



Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food)

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SCIENTIFIC REPORT OF EFSA

Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food)¹

European Food Safety Authority^{2, 3}

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ABSTRACT

Technical specifications are proposed for harmonised monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) in animals and foodstuffs by the European Union Member States in accordance with the Directive 2003/99/EC (EC, 2003). This harmonisation should facilitate a better analysis of the situation at Member State and Community levels and enable cost-effective monitoring. According to a risk-based sampling strategy, the technical specifications describe an entire survey, aiming at estimating at slaughter the prevalence of VTEC O157 contamination, primarily on the hide of young cattle and secondarily on sheep fleeces. It is proposed that all Member States carry out monitoring on a minimum three-year interval basis. In addition to the monitoring of VTEC O157, which is the most often reported serogroup in VTEC cases in humans in the European Union, Member States may extend the monitoring to the serogroups: VTEC O26, O103, O111 and O145, which are also identified as causes of human infections. Regarding foodstuffs, general guidelines are given for carrying out specific surveys on the food categories that are most likely to be sources of VTEC O157 and non-O157 infections in humans. The standardised ISO 16654:2001 (ISO, 2001) method is recommended for the detection of *E. coli* O157 in food. A method derived from the same ISO is specifically proposed for the testing of hide and fleece samples. All isolated *E. coli* O157 strains must be confirmed as VTEC by testing for the presence of VT-encoding genes (*vtx*). Regarding the detection of serogroups O26, O103, O111 and O145, the draft CEN TC275/WG6 standard, currently submitted to ISO for evaluation, is proposed to be used. Specifications are also given for the reporting of the information on the VTEC monitoring programme and its results in animals and foodstuffs by Member States in their annual zoonoses reports. It is further proposed that the technical specifications be reviewed in the light of the results of the first surveys.

KEY WORDS

Cattle, sheep, monitoring, foodstuff, fleece, hide, STEC, VTEC

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SUMMARY

The Directive 2003/99/EC (EC, 2003) lays down the Community system for the monitoring of zoonoses, which obliges the European Union Member States to collect relevant, and where applicable, comparable data on verotoxigenic *Escherichia coli* (VTEC) in animals and foodstuffs.

Based on the opinion of the scientific panel on Biological Hazards on the monitoring of VTEC and the identification of human pathogenic VTEC types (EFSA, 2007), harmonised technical specifications are proposed for the monitoring and reporting of VTEC in relevant animal populations and foodstuff categories. These technical specifications, once implemented, would facilitate a better analysis of the situation at Member State and Community levels.

According to a risk-based sampling strategy, the technical specifications describe an entire survey design, aiming at estimating at slaughter the prevalence of VTEC O157 contamination, primarily on hide of young cattle and secondarily on sheep fleeces. VTEC O157 is the serogroup most often reported in human VTEC infections, including severe HUS cases and young cattle are assumed to be the most important VTEC reservoir. Also, VTEC prevalence on hides/fleeces has been reported to be higher than in faecal samples. This risk-based approach will enable a cost-effective monitoring for public health purposes. It is recommended that all Member States carry out monitoring at minimum three-year intervals. Member States may extend the monitoring to the serogroups of VTEC O26, O103, O111 and O145, identified in some Member States as causes of human infections. Regarding foodstuffs, general guidelines are proposed for carrying out specific surveys on the food categories that are most likely to be sources of VTEC O157 and non-O157 infections in humans.

The standardised ISO 16654:2001 (ISO, 2001) method is recommended for the detection of *E. coli* O157 in food. A method derived from the same ISO is specifically proposed for the testing of hide and fleece samples. All isolated *E. coli* O157 strains must be confirmed as VTEC by testing for the presence of VT-encoding genes (*vtx*) and *eae* by means of a suitable Polymerase chain reaction, such as the one annexed to the document. Regarding the detection of serogroups O26, O103, O111 and O145, the use of the draft CEN TC275/WG6 standard, currently submitted to ISO for evaluation, is proposed. It is based on a Real-time PCR-based horizontal method for screening followed by a confirmation step aiming at the isolation of the VTEC strains. For the purpose of the harmonised technical specifications, buffered peptone water without antibiotics is proposed as a single enrichment medium for hide/fleece samples so that the same enrichment culture is used for the isolation of both O157 and non-O157 VTEC and to simplify laboratory protocol.

Technical specifications for the reporting of harmonised information on the VTEC monitoring programme in animals and foodstuffs as well as on survey results by Member States in their annual zoonoses reports are defined.

Finally, it is proposed that the technical specifications be reviewed in the light of the results of the first surveys.

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BACKGROUND AS PROVIDED BY EFSA

The Directive 2003/99/EC lays down the Community system for the monitoring and reporting of information on zoonoses, which obligates Member States (MSs) to collect relevant, and where applicable, comparable data on zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks. The European Food Safety Authority (EFSA) is assigned the tasks of examining the data collected and preparing the Community Summary Report (CSR).

So far, EFSA has published, in collaboration with the European Centre for Disease Prevention and Control (ECDC), three CSRs on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Food-borne Outbreaks in the European Union. While analysing the data received from Member States, it has become apparent that the information available on Verotoxigenic *Escherichia coli* (VTEC) was not sufficient to facilitate the analyses of the importance of VTEC findings in foodstuffs and animal populations to human VTEC cases. This information would be crucial in order to assess the potential sources of human infections.

Therefore, EFSA asked the Scientific Panel on Biological Hazards (BIOHAZ) for scientific advice regarding the identification of the strains and/or serotypes of VTEC that are pathogenic to humans, the analytical methods to be used to detect and identify the human pathogenic VTEC strains/serotypes from food and animals and the monitoring methods in animal populations and foodstuffs that are optimal from the public health point of view. The BIOHAZ panel issued its opinion on the monitoring of Verotoxigenic *Escherichia coli* (VTEC) and the identification of human pathogenic VTEC types on 18 October 2007 (EFSA, 2007).

TERMS OF REFERENCE AS PROVIDED BY EFSA

In light of the BIOHAZ panel opinion, the Task Force on Zoonoses Data Collection is asked, to:

- issue a report on specifications for harmonised monitoring and reporting of VTEC in animal populations and/or foodstuffs to be applied under the Directive 2003/99/EC;
- consider the VTEC types to be covered by the monitoring and reporting; and
- provide recommendations for further development of the monitoring, if necessary.

TECHNICAL SPECIFICATIONS

1. Introduction

The Directive 2003/99/EC (EC, 2003) on the monitoring of zoonoses and zoonotic agents obliges the European Union (EU) MSs to collect relevant, and where applicable, comparable data of zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks. In addition, MSs shall assess trends and sources of these agents and outbreaks in their territory, and transmit to the European Commission (EC), a report covering the data collected every year. Data collected in the framework of Directive 2003/99/EC relate to the occurrence of zoonotic agents isolated from animals, food, and feed, as well as to antimicrobial resistance in these agents. The information concerning zoonoses cases in humans and related antimicrobial resistance is derived from the structures and/or authorities referred to in Article 1 of the Decision No 2119/98/EC (EC, 1998) that are currently coordinated by ECDC. The latest CSR on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union is from the 2007 data (EFSA, 2009) and was prepared in collaboration with EFSA and ECDC.

Verotoxigenic *Escherichia coli* (VTEC) is one of the zoonotic agents that MSs have to report according to Directive 2003/99/EC on a mandatory basis. In the VTEC data reported by MSs there was a lack of information on serogroups and virulence factors of the VTEC isolates from food and animals, and thus, it was often not possible to estimate whether the VTEC isolates from foodstuffs and animals were potentially pathogenic to humans. This information is crucial in order to assess the potential sources of human infections and measures to protect public health. According to the Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on Verotoxigenic *E. coli* (VTEC) in foodstuffs (EC, 2003), only a small fraction of all VTEC-types isolated from animals, food, or the environment, are consistently associated with human illness. In addition, MSs used different analytical methods, some of which were only able to detect the serogroup VTEC O157, and this hampered the epidemiological analyses.

VTEC infections are important zoonotic diseases which are able to cause severe and life threatening diseases in humans. In 2007, a total of 2,905 human VTEC cases, confirmed in laboratory, were reported in 23 EU MSs (EFSA, 2009).

For the reasons described above, EFSA sought advice from its scientific Panel on Biological Hazards (BIOHAZ) regarding the identification of the strains and/or serotypes of VTEC that are pathogenic to humans, the analytical methods to be used to detect and identify the human pathogenic VTEC strains/serotypes from food and animals, and the monitoring methods in animal populations and foodstuffs that are optimal from the public health point of view. The BIOHAZ panel issued its opinion on the monitoring of VTEC and identification of human pathogenic VTEC types on 18 October 2007 (EFSA, 2007), where conclusions and recommendations on these matters are presented.

The aim is to improve the comparability of the data collected from MSs on the occurrence of zoonotic agents in animals and food. This will facilitate better analyses of data at Community level, and provide more reliable updates of the situations in MSs and the Community to risk managers, such as the EC and MSs. Currently there are no harmonised guidelines for the monitoring of VTEC in animals or food. The technical specifications presented in this guidance document provide recommendations for harmonised monitoring and reporting and are expected to improve the possibilities of analysing VTEC data at Community level.

2. Definitions

For the purpose of this document, the following definitions will apply:

eae - gene encoding intimin, an outer membrane protein that mediates the typical attaching and effacing lesions seen in the intestinal mucosa of human patients. The majority of human pathogenic VTEC harbour *eae*, which is situated on a pathogenicity island designated locus of enterocyte effacement (LEE).

Fresh meat - meat which has not undergone any preserving process other than chilling, freezing or quick-freezing including meat that is vacuum-wrapped or wrapped in a controlled atmosphere (Regulation (EC) No 853/2004 (EC, 2004a) of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin).

Meat - edible parts of the animal, including blood (Regulation (EC) No 853/2004).

Meat preparations - fresh meat, including meat that has been reduced to fragments, which has had foodstuffs, seasonings or additives added to it or which has undergone processes insufficient to modify the internal muscle fibre structure of the meat and thus to eliminate the characteristics of fresh meat (Regulation (EC) No 853/2004 (EC, 2004a)).

Meat products - processed products resulting from the processing of meat or from the further processing of such processed products, so that the cut surface shows that the product no longer has the characteristics of fresh meat (Regulation (EC) No 853/2004 (EC, 2004a)).

Minced meat - boned meat that has been minced into fragments and contains less than 1% salt (Regulation (EC) No 853/2004 (EC, 2004a)).

Monitoring - system of collecting, analysing and disseminating data on the occurrence of zoonoses, zoonotic agents and antimicrobial resistance related thereto (Directive 2003/99/EC) - conducting a planned sequence of observations or measurements with a view to obtaining an overview of the state of compliance with feed or food law, animal health and animal welfare rules (Regulation (EC) No 882/2004 (EC, 2004b)).

Retail - the handling and/or processing of food and its storage at the point of sale or delivery to the final consumer (Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety). In this document, retail covers only shops, supermarkets and other similar outlets that directly serve the final consumer. It does not include distribution terminals or centres, catering operations, factory canteens, restaurants and other similar food service operations and wholesale outlets.

Sampling frame - complete list of all units of the population, which can be sampled.

Sample size - the number of units randomly chosen from the sampling frame.

Survey - a study involving a sample of units selected from a study population. This type of study is often known as a descriptive survey. Its main objective is that of estimating the mean level of some characteristics in a defined population including a measure of the precision for those estimates.

VTEC - Verotoxigenic *Escherichia coli* that produce potent cytotoxins, termed verocytotoxin(s) (VT) that inhibit the protein synthesis within eukaryotic cells. These VTs are also named Shiga toxins (*Stx*). The terms VTEC and STEC (Shiga toxin-producing *E. coli*) are synonymous. VT is encoded by the gene *vtx* and consists of two groups VT1 and VT2, encoded by *vtx1* and *vtx2* genes, respectively.

