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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the prospection of biological hazards of interest in food safety in Spain

Section of Food Safety and Nutrition
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Abstract

In food safety, there are hazards of interest for which no specific regulation exists that may be the subject of survey programmes in order to obtain data to carry out a risk assessment.

The Scientific Committee has reviewed and identified some biological hazards, indicating which of those foods or conditions that, a priori, may involve a greater risk to consumers. The list of hazards addressed in this report is not intended to be comprehensive, given that it does not take into account new possible biological hazards. It is meant to serve as a starting point for possible prospective studies with which data will be obtained regarding the presence of these hazards in different foods.

The proposal includes viruses, bacteria and parasites:

- Foodborne viruses: Norovirus, Hepatitis A virus, and Hepatitis E virus in bivalve molluscs and fresh vegetables and Hepatitis E virus in pork meat products.
- Bacteria: *Yersinia enterocolitica* in pork meat, *Vibrio parahaemolyticus* and *V. vulnificus* in bivalve molluscs and fish products, *E. coli* (non-STEC pathotypes) in fresh vegetables and *Clostridium difficile* in fresh meat.
- Protozoan Parasites: *Toxoplasma* and *Cryptosporidium* in fresh meat and vegetables.

The report details the methodologies available to detect them in food samples, and the gaps in our knowledge of these hazards, which may be a starting point to promote research activities aimed at improving our knowledge about them. The report also includes information about the possible ways to control the transmission of these microorganisms through the food chain.

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Key words

Biological hazards, prospective studies, Norovirus, Hepatitis A virus, Hepatitis E virus, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Escherichia coli, Clostridium difficile, Toxoplasma, Cryptosporidium.*

1. Introduction

Different hazards of a chemical or biological nature which may pose a risk to the consumer may be present, occur or integrate themselves along the food chain.

Official control programmes aim to guarantee the performance of controls of the hazards of interest to food safety according to the risk, but these only affect those parameters with maximum established limits in certain foods.

However, there are other hazards of interest to food safety for which there is no specific regulation, or such regulation exists but only for certain foods. These may be the object of prospective study programmes with the aim of obtaining data that, in addition to protecting the consumer from timely exposure to a hazard, permit the performance of a risk assessment.

On the other hand, the identification of new hazards to which significant exposure may occur, or the assessment of the risk derived from new or significantly increased susceptibility or exposure to a known hazard is important. This is not only with respect to an eventual control of these emerging hazards, but also for promoting research and improving the knowledge of consumers and the scientific community.

For this reason, the Section of Food Safety and Nutrition of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has been asked to review the hazards of most relevance to food safety in Spain which are not specifically regulated, identifying them and marking those food products or conditions which, a priori, may imply increased risks for the consumer in order to eventually conduct prospective studies.

2. Biological hazards

The following microorganisms have been considered:

- Food-borne viruses:
  - Norovirus, the Hepatitis A virus and the Hepatitis E virus in bivalve molluscs and fresh vegetables.
  - Hepatitis E virus in pork meat derivatives.
- Bacteria
  - *Yersinia enterocolitica* in pork meat.
  - *Vibrio parahaemolyticus* and *V. vulnificus* in fish products and bivalve molluscs.
  - *E. coli* (non-STEC pathotypes) in fresh vegetables.
  - *Clostridium difficile* in fresh meat.
- Parasites
  - Protozoa (*Toxoplasma* and *Cryptosporidium*) in fresh meat and fresh vegetables.

2.1 Food-borne viruses (Norovirus, Hepatitis A and Hepatitis E) in bivalve molluscs, fresh vegetables and pork meat derivatives

2.1.1 General information

The viruses which may contaminate water and food and transmit diseases to new individuals are numerous and varied. They belong to different families and may cause different pathologies, ranging

from acute gastroenteritis, normally minor, to hepatitis, and even myocarditis or severe neurological infections, such as meningitis or aseptic encephalitis (Bosch et al., 2016). In spite of this diversity, the most relevant human enteric viruses in the area of food safety are the human Noroviruses (NoV), which cause gastroenteritis, and the Hepatitis A virus (HAV), the leading cause of acute hepatitis at global level. In addition, the importance of the Hepatitis E virus (HEV) has also been indicated. This also causes acute hepatitis disorders and in certain individuals may lead to chronic hepatitis with severe consequences (EFSA, 2011). As they are enteric viruses, all of these are mainly spread via the faecal-oral route.

In recent years, the rate of food-borne outbreaks caused by NoV and HAV has increased significantly in developed countries. This has mainly been associated with the consumption of bivalve molluscs, green leafy vegetables and berries, as well as ready-prepared food. In the case of HEV, in addition to these matrices, products derived from raw or undercooked pork meat should also be included as the pig is its main reservoir. Lastly, although viral contamination may occur during the pre-harvest phase, ready-to-eat foods are often contaminated during preparation by an infected food handler.

2.1.2 General characteristics and impact

The NoV are found all over the world and cause sporadic or epidemic gastroenteritis in all age groups. They are sub-divided into seven genogroups, of which genogroups I, II and IV affect humans. Of these three, genogroup II is the most prevalent followed by genogroup I; at present genogroup IV has been detected with a very low frequency. In turn, each genogroup can be subdivided by genetic variability into different genotypes. Of the more than 40 genotypes existing altogether, genotype GII.4 has been found to be the most prevalent since the end of the 80s.

At present, they are considered to be the agents causing 18 % of all the infections caused by food-borne pathogens at global level, amounting to 600 million cases every year (Lopman et al., 2016). At European level, the World Health Organisation (WHO) estimates that NoV causes around 15 million cases (and 400 deaths) per year. Epidemic outbreaks occur very frequently, in particular in the catering industry and in closed or semi-closed institutions. Of all the infections, approximately 17 % (95 % IC 16-47) of these are attributed to a food origin (Havelaar et al., 2008). Certain economic studies from our country indicate that the direct and indirect costs of an outbreak of NoV range between 3 500-4 800 € (Navas et al., 2015). At European level, although monitoring of NoV is not harmonised in the different countries, in 2015 and 2016 NoV caused 8-9 % of the food-borne outbreaks, behind *Salmonella*, bacterial toxins and *Campylobacter* (EFSA, 2016b, 2017b). Figures from Catalonia indicate that NoV was diagnosed as the cause of 128 outbreaks of acute gastroenteritis between 2010-2012, 47 % of which were food-borne, where bivalve molluscs (oysters, mussels or clams) were the contaminated food in 18 % of the outbreaks (Sabria et al., 2014). In Europe in 2015, the foods classified in the category of "crustaceans, shellfish, molluscs and derivatives" caused 27.8 % of the outbreaks of NoV, and fruit and vegetables caused 11 % (EFSA, 2016b). The largest documented food outbreak of NoV occurred in several schools in Germany in 2012, associated with the consumption of frozen strawberries imported from China, and affected almost 11 000 individuals. Since then, the

European Commission has required that 5 % of the frozen strawberries imported from China are tested for NoV and HAV (EU, 2012). The greatest outbreak of NoV in Spain occurred in 2016, caused by bottled water from a spring in Andorra, affecting more than 4 100 people, and demonstrating that bacteriological controls may not be sufficient for guaranteeing the safety of certain products.

Unlike NoV, HAV appears with a marked geographical distribution. Whereas in the developing countries, the majority of individuals are infected during the first 5 years of life and suffer a subclinical infection, in the industrialised countries individuals reach adulthood without having being exposed to the virus and, in the event of becoming infected, they present symptoms which may be particularly severe among those over the age of 60. Although not chronic, fulminant hepatitis has been described in 2 out of every 1 000 cases. Although the existence of only one serotype is known, based on the genetic variability, six genotypes of HAV can be distinguished. Genotypes I, II and III affect humans and these are in turn subdivided into subgenotypes (IA, IB, IC, IIA, IIB, IIIA and IIIB). In Spain, infection by HAV must be declared and in recent years, 543 cases were reported in 2015, 742 cases in 2016 and 3 988 cases in 2017. In comparison with NoV, it is estimated that the percentage of cases of HAV with an origin in food is lower, and some 4 % of the infections are estimated to be due to this origin. Although there is an effective vaccination for HAV and many countries include it in their systematic vaccination schedule, in Spain all the children from Catalonia have only received the vaccination since 1998, and in Ceuta and Melilla since 2000. In the rest of the country, it is selectively given to the higher risk groups.

Given the extremely low level of endemicity in the European region, the percentage of the non-immunised population susceptible to infection is high. In recent years, there have been outbreaks of high magnitude both with respect to the number of cases and the territorial impact, in some cases due to the consumption of contaminated food, although there have also been significant outbreaks among MSM (men-having sex-with-men) males. In 2013, two outbreaks caused more than 250 cases in different Northern European countries and in different states in the United States due to the consumption of frozen strawberries imported from Egypt and pomegranate seeds imported from Turkey, respectively. In 2013-14, more than 1 300 cases were declared in 11 European countries, associated with the consumption of frozen strawberries produced in Europe.

