

## SCIENTIFIC REPORT OF EFSA

# Technical specifications for harmonised national surveys on *Yersinia enterocolitica* in slaughter pigs<sup>1</sup>

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### ABSTRACT

Technical specifications are proposed for the harmonised monitoring and reporting of *Yersinia enterocolitica* in slaughter pigs in European Union Member States in accordance with Directive 2003/99/EC (EC, 2003). The aim of the technical specifications is to facilitate a better understanding of the situation in Member States and enable harmonised monitoring. According to a risk-based sampling strategy, the technical specifications describe the complete survey, aiming at the estimation at slaughter of the preliminary prevalence of *Yersinia enterocolitica* contamination in the tonsils of slaughter pigs with a live weight of between 50 kg and 170 kg, originating from all rearing systems. It is suggested that monitoring be carried out every three or four years in each Member State, depending on the prioritisation based on the epidemiological situation in the Member State. Taking into account public health relevance, the proposal focuses primarily on the estimation of the prevalence of human pathogenic *Yersinia enterocolitica* types, identified by biotype that represents the pathogenicity of *Yersinia enterocolitica*. The standardised ISO 10273:2003 method (ISO, 2003) or the direct plating method is recommended for the detection of presumptive pathogenic *Yersinia enterocolitica*. Biotyping and serotyping of all isolated strains is necessary. Specifications are also given for the reporting of information on the monitoring of *Yersinia enterocolitica* in the annual zoonoses reports to be prepared by Member States.

### KEY WORDS

Pigs, monitoring, national survey, tonsils, *Yersinia enterocolitica*

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## SUMMARY

The Directive 2003/99/EC (EC, 2003) on the monitoring of zoonoses and zoonotic agents establishes the Community system for the monitoring and collection of information on zoonoses. Member States should collect relevant, and where applicable, comparable data on some zoonoses in certain animals and foodstuffs. The monitoring of yersiniosis and agents thereof is mandatory only if the epidemiological situation in a Member State so warrants. However, yersiniosis remained the third most frequently reported zoonosis in the European Union. Therefore, more comparable data are needed on the prevalence of pathogenic *Yersinia enterocolitica* strains.

Based on the opinion from the scientific panel on Biological Hazards on the monitoring and identification of human enteropathogenic *Yersinia* spp. (EFSA, 2007c), the Task Force on Zoonoses Data Collection was asked by the European Food Safety Authority to draft harmonised technical specifications to be used for national surveys for the monitoring and reporting of *Yersinia enterocolitica* in pig populations under the Directive 2003/99/EC (EC, 2003). These surveys could contribute to the collection of information on the prevalence of the pathogenic *Yersinia enterocolitica* strains in the target animal population and enhance the optimisation of future survey designs.

According to public health relevance, the proposal focuses primarily on the estimation of the prevalence of human pathogenic *Yersinia enterocolitica* types, identified by biotype, which represents the best and most reliable indicator of *Yersinia enterocolitica* pathogenicity.

The proposed technical specifications define slaughter pigs as the target population, including animals between 50 kg and 170 kg live weight and originating from all rearing systems.

Sampling of these animals is targeted at the slaughterhouse, where the main contamination of pig meat with human pathogenic *Yersinia enterocolitica* takes place. Such a survey would provide preliminary data on prevalence in each Member State at a point in the food chain which is relevant for public health. It is proposed that sampling be performed in a limited number of slaughterhouses, located in the most important production regions for slaughter pigs in the country and covering 50% of the annual throughput in the country, and with the collection of 5 to 10 samples per visit.

Recommended samples are tonsils, which are more efficient for pathogenic strain recovery than other sample types. They are to be collected on the slaughter line.

The standardised ISO 10273:2003 method (ISO, 2003), or the direct plating method, is recommended for the detection of presumptive pathogenic *Yersinia enterocolitica*. All isolated *Yersinia enterocolitica* strains must be biotyped and serotyped. Isolates are suggested to be stored for the purpose of future typing. Serological analysis is also recommended.

Finally it is suggested that all Member States carry out this survey at regular intervals, e.g. every three or four years, depending on the prioritisation made by risk managers based on the epidemiological situation in the Member States.

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## BACKGROUND AS PROVIDED BY EFSA

### Developing specifications for harmonised monitoring surveys for *Yersinia enterocolitica* in pigs

The Directive 2003/99/EC (EC, 2003) 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 (EU) (EFSA, 2007a, 2007b, 2009). While analysing the data received from MSs, it has become apparent that the information available on *Yersinia enterocolitica* (*Y. enterocolitica*) was not sufficient to analyse the importance of the findings in foodstuffs and animal populations in relation to human yersiniosis 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 serotypes of *Y. enterocolitica* that are pathogenic to humans, the analytical methods to be used to detect and identify the human pathogenic serotypes from food and animals and the monitoring methods in animal populations and foodstuffs that are optimal for public health. The BIOHAZ panel issued its opinion on the monitoring and identification of human enteropathogenic *Yersinia* spp. (EFSA, 2007c) on 6 December 2007.