VTEC positive sample – For the purpose of this specification; a VTEC positive sample is considered to be any sample, from which at least one *E. coli* strain containing *vtx* and *eae* has been isolated. Both gene-groups must be present in the isolated *E. coli* strain, for a sample to be positive.

VTEC serogroup positive sample – Any sample containing at least one isolated *E. coli* strain of the targeted serogroup that harbours *vtx* and *eae*. In this programme, serogroups O157, O26, O103, O111, and O145 are of interest. To be considered VTEC positive both *vtx* and *eae* must be present.

vtx – gene encoding for verocytotoxin 1 (VT1) or 2 (VT2).

3. Rationale for the choices made in the technical specifications

3.1. Rationale for the choice of objectives

Currently there are no harmonised rules or recommendations for the monitoring of VTEC in animal populations and food categories in the EU, even though most MSs carry out monitoring. The stage of sampling, the types of samples taken, and the analytical methods used vary from country to country and also between investigations. This lack of harmonisation has hampered the analyses of data at Community level and therefore there is no clear picture of the occurrence of human pathogenic VTEC serogroups in the relevant animal populations and food categories in the Community. The objectives of these technical specifications are to recommend a harmonised methodology to be used in the monitoring of the most relevant animals and foodstuffs throughout the EU.

Since VTEC O157 is the most frequently reported serogroup in human VTEC infections, including Haemolytic Uremic Syndrome (HUS) cases, in the EU (CSR 2007), the primary objective of these technical specifications is to collect information on the occurrence of this serogroup on the hide of young cattle which is the animal population recognised as an important reservoir of VTEC. The data from the harmonised monitoring will be comparable between MSs and will provide an overall picture of the situation in MSs and in the EU.

Sheep and sheep meat are also considered to be reservoirs of human VTEC infections. Some MSs have a substantial sheep population and, therefore, the monitoring of VTEC O157 in sheep population is presented as a secondary objective in these technical specifications.

The point of sampling is the hide of cattle and fleece of sheep at slaughter. Such samples reflect the VTEC O157 load in slaughterhouses and give an indication of the risk of carcass contamination before de-hiding and thus of further contamination of the meat during the slaughter process. In order that the survey results mirror the VTEC load in slaughterhouses, independent samples are collected, i.e. samples originating from different slaughter batches are taken without the possibility of cross-contamination between batches. Sampling hide and fleece lowers the sample size needed, as the highest prevalence of contamination is expected at this stage of the production chain. In addition, monitoring VTEC O157 just before de-hiding is also of special interest as the implementation of good hygiene practices at this step is an important control measure of carcass contamination.

Other secondary objectives include the monitoring of serogroups other than O157 in cattle and sheep populations. Certain non-O157 VTEC serogroups are also often isolated from human cases, and there are indications that in some MSs non-O157 serogroups are important causes of severe human infection. Therefore, those MSs that have the capacity and capability to analyse for non-O157 serogroups associated with human cases within the country are encouraged to include these serogroups in the monitoring programme.

Since the prevalence of VTEC in these animal populations is not expected to change over a one- to two- year period, conducting the monitoring every three years should provide the necessary information on the situation and trends in each particular country and at EU level. It is therefore

recommended that all MSs carry out this monitoring at three-year intervals. It would also be desirable that all MSs conduct the survey in the same year.

Repeated monitoring over time will enable the following of trends of the prevalence of the different VTEC serogroups in monitored animal populations in the EU and individual MSs.

There are many food categories that may be contaminated with VTEC, and at this stage no category is singled out for more intensive monitoring and trend watching, but instead, general guidelines are given for carrying out specific surveys on the food categories that are most relevant for VTEC infections in humans. This will provide more comparable datasets at EU level that will enable better analyses of the data and the situation regarding VTEC in foodstuffs.

It is acknowledged that food is not the only source of VTEC infections in human, where environmental contamination and direct contact with animals, for example at petting farms, also play a role. However, these technical specifications focus on the food safety aspects of VTEC.

3.2. Rationale for the choice of the risk-based sampling strategy

Prevalence of VTEC O157 in cattle at slaughter has been reported by several MSs and range from <0.1% to 4.6% in faecal samples (Zschock et al., 2000; Kuhnert et al., 2005; Milnes et al., 2008), while the prevalence of VTEC O157 on beef carcasses are generally around 1% to 3% (McEvoy et al., 2003; Tutenel et al., 2003). The expected low prevalence would require large sample sizes (>1,000) to provide sufficient confidence in and accuracy of a prevalence estimate and to enable monitoring of trends over time. To ensure the usefulness of collected data and to reduce the required sample sizes needed, a risk-based sampling approach is recommended, targeting a subpopulation and a sampling stage with an expected higher prevalence. On the hides of cattle, prevalences between 7.3% and 22.2% have been reported (Reid et al., 2002; O'Brien et al., 2005).

3.2.1. Study population

Cattle are the main reservoir of VTEC O157 and will constitute the study population. All MSs have an established cattle industry and monitoring VTEC O157 contamination of cattle at slaughterhouses will provide information from a relatively stable population over time.

Sheep are also an important reservoir of VTEC O157 and some MSs have a substantial sheep industry, which contributes to red meat consumption, milk and cheese production. These MSs could also consider monitoring VTEC O157 contamination of sheep at slaughterhouses. Goats are also known as reservoirs for VTEC O157, but since the goat industry distribution within the EU is limited and very uneven in MSs, this species is not included in these specifications.

While VTEC O157 is also reported from non-ruminant animal populations, many of these animal species are believed to be transient hosts, who only excrete the organism for a short period after infection and they are thus not included in the current sample plan.

Cattle

Sampling will focus on animals between 3-24 months of age as adolescent cattle have the highest prevalence of VTEC O157, whereas cattle outside this age range are less likely to excrete the pathogen (Heuvelink et al., 1998; Paiba et al., 2003). White veal calves are not targeted by the survey because VTEC prevalence in this population is reported to be low.

Sheep

The epidemiology of VTEC O157 in sheep is less explored, but it is likely that it mirrors that of cattle, where adolescent animals are more likely to shed organisms. Thus, sheep between 4-12 months of age are recommended for sampling.

3.2.2. Sampling time

In some MSs the prevalence of VTEC O157 shed by cattle is very low in winter, but higher in summer/autumn (Schouten, 2004, unpublished surveillance data). Other MSs report a more consistent prevalence with no clear seasonal pattern (Paiba, 2003). For harmonisation and to target the high risk subpopulation throughout the EU, it is recommended that sampling of cattle is done between 1 April and 1 October in all MSs, so that the monitoring programme is more cost-effective.

Seasonal shedding patterns of VTEC O157, with a peak during summer months, have also been reported for sheep. Unlike cattle, the slaughter of sheep is seasonal in many MSs, due to traditions in meat consumption practices. Therefore, no specific season for sampling is recommended. If a MS chooses to sample sheep during a specific period of time, the defined period of sampling and justifications should accompany the results at the time of reporting.

3.2.3. Samples

Hide and fleece samples are recommended for monitoring, because risk assessments have shown that the hide/fleece is likely to be a more important source for contamination of carcasses with VTEC O157 than faeces (Teagasc, 2006). Furthermore, the prevalence of VTEC O157 on hides and fleeces in slaughterhouses has been reported to be higher than faecal and carcass prevalence in several animal populations with an increase in prevalence from <4.5% in these matrices to between 7.3% and 22.2% on hides (Reid et al., 2002; O'Brien et al., 2005). Thus, sampling these matrices will allow for a smaller sample set to be examined giving a resultant saving in time and labour. The Scientific Opinion of the Panel on Biological Hazards (BIOHAZ) (EFSA, 2007) also recommended that monitoring data on the prevalence and concentration of VTEC on ruminant coats would assist in the assessment of risk to consumers.

An estimate of VTEC O157 hide contamination prevalence will reflect the risk of cross-contamination of carcasses at dressing, if preventive control measures and stringent hygiene measures are not optimally applied to these steps.

The brisket area of the hide/fleece is chosen for sampling as this has been shown to be a highly contaminated area. This is also the initial point of skin opening and thus, presents a high risk for the transfer of pathogens during carcass dressing.

For practical reasons, sampling of the hide after exsanguination and prior to de-hiding is recommended for bovine samples, and sampling before pelt removal is recommended for ovine animals.

3.2.4. Selection strategy

It is recommended that monitoring take place at the slaughterhouse mainly for practical reasons related to the feasibility of sampling, but also because slaughterhouses are often a more consistent environment with less variation than individual farms. Samples should be distributed according to slaughterhouse throughputs in the country, to represent the number of high risk animals slaughtered at each slaughterhouse. This will eliminate the risk of under/over-representation in large/small slaughterhouses.

VTEC O157 contamination of hides can occur in slaughterhouses and clustering is expected between animals dependent on the level of contact between animals during rearing, transport, housing in lairage and even sequence of passing through the slaughterhouse (Mather, 2007). Contamination will also depend on whether any VTEC O157 shedding animals have been slaughtered earlier on the same day or not, providing additional clusters in data relating to same day slaughter. To maintain independence between samples, minimum physical contact of sampled animals is important, before sampling and ideally only one animal per slaughterhouse should be sampled on any one day. Nevertheless, to improve the feasibility of the sampling, up to three animals can be sampled on one day at one location. However, the sampler should ensure that sampled animals be slaughtered at minimum three-hour intervals to avoid direct cross-contamination. In case more than three animals are sampled during the day, a clustering effect may be introduced that should be accounted for by increasing the sample size.

3.3. Rationale for choice of number of samples to collect

The target sample size may vary depending on whether the sample size is calculated for the purpose of assessing prevalence or for the purpose of determining a trend. Moreover, the sample size may also differ greatly according to the accuracy of the prevalence estimate or the magnitude of the change that will hopefully be detected.

As the monitoring scheme is primarily aimed at estimating prevalence of hide/fleece contamination within the considered subpopulations of cattle and sheep at slaughter, the required sample size calculation is calculated based on the prior prevalence of 12% \pm 4% (absolute accuracy): a minimum of 254 eligible animals should be sampled at random from MS cattle and sheep populations. Secondly, with the aim of monitoring changes in the proportion of contaminated animals over time, the detectable difference is calculated based on the sample size recommended for estimating prevalence. For a number of MSs, the population of eligible bovine and ovine animals may be of a limited size, which may request a lower sample size. However, those 254 eligible animals have to be considered as a minimum, and MSs may wish to collect more samples, for example to compensate possible clustering.

3.4. Rationale for food surveys

The occurrence of VTEC O157 in food is generally reported to be low, even in foodstuffs that intrinsically are at high risk of being contaminated with VTEC O157, e.g. fresh bovine meat. It is not unusual to detect other VTEC serogroups in many types of food, e.g. fresh meat, given that suitable analytical methods are applied. However, the observation that VTEC bacteria are present in a foodstuff is not sufficient to assess the relevance of the findings to public health. Therefore, such findings need to be qualified by the additional typing of isolated strains (virulence characterisation and serotyping) in order to provide meaningful information. The potential low prevalence of VTEC O157 in foods and the fact that laboratory analyses for VTEC are generally laborious, make monitoring of the prevalence of VTEC O157 and VTEC non-O157 in food resources demanding.

It is clear that there is a need for data on the prevalence/emergence of human pathogenic VTEC in foodstuffs, including VTEC O157. It is therefore recommended that MSs provide such data by monitoring foodstuffs that are perceived to be sources of human VTEC infections. These products include fresh meats (cuts of and minced meat, especially beef products, that are eaten with minimal cooking), ready-to-eat fermented meats, fresh produce, raw and low heat-treated milk and derived dairy products (EFSA, 2007). Additionally, focus should be given to products that are frequently identified as the cause of human VTEC infections in MSs, or products that are identified by other means, e.g. case control studies.

It is recommended that the relevant foodstuffs be monitored by conducting specially designed surveys. These surveys should include adequate numbers of representative samples that are investigated with appropriate analytical methods. The surveys should be conducted from time to time targeting one food category at a time. It is further recommended that the surveys be carried out at retail level to estimate

better consumer exposure to VTEC. However, surveys on carcasses are best carried out in slaughterhouses, preferably sampled before chilling.