Lastly, although the number of cases of HEV diagnosed in Europe is relatively low, the figures for zero prevalence in some European countries suggest the existence of numerous subclinical infections and/or a lack of monitoring. Zero prevalence ranges between 2-20 % in the majority of European countries which have reported data, including Spain (Domanovic et al., 2017), although some studies indicate values of over 70 % in certain regions in the south of France (Mansuy et al., 2015). In Europe, the number of cases reported increased tenfold between 2005 and 2015, with 5 617 cases in 2015 (EFSA, 2017b). Of the four genotypes that may affect humans, genotypes 1 and 2 are endemic and associated with water-borne outbreaks, and genotypes 3 and 4 are associated with zoonotic infections transmitted by the consumption of products derived from raw or undercooked pork or other game meat, the consumption of other contaminated food and contact with infected animals.

The main actions for the control and prevention of food-borne viral infections must be strengthened all along the food chain (EFSA, 2011). In primary production in agriculture, it is important to

control the quality of the irrigation water, the washing water, the origin and quality of natural fertilisers and manures, and the hygiene of the installations. In addition, mollusc farmers must ensure the quality of the water in the cultivation zones. In the case of HEV, strategies directed at reducing its prevalence in pigs may be considered in order to reduce the risk of transmission to humans. The correct and rigorous hygiene of workers in the food chain is crucial for preventing the transmission of any virus to foods, or its propagation through the installations and over the surfaces. In the case of food handlers with gastroenteritis, they should return to work only after a certain period of time without any symptoms of diarrhoea or vomiting (for example, 48 hours), and in the case of hepatitis, only once the symptoms of jaundice have disappeared and a medical examination has been conducted to dismiss any possibility of contagion. Nevertheless, it should be noted that the number of asymptomatic infections may be significant, and therefore it is important to maximise hygiene practices at all times. The hygiene of hands and surfaces are also crucial measures for preventing contamination in a domestic environment. Lastly, the directives on the application of the general principles of food hygiene for the control of viruses (FAO, 2012) recommend the use of cooking processes which allow the food to reach an internal temperature of 90 ºC for 90 seconds, in particular in bivalves (EFSA, 2015). Freezing is not a suitable measure for minimizing the viral contamination of food. Washing vegetables in disinfectant may reduce the level of virus by 1 or 2 log with respect to washing with water alone, where chlorine and peracetic acid are among the best characterised products (EFSA, 2011) (Bosch et al., 2018).

2.1.3 Figures for the prevalence in the highest risk foods

The available methodology has been widely used in research laboratories for determining the prevalence of contamination, in particular of NoV (genogroups I and II) and of HAV in samples of food of risk, which may reach the consumer.

2.1.3.1 NoV, HAV and HEV in bivalve molluscs

Most of the studies are qualitative and report positivity rates for NoV in different species of bivalves of 25-76 % in European countries including Spain, Italy, United Kingdom, Ireland, Poland or Belgium, or somewhat lower (9-22 %) in France. Although HAV is rarely observed in bivalve molluscs produced in non-endemic zones, there are studies that report percentages of positive samples of up to 10 or 23 % in countries such as Spain or Italy, respectively (AESAN, 2011) (Romalde et al., 2017).

For HEV, recent studies indicate HEV prevalence rates in bivalves produced in Europe of between 4-15 %, some of the highest in Spanish mussels, although rates of 0 % have also been reported (Mesquita et al., 2016) (EFSA, 2017a).

2.1.3.2 NoV, HAV and HEV in fresh vegetables

The figures obtained from outbreaks identify berries and ready-to-eat leafy vegetables as the foods with the highest risk. Between 2004-2012, berries were responsible for more than half of the outbreaks of NoV caused by vegetables, both in Europe and in the United States (Callejon et al., 2015).

Although the number of studies is still limited, the prevalence of NoV, HAV and HEV in ready-to-eat leafy vegetables, mainly lettuces, reported in European countries is low, between 0-2 % (Kokkinos et al., 2012) (Losio et al., 2015) (Terio et al., 2017). Nevertheless, earlier samplings also showed significantly high prevalence of NoV, ranging between 12-54 % for leafy vegetables and 6-34 % for berries (Mattison et al., 2010) (Baert et al., 2011) (Loutreul et al., 2014).

2.1.3.3 HEV in pork derivatives

The hepatitis E virus has been found in pork meat (liver, sausages) sold at retail level, in several countries including European countries such as Spain, France, Italy, the Czech Republic or the United Kingdom. Detection rates for the RNA of the virus have been reported at 6.5 %, 4.0 % and 6.0 % for commercially available pig's liver, although a study not yet published as part of the research project RTA2014-00024-C04 «Analysis and integrated control of *Toxoplasma gondii* and enteric virus in the food chain» raises this percentage to above 20 % in a significant number of slaughterhouses in Spain. In pig's liver sausage and raw sausage meats from Germany, detection rates of 20 to 22 % for the HEV RNA have been described. Particularly high detection rates of 57.1-58.3 % have been described for a local liver sausage from France called «Figatelli».

2.1.4 Available methodologies

Since 2013, standardised and validated methods have been available for the qualitative and quantitative detection of NoV (GI and GII) and HAV in the food matrices of highest risk, in fomites and in bottled water (ISO, 2013, 2017). The available standardised method is based on molecular detection, and includes various controls to guarantee the total absence of possible false negatives, making the tests extremely sensitive, but with high economic costs. Another important limitation is that, as this is a molecular method, the positive result does not permit confirmation of the infectivity of the virus detected.

Using this method, in 2017, NoV was the agent identified in 29 % and 28 % of the alerts reported to the RASFF (Rapid Alert System for Food and Feed) in the fruit and vegetable category (mainly frozen berries), and bivalve molluscs (mostly live oysters and other frozen bivalves), respectively; for HAV two alerts were reported in live clams. In previous years, NoV and HAV contamination alerts were reported in cooked bivalves. However, as the method did not permit confirmation of whether the viruses detected were infectious, the meaning of these results is still uncertain.

There is still no standardised method available for HEV today, although its development has been indicated by the EFSA as one of the priority aspects in the area of virology safety of food for the immediate future (EFSA, 2016c).

2.1.5 Future steps

Although further investigations and the collection of more data are required to clearly establish the relation between the detection of the genome of this virus in food and the risk for health, the possibility of establishing a regulation for NoV and/or HAV in some of the food matrices, in particular in bivalve molluscs, is being considered. While there is considerable agreement that a negative result

should be required for HAV given the severity of the infection, a maximum acceptable level of contamination would probably be considered for NoV. This is still to be determined.

Given that a high prevalence of NoV has been detected in the bivalves tested in all the European countries, a standard based on the absence of NoV would have a significant overall impact on the production sectors. According to the figures published in the EFSA scientific opinion (2012), for example, if a maximum contamination limit was established of 100 genome copies/g of digestive tissue, 33 % of the French oysters and 65 % of the British oysters would not pass the control. Also in this respect, in 2016 the EFSA (European Food Safety Authority) started a prospective study coordinated at European level to more accurately determine the prevalence of NoV in live oysters (EFSA, 2016a). In addition to these advances, it is also of priority to be able to develop methods which allow for the infectivity of these viruses to be assessed when they are found in food, and to be able to establish a correlation between their detection and the risk they pose to consumer health (EFSA, 2016c).

For the other risk matrices, in particular for fresh or frozen berries and ready-to-eat vegetables, the collection of more data for determining the frequency and contamination levels in Spain is also of priority. As the tools are available, and in light of the recent outbreaks of HAV in Spain, prospective quantitative studies including both NoV and HAV are recommended.

For HEV, the current priority lies on the one hand in the development of standardised reference methods for food matrices of interest, in particular meat and its derivatives, and on the other hand determining the impact of HEV infection in Europe (EFSA, 2016c).

2.2 Bacteria

2.2.1 *Yersinia enterocolitica* in pork meat

2.2.1.1 General information

Yersinia enterocolitica is a bacterium widely distributed in nature that may cause infection in human beings resulting in the disease known as yersiniosis. *Salmonella* spp., *Y. enterocolitica* and *Campylobacter* spp. are the three hazards most frequently involved in food-borne diseases resulting from the consumption of pork meat (Fosse et al., 2009). The main vehicle of transmission of *Y. enterocolitica* to the human being is raw or undercooked pork meat and derivatives (Huovinen et al., 2010).

2.2.1.2 General characteristics and impact

Y. enterocolitica is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. It is a facultatively anaerobic (able to grow in food packaged in oxygen and under modified atmospheres) psychrotrophic bacteria (grows at refrigeration temperatures), and also grows in a wide range of pH values (4-10). This bacterium remains viable at freezing temperatures and survives long periods of time in frozen foods (Montville et al., 2012).