## TERMS OF REFERENCE AS PROVIDED BY EFSA

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

- issue a report on specifications for harmonised surveys on *Yersinia enterocolitica* in the pig population in EU Member States;
- consider the *Y. enterocolitica* serotypes to be covered by the surveys and the analytical methods to be applied;
- give recommendations on reporting the survey results under the Directive 2003/99/EC; 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 lays down the Community system for the monitoring and collection of information on zoonoses. MSs shall assess the 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. 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 Council Decision No 2119/98/EC (EC, 1998a) that are currently coordinated by ECDC.

EFSA has the task of examining the data collected and preparing the CSR. Data collected in this framework relate to the occurrence of zoonotic agents isolated from animals, food and feed listed in part A of Annex I of Directive 2003/99/EC (EC, 2003) and, according to the epidemiological situation, also in part B. In part B.2, referring to bacterial zoonoses, yersiniosis is also listed.

Yersiniosis in humans most often causes diarrhoea, at times bloody, and occurs mostly in young children. Symptoms may last for one to three weeks. In older children and adults the predominant symptoms may be similar to those caused by appendicitis (right-sided abdominal pain and fever); therefore the development of differential diagnosis methods in humans is prerequisite to decrease the burden of the disease. Sometimes further complications (rash, joint pain and bacteraemia) can occur. In 2007, yersiniosis was, for the third consecutive year, the third most frequently reported human zoonosis in the EU with a total of 8,792 confirmed cases (EFSA, 2009). This represents a decreasing trend in the incidence of yersiniosis cases in humans since 2003. However, the notification rate is slightly higher in 2007 (2.8/100,000 population) than the previous year (2.1/100,000 population). *Y. enterocolitica* was the most common species reported in human cases by MSs and was isolated from 93.8% of all confirmed cases. Infection is most often acquired by eating contaminated food, particularly raw or undercooked pig meat. In fact pigs have been considered to be the primary reservoir for the human pathogenic types of *Y. enterocolitica*, even if other animal species, e.g. cattle, sheep, deer, small rodents, cats and dogs may also carry pathogenic serotypes.

However, when analysing the data received from MSs, the information available on *Y. enterocolitica* was not sufficient to facilitate an in-depth analysis of the importance of the findings of *Yersinia* from foodstuffs and animal populations in human yersiniosis cases. MSs reported *Y. enterocolitica* findings from various animal species and foodstuff categories, including pigs, cattle, sheep and goats, as well as pig, bovine and poultry meat, and milk. However, there was a lack of information on the *Y. enterocolitica* serotypes isolated from food and animals and, in particular, on biotypes. Actually, this information is of paramount importance to understand strain pathogenicity. According to the opinion of the BIOHAZ panel on *Yersinia* spp. (EFSA, 2007c), the best and most reliable indicator of *Y. enterocolitica* pathogenicity is the biotype as the various biotypes are either pathogenic or non-pathogenic. The serotype is not a reliable marker of *Y. enterocolitica* pathogenicity because several serotypes are common to both pathogenic and non-pathogenic strains. The combination of biotype and serotype is used for maximal identification of the strain. Due to the lack of these data it was often not possible to estimate whether these *Yersinia* findings were pathogenic to humans or to assess the potential sources of human infections and measures to protect public health.

Even though the routine EU-wide monitoring of human-pathogenic *Y. enterocolitica* in animals and foods is not recommended in the opinion, the BIOHAZ Panel concludes in its report that more comparable data are needed on the prevalence of pathogenic *Y. enterocolitica* in the porcine reservoir. These could be obtained, depending on risk management priorities, by an EU wide baseline survey on pathogenic *Y. enterocolitica* in the pig population, or by national surveys on pathogenic *Y. enterocolitica* in the pig population following a harmonised design.

On the basis of the BIOHAZ panel opinion, EFSA decided to issue a proposal for technical specifications for a harmonised monitoring survey on *Y. enterocolitica* in pigs within the framework of Directive 2003/99/EC (EC, 2003).

## 2. Rationale for the choices made

### 2.1. The rationale for the choice of objectives

Yersiniosis is a typically sporadic food-borne disease in humans. In 2007, a total of 8,792 confirmed cases of yersiniosis were reported in the EU, from which *Y. enterocolitica* was isolated from 93.8% (EFSA, 2009). Infection is most often acquired by eating contaminated food, particularly raw or undercooked pig meat. Thus, pigs have been considered to be the primary reservoir for the human pathogenic types of *Y. enterocolitica*. Yersiniosis is listed in the annex I to Directive 2003/99/EC (EC, 2003) under the "B list" of zoonoses that are to be monitored according to the epidemiological situation. Therefore, monitoring and annual reporting is not mandatory for MSs. However, yersiniosis remained the third most frequently reported zoonosis in the EU. Therefore more comparable data are needed on the prevalence of pathogenic *Y. enterocolitica* in the porcine reservoir which can be obtained, depending on the risk management priorities, by EU-wide or national surveys following a harmonised design according to the BIOHAZ opinion (EFSA, 2007c).