3.5. Rationale for choice of VTEC serogroups to monitor

The choice of VTEC serogroups to be monitored in the food chain should ideally reflect the picture of VTEC serogroups that are causing disease in humans at any given time. This means that choice should be guided by the periodical analysis of epidemiological data on human infections in different MSs.

VTEC O157 is currently the serogroup most frequently reported as the cause of VTEC associated diseases in MSs. Moreover, it is also commonly associated with the most severe cases of disease i.e. haemorrhagic colitis (HC) and the potentially life threatening HUS (EFSA, 2009). These facts and the existence of well-adapted laboratory methods for the detection of VTEC O157, make up the primary reasons why it is recommended that monitoring initially focus on VTEC O157. The laboratory methods indicated for VTEC O157 do not allow the isolation of sorbitol-fermenting (SF) VTEC O157, which have been associated with human disease in several European countries. However, the zoonotic origin of SF VTEC O157 has not been demonstrated yet, and therefore these strains do not fit with the scope of these technical specifications. Besides, it is expected that, in the future, monitoring will gradually expand to include other VTEC serogroups that are frequently recorded as a cause of severe human diseases. According to the opinion of the BIOHAZ panel on the monitoring of VTEC (EFSA, 2007), the serogroups that currently should be considered beside O157 are: O26, O91, O103, O111, and O145. However, the epidemiology of VTEC differs from MS to MS and therefore it may well be that each MS may have to adopt different monitoring schemes.

With regard to the monitoring of non-O157 VTEC serogroups it is initially recommended to focus on serogroups O26, O103, O111, and O145 and leave out O91. The main reasons for prioritising these four serogroups are: i) their association with HUS; and ii) the fact that the European Committee for Standardization (CEN) is currently developing standard methods for their detection in foodstuffs. However, recently new methods are constantly being developed and as new methods are standardised at EU level it may be possible to expand the monitoring programme to include other relevant serogroups (e.g. O91).

3.6. Rationale for choice of laboratory methods

3.6.1. VTEC O157

Internationally recognised standard methods for the detection of *E. coli* O157 in food (and feed) are based on specific immunomagnetic separation (IMS) after an enrichment step. It is acknowledged that other methods that are validated against ISO 16654:2001 (ISO, 2001) in accordance with ISO 16140:2003 (ISO, 2003) and certified by relevant bodies, are in use in some MSs. However, for harmonisation and comparison purposes, the use of ISO 16654:2001 (ISO, 2001) is recommended in the current monitoring programme.

Since there is no ISO method for detecting *E. coli* O157:H7 from animal samples, the ISO 16654:2001 (ISO, 2001) method has been adapted for the examination of animal faeces in many investigations. This method could also be used for other animal samples, such as contaminated hides and fleeces as recommended samples in the present programme.

Different media and conditions have been successfully used for the enrichment of animal samples. Enrichment without antibiotics, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004, Chapter 2.10.13, have been used in several reported investigations in MSs. Conversely, other studies adopted media containing selective antibiotics such as novobiocin or vancomycin, with or without the addition of cefixime-tellurite. For the purposes of the present technical specifications, the choice of buffered peptone water (BPW) without antibiotics as an enrichment medium for hide/fleece samples has been made to allow use of the same enrichment

culture for isolation of both VTEC O157 and VTEC non-O157. An increase in the incubation temperature to 41.5°C from 37°C, as recommended by the OIE method, has been included in the protocol to improve the selectivity of the enrichment step.

After detection and isolation of *E. coli* O157 by ISO 16654:2001 (ISO, 2001), the isolates must be confirmed as both *eae* positive and *vtx* positive as *E. coli* O157 may be present without these genes and only *eae* positive VTEC are to be reported as positive.

3.6.2. VTEC non-O157

The Working Group 6 of the Technical Committee 275 of the European Committee for Standardization (CEN TC275/WG6) has drafted a Real-time PCR-based horizontal method for the detection of VTEC belonging to O26, O103, O111, O145 and O157, including SF VTEC O157, serogroups in food (and animal feeding stuffs). The draft has been submitted to ISO in the form of “Technical Specification” and is presently under evaluation.

The method targets a combination of both virulence genes and serogroup specific genes (Nielsen and Andersen, 2003; Perelle et al., 2004; Perelle et al., 2005) and it is based on experiences proposed by the CEN. The virulence genes include those encoding VT (*vtx1* and *vtx2*) and *eae*. The “Technical Specification” is based on the Real-time PCR screening of samples, followed by a confirmation step with the isolation of the VTEC strains responsible for the positive PCR reactions.

The draft “Technical Specification” is intended for the examination of food (and feed), but it can also be applied to samples such as contaminated hide and fleece. In this case, since there is no enrichment protocol validated for all four serogroups, BPW without antibiotics is chosen for the enrichment step. This choice allows the use of the same enrichment culture used for the isolation of VTEC O157.

3.6.3. Storage of isolates

Storage of isolates is recommended so that further characterisation and typing could be performed as it is relevant for comparison with strains causing human infection. It is recommended that National Reference Laboratories for VTEC (NRLs) should store isolates so that they can be made available for further characterisations and typing.

3.7. Rationale for choice of reporting

The monitoring of VTEC is reported to the EC in accordance with Directive 2003/99/EC. To ensure sufficient information on VTEC, facilitating a proper summary and assessment of the importance of the findings of VTEC from foodstuffs and animal populations to human VTEC cases, reporting needs to be strictly harmonised.

According to the Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on Verotoxigenic *E. coli* (VTEC) in foodstuffs (EC, 2003), only a small fraction of all VTEC isolated from animals or food are associated with human illness. As the majority of VTEC considered potentially pathogenic to humans carries both the *eae* in addition to the virulence genes encoding VT (*vtx1* and/or *vtx2*), only *eae* positive VTEC are to be reported under these specifications.

The severity of human disease is related to the *vtx* variant present in the disease causing VTEC. Information on *vtx* variants, *vtx1* and *vtx2*, is therefore important to estimate whether the VTEC isolates from foodstuffs and animals are pathogenic to humans, and are therefore to be reported.

Some MSs probably carry out further typing methods than recommended through these technical specifications. To keep the reporting harmonised, such data are not to be reported under these specifications. Over time more MSs may start further typing as well. The current specifications should

be reviewed within a few years, and the reporting of further typing methods should then be considered.

3.8. Rationale for review after first survey

It is recommended that these technical specifications be reviewed when the first survey is completed. This is necessary because the survey will add new experience and knowledge to an emerging and evolving area of food-borne VTEC infections. At the time of the development of the technical specifications, some areas are well-tested and well-known. These include, in broad terms, the epidemiology of VTEC O157 in cattle and laboratory methods for VTEC O157. Other areas are less explored, including the epidemiology of all VTEC serogroups in sheep and of non-O157 in cattle, and laboratory methods for non-O157 serogroups. The use of BPW without antibiotics as an enrichment medium for hide and fleece samples is also a novel approach.

A review of the technical specifications after the first survey in MSs will allow the adjustment of sample sizes and data collection procedures and will ensure that any improvement in laboratory methods be incorporated into the programme. This will increase the precision of prevalence estimates in future surveys. Also, a review could consider the inclusion of further typing methods (H-typing, *vtx* subtyping, pulsed field gel electrophoresis (PFGE), etc.).

4. Objectives

The general objective of these technical specifications is to define a harmonised scheme for the monitoring and reporting of the occurrence of VTEC in certain animal populations and/or foodstuffs in order to obtain comparable data from EU MSs. Results from such harmonised methodology should provide data for analyses of trends in the occurrence of VTEC in specific MSs and at EU level.

The primary objective is to

- determine a standardised and comparable estimate of the occurrence of VTEC O157 on the hide of young cattle at slaughter within the MSs and in the EU.

The secondary objectives are to:

- determine a standardised and comparable estimate of the occurrence of VTEC O157 on the fleece of young sheep at slaughter within the MSs and in the EU;
- determine the occurrence of the VTEC non-O157 serogroups of O26, O103, O111, and O145 on the hide of young cattle and fleece of sheep at slaughter within the MSs and in the EU;
- gather data on the occurrence of VTEC O157, and when possible of VTEC O26, VTEC O103, VTEC O111, and VTEC O145 in food categories that are most likely to be contaminated with VTEC; and
- assess temporal trends in VTEC occurrence on hide of cattle and fleece of sheep and, if possible, in foodstuffs.

Initially, monitoring should concentrate on VTEC O157 since this serogroup is most frequently associated with severe human VTEC infections (including HUS) in the EU. However, individual MSs could extend the monitoring to those non-O157 serogroups that are known frequently to cause human infections in the EU (i.e. O26, O103, O111 and O145).

Recommended monitoring takes the form of regular surveys based on representative samples.

5. Survey of contamination on animals at slaughter

5.1. Animal population

5.1.1. Cattle

Eligible animals

Cattle at 3-24 months of age slaughtered from 1 April to 1 October are eligible for sampling. However, white veal calves are excluded from the survey.

Slaughterhouse selection

Each MS will rank all slaughterhouses by throughput of cattle aged 4-12 months between 1 April and 1 October in the previous year. Starting with the slaughterhouses of largest throughput, sufficient slaughterhouses should be enrolled to cover at least 60% of the national throughput of eligible animals. In MSs that have many small slaughterhouses, an upper limit of 10 slaughterhouses can be considered for logistical reasons.

Animal selection

A list of participating slaughterhouses is then compiled, and the predetermined number of animals to be sampled is distributed according to the proportional throughput from 1 April to 1 October in the previous year. See the example in Table 1.

The sample size for each slaughterhouse is allocated proportionally to the slaughterhouse throughput in the sampling period. Preferably, a maximum of one animal per slaughterhouse is sampled per day to ensure that there is no correlation between positive results that may derive from direct or indirect contact between sampled animals before slaughter. Samples at each slaughterhouse are distributed randomly to a list of fully operational slaughter days. For this purpose, a number of sampling days (equal to the sample size) is randomly selected from the operating days of the slaughterhouse. However, it may be necessary to exclude Fridays due to laboratory and courier limitations. If applicable, “industrial holidays” where slaughterhouses may not be fully operational, should also be excluded. The animal to be sampled is chosen at random from the animals slaughtered on the selected day.

Where it is not possible to sample only one animal per day per slaughterhouse due to logistical reasons, up to three animals per day can be sampled, in a way that ensures that sampled animals are slaughtered at minimum three-hour intervals to avoid direct cross-contamination.

In exceptional cases, when it appears, for particular economical and organisational reasons, that it would be more cost-effective to collect a greater number of samples in a day, more than three animals may be sampled per day per slaughterhouse, provided that a potentially introduced clustering effect is accounted for by increasing the sample size of the survey.

In all cases, operators should check that animals to be sampled originate from different herds, that they have been transported in different lorries and stocked in different lairage areas, to limit the probability of cross-contamination between the sampled animals.

5.1.2. Sheep

Eligible animals

All sheep slaughtered at the age of 4-12 months are eligible. Slaughter patterns of sheep are very MS-specific and a MS may choose to sample only during high season slaughtering. However, the defined period of sampling and justifications should accompany results at the time of reporting.

Slaughterhouse selection

Each MS will rank all slaughterhouses by throughput of sheep aged 3-12 months during the relevant slaughter season of sheep in the previous year. Starting with the slaughterhouses of largest throughput, sufficient slaughter houses should be enrolled to cover at least 60% of the national throughput of eligible animals. In MSs that have many small slaughterhouses, an upper limit of 10 slaughterhouses can be considered for logistical reasons.

Animal selection

The sample size for each slaughterhouse is allocated proportionally to the slaughterhouse throughput in the sampling period (see the example in Table 1). The samples are distributed randomly from a list of fully operational slaughter days. It may be necessary to exclude Fridays due to laboratory requirements. If applicable, “industrial holidays” where slaughterhouses may not be fully operational, should also be excluded. Preferably, a maximum of one animal per slaughterhouse can be sampled per day to avoid any previous physical contact between sampled animals before slaughter. The animal to be sampled is chosen at random from the animals slaughtered on the selected day.