Y. enterocolitica is sensitive to heat, and is destroyed with a heat treatment of 71.8 ºC (Montville et al., 2012). Therefore, it is important to reach temperatures of 70 ºC in the preparation of pork meat.

There are six biotypes of *Y. enterocolitica*: 1A, 1B, 2, 3, 4 and 5. Biotypes 1B, 2, 3, 4 and 5 are considered to be pathogenic for the human being and animals. Strains of biotype 1A are considered to

be non-pathogenic, although they may have pathogenic potential. In fact, certain outbreaks caused by *Y. enterocolitica* have been associated with biotype 1A (Batzilla et al., 2011) (Sabina et al., 2011).

The pathogenicity of *Y. enterocolitica* is associated with the presence of virulence factors encoded both in a plasmid and in the chromosome. The pathogenic biotypes (1B, 2, 3, 4 and 5) possess the pYV plasmid (*Yersinia* Virulence), of 70 kb, which contains genes that encode pathogenicity factors essential for its virulence. The *yadA* gene encodes the protein YadA (*Yersinia* Adhesin A) which permits the adhesion and invasion of cells. The *yop* gene encodes the production of Yop proteins (*Yersinia* outer membrane proteins), proteins which determine the resistance of the bacteria to the immune system. The *ysc* gene encodes the production of Ysc proteins (*Yersinia* secretion complex). VirF is a transcriptional regulator of other plasmid genes (*Yersinia* Virulence) (Bancerz-Kisiel and Szweda, 2015) (Gnanasekaran et al., 2017). The pathogenic strains which possess the pYV plasmid have common properties such as needing calcium for growth, the capacity to absorb Congo red and autoagglutination (Zadernowska et al., 2014).

In addition, the pathogenic strains have chromosomic genes which encode virulence factors, and which are more stable than those located in the pYV plasmid. These genes include the *ail* gene which encodes the production of the protein ail (attachment-invasion locus), an external membrane protein necessary for adhesion and entry to the cells. The *inv* gene encodes the production of a protein (invasion) required by the bacteria for its translocation (passage) through the cells. The *myfA* (mucoid *Yersinia fibrillae* A) gene encodes the production of fimbria for adhesion. The *ystA, ystB* and *ystC* genes (*Yersinia* heat-Stable Enterotoxin) encode the production of the heat stable enterotoxin of *Yersinia* A, B and C, respectively (Sabina et al., 2011) (Bancerz-Kisiel and Szweda, 2015).

The biotype 1B is considered as one the most pathogenic for the human being. The strains belonging to this biotype possess the virulence determinant: a high pathogenicity island (HPI) which encodes genes for iron uptake (Zadernowska et al., 2014).

The strains belonging to the 1A biotype do not possess the pYV plasmid, and lack the majority of the chromosomic virulence genes, although occasionally they may have some of the chromosomic genes which encode virulence factors such as *ail, inv, ystA, ystBmyfA* (Bonardi et al., 2010) (Sabina et al., 2011) (Zadernowska et al., 2014).

Although there are more than 70 O serotypes, only some of these serotypes are considered pathogenic for humans (O:3, O:5, O8, O:9, O:13, O:21,O:27) (EFSA, 2007) (Zadernowska et al., 2014). The O:3 serotype is the one most frequently isolated in humans (Montville et al., 2012). Some serotypes are common in pathogenic and non-pathogenic strains. Therefore, the serotype is not considered as a reliable marker of pathogenicity, and it is thought necessary to know both the biotype and the serotype of *Y. enterocolitica* (EFSA, 2007) (Bancerc-Kisiel and Szweda, 2015).

The strains of *Y. enterocolitica* which most frequently cause disease in humans belong to the following biotypes and serotypes: 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3, and less frequently to the bioserotype 3/O:5,27 and other serotypes of the biotypes 1B (O:7, O:13, O:21, among others) (EFSA, 2007) (Zadernowska et al., 2014). In Europe, the biotypes and serotypes most frequently associated with infection by *Y. enterocolitica* in humans are 4/O:3 and 2/O:9 (EFSA, 2007), while in the United States and Canada the most frequent biotype is the 1B, serotypes O:4,32; O:8, O:13; O:18; O:20 and

O:21. The biotype 3 serotype O:3 has been isolated in Japan and China (Petsios et al., 2016). The biotype 5 is rarely isolated (EFSA, 2007).

Y. enterocolitica has been isolated from environmental samples, foods including meat (pork, beef), milk, cheese, fish and vegetables (EFSA, 2007) (Sabina et al., 2011). The majority of the isolated strains have not been catalogued as pathogenic (ICMSF, 1996) (EFSA, 2007). The principal reservoir of the pathogenic strains of *Y. enterocolitica* for the human being is pig farming (Falcao et al., 2006) (Fredriksson-Ahomaa et al., 2006) (Bancerc-Kisiel and Szweda, 2015).

In animals, infection by *Y. enterocolitica* is usually asymptomatic. In particular, clinical symptoms are rarely observed in pork, except for diarrhoea in animals under the age of 8 weeks (Bancerc-Kisiel and Szweda, 2015).

Outbreaks of yersiniosis are often associated with the consumption of raw or uncooked pork and its derivatives (Bancerc-Kisiel and Szweda, 2015). There is a correlation between the strains isolated in pork and the strains isolated in clinical cases of yersiniosis in humans (Bancerc-Kisiel and Szweda, 2015). Nevertheless, it should be noted that it can also be transmitted through the consumption of beef, sheep or goat, contaminated water and untreated water (Zadernowska et al., 2014). Contamination of food by food handlers has been indicated; however, person-person transmission rarely occurs (Sabina et al., 2011).

Yersiniosis particularly affects children, specifically under the age of 5 years old. *Y. enterocolitica* causes gastrointestinal syndromes of varying intensity, from mild diarrhoea to mesenteric adenitis simulating appendicitis. The symptoms of this disease include fever, abdominal pain and diarrhoea, with haemorrhagic frequency. The infection on rare occasions advances and gives rise to articular problems which may remain for several months. The incubation period is from 4-7 days, after exposure, and symptoms may last from 1 to 3 weeks, and even longer on rare occasions. In adults, the predominant symptoms may include abdominal pain on the right side of the abdomen and fever, which may lead to confusion with appendicitis. These symptoms may also be present in children (Montville et al., 2012) (Bancerc-Kisiel and Szweda, 2015).

2.2.1.3 Epidemiological and prevalence in food data

In the European Union (EU), yersiniosis is the third highest zoonotic disease in terms of the number of most frequent food-borne cases, with 6 861 confirmed cases in 2016 (EFSA, 2017). The notification rate in the EU is 1.82 cases per 100 000 inhabitants. A declining trend has been observed between 2008 and 2016. Nevertheless, no significant increase or decrease was observed in the 2012-2016 period. It should be noted that the notification of cases of yersiniosis is not obligatory in all Member States. The notification rates of yersiniosis are higher in the countries in the north-east of Europe. The countries with the highest notification rates in 2016 were Finland (7.42 cases per 100 000 inhabitants) and the Czech Republic (5.76 cases per 100 000 inhabitants). In 2016, the figures registered by the EFSA indicated 485 confirmed cases in Spain (EFSA, 2017).

Y. enterocolitica is the most frequently isolated species in the EU (in 99.1 % of the cases in 2016). Information about the serotypes of *Y. enterocolitica* concerned in 2016 was only given in 39.5 % of the confirmed cases by 14 countries. According to the figures collected, the most common serotype

is the O:3 (84.6 %) followed by O:9 (11.8 %) and O:8 (1.7 %). Biotype information was only collected in 4.6 % of the confirmed cases by five countries (Austria, Denmark, Finland, Lithuania and Poland). The dominant biotype among those reported in 2016 was biotype 4 (79.6 %) followed by biotype 2 (16.9 %) and biotype 3 (2.5 %) (EFSA, 2017).

With respect to hospitalisation in 2016 in the EU, data has been received from 14 countries with a total of 1 653 cases (24.1% of the total), where the percentage of hospitalisation came to 31.5 %. The highest percentage of hospitalisation (54.8-91.7 %) was observed in Lithuania, Poland and Romania (EFSA, 2017).

In relation to the severity of the infection by *Y. entecolitica,* it should be noted that in the United States the percentage of hospitalisations is 34.4 %, with a 2 % mortality rate (Scallan et al., 2011).

In Spain, in 2015, 478 cases of yersiniosis were reported. The highest incident rate of yersiniosis in Spain is observed in children under 15 years old and in particular, in children under the age of 5 years (CIBERESP, 2017). According to the annual report from the SIM (Microbiological Information System) of 2015, from a total of 345 isolations the 0:3 serogroup was identified in 40. The presence of other serogroups was not reported. In 2016 a total of 378 isolations of *Y. enterocolitica* were reported from 44 laboratories. In 55 of these *Y. enterocolitica* O:3 was identified (SIM, 2017). No information is available for the biotypes of *Y. enterocolitica*.