The aim of these technical specifications is to define a design for harmonised national surveys to make a preliminary estimation on the prevalence of human enteropathogenic *Y. enterocolitica* in slaughter pigs.

The survey covers only human pathogenic *Y. enterocolitica* types as proposed in the recommendations made in the BIOHAZ opinion on human enteropathogenic *Yersinia* spp. (EFSA, 2007c).

Since the contamination of pig meat with human pathogenic *Y. enterocolitica* takes place mainly during the slaughter process, sampling at the slaughterhouse will provide preliminary data on the prevalence in each MS at a point in the food chain, which is relevant to public health. In order to make the survey resource-efficient but at the same time to guarantee the best possible pragmatic randomisation of the samples, sampling should be performed in a limited number of slaughterhouses and during each visit to a selected slaughterhouse the collection of different samples (e.g. each of the 5-10 collected samples originated from pigs belonging to different slaughter batches) is advisable. The results of these surveys may be used to optimise the design of future surveys.

The BIOHAZ opinion states that (regular) monitoring of pathogenic *Y. enterocolitica* in the pig population is not considered necessary, but surveys providing estimates on the prevalence of pathogenic *Y. enterocolitica* in the pig population would be useful. Therefore, it is recommended that the survey presented could be carried out at regular intervals, e.g. every three or four years, depending on the prioritisation to be made by risk managers based on the epidemiological situation in MSs.

The feasibility and the opportunity to combine sampling with other monitoring programmes and surveys such as for *Salmonella* spp. or indicators of antimicrobial resistance (*Enterococcus*, *E. coli*) can be considered at national level.

### 2.2. The rationale behind the choice of culture from tonsils

*Y. enterocolitica* is found in the tonsils of pigs, even at slaughter. In general, tonsils are more productive for the recovery of *Y. enterocolitica* strains than are tongues, and tissue samples yield higher isolation rates than do swabs (Nesbakken, 1985). In the study of Nesbakken et al. (2003), the proportion of pathogenic *Yersiniae* detected in tonsils compared to faeces was six to one. These proportions are similar to findings by Frederiksson-Ahomaa et al. (2007) and Grtler et al. (2005), who reported that the frequency of isolated pathogenic *Yersiniae* was significantly greater from tonsils than that obtained from faeces at slaughter. In conclusion, when the pigs are slaughtered at the age of

135 days or more, the tonsils are a more significant source of human pathogenic *Y. enterocolitica* than faeces (Nesbakken et al., 2006). However, when sampling is done on the slaughter line, the possibility exists that *Y. enterocolitica* in the tonsils is a result from contamination from another animal during transport, in lairage or even on the slaughter line. This is not a great problem in the suggested survey as the objective of this survey is to evaluate contamination at slaughter level.

### 2.3. The rationale for free choice between two analytical methods

A wide variety of methods for the detection of *Y. enterocolitica* are described in scientific literature. In 2003, the International Organization for Standardization (ISO) published the second edition of a method (ISO 10273) for the detection of presumptive pathogenic *Y. enterocolitica* in food and animal feedingstuffs (ISO, 2003). The opinion of the BIOHAZ panel on *Yersinia* spp. (EFSA, 2007c) recommended this ISO method as the method of choice for the detection of human pathogenic strains from pig tonsils. However, the method according to ISO 10273 is laborious, making the analysis of a sample rather expensive and time-consuming. ISO has started the revision of ISO 10273 and a revised version of this standard method can be expected in the near future.

Published data indicate that infected pig tonsils generally contain a large number of *Y. enterocolitica* (Nesbakken, 1988, Shiozawa et al., 1991, Van Damme et al., 2009).

Recently it has been shown, that direct plating resulted in a similar percentage of positive pig tonsils as obtained by the use of ISO 10273 (Van Damme et al., 2009). The applied direct method is simple to perform (homogenisation of the sample and consequently direct plating on selective plates) and results in low cost analysis and a shorter analysis time.

Therefore, it is recommended that the decision to use either ISO 10273 or the direct plating method should be made by each MS. In order to collect more data on the relation between both methods, MSs should consider applying both analytical methods in parallel. However, in each case the applied analytical method(s) should be indicated when reporting the survey results.

For confirmation of the suspected *Y. enterocolitica* colonies, biochemical tests, PCR or commercial kits can be used. The European Committee for Standardization (CEN) has started the preparation of a Real-Time PCR-based technical specification for the detection of *Y. enterocolitica* and *Y. pseudotuberculosis* and a standard method can be expected in the near future.

### 2.4. The rationale for using additional serological analysis

Serological analysis could be used as an alternative method for the estimation of the prevalence of *Y. enterocolitica* in pig herds (Nielsen et al., 1996). Serological diagnosis is not equivalent to classical microbiological detection of the organism, as the serological response of producing antibodies is delayed in comparison to the infection. This is an advantage as it allows identification of herds that have been, or are presently, infected with *Y. enterocolitica*. It is also an advantage as infection during transport and lairage will not interfere with the results, nor will contamination occur during slaughtering. However, as the result is not equivalent to classical detection, serological analyses should be performed as an additional method and should not stand alone, if *Y. enterocolitica* strains for verification and typing may be of interest.