Where it is not possible to sample only one animal per day per slaughterhouse due to logistical reasons, up to three animals per day can be sampled, in a way that ensures that sampled animals are slaughtered at minimum three-hour intervals to avoid direct cross-contamination.

In exceptional cases, when it appears for particular economical and organisational reasons that it would be more cost-effective to collect a greater number of samples in a day, more than three animals may be sampled per day per slaughterhouse, provided that a potentially introduced clustering effect is accounted for by increasing the sample size of the survey.

In all cases, operators should be asked to check previously that animals to be sampled originate from different herds, have been transported in different lorries and stocked in different lairage areas, to avoid the risk of sampling animals becoming cross-contaminated.

Table 1: Example of the selection of animals to be sampled in the slaughterhouse

| Slaughterhouse | Throughput in last year | Proportion of throughput | Contribution to 60%* of national throughput | Proportion of 60%* of throughput | Number of animals sampled |
|----------------|-------------------------|--------------------------|---|----------------------------------|---------------------------|
| 1 | 11,000 | 0.22 | 11,000 | 0.323 | 82 |
| 2 | 10,000 | 0.2 | 10,000 | 0.294 | 75 |
| 3 | 7,500 | 0.15 | 7,500 | 0.221 | 56 |
| 4 | 5,500 | 0.11 | 5,500 | 0.162 | 41 |
| 5 | 5,000 | 0.1 | 0 | 0 | 0 |
| 6 | 4,000 | 0.08 | 0 | 0 | 0 |
| 7 | 2,500 | 0.05 | 0 | 0 | 0 |
| 8 | 500 | 0.01 | 0 | 0 | 0 |
| Total | 50,000 | 1 | 34,000 | 1 | 254 |

* at least 60%

5.2. Sample size

The sample size (number of eligible animals to be tested for VTEC O157) should, primarily, enable calculation of the contamination prevalence of VTEC O157 on cattle hides and sheep fleeces in MSs within a predetermined accuracy and, secondarily, detect changes in this prevalence over time (see Appendix A).

For the purpose of prevalence assessment, it is recommended that the minimum number of eligible animals tested annually for VTEC O157 per study population in each MS be 254. This sample size provides an accuracy of +/-4% around an assumed 12% prevalence of contaminated hides/fleeces at slaughter.

This sample size enables detection of a change of +/-7% from one survey to the following, under the assumption of an initial VTEC O157 prevalence of 12%. In the situation of a very low prevalence (0.1%), an increase of +4.5% from one survey to another is detectable.

In case a linear trend exists within a country, smaller changes in proportion can be detected over time. In the case of three-yearly monitoring:

- starting from an initial proportion of contamination of 12%: a 3% decrease in the proportion of contamination per year can be detected; and
- starting from an initial proportion of contamination of 1%: an increase by 3% per year can be detected.

MSs are recommended to include a minimum of 254 eligible animals from each study population (i.e. cattle, sheep), as shown in Table 2.

Table 2: Sample sizes for cattle and sheep populations

| Target population | | No. of eligible animals |
|-------------------|-------------------------------------|-------------------------|
| Animal species | Study population (eligible animals) | |
| Cattle | Animals of 3-24 months of age | 254 |
| Sheep | Animals of 4-12 months of age | 254 |

A minimum number of 254 eligible animals shall be sampled at random based on an infinite population, an estimated prevalence of 12%, a confidence level of 95%, and an accuracy of 4%. Non-response shall be anticipated, e.g. by increasing the sample size by 10% when implementing the survey. However, the population of eligible cattle and sheep may not be infinite for some MSs. In the case of a finite population, the sample size should be adapted, i.e. whenever the calculated sample size is greater than 10% of the eligible population. Appendix A contains the formula on how to make the calculation.

The sample size of 254 samples should be considered as a minimum and cost-effective sample size in the framework of an initial survey, because little information on the variation of the level of contamination between MSs is available. The prior 12% was chosen to prioritise good estimates from MSs with low prevalence of VTEC O157 and may present an under-estimate for high prevalence MSs. However, for the particular case of the smallest MSs, a smaller sample size reflecting cattle or sheep population can be applied.

5.3. Sample collection

5.3.1. Type and detail of sample

Sampling shall be performed by the competent authority or under its supervision, by bodies to which this responsibility has been delegated. The material collected for bacteriological analysis will originate from the coat (hide or fleece) which is representative of the amount of contamination which is on animals being presented for slaughter.

Hide swabs of cattle

Swabs have to be taken from the brisket area of the animal after exsanguination and prior to de-hiding.

The swab should be taken using a pre-moistened sponge swab (polyurethane sponges, 100 mm x 100 mm x 10 mm) which have been prepared by placing in an autoclavable stomacher bag with 10 ml sterile Maximum Recovery Diluent (0.1% Peptone, 0.85% NaCl), sealed with autoclave tape and autoclaved for 15 minutes at 121°C.

The bag is either held outside and turned inside out (used as a glove) or a fresh pair of sterile gloves are used to wipe the sponge swab with firm pressure over the test surface (400 cm²), 10 times in the horizontal direction, and then 10 times in the vertical direction (EFSA, 2006). The bag should then be re-inverted over the sponge and resealed. All swabs must be placed in separate bags avoiding cross-contamination.

Fleece samples

Samples have to be taken from the brisket area of the animal before pelt removal. A sterile stomacher bag is inverted over one hand so that the inside of the bag can be used to grasp a handful of fleece/wool. Sharp sterile scissors are used to cut at least 10 g of fleece into the bag. The bag is re-inverted and then secured with an elastic band. In the case that there is a lack of fleece in the relevant brisket area, a swab can be used to wipe the brisket area instead of collecting fleece samples, in accordance with the specifications made for cattle.

5.3.2. Sample information

All relevant information should be recorded on a sampling form produced by the competent authority to fulfil data requirements set out in the section “Reporting”.

Each sample and its sample form should be labelled with a unique number. The competent authority must arrange for the issue and use of a unique numbering system.

5.3.3. Transport of samples to laboratory

All samples should be stored preferably between +2°C and +8°C and free of external contamination during storage and transportation. Samples should be sent for analysis as quickly as possible.

6. Surveys of VTEC in foodstuffs

Surveys on VTEC in food should focus on foodstuffs that are perceived to be the most important sources of human VTEC infections. It is advisable to conduct these surveys from time to time targeting one food category at a time and including an adequate number of representative samples. With the exception of carcasses, surveys should be carried out at retail level.

6.1. Food categories and types of samples

The relevant food categories recommended for survey include:

Carcasses

- Samples from ruminants – bovines, sheep, goats and game ruminants
- Samples should be taken after dressing but before chilling
- Samples should be taken in accordance with European Commission Regulation (EC) No 2073/2005 (EC, 2005) (swabbing or excision - technique for carcass sampling)

Fresh meat from ruminants

- Meat from bovines, sheep, goat or deer
- Cuts of meat (large/small) with special focus on meat intended to be eaten raw or with minimal cooking

Minced meat and meat preparations

- Meat from bovines, sheep or mixed meat including bovine or sheep meat
- Special focus on minced meat and meat preparations intended to be eaten raw or with minimal cooking and meat tenderised with “needle techniques”

Ready-to-eat dried or fermented meat products

- Fermented sausages, such as salami, pepperoni, containing ruminant meat
- Products made from bovine, sheep or mixed meats including bovine or sheep meat

Fresh vegetables and salads

- Ready-to-eat pre-cut vegetables (leaf salads, spinach etc.)
- Ready-to-eat sprouted seeds

Raw and low heat-treated milk and dairy products thereof

- Raw and low heat-treated milk from cows, goats, and sheep intended for direct human consumption
- Cheeses, especially soft or semi-soft, made from raw and low heat-treated milk

6.2. Sample size

Guidance on the selection of the number of samples needed in a targeted survey on foodstuffs is provided in Appendix B. Due to the low estimated occurrence of VTEC O157, the sample size is likely to be rather high, in the magnitude of 500 to 3,000 samples per survey depending on the expected proportion of positive samples, and the desired accuracy and level of confidence in the results.

6.3. Sample collection

6.3.1. Type and detail of sample

The sampling strategy in the survey should ensure that the results of the investigations give a representative picture of the proportion of VTEC positive samples in the foodstuff category investigated.

The objective is to collect randomly selected samples of the categories of the chosen foodstuffs, for example, randomly chosen carcasses at slaughter, or randomly chosen foods at customer display e.g. refrigerator shelves/freezer cabinets in the selected retail outlet, where possible whole retail packs should be collected.

Samples, which are packaged, must not show evidence of damage. If the label on the product is not clear or is damaged then the sample should not be used. When fresh, unpackaged meat, minced meat or meat preparations are collected, e.g. from smaller butchers, the sampling officer may need to inquire about the best before/use by date and other information, which is normally on the label of packaged meat so that this can be recorded.

It is essential that cross-contamination be avoided during the collection of samples. Precautions must therefore be taken at all stages to ensure that the equipment used during sampling, transport and storage are not contaminated with VTEC. Each sample should consist of at least 25 g of material of interest.

6.3.2. Sample information

All relevant information available from the sample should be recorded on a sampling form produced by the competent authorities to fulfil the data requirements set out in the section “Reporting”.

6.3.3. Transport of samples to laboratory

Once collected, each sample should be placed in a separate sampling bag to avoid the risk of cross-contamination, and sent to the laboratory for testing. The samples should be kept at the temperature recommended by the food producer during shipment, i.e. chilled samples must be kept chilled and frozen samples should be kept frozen. All samples should reach the laboratory before the end of the shelf life of the products.

7. Laboratory analytical methods

7.1. Laboratories

National Reference Laboratories for *E. coli* (NRLs) are the laboratories responsible for the analyses described below (detection, identification, typing). However, the competent authorities may decide to designate other laboratories involved in official controls of food and animal samples to perform the analyses, under the supervision of the NRLs. These laboratories should have proven experience of using the required detection method, and have an accredited quality assurance system complying with ISO 17025 (ISO 2005).

7.2. Preparation of specimen

Ideally, laboratory analyses commence within 24 hours of sampling. In exceptional situations (e.g. long journeys, weekends and public holidays) this period may be extended to 80 hours. All samples received shall be examined to ensure that the transport packaging is intact and that the temperature of the samples is acceptable before analyses are started. The laboratory should ensure that the samples have been maintained at the appropriate temperature during transport and that the analysis is started before the end of the shelf life of the product. Handlers must take care to avoid cross-contamination between samples and from the surrounding environment at all stages.

7.2.1. Hides and fleeces

Plastic bags containing the samples (see paragraph 5.3.1), are opened using sterile scissors. The hide sponge is taken from the bag and put inside a stomacher bag before the addition of pre-warmed enrichment broth. For fleece samples, 10 g are placed inside a stomacher bag before the addition of the broth.

7.2.2. Foodstuffs

Plastic bags containing the samples (see paragraph 6.3), are opened using sterile scissors. In general, it is recommended that 25 g of the sample be analysed. The sample for analysis should be representative of the whole food product. When analysing cuts of meats it is important to analyse as much of the surface of the meat as possible. Before enrichment is started, the samples for analysis should be treated according to ISO 6887-1/4 (ISO, 1999, 2003) which specify preparation of food samples and their suspension for microbiological examination, and notably include thorough mixing of samples by a stomacher.

7.3. Detection and identification methods

The sequence of the analyses is displayed in Figures 1 and 2 for the animal and food samples, respectively.