Although *Y. enterocolitica* is found in all climate zones, the different bioserotypes have been associated to specific geographical regions. Thus *Y. enterocolitica* 4/O:3 and 2/O:9 have been mainly isolated in Europe and *Y. enterocolitica* 1B/O:8 in the United States. *Y. enterocolitica* 4/O:3 is predominant in the majority of European Countries, while *Y. enterocolitica* 2/O:9 is predominant in the United Kingdom (EFSA, 2007). As a consequence of the flow of raw materials, feed and food, and of tourism, an increase in the number of cases of disease caused by *Y. enterocolitica* 1B/O:8 has been observed in zones where it had not previously been isolated, including Poland and Germany (Rastawicki et al., 2009) (Zadernowska et al., 2014). This fact is of concern, as the strains of *Y. enterocolitica* of the 1B biotype and O:8 serotype are considered to be more dangerous and virulent for humans (Barcercz-Kisiel et al., 2015). According to figures from the EFSA in 2016 the O:8 serotype was isolated in 1.7 % of the cases reported (EFSA, 2017). It would seem advisable to monitor the presence of *Y. enterocolitica* 1B:O:8, given its special virulence.

In a study conducted in the North of Spain on infections in humans by *Y. enterocolitica* during the 1985-2014 period, 99 % of those isolated were found to be from the O:3 serotype. In the isolated cases in which the biotype was observed, it was found to be biotype 4. Nevertheless, it should be noted that five isolates of 2/O:9 and one isolate of 1B/O:8 were identified (Marimon et al., 2017).

In the United States, a study on food-borne diseases conducted by Scallan et al. (2011) estimates that 90 % of the cases of infection by *Y. enterocolitica* are food-borne, although it should be noted that the authors indicate that all the outbreaks reported in the United States were associated with contaminated food. Studies carried out in the United Kingdom and France estimate that the percentage of cases of *Y enterocolitica* attributed to the consumption of food is 90 % (Adak et al., 2002) (Vaillant et al., 2005).

In relation to the presence of isolated pathogenic strains found in samples taken from humans, animals and the environment, the study conducted by Le Guern et al. (2016) is of relevance, with 19 670 strains of *Yersinia* isolated in France over more than 50 years. The authors observed that the majority of the strains of human origin were pathogenic (59 %), predominantly *Y. enterocolitica* bioserotype 4/O:3 (66.8 %), followed by *Y. enterocolitica* 2/O:9 (23.8 %). Only *Y. enterocolitica* 4/O:3 was found in pigs and pork meat. In environmental and food samples, pathogenic strains were rarely found (0.2 %). The largest source of pathogenic strains of *Yersinia* were animals, with a significant association between *Y. enterocolitica* 4/O:3 and pig farming.

It is estimated that between 86.1 and 100 % of the outbreaks due to *Y. enterocolitica* in humans are associated with pork meat (Painter et al., 2013). There is a correlation between the strains isolated in pork and the strains isolated in clinical cases of yersiniosis in humans (Bancerc-Kisiel and Szweda, 2015).

The EFSA report for 2016 only includes figures for the presence of *Y. enterocolitica* in livestock from one country (Italy) with a total of 100 samples analysed. With respect to the presence in meat and meat products, it only includes information from five countries with a total of 971 samples analysed. The presence in meat was low (>1-10 %) to high (>20-50 %) (EFSA, 2017).

Although an EFSA report suggested technical specifications to harmonise the monitoring and notification of *Y. enterocolitica* in pork at slaughterhouse level (EFSA, 2009), the information included in the latest report of the EFSA indicates that the figures presented do not follow a harmonised pattern (EFSA, 2017), the prevalence of pathogenic strains of *Y. enterocolitica* in pork vary according to the methodology used, the sampling zone and geographical area (Petsios et al., 2016).

Y. enterocolitica is frequently found in the oral cavity (especially in tonsils), faeces and the gastrointestinal track of pigs (EFSA, 2007) (Fredriksson-Ahomaa et al., 2009). Pig tonsils are a significant source of contamination in the slaughterhouse. Fredriksson-Ahomaa et al. (2009) detected *Y. enterocolitica* in 62 % of the tonsils analysed, whereas in faeces it was observed in 16 %. In a study carried out by Bonardi et al. (2013), the presence of this bacteria was detected in 10.8 % of tonsils, 17.1 % of faeces, 11.8 % of blanching water and 2.4 % of carcasses. 24.4 % of the isolated strains were identified as pathogens. These pathogenic strains were isolated from 8 % of tonsils, 1.1 % of faecal samples and 0.7 % of carcasses. Other studies indicate different prevalence of *Y. enterocolitica* in tonsils samples at pig slaughterhouses. A prevalence of 93 % was observed in Spain, 44-55.3 % in Belgium and 32 % in Italy, where the most frequently isolated bioserotype is the 4/O:3 (Ortiz Martínez et al., 2011) (Van Damme et al., 2015). Although the biotype 4 serotype O:3 is the one which is most frequently isolated in pigs, it should be noted that the following minority biotypes have also been identified: 2/O:9 2/O:5, 27 and 3/O:9 (Bonardi et al., 2003, 2007, 2013) (Fredriksson-Ahomaa et al., 2007) (Ortiz Martínez et al., 2011). In fact, in England the most frequently isolates are 2/O:9 and 2/O:5 (Ortiz Martínez et al., 2010).

In relation to the presence of genes, which code virulence factors in strains of *Y. enterocolitica* isolated in pig slaughterhouses of the bioserotype 4/O:3, *ystA* (100 %), *inv* (95.8 %), *ail* (87.5 %) and *yadA* (54.2 %) have been identified. In the isolates of *Y. enterocolitica* 2/O:9 the genes *ail*, *inv* and *ystA* were identified in 100 % of the isolates, while the *ystB* gene was isolated in 25.0 %. The gene

yadA associated with the plasmid pYV was not found in the 2/O:9 isolates (Bonardi et al., 2013). In the strains of *Y. enterocolitica* of the biotype 1A, considered as non-pathogenic, the following genes have been identified, *inv* (95.4 %), *ystB* (72.4 %), *ystA* (11.5 %) and *ail* (6.9 %) (Bonardi et al., 2013).

Some studies of the prevalence of pathogenic *Y. enterocolitica* are based on the detection of the *ail* gene for discriminating between pathogenic and non-pathogenic strains. Other authors use as a criterion for the classification of pathogenic *Y. enterocolitica* the presence of both the *ail* gene and the *ystA* gene (Van Damme et al., 2015) (Lorencova and Slany, 2016). However, in certain 4/O:3 isolates, the *ail* gene has not been identified. On the other hand, the presence of the *ail* gene is not considered sufficient to ensure the pathogenicity of the strains (Sihvonen et al., 2011). This fact must be considered when comparing the results of the different studies.

The available studies indicate that the tonsils are the main source of pathogenic strains of *Y. enterocolitica* for humans (Bonardi et al., 2013) (Nesbakken et al., 2003, 2006). During the processing of pork meat in the slaughterhouse, *Y. enterocolitica* present in the oral cavity or intestinal content may contaminate the carcass and the slaughterhouse environment (Laukanen-Ninios, 2014). Counts of 4.4 log CFU/g have been found in tonsils and 3.8 log/cfu in faeces, while the carcass count is, for the majority of the cases, lower than the detection limit (Van Damme et al., 2015). As a result, there is a need to adopt measures at pig slaughterhouse level in order to minimise the contamination of the carcasses with *Y. enterocolitica* from the oral cavity, faeces, and intestinal content (Laukanen-Ninios, 2014).

Studies on the presence of *Y. enterocolitica* in pork and pork meat products indicate a prevalence of 15.2 %, with very low counts (Bonardi et al., 2010). A higher prevalence of *Y. enterocolitica* has been found in minced pork (20 %) than in fresh sausages (10.9 %) (Bonardi et al., 2010). With respect to the prevalence of pathogenic *Y. enterocolitica* in pork, limited information is available. The highest figures were found in tongue at 40 %, followed by heart 18 %, in minced meat the values indicated by different authors range between 4.9 and 17.2 % (Messelhäusser et al., 2011) (Lorencova and Slany, 2016). Some studies indicated prevalence rates of 11 % in fermented pork sausages (Lambertz et al., 2007). Other authors have also indicated the prevalence of high pathogenic *Y. enterocolitica* in fresh pork products (Johannessen et al., 2000). The methodology used to determine pathogenic *Y. enterocolitica* may have a significant impact on the prevalence rates found in scientific literature.

