, geographic origin, outbreak investigation, medical conditions
- 23 laboratories testing animal samples indicated they also receive some or all of the following metadata: date of sampling, date of receipt in the laboratory, animal species, type of sample, geographic origin
- 41 laboratories indicated they have a specific system for data management and all of these except 1 store information about the samples
- All use electronic data storage systems while 10 laboratories also use a paper-based system
- 28 laboratories report their data: 21 to national authorities only, 3 to regional authorities only and 6 to both authorities; 23 laboratories report to ECDC and/or EFSA

AMR for Campylobacter (n=37):

- The laboratories test mostly for human samples only (20) or animal samples (including poultry) (16)
- 36 laboratories indicated they record the *Campylobacter* species tested
- A range of testing methods are used for human isolates including disk diffusion, broth dilution, agar dilution, gradient strips (eg. the E-test) and WGS based methods (1 laboratory)
- Disk diffusion, broth dilution, agar dilution and gradient strips (eg. the E-test) are also used for testing animal, poultry, food and environmental isolates, while 1 laboratory indicated they also use WGS based methods for testing poultry and food isolates
- 26 laboratories were accredited for AMR testing to ISO/IEC 17025, ISO 15189, ISO 20776, CLSI or EUCAST
- The total number of isolates tested in 2019 in a given laboratory ranged from 0 to 680 with the majority (19/36) testing less than 100 isolates
- 14 out of 37 laboratories test for the presence of specific AMR genes or point mutations, with 11 testing for at least one of fluoroquinolones/macrolides-resistance associated point mutations and tetracycline/aminoglycosides resistance genes, while 2 test also for all resistance determinants through bioinformatic analysis
- 22 laboratories indicate they always store isolates, 7 sometimes and 8 never store. Isolates are stored in their own laboratory only (14), the National Reference Laboratory only (6) or both (9)

- The metadata included with human samples includes date of sampling, date of receipt in the laboratory, specimen type, age, gender, geographic origin and/or outbreak information. Animal samples typically include information on the data on date of sampling, date of receipt in the laboratory, animal species and/or geographic origin
- 33 laboratories indicated they have a data storage system while 4 do not
- 33 laboratories indicated they store information about the samples with all using electronic formats while 7 also use a paper-based system
- 20 laboratories report their data: 17 to national authorities only, 1 to regional authorities only and 2 to both authorities; 18 laboratories report to ECDC and/or EFSA

5.2 Sampling including best practice in designing statistically based sampling plans & most appropriate sampling methods

As part of their legal obligation (Directive 2003/99/EC) to monitor zoonoses and zoonotic agents, EU member states must also ensure they provide data on the occurrence of AMR using methodologies and data recording that facilitates comparison with other MSs. Moreover, Decision 2013/652/EC lays down specific technical requirements for AMR testing of zoonotic bacteria and reporting the information obtained. In 2014, EFSA provided recommendations on harmonised procedures for AMR monitoring pursuant to Decision 2013/652/EC which provides procedures for randomised sampling of animal and meat samples to provide representative and comparable data on *Salmonella*, *Campylobacter* and other bacterial pathogens along the food chain (EFSA, 2014). This document contains the following recommendations:

- isolates tested for AMR should be from active monitoring programmes
- randomised sampling strategies should be used to ensure a representative sample is obtained, avoiding bias and facilitating proper statistical data analysis
- bacterial isolates tested should be from healthy animals sampled from randomly selected epidemiological units (eg. poultry flocks, slaughter batches, etc. randomly selected in slaughterhouses)
- samples should be equally distributed over the year to ensure the different seasons are covered
- the number of isolates selected for AMR testing should allow; [1] the calculation of the proportion of the population that is resistant (for a given bacterial species-animal population/food category combination) to a given antimicrobial within predetermined accuracy targets and [2] allow the detection of changes in this proportion over time
- at least 170 isolates of each bacterial species should be tested for AMR for each type of domestic animal production type but may be reduced to 85 isolates for pigs and poultry if annual production in that MS is less than 100,000 tonnes per annum
- the sample size should be revised if there is a low prevalence of that bacterial species and/or very small production sectors

- for *Salmonella* at least 170 isolates from national control programmes (NCPs) should be tested from poultry. If there is a high number of *Salmonella* isolates available, the 170 isolates should be randomly selected from the collection for a given year. If there is a low prevalence of *Salmonella*, all isolates (excluding clinical isolates) should be tested for AMR
- AMR testing should be undertaken annually although biennial monitoring may be considered if the testing capacity in the MS is limited.

The document also provides numerical simulations to evaluate the relative statistical power of different sample sizes when assessing the occurrence and trends in AMR. For further information the reader is advised to carefully read this document, available at: https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2019.5709.

Reference

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5.3 Detection including currently available detection methods & how these are applied as part of ISO or other peer reviewed methods

The emergence and dissemination of antimicrobial resistance (AMR) is a major public health issue. In the European Union it is estimated that the healthcare costs associated with AMR are in excess of €1.5bn per annum (WHO, 2014). AMR susceptibility testing methods are used to determine susceptibility or resistance of an isolate against a range of relevant antimicrobial agents. The results are used to select the most appropriate antibiotic treatment for a patient but may also be used to monitor the emergence and dissemination of resistant within bacteria populations and of resistant bacteria in the animal or human population.