7.3.1. Hides and fleeces

7.3.1.1. Detection of VTEC O157

The ISO 16654:2001 (ISO, 2001) standard method for the detection of *E. coli* O157 in food and feed, shall be used for hide and fleece with the following modifications, concerning the enrichment step:

- cattle hide swabs: the hide sponge shall be mixed in 90 ml of pre-warmed BPW and incubated at $41.5 \pm 1^\circ\text{C}$ for 18 hours; and
- sheep fleece samples: 10 g of fleece shall be mixed and homogenized in 90 ml of pre-warmed BPW and incubated at $41.5 \pm 1^\circ\text{C}$ for 18 hours.

The immunomagnetic separation and isolation steps will be performed at the end of the enrichment stage according to the protocol described in ISO 16654:2001, as well as the identification of suspected colonies. The *E. coli* O157 strains isolated must be confirmed as VTEC by testing for the presence of *eae* and *vtx*. A suitable PCR method for detecting *eae* and *vtx* genes is provided in Appendix C.

7.3.1.2. Detection of non-O157 VTEC

The recommended detection method is a Real-time PCR protocol submitted to ISO in the form of “Technical Specification” by Working Group 6 of the Technical Committee 275 of the European Committee for Standardization (CEN TC275/WG6). The method targets both virulence genes (*vtx1* and *vtx2*, and *eae*) and serogroup-specific genes for O26, O103, O111 and O145. It can be used for screening samples, but requires a confirmation step with the isolation of the VTEC strains responsible for the positive PCR reactions.

The method is applied to the same enrichment culture performed for the isolation of VTEC O157. One millilitre aliquot of such a culture is used for deoxyribonucleic acid (DNA) extraction and purification. This step shall be accomplished according to ISO 20837:2006: “Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for sample preparation for qualitative detection” (ISO, 2006). The remaining culture shall be stored at 4°C for the isolation steps that will follow a positive PCR result.

A Real-time PCR is performed according to the above-mentioned experiences (Nielsen and Andersen, 2003; Perelle et al., 2004; Perelle et al., 2005). A synopsis of the primer and probe sequences is provided in a guideline included as Appendix 4. Amplification conditions to be applied may vary depending on the system used, and will refer to the instructions supplied with the instrument and kit of choice.

The method is sequential:

- Step 1: Enrichment of the sample.
- Step 2: Detection of the genes *vtx1*, *vtx2* and *eae*.
- Step 3: Samples positive for both *vtx* and *eae* at the second step are tested for the serogroup-associated genes (molecular serogrouping).
- Step 4: Isolation of the VTEC strain; samples positive at the same time for *vtx*, *eae*, and serogroup-associated genes are submitted to a further step aimed at the isolation of the VTEC strain. This requires serogroup-specific enrichment based on IMS or other immuno-capture suitable approaches. A guideline for the isolation of the different VTEC serogroups is included as Appendix E.
- Step 5: Characterisation of the isolate i.e. identification, detection of *vtx* genes, the *eae* gene and the serogroup gene.

7.3.2. Foodstuffs

7.3.2.1. Detection of VTEC O157

The detection of VTEC O157 in all types of foods is done according to ISO 16654:2001 (ISO, 2001), which employs modified Tryptone-soy broth (TSB) supplemented with 20 mg/l novobiocin as enrichment broth. The *E. coli* O157 strains isolated must be confirmed as *eae* positive VTEC by testing for the presence of *eae* and *vtx* genes, as described for animal samples.

7.3.2.2. Detection of non-O157 VTEC

The detection of non-O157 VTEC serogroups in foodstuffs is done according to the Technical Specification prepared by the VTEC ad hoc Group of the CEN TC275/WG6. With the exception of the enrichment step, the same Real-time PCR protocol described for identifying *eae* and *vtx* genes in animal samples are used.

For food samples, the enrichment culture prepared for the detection of VTEC O157 cannot be used, since the novobiocin concentration of 20 mg/l can inhibit the growth of many non-O157 strains (Vimont et al., 2007), increasing the risk of false negative results. Therefore, according to the Technical Specification, samples are mixed at a dilution of 1/10 in TSB modified by the addition of 1.5 g/l bile salts no. 3 and supplemented with either 16 mg/l of novobiocin or, for dairy products, 12 mg/l of acriflavin. When frozen samples are processed, pre-warmed BPW without antibiotics are used as an enrichment medium. Enrichment cultures are incubated at 37±1°C for 18 hours.

7.4. Storage of isolates

It is recommended that all the VTEC isolates be sent to the NRL for storage. Storage of isolates is important, since it will allow further characterisation and typing relevant for comparison with strains causing human infections.

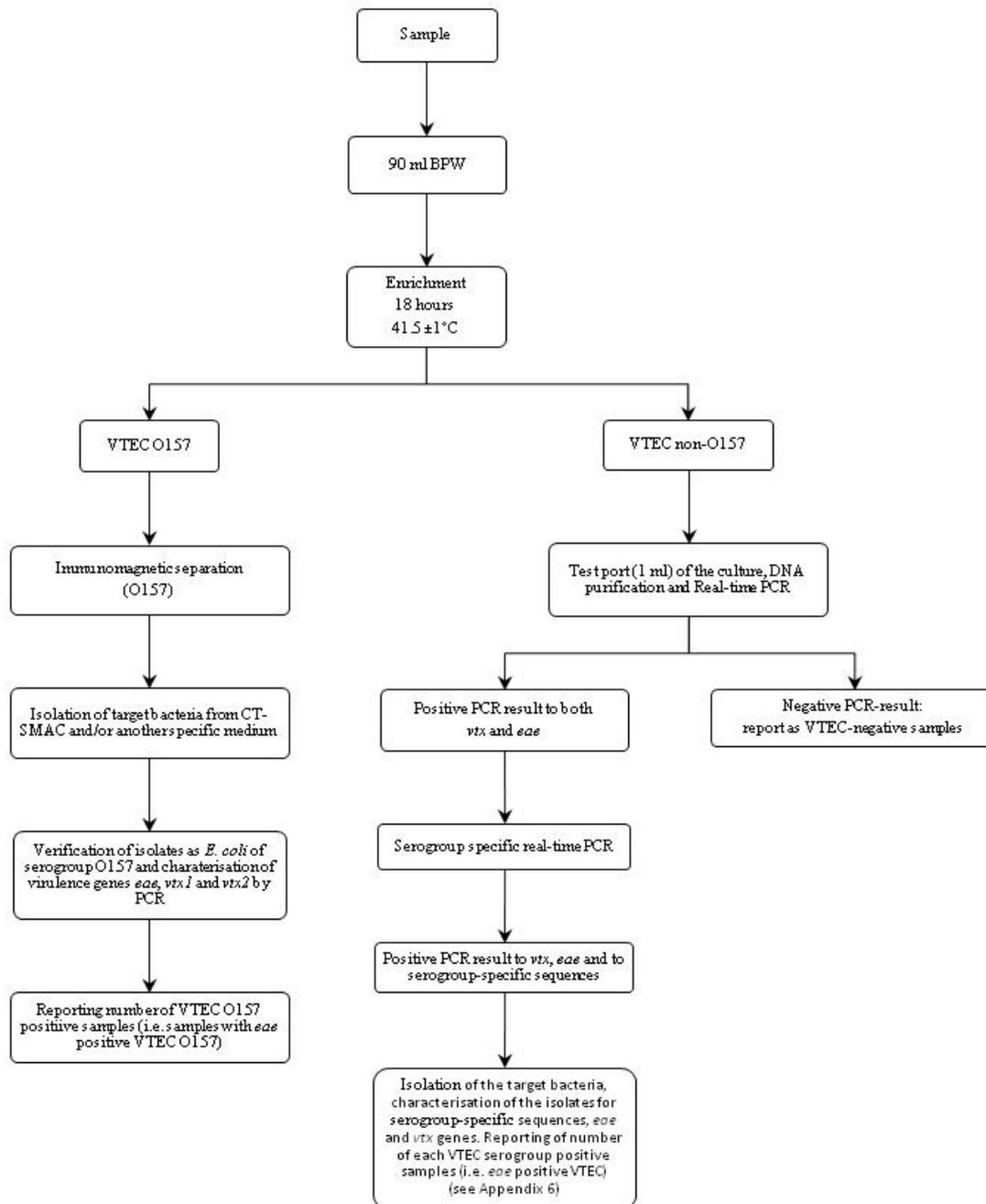


Figure 1: Flow diagram for the detection of VTEC on hides and fleeces

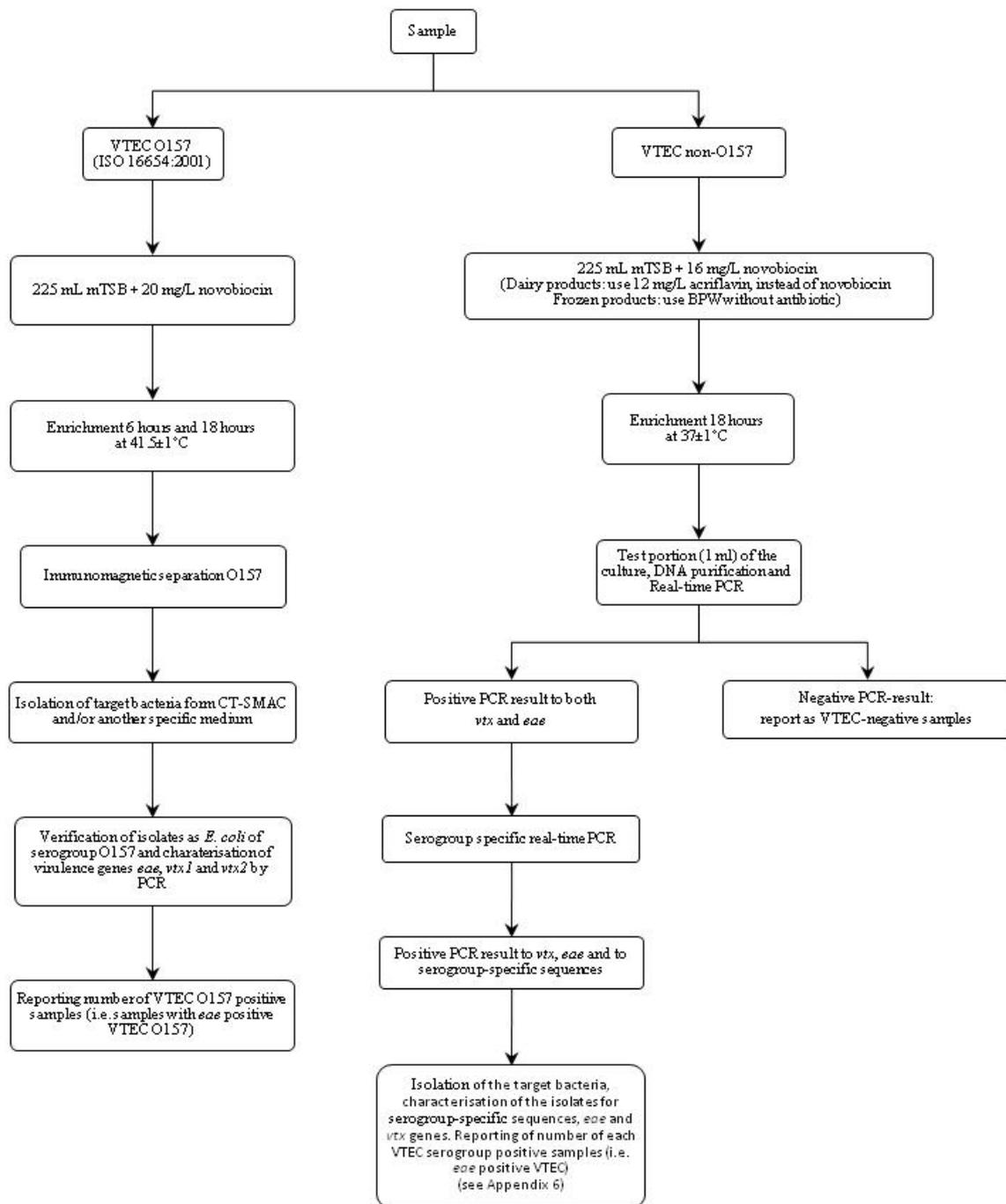


Figure 2: Flow diagram for the detection of VTEC in foodstuffs

8. Reporting from MSs

8.1. General provisions

The competent authority responsible for the preparation of the yearly national report on zoonoses pursuant to Article 9 of Directive 2003/99/EC should ensure that the results of the surveys are collected, evaluated and reported to the annual Zoonoses report to the Commission and EFSA.