In pork meat, a high percentage of the biotype 1A (90.9 %) has been observed, although biotype 2 serotype O:9 has also been identified (Bonardi et al., 2010). Pork may be a major source of *Y. enterocolitica* 4/O:3 (EFSA, 2007). In the isolated strains of the biotype 1A the genes which encode virulence factors, *ail*, *inv*, *ystA* and *ystB* have been detected. Some authors have indicated that the majority of the strains of the biotype 1A possess the gene *ystB* (90 %) (Bonardi et al., 2010). The strains of the biotype 1A are generally considered non-pathogenic; however, they have been isolated in clinical cases of yersiniosis, and been associated with certain outbreaks of yersiniosis (Ratnam et al., 1982) (Greenwood and Hooper, 1990) (Tennant et al., 2003). Consequently, some authors indicate that the strains of the biotype 1A may be considered as emerging pathogens (Batzilla et al., 2011) (Bancerc-Kisiel and Szweda, 2015). It is necessary to reconsider the potential pathogen of the strains of *Y. enterocolitica* of the biotype 1A, investigating whether it is limited to a small proportion or is spreading.

2.2.1.4 Methodologies for detection

The prevalence of pathogenic *Y. enterocolitica* in pork meat and carcasses may be underestimated as a consequence of the limitations of the detection methods due to the low concentration of pathogenic strains present in food, the similarities with other Enterobacteriaceae, and the heterogeneity of *Y. enterocolitica* as this includes both pathogenic and non-pathogenic strains (EFSA, 2007) (Petsios et al., 2016). In samples taken in the slaughterhouse of tonsils and faeces, *Y. enterocolitica* is present in higher concentrations than in the carcasses and consequently its detection is more likely (Zadernowska et al., 2014).

For the detection of *Y. enterocolitica* traditional culture techniques have been used, proceeding to the subsequent identification using biochemical, technical, serological and microscopic tests. In foods *Y. enterocolitica* is present in low numbers and there are often a large variety of microorganisms present. Therefore, direct isolation on selective media may not be suitable. The isolate methods usually include an enrichment stage, plating on selective media and subsequent confirmation of selected typical colonies. The isolated strains are subsequently characterised: biotype, serotype, virulence factors (EFSA, 2007). It should be noted that the methods used may result in the isolation of non-pathogenic strains of *Y. enterocolitica*. The use of suitable enrichment procedures together with chromogenic media may improve the isolation of pathogenic strains of *Y. enterocolitica* (Zadernowska et al., 2014) (Petsios et al., 2016). Biotyping is essential for differentiating between pathogenic and non-pathogenic strains (EFSA, 2007).

Molecular biology techniques help to resolve the limitation of the traditional culture methods. The use of the polymerase chain reaction (PCR) for the detection of *Y. enterocolitica* in foods detects higher prevalence rates than traditional culture methods. However, it does have the limitation of not differentiating between viable and non-viable cells (Bonardi et al., 2014) (Zadernowska et al., 2014). The development of methods which permit the detection only of viable cells such as real-time PCR, is a major advance (Lambertz et al., 2008).

The EFSA has recommended the sampling of tonsils in pig slaughterhouses. Among the methodologies to be used, the ISO 10273:2003 (ISO, 2003) standard is recommended. In addition, the biotyping and serotyping of the strains is also recommended (EFSA, 2007, 2009). The ISO 10273:2003 methodology for the detection of pathogenic strains of *Y. enterocolitica* is considered to have limitations, and therefore alternative methodologies have been suggested (Bonardi et al., 2016). In 2017, the ISO 10273 standard was updated for the detection of pathogenic *Y. enterocolitica* (ISO, 2017). This standard introduces changes in relation to the culture media, incubation times, and confirmation tests. For the confirmation of pathogenic *Y. enterocolitica,* it adds biochemical tests that permit the differentiation between pathogenic and non-pathogenic strains, and the alternative optional confirmation using the detection of the *ail* gene using real-time PCR techniques.

The determination of the biotype both in the ISO 10273:2003 standard and in the ISO 10273:2017 standard is based on the following tests: fermentation of the xylose and trehalose, esculin hydrolysis and detection of pyrazinamidase, lipase and indole (ISO, 2003, 2017).

The detection of the serotypes based on more frequent O antigens (O:3, O:5, O:8, O:9, O:5 and O:27) uses specific antisera agglutination techniques (Zadernowska et al., 2014) (Petsios et al., 2016). For

the differentiation of pathogenic strains of *Y. enterocolitica*, techniques have been used based on the determination of phenotype characteristics associated with the pYV virulence plasmid such as calcium dependent growth, Congo red absorption and autoagglutination. In this respect, the Congo red-magnesium oxalate (CR-MOX) agar has been used to differentiate the pathogenic strains (Petsios et al., 2016).

The serological test using ELISA (Enzyme-linked Immunosorbent Assay) techniques may be used for the estimation of the prevalence of *Y. enterocolitica* on pig farms and in slaughter houses (Nielsen et al., 1996) (Bonardi et al., 2016).

The PCR technique has been used for the detection of genes associated with the virulence of *Y. enterocolitica* and for the identification of pathogenic isolates (Fredriksson-Ahomaa and Korkeala, 2003). Nevertheless, its effectiveness is limited, as in the non-pathogenic strains (biotype 1A) some of the virulence genes may be found. The differentiation between pathogenic and non-pathogenic strains has been carried out with the detection of the plasmid genes of pathogenicity or of chromosomic genes (Bonardi et al., 2010, 2013). It should be noted that the loss of the pYV virulence plasmid is common in *Y. enterocolitica*, while the loss of the chromosomic genes of virulence is uncommon (Zheng et al., 2008). If the use of the pYV virulence plasmid is selected, the tests must be conducted at an early stage of the confirmation. The *yadA, virF* genes found in the pYV plasmid have been used to discriminate between pathogenic and non-pathogenic strains (Bonardi et al., 2014). The *ail* gene located in the chromosome of pathogenic strains of *Y. enterocolitica* is the most frequently used gene for discriminating between pathogenic and non-pathogenic strains (Petsios et al., 2016). Some authors have isolated this gene in all the isolates of *Y. enterocolitica* 4/O:3. However, in certain 4/O:3 pathogenic isolates, the *ail* gene has not been identified. On the other hand, the presence of the *ail* gene is not considered sufficient to ensure the pathogenicity of the strains, as it is also present in some strains of the biotype 1A (Sihvonen et al., 2011). Other chromosomic genes present in pathogenic strains of *Y. enterocolitica* detected using PCR techniques include the *inv* gene and *ystA* gene (Van Damme et al., 2015) (Lorencova and Slany, 2016).

2.2.1.5 Future steps

Given that the pig farm is the principal reservoir of pathogenic *Y. enterocolitica,* it is important to obtain information about the prevalence of this pathogen at slaughterhouse level, including information about the biotypes and serotypes present.

In pork meat the strains of isolated *Y. enterocolitica* are mainly of the 1A biotype. The pathogenic biotypes are occasionally isolated in pork meat. Nevertheless, it is important to obtain more information about the prevalence of pathogenic *Y. enterocolitica* in pork meat, including information about the biotypes and serotypes. Given the limitations of the methodologies used and the low counts in pork, prevalence may be underestimated. Given the higher prevalence in minced pork observed in the scientific literature and its association with cases of yersiniosis, it is thought that a good objective would be to make a control plan, and both the biotype and the serotype should be determined.

It is important to harmonise the methodology in order to be able to compare the results obtained. Special attention must be given to the limitations of the different methodologies used. The ISO 10273:2017 standard includes the detection of pathogenic *Y. enterocolitica*, and adds pathogenicity-

related tests that permit the differentiation between pathogenic and non-pathogenic strains. This standard includes the alternative optional confirmation using the detection of the *ail* gene using real-time PCR techniques, although it is essential to consider that this gene has certain limitations as it has not been identified in certain 4/O:3 pathogenic isolates. In addition, the presence of the *ail* gene is not considered sufficient to ensure the pathogenicity of the strains, as it is also present in some strains of the biotype 1A.

In order to minimise the contamination of pork carcasses in slaughterhouses, special attention must be given to the hygiene measures adopted.

Heat treatment plays an important role in the control of *Y. enterocolitica.* Therefore, campaigns may be carried out directed at the consumer to ensure the meat is correctly cooked.

2.2.2 *Vibrio parahaemolyticus* and *V. vulnificus* in fish products and bivalve molluscs

2.2.2.1 General information

The bacteria of the *Vibrio* genus are Gram-negative bacilli, mainly related to aquatic habitats. There are more than 100 species in this genus, of which just over 10 have been associated with human disease (Janda et al., 2015). The species relevant to human health, apart from *V. cholerae* (*V. parahaemolyticus* and *V. vulnificus*) are mainly associated with warm water (> 15 ºC) and low salinity (<25 ‰ NaCl), conditions which can be presupposed as possible in many regions of Europe as a consequence of global warming (Baker-Austin et al., 2013) (Roux et al., 2015).