The ISO method 20776-1:2006, based on broth microdilution, is the reference method for the *in vitro* testing of infectious bacteria for resistance to antimicrobials. From this a minimum inhibitory concentration (MIC), the lowest concentration at which the isolate is completely inhibited, is determined. Agar dilution is a similar procedure, for establishing the lowest concentration of a serially diluted antibiotic concentration that still inhibits the bacteria. However, disk diffusion is probably a more preferred method because of convenience, efficiency and cost and is widely used in private veterinary clinics. This method uses commercially prepared disks, pre-impregnated with a standard concentration of a specific antibiotic, which are lightly pressed onto the agar surface. The antibiotic immediately diffuses outward from the disk, creating a gradient of antibiotic concentration in the agar from higher to lower concentration the further from the disk. After an overnight incubation, the zone of no bacterial growth, referred to as the zone of inhibition, is measured in millimetres and interpreted using a standard interpretation chart used to categorize each isolate as susceptible, intermediately susceptible or

resistant. A similar principle is used in E-tests (AB Biodisk, Solna, Sweden), a commercially available test where a plastic test strip impregnated with a gradually decreasing concentration of a specific antibiotic is used to provide a quantitative test of antibiotic resistance. Molecular methods for AMR determination are also available based on PCR assays and DNA hybridization targeting specific genes encoding resistance. In recent years as the technology has become more widely available and cheaper, many laboratories have employed WGS technologies to identify the molecular mechanisms in bacteria, such as *Salmonella* spp. and *Campylobacter* spp., underlying AMR (Köser et al., 2012; McDermott et al., 2016). WGS is especially useful as in addition to predicting AMR, subspecies typing and phylogenetic source attribution information is also obtained. Moreover, WGS informatics pipelines for surveillance have been developed and are available for research and diagnostic laboratories (ECDC, 2017).

Regardless, the broth microdilution is the method mandated for AMR reporting in the EU harmonised surveillance programme (ECDC, 2016). AMR testing of human isolates is usually designed to identify clinical breakpoints (a threshold value or chosen concentration (mg/L) of an antibiotic which defines whether a species of bacteria is susceptible, intermediate or resistant to a given antibiotic). In contrast, animal and food isolates, tested as part of ongoing AMR monitoring programmes, are generally interpreted based on epidemiological cut-off (ECOFF) values (measures of a drug MIC distribution that separate bacterial populations into those representative of a wild type population, and those with acquired or mutational resistance to the drug). The concentration ranges to be tested for each antimicrobial should therefore be wide enough to encompass both the clinical breakpoints and the ECOFF-values, thereby facilitating comparison between human, animal and food isolates.

References

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5.4 Latest sampling/technological developments that should be incorporated into future sampling & testing activities

Campylobacter and *Salmonella* are the most commonly reported zoonoses in humans in the EU/EEA, altogether representing 94% of all reported cases in 2018. Both these pathogens are transmitted through consumption of contaminated food or water. *Salmonella* and *Campylobacter* are also responsible for most of the foodborne outbreaks in the EU/EEA. Since 2003 (Directive 2003/99/EC), both zoonoses are included in the compulsory annual monitoring and many Member States are also currently monitoring antimicrobial resistance in both zoonotic agents. Surveillance of the antibiotic resistance of *Salmonella* and *Campylobacter* isolated from human samples are gathered in the European Surveillance System (TESSy) and analysed each year.

In 2016, ECDC published a harmonized protocol for monitoring of antimicrobial resistance (AMR) in *Salmonella* and *Campylobacter* from human isolates that takes into account new interpretive criteria and recommendations from EUCAST. The protocol was aimed not only at improving the quality and comparability of data between MSs, but also to achieve harmonisation between human and veterinary surveillance. The gold standard for antimicrobial susceptibility testing (AST) is the determination of the minimum inhibitory concentration (MIC), although the most widely used and well accepted method when testing human isolates is disk diffusion, given that this method is extremely inexpensive and simple to use in a routine laboratory. Nevertheless, the fact that only dilution susceptibility test data are accepted in the monitoring in animals and food is still a major setback when comparing resistance with human isolates.

In recent years, the development of the Next Generation Sequencing technology has made it possible to sequence whole genomes at an affordable price and fairly quickly. Databases such as CARD and Resfinder have been developed to submit raw reads or contigs and allow naive users to determine the present of antibiotic resistance factors (genes or mutations) and predict the phenotype. Research evidence suggests there is a strong correlation between *in silico* resistance and phenotypic data for both *Salmonella* and *Campylobacter*. The application of these technologies to the detection and monitorization of AMR across different sectors should overcome the difficulties still associated with

phenotypic testing. However, if a successful transition is to be made, standardization of pipelines and databases should become a priority.

Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs and subsequent amendments, lays down the microbiological criteria for certain foodborne bacteria, such as *Salmonella* and *Campylobacter*, and the rules to be followed in the implementation of the hygiene measures established in Regulation (EC) No 852/2004. Regarding *Salmonella*, the specific requirements for compliance with the regulation, must be 'not detected' in 10 or 25 g, depending on the foodstuffs, with the exception of fresh poultry meat, for which compliance is met if *S. enterica* serovar Typhimurium, *S. enterica* serovar 1,4,[5],12:i:-, and *S. enterica* serovar Enteritidis is 'not detected' in 25 g. This means that if any other serovar is detected in the sample of fresh poultry meat, it will be in compliance with the EC regulation and will enter the European market. In fact, both Portugal and the Netherlands have isolated multiresistant *S. enterica* serovar Heidelberg and *S. enterica* serovar Minnesota in imported fresh poultry meat (Silveira L, 2019; Berg, R. R. Van Den, 2019). An additional problem is the lack of harmonisation of these data across MSs, due to different sampling objectives, frequency and place of sampling. For *Campylobacter*, the microbiological criterion is only applied to food business operators and a limit of 1,000 CFU/g is set for 50 samples of broiler carcasses derived from 10 different sampling sessions. Given the continued presence of multiresistant isolates in foodstuffs in compliance with the criteria laid down by these regulations, especially in imported foodstuffs, an amendment considering not only the presence of such pathogens but also their resistance profile may be in order.

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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

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 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

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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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