The information to be reported by MSs is outlined in subsections 8.2.1. and 8.2.2. for the monitoring of animals, i.e. cattle hides and sheep fleeces, at slaughter and in 8.3.1. and 8.3.2. for the monitoring of foodstuffs, and consists of two broad categories: description of the monitoring programme and survey results. In addition, Appendix F provides some information that may be useful to be collected at national level as a supplement, but which is not to be reported in the framework of Directive 2003/99/EC.

8.2. Survey of contamination on cattle hides and sheep fleeces at slaughter

8.2.1. Overview of the survey

The description of the survey should provide an overview of the monitoring in its entirety in the country. These descriptions are included in the zoonoses web reporting application (www.efsa.europa.eu/zoonoses) in the text forms.

- If the sampling strategy, the methods of sampling, and the analytical methods applied were according to all technical specifications given in this report, e.g. sampling regimen, frequency of sampling, or type of sample, reference to this report is sufficient. However, for the sake of clarity the animal species, age of animals, time of sampling, and VTEC serogroups analysed are specified in the text.
- If any modification to the sampling strategy, methods of sampling or laboratory methods presented in these technical specifications was made, reference to the technical specifications is given. However, but these modifications are explained in detail in the text form.
- If other sampling strategies, methods of sampling or laboratory methods were applied, a description of sampling (i.e. sampling regimen, frequency of sampling, type of sample, method of sampling, case definition etc.) are given.

The text form(s) are to be created and filled in as specified in the following. One form for each animal species is made.

Zoonotic agent/zoonoses

“*eae* positive VTEC” since only *eae* positive VTEC are reported.

Animal species

“Cattle” and/or “sheep” and further subcategories “young cattle 3-24 months” and/or “lambs 4-12 months”, respectively.

Sampling strategy

Fill in text field.

Example: “Sampling strategy, methods of sampling and analytical methods applied according to all technical specifications given in the "EFSA Guidance on surveys on VTEC in animals and food, EFSA Journal 2009, xxx". Any deviations from the specifications are described.

In addition, the following are specified:

- animals sampled: cattle and/or sheep and their age;
- time of sampling: cattle during the months [MS to insert corresponding month], sheep during the months [MS to insert corresponding month]; and
- serogroups analysed: O157, O103 and O26.

Frequency of sampling

Animals at slaughter: for cattle select “Sampling takes place during the months” and specify the months. Select the same for sheep if sampling is restricted to a specific time period, otherwise select “Sampling distributed evenly throughout the year”.

Type of specimen taken

Animals at slaughter: “other” and specify “cattle hide” and/or “sheep fleece”.

Methods of sampling/Animals at slaughter

A reference to the EFSA Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food, EFSA Journal 2009, 7(11).

Case definition

Animal where *eae* positive VTEC is found from a hide/fleece sample.

Laboratory methods used

“ISO 16654:2001” for VTEC O157 and/or “Molecular and Bacteriological method” for non-O157 VTEC.

Remaining items

Fill in text fields where relevant and as appropriate.

8.2.2. Overview of the results in the tables

The results of testing of the samples are reported in tables provided by the zoonoses web reporting application (www.efsa.europa.eu/zoonoses).

The sample information accompanying the results shall include the animal species tested i.e. cattle and/or sheep and the animal subcategory “young cattle 3-24 months” and/or “lamb 4-12 months”, respectively.

Results table specifications

- Sampling stage: “slaughterhouse, hide/fleece”
- Sampling context: “Survey, national”
- Sampling unit: “animal”
- Sample weight: fill in sample weight used in the laboratory (i.e. swab/fleece 10 g)
- Number of units tested: fill in the number of animals tested
- If for some reason only a few units (animals) have been analysed for a specific serogroup, the results of these units are reported on a separate row

Total number of units positive for:

- *eae* positive VTEC: number of units positive for *eae* positive VTEC (all serogroups).

The breakdown of these positive units into different *eae* positive VTEC serogroups with *vtx* profiles:

- only *eae* positives are reported. The correct VTEC O-group are added to the table from a drop down list;
- for serogroups analysed with none *eae* positive VTEC positives, fill in number “0”.

Example for *eae* positive VTEC O157:

| | |
|---|--|
| <i>eae</i> positive VTEC O157 that are <i>vtx1</i> positive | select “ <i>eae</i> positive VTEC O157” and subgroup “ <i>vtx1</i> ” |
| <i>eae</i> positive VTEC O157 that are <i>vtx2</i> positive | select “ <i>eae</i> positive VTEC O157” and subgroup “ <i>vtx2</i> ” |
| <i>eae</i> positive VTEC O157 that are <i>vtx1</i> & <i>vtx2</i> positive | select “ <i>eae</i> positive VTEC O157” and subgroup “ <i>vtx1</i> & <i>vtx2</i> ” |

8.3. Survey of foodstuffs

8.3.1. Overview of the monitoring programme (surveys)

The description of the programme should provide an overview of the monitoring programme and surveys carried out in their entirety in the country. These descriptions are included in the zoonoses web reporting application in the text forms.

- Description should include information on sampling regime, frequency of sampling, foodstuffs categories sampled, analytical methods, serogroups analysed etc.

Text form specifications:

- Zoonotic agent/zoonoses: “*eae* positive VTEC” since only *eae* positive VTEC are reported
- Foodstuff category: “food” or specific food category
- Sampling strategy: fill in text field
- Frequency of the sampling: fill in text field
- Type of specimen taken: select correct foodstuff category from drop-down menu, for the selection “other”, specify foodstuff category on text
- Methods of sampling: fill in text field
- Definition of positive finding: food sample where *eae* positive VTEC is found

For the other fields the same applies as to animals.

8.3.2. Overview of the results in the table

The testing results of the samples are reported in tables provided by the web reporting application.

The sample information accompanying the results shall include details on the foodstuff category sampled and the sampling stage.

Results table specifications

Sampling stage: “slaughterhouse” or “retail”.

Sampling context: “Survey, national”.

Sampling unit: “single” or “batch”.

Sample weight: fill in sample weight (typically 25 g).

- If for some reason only a few units have been analysed for a specific serogroup, add this in the comments field.

The number of positive units is reported in the same way as for animals.

9. Review of the monitoring scheme

It would be desirable to review the technical specifications and the monitoring scheme when the first survey is completed as this would increase the precision of VTEC prevalence estimates in future surveys. Special reference should be paid to VTEC serogroups and food categories to monitor, survey and design laboratory methods.

Analysis of serogroups occurring in human VTEC infections in different MSs should be carried out and serogroups to monitor in animals and food categories adjusted according to these analyses. Rapidly evolving new methods to detect different VTEC serogroups might make it possible to expand the technical specifications to include other relevant serogroups. Periodical analysis of foodstuffs identified as sources of human infections in MSs should be carried out and focus should be given to these in food surveys. The minimum number of hide and fleece samples analysed should be adjusted, if necessary, to meet the primary and secondary objectives of the technical specifications. The possible effect of using BPW without antibiotics as an enrichment broth for VTEC O157 in hide and fleece samples for the prevalence of this bacterium should be carefully evaluated. If any new methods for the detection of VTEC or modifications to the existing methods should be given by European standardisation bodies, the laboratory methods stated in this monitoring programme should be re-examined. Also, it should be evaluated whether the technical specifications should be extended to include further characterisation or typing methods (*vtx* subtyping, H-typing, PFGE etc.).

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APPENDICES

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A. SAMPLE SIZE CALCULATIONS FOR MONITORING VTEC

The following assumptions were used for the calculations:

- (a) infinite population size for the number of eligible animals in each study population and MS;
- (b) a confidence level of 95% and a power of 80%;
- (c) perfect sensitivity and specificity of the diagnostic test (VTEC O157 testing).

1. Primary objective

- **Precision of proportion of VTEC O157 contamination estimate**

The following table lists the expected precision of the proportion of VTEC O157 contamination estimates for different sample sizes and proportions of contamination:

| VTEC O157 prevalence | Absolute precision | | | | |
|----------------------|--------------------|------|-----|------|-----|
| | 2% | 3.5% | 4% | 4.5% | 5% |
| 15% | 1,225 | 400 | 306 | 241 | 196 |
| 12.5% | 1,050 | 343 | 263 | 207 | 168 |
| 12% | 1,014 | 331 | 254 | 200 | 162 |
| 10% | 864 | 282 | 216 | 171 | 138 |
| 8% | 707 | 231 | 177 | 140 | 113 |
| 7.5% | 666 | 218 | 167 | 132 | 107 |
| 5% | 457 | 149 | 114 | 90 | 73 |

2. Secondary objectives

- **Detection of differences between prevalence (from survey to survey)**

The magnitude of change in the prevalence of VTEC O157 detectable by any given sample size depends on initial prevalence. The table below lists the required sample size to detect changes in the prevalence (differences between initial and final prevalences) for the situation of widespread contamination (initial prevalence 12%) and rare contamination (initial prevalence 0.1%):

| Initial prevalence | Final prevalence | Sample size | Initial prevalence | Final prevalence | Sample size |
|--------------------|------------------|-------------|--------------------|------------------|-------------|
| 12% | 5% | 245 | 0.1% | 2% | 449 |
| | 4% | 177 | | 3% | 282 |
| | 3% | 131 | | 5% | 160 |
| | 2.5% | 113 | | 10% | 74 |
| | 1% | 75 | | | |

If the same number of eligible animals is tested each year, a change in the prevalence situation within one MS from one year to the following year can be detected with the sample size listed in the table (e.g. a change from a 12% to 4% positive proportion with a sample size of 177 isolates).

• **Detection of trend in prevalence over several years**

A monitoring programme, which is designed to detect changes (trends) in prevalence after two or more years requires a decreasing sample size. For instance, a trend in the prevalence of a 5% decrease per year can be detected after two years with a monitoring programme designed to detect a 10% decrease, or after three years with a programme designed to detect a 15% decrease. The table below lists the required sample size to detect changes in prevalence for the situation of high contamination (initial prevalence 12%) and rare contamination (initial prevalence 1%):

| Initial prevalence | Trend | Required sample size to detect trend over: | | | |
|--------------------|-----------------------------|--|---------|---------|---------|
| | | 2 years | 3 years | 4 years | 5 years |
| 12% | <i>2% decrease per year</i> | 879 | 353 | 177 | 99 |
| | <i>3% decrease per year</i> | 353 | 131 | - | - |
| 1% | <i>1% increase per year</i> | 765 | 421 | 282 | 208 |
| | <i>2% increase per year</i> | 282 | 164 | 113 | 85 |
| | <i>3% increase per year</i> | 164 | 97 | 67 | 42 |

The calculations assume that the change in prevalence over time is linear and that it is sufficient to demonstrate statistical significance of the change in prevalence after 2, 3, 4 or 5 years, respectively. This could be achieved by applying an interval of two or more years for the testing of VTEC O157 in a given study population.

Nevertheless, testing samples every year is recommended, because trends in proportion to contamination might be detected earlier, even if the statistical power is not sufficient to reliably detect a trend after that time. For example, with a sample size of 230 there is a 15% chance (power) that a decrease of 3% per year from an initial prevalence of 12% will already be detected as statistically significant after one year. In the second year of monitoring, the power to detect this decrease as significant is 55%, and in the third year 94%. The same sample size results in a power of 42% for the detection of a 3% increase from an initial prevalence of 1% after one year, and in a power of 86% to detect the same trend after two years.

3. Calculation of sample size for finite population:

In case of finite population of eligible cattle and sheep, i.e. when the sampling fraction n/N is greater than 10%, the adjusted sample size n_c should be used.