2.2.2.2 General characteristics

V. parahaemolyticus causes acute gastroenteritis characterised by diarrhoea, nausea, vomiting, abdominal pain, headaches, fever and chills, from the consumption of raw fish and molluscs (Nelapati et al., 2012). Although on the majority of occasions, this is a self-limiting aqueous diarrhoea, occasionally it may cause bloody diarrhoea and, on rare occasions, cardiac arrhythmia (Honda et al., 1976). It is the main cause of bacterial gastroenteritis from the consumption of fish all over the world (Rippey, 1994). It also causes travellers' diarrhoea, wound infections, otitis and secondary septicaemia (Pavia et al., 1989).

The minimum quantity necessary to develop gastroenteritis is between 2×10^5 and 3×10^7 bacteria, with an incubation period of 3 to 24 hours, normally between 10 and 15 hours (Nelapati et al., 2012).

To date, 13 O serotypes and around 70 K serotypes have been identified in *V. parahaemolyticus*, based on the somatic (O) and capsular (K) antigen (Jones et al., 2012), where the O3:K6 serotype has been responsible for the majority of outbreaks around the world since 1996 (Hara-Kudo et al., 2012).

Its pathogenicity is closely linked to the synthesis of thermostable direct haemolysin, TDH, known as Kanagawa haemolysin (Honda e Iida, 1993). Other serotypes produce a thermostable related haemolysin, TRH, which is also linked to the pathogenicity (Honda et al., 1988). TDH and TRH, respectively encoded by the *tdh* and *trh* genes, are important virulence factors of this microorganism, with cytotoxic, cardiotoxic and enterotoxic properties (Honda e Iida, 1993). The O3:K6 serotype, one of the most prevalent in gastrointestinal infections, for example, is a carrier of the *tdh* gene (Matsumoto et al., 2000).

V. vulnificus is a species which is associated with cases of disease in animals and humans. Three different biotypes have been described, of which biotype 1 is the one which is linked to the severe human cases, while biotype 2 is associated with the cultivation of eels and biotype 3, very rare, is considered as a hybrid of the previous two, and to date has only been detected in Israel (FAO/WHO, 2005) (Horseman and Surani, 2011). In humans, it may cause gastrointestinal disease, although the most common symptoms are the infection of wounds (due to exposure to contaminated water) or primary sepsis due to the intake of molluscs, mainly contaminated oysters (SCVMPH, 2001) (Janda et al., 2015). The symptoms of the infection appear quickly and can be fatal, especially in immunocompromised males or males with health problems that result in high levels of serum iron (hemochromatosis), including liver disease, diabetes or alcoholism. The mortality rate ranges between 50 and 60 % of patients with septicaemia.

There are a number of studies aimed at explaining the mechanisms used by *V. vulnificus* to produce such severe disease. Numerous factors are involved, including the production of lysine decarboxylase, which permits the neutralisation of stomach acid, the presence of polysaccharide capsules that prevents phagocytosis, or the production of siderophores and a haemolytic toxin, involved in iron uptake (Horseman and Surani, 2011). These factors, among others, contribute to the development of the infection; however, the fatal outcome of the infection appears to be due to the lipopolysaccharide (LPS), as when it is injected in animals used for research it results in rapid death. This effect is not observed when neutralised LPS is used. In addition, it also offers an explication to the fact that infection by *V. vulnificus* almost exclusively affects males, as an oestrogen-mediated inhibition of the action of the LPS has been observed (Oliver, 2013).

2.2.2.3 Epidemiological and prevalence in food data

Both *V. parahaemolyticus* and *V. vulnificus* are widespread in coastal environments, where the ambient conditions of the water, mainly salinity (they are halophilic microorganisms, which grow at between 2 and 25 ‰ NaCl, with an optimum salinity of between 10 and 18 ‰ NaCl) and temperature (temperatures above 20 ºC are of particular concern, although above 30 ºC they are negatively affected; a temperature of 13 ºC is considered as the lower limit of growth, although they may be found at lower temperatures in a viable but not cultivable state) are of importance (Kaneko and Colwell, 1975) (Oliver, 2015). Vibrios are bacteria predominantly found in the digestive tract of oysters, clams, mussels and prawns, and the consumption of raw molluscs and raw or undercooked fish or shellfish are the main sources of the disease (Nelapati et al., 2012).

The incidence of *V. parahaemolyticus* is markedly seasonal, and is restricted to the summer months, due to the sensitivity of the microorganism to low temperatures (Kaneko and Colwell, 1975). In tropical zones, it should be noted that it is found all year round (Elhadi et al., 2004).

V. parahaemolyticus was first isolated after an outbreak associated with the consumption of sardines (272 cases and 20 deaths) in Japan in 1950 (Fujino et al., 1953). Its incidence rate has increased in many parts of the world (Hara-Kudo et al., 2012). Its spread around the world has been facilitated by many paths. Factors such as climate change, which has resulted in new oceanic currents and a warming of coastal waters (Vezzulli et al., 2013) (Burge et al., 2014), the inadequate transport and

storage of fish and fish products over long distances, or the seawater used by vessels as ballast, have facilitated its expansion (Martínez-Urtaza et al., 2016, 2018). Once introduced to a new region, it becomes endemic. In a simulation developed recently using different future climate scenarios and time horizons, and considering the effect of the factors which contribute to the growth of *V. parahaemolyticus* from the seawater to consumption, it has been observed that as coastal waters heat up, the risk of outbreaks of this microorganism continue to rise (Ortiz-Jiménez, 2018). To these facts, it is necessary to add the increase in the resistance to antibiotics, also observed in *V. parahaemolyticus*, having found that 90 % of the strains isolated in a recent study were resistant to streptomycin, and to other antibiotics (Xie et al., 2017).

In Europe, the main outbreaks related to *Vibrio* occurred in the Baltic Sea region during the summer, due to the recreational use of the sea (Baker-Austin et al., 2010). Clinical cases were also found in Italy and France due to the O3:K6 serotype of *V. parahaemolyticus*, linked to the consumption of molluscs (Baker-Austin et al., 2010). In their annual report for 2015 on outbreaks of food-borne disease, the EFSA listed four outbreaks due to *V. parahaemolyticus* in France, with 29 cases. The food responsible for two of these outbreaks were crustaceans, shellfish, molluscs and derivatives (EFSA, 2016). These figures were similar to those reported in 2014. In 2016, eight outbreaks were reported, with 76 cases in total (EFSA, 2017). However, this latest report does not differentiate between the species of *Vibrio.*

In Spain, and more specifically in Galicia, there have been three major outbreaks in the last 20 years, one in 1999, with 64 cases due to the consumption of raw oysters, another in 2004, with 80 cases related to the serotype O3:K6 and another, with almost 100 cases, from the consumption of prawns in 2012 (Martinez-Urtaza et al., 2016). Nevertheless, since 2012, a clear transition has been observed in the epidemiology of this pathogen, observing isolated sporadic cases along the coast instead of major outbreaks, caused by different unrelated strains, typically associated with the consumption of local fish products. This would be indicative of frequent episodes of introduction from remote and separate zones, always associated with the increase of the temperature of the coastal waters (Martínez-Urtaza et al., 2018). In any case, in 2015 no cases of infection by *V. parahaemolyticus* were reported to the Microbiological Information System (SIM, 2016).

Of note is the case of Japan, in which *V. parahaemolyticus* caused 70 % of the cases of gastroenteritis associated with fish products (Kaneko and Colwell, 1975). Nevertheless, in the period between 1999 and 2009, a sharp decline has been observed in the number of infections caused by this microorganism (Hara-Kudo et al., 2012). This decline is not correlated with changes in the contamination of the fish products with *V. parahaemolyticus,* which remains at approximately the same levels (Hara-Kudo et al., 2012). However, the establishment of hygiene measures for fish products during production and marketing (distribution and storage at temperatures below 10 ºC, use of hygenised seawater or drinking water and the establishment of microbiological limits of absence at 25 g in ready-to-eat cooked products and of <100 MPN/g in raw products throughout the distribution chain), and the recommendation to consumers to eat fish products within two hours of removing them from the fridge, would appear to be responsible for this decline (Hara-Kudo et al., 2012). Other preventive measures which could help to reduce the risk of outbreaks due to this microorganism include adequate cooking

of the fish products prior to consumption, and the application of adequate hygiene measures to prevent cross-contamination between raw and cooked food (Nelapati et al., 2012).