The serological test only covers serotypes O:3 and O:9, which is a drawback although the vast majority of European *Y. enterocolitica* strains belong to these serogroups.

### 2.5. The rationale for typing

It is crucial that the investigation include discrimination between strains that are pathogenic to humans and strains that are not, in order to evaluate human health significance of the results. Within *Y. enterocolitica*, the majority of isolates from food and environmental sources are non-pathogenic types. The BIOHAZ opinion (EFSA, 2007c) concluded that biotyping of the isolates is essential to

determine if the *Y. enterocolitica* isolate may be a human pathogen, and it should be accompanied by serotyping. In Europe, the majority of human pathogenic *Y. enterocolitica* belongs to biotype 4 (serotype O:3), followed by biotype 2 (serotype O:9). EFSA (2007c) has described the biotype/serotype combinations most relevant for Europe (Table 1).

**Table 1:** Pathogenic potential of the various biotypes and serotypes of *Y. enterocolitica*

Species	Biotype	Serotypes	Virulence for humans	Frequency in Europe	Pathogenicity determinants	
					pYV	HPI
<i>Y. enterocolitica</i>	4	O:3	P	++++ <sup>(a)</sup>	Yes	No
	2	O:9; O:5,27	P	++ to +++	Yes	No
	3	O:3; O:5,27	P	+	Yes	No
	1B	O:8; O:21; O:13; O:7 (and others)	HP	≈0	Yes	Yes
	5	O:3; O:2,3; O:1,2,3	P	≈0	Yes	No
	1A	Numerous (including O:8; O:5; O:7; O:13; ...)	NP	++++	No	No

pYV: virulence plasmid, HPI: High-Pathogenicity Island.

HP: Highly pathogenic, P: pathogenic, NP: non-pathogenic.

<sup>(a)</sup>: From 0 to ++++ indicates the degree of frequency of the various subgroups.

### 3. Objectives

The general objective is to provide MSs with harmonised specifications for national surveys on the prevalence of *Y. enterocolitica* in slaughter pigs.

The primary objective of these national surveys is to estimate, on a preliminary basis, the prevalence of human enteropathogenic *Y. enterocolitica* in slaughter pigs within MSs.

The secondary objective is to obtain comparable results among MSs and estimate the occurrence of the *Y. enterocolitica* biotype distribution in slaughter pigs within MSs. The aim is to report only pathogenic biotypes and their distribution in slaughter pigs, therefore the biotyping of all strains of *Y. enterocolitica* is necessary.

Results from such harmonised surveys should provide data for further analyses such as the analysis of trends in the occurrence of *Y. enterocolitica* as a long-term goal.



## 4. Sampling frame

### 4.1. The population

In the survey, the population addressed in selected slaughterhouses included pigs slaughtered with a live weight of between 50 kg and 170 kg. Slaughter pigs originating from all rearing systems are targeted, such as farrow to finish or weaner to finish. Specific Pathogen Free (SPF), conventional, free-range or organic holdings, as well as high and low capacity production shall be included. The data obtained allows the prevalence of pathogenic *Y. enterocolitica* to be estimated in MSs. Moreover, the use of the harmonised survey protocol makes possible the comparison of preliminary prevalence data between different countries. The point in the food chain where sampling is to be carried out is at the slaughterhouse, where tonsil samples should be collected after removal of the pluck set on the slaughter line.

### 4.2. Sampling design

The sampling plan of the survey is based on harmonised specifications. As the prevalence of slaughter pigs contaminated with pathogenic *Y. enterocolitica* may vary over the year, a 12-month period is divided into four periods of three months. In each of those periods one quarter of the total sample size should be taken.

#### Choosing slaughterhouses

For the selection of slaughterhouses implemented in the sampling scheme, the following items should be taken into account:

- At least five and a maximum of 10 slaughterhouses must be included.
- This subset of slaughterhouses should be located in different parts of the country and should be located in the most important slaughter pig production regions in the country. In each of the selected regions only large slaughterhouses should be selected.
- It is recommended that slaughterhouses be selected where the capacity totals at least 50% of the annual throughput in the country.
- However, when a maximum of 10 of the biggest slaughterhouses are selected, these 10 slaughterhouses may cover less than 50% of the annual throughput in the country.

#### Choosing slaughter pigs/batches

- It is recommended that the annual throughput of each slaughterhouse be taken into account in the distribution of samples between slaughterhouses. The sample size per slaughterhouse should be proportional to its share of the total throughput of the selected slaughterhouses. If information on the throughput is not available, the same number of samples is collected in all slaughterhouses.
- The sampling visits to each selected slaughterhouse should be distributed over the year.
- In each slaughterhouse the number of samples to be taken should be equally spread over each of the four periods.
- On each sampling visit to a slaughterhouse, a collection of five samples is recommended, but when necessary up to a maximum of 10 samples may be collected.
- Each sample must be taken from a slaughter pig originating from a different slaughter batch.
- Sampling days should be randomised taking the following limitations into account:
  - days of slaughter activities in the selected slaughterhouses;
  - number of batches slaughtered on slaughter days; and
  - exclusion of some days such as Fridays due to transport limitations.