To calculate the adjusted sample sizes (n_c) for finite population sizes, the following formula may be used:

$$n_c = n / (1 + n/N),$$

where n is the sample size for infinite population and N the finite population size.

For an estimated prevalence of 12% with an absolute precision of 4%, the calculated value of n is 254 in an infinite population. Assuming that the total population size was for example 500, the adjusted sample size would be $n_c = 254 / (1 + 254/500) \approx 168$.

B. GUIDANCE ON THE SELECTION OF THE NUMBER OF SAMPLES NEEDED IN A TARGETED SURVEY IN FOODSTUFFS

In the following table some guidance can be found regarding the number of samples to be collected in the foodstuffs surveys. The expected prevalence is the occurrence of VTEC O157 or other targeted serogroups in the food category, i.e. the proportion of positive samples expected to be found. Then the desired level of confidence and the desired accuracy are chosen from the table.

It should be borne in mind that the chosen accuracy has to be in relation to the expected prevalence, for example choosing the combination of 1% expected prevalence and accuracy of 1% would result in a precision of results of 1% +1% and by choosing the 0.1% accuracy, the precision would be increased to 1% +0.1%.

| Expected prevalence | Level of confidence | | | | | | | | | | | |
|---------------------|---------------------|-----|-------|---------|------------------|-----|-------|---------|------------------|-----|--------|-----------|
| | 90% | | | | 95% | | | | 99% | | | |
| | Desired accuracy | | | | Desired accuracy | | | | Desired accuracy | | | |
| | 10% | 5% | 1% | 0.1% | 10% | 5% | 1% | 0.1% | 10% | 5% | 1% | 0.1% |
| 0.1% | - | - | - | 2,687 | - | - | - | 3,838 | - | - | - | 6,650 |
| 0.5% | - | - | - | 13,381 | - | - | - | 19,112 | - | - | - | 33,116 |
| 1% | - | - | 266 | 26,627 | - | - | 380 | 38,032 | - | - | 659 | 65,898 |
| 5% | - | 51 | 1,278 | 127,756 | - | 73 | 1,825 | 182,476 | - | 126 | 3,162 | 316,179 |
| 10% | 24 | 97 | 2,421 | 242,064 | 35 | 138 | 3,457 | 345,744 | 60 | 240 | 5,991 | 599,076 |
| 50% | 67 | 269 | 6,724 | 672,400 | 96 | 384 | 9,604 | 960,400 | 166 | 666 | 16,641 | 1,664,100 |

C. MULTIPLEX PCR FOR THE CHARACTERISATION OF VTEC VIRULENCE GENES: *VTX1*, *VTX2* AND *EAE*

4. Principle of the method

The method is based on PCR amplification of specific DNA regions from a DNA template, with oligonucleotides triggering the start of the PCR reaction. Detection of *vtx1*, *vtx2* and *eae* is performed by a multiplex PCR reaction using specific primers (Table 1).

The primer pairs used, *stx1F/stx1R* and *stx2F/stx2R* (Paton & Paton, 1998), are able to detect the genes *vtx1* and *vtx2*, respectively. The latter recognise all the variants of *vtx2*, except *vtx2f*. The primers used for the detection of the intimin-coding gene *eae* (Paton & Paton, 1998) recognise all the reported polymorphic variants of this gene.

The method is composed of the following steps:

- template preparation;
- setting-up of the PCR reaction; and
- determination of the PCR results by agarose horizontal gel electrophoresis.

5. Template preparation

Cultures streaked onto solid media (e.g. TSA) are processed as follows:

- pick a single bacterial colony up with a sterile 1 µl loop; and
- prepare the template by suspending the bacteria in 100 µl of 0.22 µm filter-sterilised MilliQ water and boil for 10 minutes.

6. Setting up the PCR reaction

For each sample, set up a 50 µl reaction (reaction buffer 1X, MgCl₂ 1.2 mM, dNTPs 0.2 mM each, 50 pmoles of each primer, 2 Uts of *Taq* polymerase and 10 µl of DNA template). The volume of the reagents can be scaled according to the final volume of reaction.

In each PCR assay, a positive and two negative controls must be included. The positive controls are DNA templates obtained from *E. coli* strains possessing the virulence genes tested, while a negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes harboured) and the other is constituted by a sample without template added.

The reactions are incubated in a thermal cycler programmed with the thermal profile described by Paton & Paton (1998): 35 PCR cycles, each consisting of 1 minute of denaturation at 95°C; 2 minutes of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 minutes of elongation at 72°C, incrementing to 2.5 minutes from cycles 25 to 35.

7. Agarose gel electrophoresis

Prepare a 2.5% (w/v) agarose gel in 1X Tris/Borate/EDTA (TBE) or Tris/Acetate/EDTA (TAE). Each well of the gel is loaded with 15 µl of each reaction added with loading dye at 1X final concentration. Run the samples in 1X running buffer (TBE or TAE) in constant voltage (100 V). Use a molecular weight marker suitable for the assignment of the correct molecular weights to the amplicons produced (refer to Table 1 in this appendix). Consider that a correct band assignment is a crucial point in the assessment of the presence of the virulence genes. Make sure that the bands produced by the reference strains match exactly the expected molecular weight.

Ethidium bromide should be added to agarose gels to allow the visualisation of DNA. This reagent is a DNA intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories. When exposed to ultraviolet light, it will fluoresce with a red-orange colour. Ethidium bromide should be added to a final concentration of 0.5 µg/ml before pouring the agarose gel into the electrophoresis gel cast. Alternatively the agarose gel can be stained after electrophoresis in a 0.5 µg/ml ethidium bromide aqueous solution.

8. Reference strains

A VTEC strain harbouring *vtx1*, *vtx2* and *eae* genes should be used as positive control for all these genes. An example is the *E. coli* O157 EDL933 reference strain (ATCC no 43895).

Any *E. coli* K12 strain such as LE392 can be used as negative control.

PCR controls are prepared as described in section 5.2 (Template preparation). The control templates can be prepared in advance and stored in 10 µl ready-to-use aliquots at -20°C for 8 months.

9. Interpretation of the results

Samples showing amplification fragments of the expected size (see Table 1) are considered as positive for related target genes. Positive and negative controls must be included in each reaction and give positive and negative results, respectively.

Table 3: Primer sequences and amplicon sizes

| Target gene | Primer Name (reference) | Primer Sequence | Amplicon Size (bp) |
|---------------------|--|-------------------------|--------------------|
| <i>eae</i> | <i>eae</i> AF (Paton & Paton, 1998) | GACCCGGCACAAGCATAAGC | 384 |
| | <i>eae</i> AR (Paton & Paton, 1998) | CCACCTGCAGCAACAAGAGG | |
| <i>vtx1</i> | <i>stx1</i> F (Paton & Paton, 1998) | ATAAATCGCCATTCGTTGACTAC | 180 |
| | <i>stx1</i> R (Paton & Paton, 1998) | AGAACGCCCACTGAGATCATC | |
| <i>vtx2</i> (group) | <i>stx2</i> F (Paton & Paton, 1998) | GGCACTGTCTGAAACTGCTCC | 255 |
| | <i>stx2</i> R (Paton & Paton, 1998) | TCGCCAGTTATCTGACATTCTG | |

D. REAL-TIME PCR FOR DETECTION AND IDENTIFICATION OF VTEC

1. Principle of the method

This Real-time PCR protocol aims at the detection and identification of the major virulence genes and serogroup-associated genes characterising the VTEC strains that are considered to be pathogenic to humans. These genes include:

- *vtx* genes (*vtx1*, *vtx2* and its variants) encoding the Verocytotoxins (Shiga toxins), the main virulence factors of VTEC (Table 1);
- the *eae* gene, encoding a 90KDa protein, the intimin, which is the key factor for the induction of the “attaching and effacing” lesion on the enterocyte, a typical feature of the pathogenic VTEC strain (Table 1);
- genes associated with the VTEC serogroups that are mainly isolated from human cases of severe disease: O157, O26, O111, O103, and O145. The genes are either comprised in the operons encoding the different lipopolysaccharides (LPS) constituting the O antigens or are anyhow associated to each serogroup in a unique manner (Table 2).

2. Operating procedures

The protocol is based on the 5' nuclease PCR assay. Considering that Real-time PCR may use different instruments and probes labelling chemistry, the amplification conditions to be applied may vary depending on the system used. Refer to the instructions supplied with the instrument and kit of choice.

The primers and probes to be used are listed in the tables below together with the related references. The chemistry of the reporter and quencher fluorophores are not indicated being largely dependent on the Real-time PCR systems available in each laboratory. The method has recently been evaluated by examining a large number of VTEC belonging to serogroups O26, O103, O111, O145 and O157 (Beutin et al., 2009).

3. Reference strains

A VTEC strain harbouring *vtx1*, *vtx2* and *eae* genes should be used as positive control for the virulence genes. An example is the *E. coli* O157 EDL933 reference strain (ATCC no 43895). For serogroup-associated genes, reference strains belonging to each of the serogroup should be used.

The Real-time PCR procedure requires an **inhibition/extraction control**. Details on the possible systems to be used as inhibition/extraction control are given below. In particular, two different internal amplification controls (IACs) can be used alternatively:

- a commercially available TaqMan[®] Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The kit includes all reagents necessary (primers, a Vic[™]probe, IAC target DNA and blocking solution). The IAC target DNA must be diluted 10 times to achieve a copy number of approximately 100 per PCR reaction. The PCR product length is not declared to the customer; and
- the open formula pUC 19 based internal amplification control IAC developed by Fricker et al. (2007. Appl Environ Microbiol 73, 1892-1898). Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC is 119 bp.

The latter may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid to the sample aliquot prior to the DNA purification step.

4. Interpretation of the results

Table 4: Degenerate primers and TaqMan probes used in 5' nuclease PCR assays for the detection of virulence genes

| Target gene (Ref.) | Forward primer, reverse primer and probe sequences (5'-3') ^(a) | Amplicon size (bp) | Location within sequence | GenBank accession number |
|----------------------------|---|--------------------|--------------------------------|--------------------------|
| <i>vtx1</i> ⁽¹⁾ | TTTGTACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC Probe- CTGGATGATCTCAGTGGGCGTTCTTATGTAA | 131 | 878–906 983–1008 941–971 | M16625 |
| <i>vtx2</i> ⁽¹⁾ | TTTGTACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC Probe-TCGTCAGGCACTGTCTGAAACTGCTCC | 128 | 785–813 785–813 838–864 | X07865 |
| <i>Eae</i> ⁽²⁾ | CAT TGA TCA GGA TTT TTC TGG TGA TA CTC ATG CGG AAA TAG CCG TTA Probe- ATAGTCTCGCCAGTATTCGCCACCAATACC | 102 | 899-924 1000-979 966-936 | Z11541 |

^(a) In the sequence Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C)

⁽¹⁾ Perelle S. et al. Mol Cell Probes 2004 18: 185–192

⁽²⁾ Møller Nielsen E. and Thorup Andersen M. J Clin. Microbiol. 2003 41: 2884-2893

Table 5: Primers and probes used for the amplification of O antigen-specific genes in 5' nuclease PCR assays

| Target gene (serogroup) | Forward primer, reverse primer and probe sequences (5'-3') | Amplicon size (bp) (Ref.) | Location within sequence | GenBank accession number |
|-------------------------|--|---------------------------|-------------------------------------|--------------------------|
| <i>rfbE</i> (O157) | TTTCACACTTATTGGATGGTCTCAA CGATGAGTTTATCTGCAAGGTGAT Probe- AGGACCGCAGAGGAAAGAGAGGAATTAAGG | 88 ⁽¹⁾ | 348–372 412–435 381–410 | AF163329 |
| <i>wbdI</i> (O111) | CGAGGCAACACATTATATAGTGCTTT TTTTTGAATAGTTATGAACATCTTGTTTAGC Probe- TTGAATCTCCAGATGATCAACATCGTGAA | 146 ⁽¹⁾ | 3464–3489 3579–3609 3519–3548 | AF078736 |
| <i>wzx</i> (O26) | CGCGACGGCAGAGAAAATT AGCAGGCTTTTATATTCTCCAAC TTT Probe- CCCCGTTAAATCAATACTATTTACGAGGTTG A | 135 ⁽¹⁾ | 5648–5666 5757–5782 5692–5724 | AF529080 |
| <i>ihpI</i> (O145) | CGATAATATTTACCCACAGTACAG GCCGCCGAATGCTT Probe-CCGCCATTCAGAATGCACACAATATCG | 132 ⁽¹⁾ | 1383–1408 1500–1514 1472–1498 | AF531429 |
| <i>wzx</i> (O103) | CAAGGTGATTACGAAAATGCATGT GAAAAAAGCACCCCGTACTTAT Probe-CATAGCCTGTTGTTTTAT | 99 ⁽²⁾ | 4299–4323 4397–4375 4356–4373 | AY532664 |

⁽¹⁾ Perelle S. et al. Mol Cell Probes 2004 18: 185–192

⁽²⁾ Perelle S. et al. J. Appl. Microbiol. 2005 98: 1162–1168

E. ISOLATION OF VTEC O26, O103, O111 AND O145 FROM REAL-TIME PCR POSITIVE SAMPLES

The Real-time PCR method proposed for the detection of VTEC belonging to the pathogenic serogroups O26, O103, O111 and O145 is based on screening samples for the presence of *vtx* (VT-coding genes), *eae* (intimin-coding gene) and specific serogroup-associated genes. Samples positive at the same time for *vtx*, *eae*, and a serogroup-associated gene must be submitted to a further step aimed at the isolation of the VTEC strain, to confirm the simultaneous presence of the genes in the same live bacterial cell.