V. vulnificus has been isolated from water, marine sediments and various aquatic animals, mainly oysters and other molluscs (Jones and Oliver, 2009). In addition, the geographical area of distribution is expanding as a consequence of climate change and *V. vulnificus* has been found in regions to date unaffected, such as the Baltic Sea (Baker-Austin et al., 2013). In Spain, the presence of *V. vulnificus* had already been described in the 90s, as a pathogen of eels and its detection on the Mediterranean coasts (Biosca et al., 1991). The first studies on its presence in water and bivalve molluscs indicated a low prevalence (Arias et al., 1999), later investigations revealed its regular presence in water and molluscs on the Mediterranean coast (Cañigral et al., 2010) and some cases of wound infection as a consequence of exposure to water in the Mediterranean (Torres et al., 2002), but also in the Cantabrian Sea (Martínez-Rienda et al., 2007).

The food-borne disease almost always occurs in individuals with the afore-mentioned health problems, mainly liver diseases, and is associated with the consumption of raw oysters and other shellfish (Strom and Paranjpye, 2000) (Jones and Oliver, 2009) (Janda et al., 2015). Nevertheless, exposure to *V. vulnificus*, even in predisposed persons, does not imply the development of the disease, as the existence of two types of *V. vulnificus* has been demonstrated. These are called "C" (clinical) and "E" (environmental), and can be differentiated based on their genetic characteristics, and the strains belonging to type C are those that would provoke human infection (Oliver, 2013).

V. vulnificus mainly affects males with a physiological predisposition, which is the reason that it is not associated with outbreaks of the disease, but is found in isolated cases. In the United States infections from *V. vulnificus* are voluntarily registered in the COVIS (*Cholera* and Other *Vibrio* Illness Surveillance System; https://www.cdc.gov/vibrio/surveillance.html). The figures registered in recent years indicate average values of 126 cases/year and 30 deaths/year, although the true number of cases associated with the consumption of oysters may be up to 2.5 times higher (FAO/WHO, 2005). In Spain, it is not obligatory to declare infection by *V. vulnificus* and the data existing in scientific literature registered a total of six cases declared, of which four were caused by wound infections after exposure to seawater and two can be attributed to the intake of raw oysters and steamed mussels (Martínez-Rienda et al., 2007). In other European countries, the incidence of cases of infection by *V. vulnificus* is also low, appearing sporadically (Baker-Austin et al., 2010). The majority of cases of infection were recorded in the hottest months, which also coincide with the highest rates of isolation of the bacteria from environmental samples and with a highly significant correlation with the water temperature (Baker-Austin et al., 2010) (Oliver, 2015).

In an estimate carried out based on the data collected in the United States on the coast of the Gulf of Mexico, it was concluded that the concentration of *V. vulnificus* in oysters at the time of consumption could be over 4 log CFU/g (FAO/WHO, 2005). Although the majority of the isolates obtained from oysters belong to type E strains, the intake of just one oyster contaminated by type C by a predisposed person could be enough to develop the infection (Oliver, 2013).

There is no legal requirement for monitoring the presence of *Vibrios* in bivalve molluscs in the EU, as they are not included in the current microbiological criteria for food (EU, 2005). This circum-

stance, combined with the low incident rate of the disease and the absence, until recently, of techniques of reference for its detection, has meant that many countries do not monitor this bacterium on a regular basis (Baker-Austin et al., 2010). Nevertheless, in the case of *V. parahaemolyticus,* the Regional Government of Galicia was a pioneer in making it obligatory to declare its infections since 1995 (Marínez-Urtaza et al., 2016), and since 2013 they must be notified as a matter of urgency (DOG, 2013). In light of the above, the incidence is probably underestimated.

In addition, it is important to remember that the presence of *V. parahaemolyticus* and of *V. vulnificus* in waters is not associated with the faecal contamination indicators (coliform microorganisms, *E. coli*). Consequently, the absence of these indicators in water is not an effective measure for guaranteeing the absence of contamination by *Vibrio* in molluscs. Other parameters such as the monitoring of the temperature and salinity of the water are of more use (FAO/WHO, 2005). In this respect, the European Environmental and Epidemiological network (https://e3geoportal.ecdc.europa.eu) has prepared a tool (*Vibrio* Map Viewer; https://e3geoportal.ecdc.europa.eu/SitePages/Vibrio %20Map %20Viewer.aspx) which monitors the temperature and salinity of the waters in order to establish a risk probability model of *Vibrio* spp.

2.2.2.4 Methodologies for detection

PCR methods have been designed which permit the detection of the *tdh* and *trh* genes in fish products contaminated with pathogenic strains of *V. parahaemolyticus* (Nordstrom et al., 2007) (Nemoto et al., 2009). These methods are very sensitive and specific, however, they are unable to differentiate between whether the DNA is from live or dead cells, which may result in the generation of false positives (Sakata et al., 2018).

Given that both TDH and TRH are exotoxins, when a food containing pathogenic strains of *V. parahaemolyticus* is incubated in a broth medium, they are released to the medium. Consequently, its presence in culture mediums serves as a specific marker of the presence of the viable pathogenic strains of this microorganism (Honda e Iida, 1993). This has permitted the development of immunological methods including ELISA. Nevertheless, these methods are relatively slow and complicated to perform.

Immunochromatographic techniques have also been designed which are faster and simpler. These permit the individual and simultaneous detection of TDH and TRH (Kawatsu et al., 2006) (Sakata et al., 2018). These techniques permit the detection of the fish products contaminated with enteropathogenic strains of *V. parahaemolyticus* in a period of 8.5 hours, including the growth time in the culture medium.

Standard ISO 21872 was recently updated, and includes techniques for the detection of potentially pathogenic species of *Vibrio* spp. And, among these, *V. parahaemolyticus* and *V. vulnificus*. This method consists in a preliminary double enrichment of the sample in alkaline saline peptone water, optional screening of the samples using PCR, planting onto the solid medium (thiosulfate, citrate, bile salts and sucrose agar; TCBS) and a second isolation medium chosen by the laboratory, and confirmation using biochemical testing and/or the detection of the genes of thermostable direct haemolysin, *tdh,* and thermostable related haemolysin TDH, *trh* (*V. parahaemolyticus*) and haemolysin *vvha* (*V. vulnificus*) (Hill et al., 1991) (Campbell and Wright, 2003) (UNE-EN ISO, 2018).

2.2.2.5 Future steps

The presence of *V. parahaemolyticus* and *V. vulnificus* in Spanish coastal waters, both on the Mediterranean and Cantabrian coasts, and their implication in human infections has already been confirmed and an increase in incidence is to be expected as a consequence of the phenomena associated with global warming (Baker-Austin et al., 2010). The European Centre for Disease Prevention and Control (ECDC) also considers that climate change may have repercussions on the proliferation of *V. parahaemolyticus* and *V. vulnificus* and the development of infections as a result of recreational water use (https://ecdc.europa.eu/en/climate-change/climate-change-europe/water-borne-diseases). However, at present, in accordance with the work programmes of the European Union Reference Laboratory (https://eurlcefas.org/), no routine monitoring of *V. vulnificus* in bivalve molluscs exists.

A standardised test method is available for monitoring the presence of *V. vulnificus* in coastal waters and in bivalve molluscs. However, it should be remembered that the mere presence of *V. vulnificus* does not indicate that the strains are of the clinical type. Therefore, further investigation would seem necessary into the available methodologies for differentiating between the environmental and clinical types. In this respect, techniques have been described based on the detection of the variants of the virulence-correlated-gene, *vcgC* and *vcgE* related to the clinical and environmental types respectively (Warner and Oliver, 2008). These have been used successfully for distinguishing between the two types of strains in samples of oysters which are carriers of strains of *V. vulnificus* with huge genetic diversity (Guerrero et al., 2015).

2.2.3 Foodborne non-STEC pathotypes of *E. coli*

2.2.3.1 Introduction

Although *Escherichia coli* is a microorganism normally found in the human gastrointestinal tract, there are some types which have acquired virulence attributes and are able to cause both enteric and extraintestinal disease (ExPEC), mainly infections of the urogenital tract (Kaper et al., 2004). Six pathotypes of diarrheagenic *E. coli* are now recognised: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC) and Shiga toxin producing (STEC), which includes the enterohaemorrhagic types (EHEC) (Nataro and Kaper, 1998) (Kaper et al., 2004) (Tozzoli and Scheutz, 2014):

- STEC is characterised by the production of Shiga toxins (Stx); it is also known as VTEC (verotoxigenic, cytotoxin producing Vtx, which affects the cells of the Vero line). The EHEC strains, in addition to producing these toxins, are carriers of the *eae* gene which produces an intimin involved in the attaching and effacing lesions in the infected cells.
- The EPEC strains have the *eae* gene, but do not produce Stx toxins. They are divided into two groups, typical and atypical strains, based on the presence of an adherence plasmid, which includes the "pili" genes involved in the attachment to intestinal cells. The typical strains are plasmid carriers and are of human origin, whereas the atypical strains, lacking the plasmid, have also been isolated in production animals (Hernandes et al., 2009).
- The ETEC category includes thermolabile (LT) and thermostable (ST) toxin-producing strains and are associated with travellers' diarrhoea. Although it colonises the animal and human in-

testine, there are differences between the virulence factors which cause it, indicating a certain host specificity.