### 4.3. The sample size (number of samples)

The sample size of a survey is constituted by the number of observations (samples, sampling units). When planning a survey, it is mandatory to estimate (calculate) the minimum sample size that is

required to yield an outcome with a predetermined level of precision (precision is the lack of random error, i.e. error due to chance alone). These calculations require a number of values to be agreed upon beforehand and input into an equation. The calculated sample size is used as a reference or target number of samples (sampling units) to be collected in the field, in order to provide an outcome (an estimate of the parameter) with the desired and predetermined (hypothetical) level of accuracy.

The sample size in this survey is understood to be the number of slaughter pigs that needs to be investigated and it is determined considering the following criteria, assuming simple random sampling:

- the total number of slaughter pigs;
- annual expected prevalence (p): 50% (\*);
- desired confidence level (Z): 95%, corresponding to a  $Z_{\alpha}$  value of 1.96;
- accuracy (L): 5%; and

using these values and the formula:  $n_{\infty} = \frac{(Z_{\alpha})^2 p(1-p)}{L^2}$ .

(\*) There is no information available on the prevalence of *Y. enterocolitica* in infected slaughter pigs that provides an estimate of the likely prevalence for each of the MSs, and thus it was decided to use a 50% assumed prevalence (worst case scenario with highest sample size). In the light of the results of the first national surveys, the sample size may be adjusted to the expected prevalence.

For an infinite population size of slaughter pigs (i.e. more than 100,000 units infinite<sup>4</sup>) the sample size is 384, assuming the above-mentioned criteria.

Specifically for this survey and the preliminary estimation of the prevalence of *Y. enterocolitica* in infected slaughter pigs, it is proposed to round up the sample size to 400.

In case the same number of samples is collected in all slaughterhouses, this number needs to be equally divided between the selected (5-10) slaughterhouses and between the four sampling sessions during the year. For example, in case 10 slaughterhouses were selected, 40 samples need to be collected during each year in every slaughterhouse, and consequently 10 slaughter pigs could be sampled on each of the four samplings.

## 5. Sample collection

### 5.1. Type and detail of sample

#### 5.1.1. Sampling for microbiological analysis

Only one sample is collected from each slaughter batch and the two tonsils of the pig are regarded as one sample.

On the slaughter line, tonsil samples are collected from plucks with intact tonsils (see Appendix A: Figures 1 and 2). The plucks may be conveniently transferred to a tray or hook during sampling. Using disposable plastic gloves, the tonsils are removed using sterile utensils, i.e. scissors (Appendix A: Figure 3). In case the slaughter procedure leaves the tonsils in the head region of the carcass, samples must be taken from the carcass. In this case, sampling should also be made from intact tonsils (Appendix A: Figures 4 and 5). The removed tonsils are placed in a container for transport to the laboratory (Appendix A: Figures 6 and 7).

Samples may conveniently be collected at the platform used by meat inspection. Sinks with soap and hot water should be within easy reach, as well as a steriliser with running hot water (82°C). Gloves

<sup>4</sup>A population is considered infinite when it is above 100,000.

should be changed between each sample. The gloves may be ordinary disposable gloves, used routinely for laboratory work, as they may be regarded as 'commercially sterile'.

The utensils used for collecting the tonsils should be sterile. This may be achieved by using disposable utensils, or by sterilising the utensils between operations. Sterilisation may be carried out using ethanol or hot water. The utensils should be cleaned by washing prior to sterilisation.

### **5.1.2. Sampling for optional serological analysis**

A meat sample consisting of at least 10 grams is collected from the diaphragm pillar. The sample is tagged for identification and frozen for later shipment to the laboratory.

## **5.2. Sample information**

Information on the sample should be recorded on a sampling form produced by the competent authority to enable the data requirements in Section 7 to be fulfilled. For example, this includes information on the date, time and place of sampling. Additional requirements according to Appendix B can also be recorded for use at national level.

Each sample and its sample form should be labelled with a unique number which should be used from sampling to analysis.

## **5.3. Transport of samples**

Samples for microbiological analysis must be transported at a temperature between 1°C to 8°C. The samples should reach the laboratory within 24 hours of sampling or in exceptional circumstances, i.e. because of a long distance to the laboratory, within 48 hours from the time of sampling.

Samples for the serological analysis must be shipped frozen to the laboratory.

# **6. Laboratory analytical methods**

## **6.1. Laboratories**

Laboratories shall have proven experience in using the required methods to detect *Y. enterocolitica* and have a quality assurance system complying with EN/ISO standard 17025 (ISO, 2005).

## **6.2. Receipt of samples**

### **6.2.1. Sample for microbiological analysis**

On receipt of the samples, laboratories shall check the information recorded by the sampler and complete the relevant sections of the sample form.

Samples shall be kept refrigerated below 5°C at the laboratory until examination, which shall commence within 24 hours after receipt.