Flow of the operations may include:

1. Submit the enrichment culture maintained as a back-up to serogroup-specific enrichment (SSE), using immuno-capture systems such as immunomagnetic separation (IMS) or equivalent following instructions supplied by the manufacturer.
2. Streak the SSE onto Tryptone-bile-glucuronic medium (TBX) or onto a specific selective medium where available (see Note 1) and incubate for 18 to 24 hours at 37°C.
3. Pick from 10 to 50 colonies with *E. coli* morphology or with characteristic aspect according to the medium used (see Note 1) and point-inoculate on nutrient agar (NA) (see Note 2) and H₂O (the colonies may be pooled in water up to a number of 10 per pool).
4. Perform conventional PCR (Appendix C) or Real-Time PCR (Appendix D) on the H₂O pools to assess the presence of the *vtx* and the *eae* genes.
5. If a PCR pool is positive, go back to NA and assay the individual colonies forming the positive pool in order to select one single positive colony.
6. Identify the isolates as *E. coli* and confirm the serogroup the sample was positive for in the screening PCR assay (see Note 3).
7. Isolates are recommended to be sent to the National Reference Laboratory for storage.

NOTE 1: For VTEC O26 isolation, a differential solid media (MacConkey) containing rhamnose instead of lactose is commercially available (RMAC). It is very effective in distinguishing VTEC O26 strains, which do not ferment rhamnose, from other *E. coli*. Differential and confirmation plating media for VTEC serotypes O26, O103, O111, O145, and sorbitol-positive and -negative O157 have been recently proposed by Posse et al. (2008a and 2008b). Enterohaemolysin Agar can also be used. It detects Enterohaemolysin production, which is a common feature of pathogenic VTEC in humans (Beutin et al., 1996).

NOTE 2: There are several types of nutrient agar media available commercially either ready-to-use plates or prepared in-house from dehydrated powders. Every type of non-selective nutrient agar media (e.g. TSA) is suitable for the purpose of maintaining the colonies for further characterisation. Enterohaemolysin Agar, can also be used, which gives the advantage of detecting the Enterohaemolysin production.

NOTE 3: Colony confirmation as *E. coli* may be achieved by using commercial biochemical galleries or by assessing the indole production. Confirmation of the serogroup the sample was positive to, in the screening PCR assay, may be achieved either by PCR or by agglutination with commercial antisera.

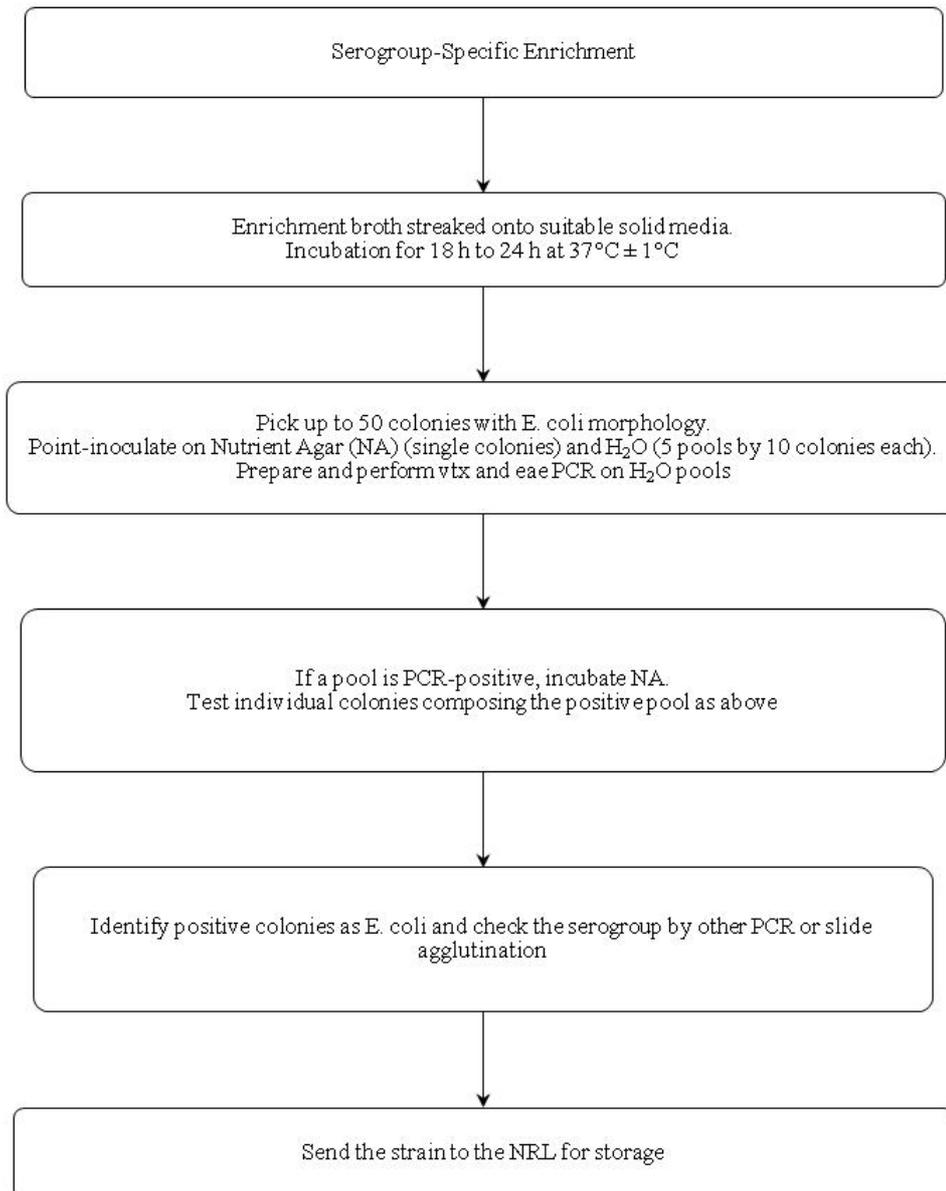


Figure 3: Flow diagram of the isolation procedure

F. SUGGESTION OF ADDITIONAL INFORMATION TO BE RECORDED FOR OTHER USES

This appendix provides suggestions of additional data that may be useful at national levels or within groups of countries, or within the network constituted by the NRLs and the CRL. These are not to be reported in the framework of Directive 2003/99/EC.

Overview of the laboratory involved

- For each laboratory involved
 - laboratory identifier code
- NRL for this organism

Sample level variables for cattle hide and sheep fleece samples

- Sample taken according to all specifications given in the protocol, i.e. sampling regime, analytical methods, time, temperature etc.
- Abattoir code from where sample was taken
- Date of sampling
- Hours the animal has spent in slaughterhouse before sampling
- Age of animal
- State of hide/fleece i.e. wet/dry, clean/dirty
- Herd/flock of origin
- Size of herd/flock of origin

Sample level variables for foodstuff samples

- Precise description of the foodstuff, including frozen or not frozen
- Date of sampling
- Length of shelf life
- Town/region of sampling

Sample level variables and results from laboratory analysis

- Date laboratory analysis began
- Code of laboratory involved in detection and isolation
- Code of laboratory involved in virulotyping
- Serogroup(s) detected (may be more than one)
- Result for VTEC O157 analysis:
 - qualitative result (positive/negative);
 - specific results from virulotyping of *E. coli* O157 (i.e. details on *eae*, *vtx1*, *vtx2* resulting in *E. coli* O157, *vtx* positive *eae* negative *E. coli* O157, *vtx* positive *eae* positive *E. coli* O157; may be more than one virulotype per sample).
- Result for non-O157 VTEC analysis
 - Specific results from Real-time PCR screening of samples:
 - step 1: *eae*, *vtx1*, *vtx2*;
 - step 2: serogroup-associated genes (may be more than one per sample);
 - step 3: isolation of *E. coli* of serogroup(s) expected from step 2, qualitative result (positive/negative).
 - Specific results from virulotyping of non-O157 *E. coli* (i.e. details on *eae*, *vtx1*, *vtx2* resulting in *E. coli*, *vtx* positive *eae* negative *E. coli*, *vtx* positive *eae* positive *E. coli* of serogroup(s) expected from step 2; may be more than one virulotype per sample).
- Other typing results (i.e. H-typing, *eae* subtyping, *vtx* subtyping, PFGE etc.).

ABBREVIATIONS

| | |
|----------------|---|
| BIOHAZ | Biological Hazards |
| BPW | buffered peptone water |
| CEN | European Committee for Standardization |
| CSR | Community Summary Report |
| DNA | deoxyribonucleic acid |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| <i>eae</i> | gene encoding attaching/effacing adhesion factor intimins |
| ECDC | European Centre for Disease Prevention and Control |
| EFSA | European Food Safety Authority |
| EU | European Union |
| HC | haemorrhagic colitis |
| HUS | Haemolytic Uremic Syndrome |
| <i>ihp1</i> | gene specific for <i>E. coli</i> O145 |
| IAC | internal amplification control |
| IMS | immunomagnetic separation |
| IMS | immunomagnetic separation |
| ISO | International Organization for Standardization |
| LEE | locus of enterocyte effacement |
| MS | Member State |
| NA | nutrient agar |
| NRL | National Reference Laboratories |
| OIE | World Organisation for Animal Health |
| PCR | polymerase chain reaction |
| PFGE | pulsed field gel electrophoresis |
| <i>rfbE</i> | gene specific for <i>E. coli</i> O157 |
| RMAC | rhamnose MacConkey agar |
| SF | sorbitol-fermenting |
| SSE | serogroup-specific enrichment |
| STEC | Shiga toxin-producing <i>E. coli</i> |
| <i>Stx</i> | Shiga toxin |
| TAE | Tris/Acetate/EDTA |
| TBE | Tris/Borate/EDTA |
| TBX | tryptone-bile-glucuronic medium |
| TSA | Tryptic Soy Agar |
| TSB | tryptone-soy broth |
| VT | verocytotoxin |
| VTEC | verotoxigenic <i>Escherichia coli</i> |
| <i>vtx</i> | gene encoding verocytotoxins |
| <i>wbdI</i> | gene specific for <i>E. coli</i> O111 |
| <i>wzx</i> | gene specific for <i>E. coli</i> O103/O26 |