### **6.2.2. Sample for serological analysis**

At the laboratory, the frozen samples are thawed out and the meat juice is collected for serological testing for antibodies against *Y. enterocolitica* serotype O:3 and O:9.

### 6.3. Microbiological analysis

#### 6.3.1. Sample preparation

It is essential that handlers take care in avoiding cross-contamination between samples.

Using a sterile scalpel or knife, the tonsil tissue collected from one pig is cut into small pieces or lacerated by approximately 20 parallel incisions (Appendix A: Figure 8) and transferred into a sterile plastic bag. This is the test sample.

#### 6.3.2. Detection methods

Detection is carried out either according to the horizontal method for the detection of presumptive pathogenic *Y. enterocolitica* ISO 10273:2003 (ISO, 2003) or by using a direct plating method. The application of both methods in parallel for each sample is encouraged.

##### 6.3.2.1. Detection according to ISO 10273:2003

The test sample (approximately 10 g) is weighed into a sterile plastic Stomacher bag and nine times the mass of PSB (peptone, sorbitol and bile salts) broth is added to give a 1/10 dilution. The PSB broth must be pre-warmed to room temperature before use. The suspension is homogenised using a peristaltic blender for four minutes.

The second initial suspension is prepared by transferring 10 ml of the initial suspension in PSB into 90 ml of ITC (irgasan, ticarcillin and potassium chlorate) broth, pre-warmed to room temperature before use, so as to obtain a 1/100 dilution.

ISO 10273:2003 is followed to proceed with the enrichment of the suspensions in PSB broth and ITC broth and subsequent plating out.

The confirmation, biotyping and serotyping of isolates are completed as described in sections 6.3.3 - 6.3.5.

##### 6.3.2.2. Detection using a direct plating method

The test sample (approx. 10 g) is weighed into a sterile plastic Stomacher bag and nine times the mass of PSB broth is added to give a 1/10 dilution. The PSB broth must be pre-warmed to room temperature before use. The suspension is homogenised using a peristaltic blender for four minutes.

One millilitre of the suspension is spread over three plates (Ø 9 or 10 cm) with cefsulodin, irgasan and novobiocin (CIN) agar (= 2 x 0,3 ml and 1 x 0,4 ml).

The CIN plates are incubated at 30°C, then examined after 24 hours and, if necessary, after 48 hours, to check if any characteristic colonies of *Y. enterocolitica* are present.

The confirmation, biotyping and serotyping of isolates are completed as described in sections 6.3.3 - 6.3.5.

##### 6.3.2.3. Detection using both ISO 10273:2003 and a direct plating method

In the case of simultaneous detection using ISO 10273:2003 and the direct plating method, an initial suspension in PBS is prepared according to 6.3.2.1. After homogenisation, one millilitre is withdrawn for detection using the direct plating method (6.3.2.2) and the rest of the initial suspension is used for detection according to ISO 10273:2003 (6.3.2.1).

### 6.3.3. Confirmation

Suspect colonies of *Y. enterocolitica* have to be confirmed by biochemical confirmation tests as described in ISO 10273:2003. Confirmation can also be realised using published PCR or commercialised biochemical identification kits.

### 6.3.4. Biotyping

Further confirmation such as presumptive pathogenic *Y. enterocolitica* must be carried out by estimating the biotype through specific tests according to Table 2 and as described in ISO 10273:2003.

**Table 2:** Biovars (Biotyping) of *Y. enterocolitica*

Biotypes	Tween <sup>TM</sup> -esterase	Aesculin	Pyrazinamidase	Indole	Xylose	Trehalose
1A <sup>(a)</sup>	+	+	+	+	+	+
1B	+	-	-	+	+	+
2	-	-	-	+ <sup>(b)</sup>	+	+
3	-	-	-	-	+	+
4	-	-	-	-	-	+
5	-	-	-	-	D <sup>(b)</sup>	-

<sup>(a)</sup>: Non-pathogenic.

<sup>(b)</sup>: Often weak or delayed.

### 6.3.5. Serotyping

Serotyping for the serotypes O:3, O:9, O:5, O:27 and O:8 should be done on a mandatory basis, as a complementary test to biotyping. Monospecific test sera for these serotypes are commercially available.

### 6.3.6. Storage of isolates

All the isolates, or at least one per sample are stored in an appropriate medium (example: peptone-glucose-buffered medium (PGB)) in appropriate conditions (example -80°C) as long as it ensures viability of the strains for a minimum of two years.

## 6.4. Serological analysis

Serological analysis is performed on a voluntary basis according to the procedure described by Nielsen et al., (1996) with the modification that meat juice is used instead of blood. Meat juice has been shown to be equivalent to blood for the serological analysis for *Salmonella* (Mousing et al., 1997) as well as for *Y. enterocolitica* (Niels Christian Feld, National Veterinary Institute, Denmark, personal communication).

## 7. Reporting from the Member States

### 7.1. 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 (EC, 2003), shall ensure that the results of the surveys be collected, evaluated and reported in the annual Zoonoses report to the Commission and EFSA.

The information to be reported by MSs is outlined in subsections 7.2 and 7.3, and consists of two broad categories: a description of the monitoring programme and survey results. In addition, Appendix B provides information that may be useful for collection at national level, but which is not to be reported in the framework of Directive 2003/99/EC (EC, 2003).

## 7.2. Overview of the survey

This description provides an overview of the survey in its entirety in MSs. The following descriptions are included in the zoonoses web reporting application text forms ([www.efsa.europa.eu/zoonoses](http://www.efsa.europa.eu/zoonoses)).

- If the sampling strategy, the methods of sampling, and the analytical methods applied were according to all 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 sample type and time of sampling analysed are specified in the text.
- If any modifications to the sampling strategy, methods of sampling or laboratory methods presented in this report were made, a reference to the report is made 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, analytical methods etc.) is given.
- The slaughterhouse selection process should be described in detail. More precisely, it should be reported whether selection was random, or not, and whether large capacity or low capacity slaughterhouses were surveyed. Also, whether the sample size per slaughterhouse was adjusted according to the annual throughput, should be mentioned.

The text forms are to be created and completed as specified in the following.

### Zoonotic agent/zoonoses

“*Y. enterocolitica*, human pathogenic biotypes”.

### Animal species

“Pig” and further subcategories “slaughter pigs”.

### Sampling strategy

Fill in text field.

Example: “*Sampling strategy, methods of sampling and analytical methods applied according to all specifications given in the "Technical specifications for harmonised national surveys on Yersinia enterocolitica in slaughter pigs, The EFSA Journal 2009; 7(11):1374"*. Any deviations from the specifications are described.

In addition, the following are specified:

### Frequency of sampling

Enter “*Sampling takes place during the months xxxx*” and specify the months, if sampling is restricted to a specific time period; otherwise enter “*Sampling distributed evenly throughout the year*”.

### Type of specimen taken

Animals at slaughter: “*tonsils*”.

### Methods of sampling

A reference to the "Technical specifications for harmonised national surveys on *Yersinia enterocolitica* in slaughter pigs, The EFSA Journal 2009; 7(11):1374" is made.

### Case definition

"Animal where human pathogenic *Y. enterocolitica* is isolated from tonsils".

### Analytical methods used

For a microbiological analysis: "ISO 10273:2003" and/or "direct plating method".

For a serological method: "procedure described by Nielsen et al. (1996)".

### Remaining items

Fill in text fields where relevant and as appropriate.

## **7.3. Overview of the results in the tables**

The results of the sample testing are reported in the table (*Yersinia* in animals) provided by the zoonoses web reporting application ([www.efsa.europa.eu/zoonoses](http://www.efsa.europa.eu/zoonoses)), whereas population data are provided in the animal population table.

The sample information accompanying the results shall include the animal species tested (*pig*) and the animal subcategory (*slaughter pigs*).

### Results table specifications

- Sampling stage: "at slaughterhouse/animal sample/tonsil"
- Sampling context: "survey, national"
- Sampling unit: "animal"
- Number of units tested: fill in the number of animals tested
- Total number of units positive for *Y. enterocolitica*

The breakdown of these positive units into different biotype/serotype combinations is requested. For example:

- biotype 4/O:3
- biotype 2/O:9
- biotype 2/O:5,27
- biotype 3/O:3
- biotype 3/O:5,27
- biotype 1B/O:7
- biotype 1B/O:8
- biotype 1B/O:13
- biotype 1B/O:21
- biotype 5/O:3
- biotype 5/O:2, 3
- biotype 5/O:1, 2, 3

Other biotype/serotype combinations are added to the table if needed.

The breakdown of the results by season is requested, if possible.

Note that:

- the value contained in the columns “*Y. enterocolitica*” should also be included in the column “Total units positive for *Yersinia* spp.” (which is the sum of “*Y. enterocolitica*”, “*Y. pseudotuberculosis*” and “*Yersinia* spp., unspecified”)
- the value in the column “*Y. enterocolitica*” is the sum of the values in the columns “*Y. enterocolitica* biotype 4/O:3”, “*Y. enterocolitica* biotype 2/O:9” etc. and “*Y. enterocolitica*, unspecified”.

#### Population table specifications

- Number of existing slaughter pigs, i.e. the slaughter pig population.



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## APPENDICES

### A. INSTRUCTIONS FOR TONSIL SAMPLING



**Figure 1.** The plucks, in this case hung from the throat, consisting of tongue, lungs, heart and liver.



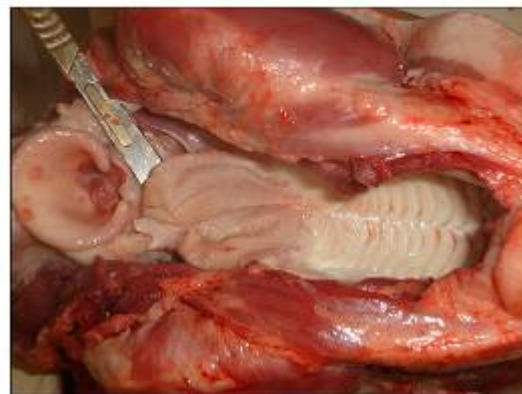
**Figure 2.** The tonsils are found at the base of the tongue (shown between thumb and index finger).



**Figure 3.** The tonsils are in this case removed by using scissors.



**Figure 4.** In case the tonsils are in the head region of the carcass, the tongue is lifted.



**Figure 5.** Tonsils are found between the hard palate and the epiglottis.



**Figure 6.** The removed tonsils.



**Figure 7.** The tonsils have been placed in a container suitable for transport to the laboratory.



**Figure 8.** The tonsils are lacerated by approximately 10 slashes in each tonsil to enhance isolation of *Y. enterocolitica* from the deeper layers of the tonsilary tissue.

## **B. SUGGESTION OF ADDITIONAL INFORMATION TO BE REPORTED FOR NATIONAL USE ONLY**

This appendix provides suggestions of additional data that may be useful for collection at national level (or within groups of countries). These are not to be reported in the framework of Directive 2003/99/EC (EC, 2003).

### **Overview of the laboratory involved**

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

### **Sample level variables for samples**

- Sample taken according to all specifications given in the protocol, i.e. sampling strategy, analytical methods, transport and storage temperature etc.
- Code of slaughterhouse where sample was taken
- Annual throughput of the slaughterhouse
- Date of sampling
- Hours the animal has spent in slaughterhouse before slaughtering
- Weight of animal
- Animal origin (domestic or imported)

### **Herd level variables for samples (optional)**

- Herd of rearing
- Herd size
- Type of rearing:
  - farrow to finish holding
  - weaner to finish holding
  - finisher pig holding
  - organic farms
  - outdoor housing
  - SPF holding

### **Sample level variables and results from microbiological analysis**

- Starting date of laboratory analysis
- Code of the laboratory involved in detection and isolation
- Code of the laboratory involved in biotyping and serotyping
- Detection method used (ISO or direct plating)
- Biotype(s)/serogroup(s) detected (maybe more than one)
- Result:
  - qualitative result (positive/negative sample)
  - specific results from biotyping / serotyping (i.e. biotype 2/O:9; biotype 4/O:3 etc.)
- Other typing results (i.e. PFGE etc.)

### **Sample level variables and results from serological analysis**

- Starting date of laboratory analysis
- Code of the laboratory involved in serological analysis
- Result:
  - qualitative result (positive/negative sample)

## GLOSSARY

**Farrow to finish holding** - a pig raising system in which piglets are born, reared, weaned, grown and fattened in the one holding.

**Finisher holding** - a pig raising system where pigs are fattened until they reach market weight in the one holding.

**Holding** - any establishment, construction or, in the case of an open air farm, any place in which animals are held, kept or handled (Directive 92/102/EEC (EC, 1992)).

**Monitoring** - a system of collecting, analysing and disseminating data on the occurrence of zoonoses, zoonotic agents and antimicrobial resistance related thereto (Directive 2003/99/EC (EC, 2003)). As opposed to surveillance, no active control measures are taken when positive cases are detected.

**Pathogenic *Y. enterocolitica* strain** - strain of biotypes 1B, 2, 3, 4 and 5 (Scientific Opinion of the Panel on BIOHAZ on a request from EFSA on monitoring and identification of human enteropathogenic *Yersinia* spp. (EFSA, 2007c)).

**Population** - the entire set of subjects (items, batches) to which findings of a study are to be extrapolated or from which information is required.

**Prevalence** - the total number of infected animals that are present in the population under consideration.

**Random sample** - sample in which the characteristics of the batch from which it is drawn are maintained. (Codex General Guidelines on Sampling CAC/GL 50- 2004, (CAC, 2004)); a sample which is taken under statistical consideration to provide representative data (Decision 98/179/EC (EC, 998b)).

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

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

**Slaughter batch** - a delivery of pigs, which have been raised in the same holding, to a slaughterhouse on one single day.

**Slaughter pig** - slaughtered pigs with a live weight of between 50 kg and 170 kg (Report on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, in the EU, 2006-2007. Part A: *Salmonella* prevalence estimates (EFSA, 2008)) - swine farmed not for reproductive purposes, but intended to be taken to a slaughterhouse for the production of meat and meat products (Annex III of the Opinion on “Risk assessment and mitigation options of *Salmonella* in pig production” (EFSA, 2006)).

**Weaner** - a young piglet being removed from the sow to switch from sow’s milk to dry feed.

**Weaner to finish holding** - a pig raising system where piglets are grown and fattened until they reach market weight in the one holding.

## ABBREVIATIONS

BIOHAZ	Biological Hazard
CEN	European Committee for Standardization
CIN	cefsulodin, irgasan and novobiocin
CSR	Community Summary Report
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EU	European Union
ISO	International Organization for Standardization
ITC	irgasan, ticarcillin and potassium chlorate
MS	Member State
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
PGB	peptone-glucose-buffered (medium)
PSB	peptone, sorbitol and bile (salts)
SPF	Specific Pathogen Free