- EAEC is characterised by the production of adherence fimbria which result in an ordered aggregation of bacteria on the cell surface. They also produce an enteroaggregative toxin encoded in a plasmid.
- The enteroinvasive strains (EIEC) carry a virulence factor plasmid which encodes certain invasion antigens. These are very similar to *Shigella* spp. No animal reservoir is known, and therefore it is assumed that transmission occurs via infected humans.
- Lastly, DAEC is a heterogeneous group of strains which attach to cells from the HEp-2 line following a diffuse pattern.

From these groups, some strains of the STEC type and a particular pathotype of EAEC (of the O104:H4 serotype), involved in the outbreak in 2011 are of particular relevance to health (EFSA, 2013, 2015). It is also important to remember that strains of a certain serogroup or serotype may belong to more than one pathotype. For example, the O5 and O26 serotypes include EPEC and STEC strains and the strains of O104:H4, the cause of the outbreak in 2011, also have EAEC and STEC characteristics (EFSA, 2013, 2015) (Álvarez-Suárez et al., 2016).

The main host of the diarrheagenic strains are humans, although some clones of EHEC, EPEC and ETEC may be present in animals and cause disease for animals (Kaper et al., 2004).

2.2.3.2 Hazard identification

It is not easy to find information about outbreaks and cases of human disease caused by *E. coli*. Very often, the disease is resolved spontaneously without severe symptoms, and therefore no microbiological tests are made of clinical and food samples (Nataro and Kaper, 1998). The identification of the different pathotypes is based on the presence of certain virulence factors. Consequently, the characterisation process normally involves the isolation and identification of *E. coli* and the subsequent analysis of the characteristic virulence factors (Feng et al., 2017). Since the appearance of STEC, specifically the O157:H7 serotype, specific culture methods have been developed and detection in clinical and food samples improved; in addition, the severity of the syndromes caused by EHEC demand medical care and therefore the isolation and identification of strains of this type is increased, enabling the epidemiological studies to be extended.

Other pathotypes of *E. coli* are not reported, go unnoticed or are grouped in the reports as other aetiological agents. Thus, for example, in the EU annual report it appears as "Other bacterial agents" (EFSA, 2017) and the data available on the EFSA web page shows "*Escherichia coli*" and "pathogenic *Escherichia coli*" in the list of agents causing outbreaks without specifying what each one refers to (http://www.efsa.europa.eu/en/data/biological-hazards-data). A report drafted in England and Wales differentiates between VTEC O157, VTEC no-O157 and "other *E. coli*" (Adak et al., 2002), the United States distinguishes between "*E. coli*, ETEC", "*E. coli*, O157 STEC", "*E. coli*, non-O157 STEC" and "*E. coli*, other" (Painter et al., 2013). Lastly, the report which appears in the National Epidemiology Bulletin on the outbreaks of food-borne diseases between 2008 and

2011 distinguishes between "*E. coli*, verotoxigenic O157" and "pathogenic *E. coli*" (Espinosa et al., 2014).

The report on the figures for outbreaks of disease in Spain between 2008-2011 considers 13 outbreaks due to pathogenic *E. coli*, with a total of 413 cases, but does not distinguish between types of *E. coli* or attribute them to a specific food (Espinosa et al., 2014). The figures which appear in the EU report on outbreaks in 2016, indicate three outbreaks due to *E. coli* pathotypes, one due to EIEC in the United Kingdom, one due to EPEC in Slovakia and another due to ETEC in Poland; of these, the outbreak caused by EIEC was the most numerous and it appears that it is linked to an emerging clone that has caused several outbreaks and sporadic cases in Europe (including Spain) and belongs to the O96:H19 serotype (Michelacci et al., 2016) (EFSA, 2017). One of the most studied outbreaks attributed to EIEC occurred in Italy in 2012, affecting more than 50 employees of the fire-fighting brigade in Milan. With a retrospective study of cases and controls, it was linked to the consumption of cooked vegetables (beetroot leaves, green beans), although the possibility of person-person transmission or through contaminated environments (services) was considered. An asymptomatic handler, carrier of EIEC of the same serotype (O96:H19) as that identified in some of the patients was identified as the most likely source of contamination (Escher et al., 2014).

The atypical strains of enteropathogenic *E. coli* (aEPEC) seem to be an important etiologic agent of diarrhoea acquired in the community in EU, as shown in a recent multi-centre study which analysed samples from 10 EU countries, and in which EPEC was the most frequently detected microorganism (Spina et al., 2015). Similar figures were obtained in a study conducted in Lugo (Spain) on more than 2 000 samples taken from patients with diarrhoea or other gastrointestinal disorders, the presence of aEPEC was observed in 5.2 % of the samples, the second pathogenic bacteria after *Salmonella* (Blanco et al., 2006). Likewise, in Norway, where a similar study was conducted, in this case on children under the age of 2 years, detecting aEPEC in 10 % of the samples, indicating a high prevalence of this pathotype (Afset et al., 2003). Although several outbreaks of the disease caused by EPEC have been reported, it has not been possible to confirm the food involved; in this respect it is important to remember that transmission may be from human carriers, but there are several papers which describe the isolation of aEPEC strains, belonging to serotypes involved in disease in humans, from production animals, suggesting that these animals may act as a reservoir of pathogenic strains that may be transmitted to humans (Trabulsi et al., 2002) (Hernandes et al., 2009).

Enterotoxigenic *E. coli* (ETEC) is another pathotype involved in the production of gastrointestinal diseases, mainly aqueous diarrhoea related to trips abroad, although not the sole cause of this disease (Nataro and Kaper, 1998) (Kaper et al., 2004). Some studies conducted in Europe indicate a significant prevalence in patients with diarrhoea, especially in co-infections with other pathotypes of *E. coli* or with other agents, such as NoV (Ethelberg et al., 2010) (Spina et al., 2015). A number of outbreaks attributed to ETEC have been reported, some of these affecting hundreds of people. Although it was not possible to confirm the food involved, vegetable products such as spring onions, basil and lettuce, were among the most mentioned foods consumed by those affected (Pakalniskiene et al., 2009) (Ethelberg et al., 2010) (MacDonald et al., 2015).

EAEC is another pathotype of *E. coli* of growing importance, especially following the outbreak which started in Germany in 2011, associated with fenugreek sprouts; this outbreak was caused by a strain of EAEC, but it was also a carrier of a prophage which encoded the Stx2 toxin (EFSA, 2015). Although it was considered to be an agent causing persistent diarrhoea in children, its importance is currently recognised in the development of acute diarrhoea in adults (Okeke y Nataro, 2001). Several outbreaks of gastrointestinal disease caused by EAEC in Europe have been described (in addition to the outbreak already mentioned in 2011), but it has not been possible to confirm the association with food; vegetable products are considered to be important vehicles, either contaminated at origin or by handlers. An outbreak has also been described in Italy in which goats cheese made from raw milk was suspected as a possible source of the microorganism (Scavia et al., 2009) (Dallman et al., 2014) (EFSA, 2015). As occurred with ETEC, this is a pathotype which frequently appears linked with co-infections with other agents (Spina et al., 2015).

2.2.3.3 Detection and monitoring

In the EU, the monitoring of pathotypes of *E. coli* in food is not established, with the exception of Shiga toxin-producing (STEC) *E. coli* belonging to the O157, O26, O111, O103, O145 and O104:H4 serotypes and only in outbreaks (EU, 2005). In addition, there is a shortage of information about the microorganisms present in cases of diarrhoeal disease, as the majority of the infections are resolved spontaneously, without needing medical care and even when care is required, the responsible microorganisms are not always detected, due to a lack of standardised protocols for their analysis (Nataro and Kaper, 1998) (EFSA, 2015) (Spina et al., 2015).

The phenotypic identification is difficult to address, as there are few specific biochemical tests which permit the differentiation of pathotypes, the most characteristic is the absence of mobility and the inability to ferment lactose from EIEC, the serotypes, with some exceptions, are not totally related to specific virulent types and the tests using cellular lines are complex to perform and are not within the scope of all the laboratories (Nataro and Kaper, 1998).

Various protocols for the detection of the pathotypes of *E. coli* can be found in the scientific literature, mainly based on the molecular detection of characteristic genes of the same, using multiple amplification systems or arrays (Bekal et al., 2003) (Anjum et al., 2007) (Spina et al., 2015). The European Union Reference Laboratory for VTEC (http://old.iss.it/vtec/) also has protocols for the detection of certain pathotypes in food samples, based on amplification using Real Time-PCR of the characteristic genes, following enrichment of the samples in buffered peptone water. The detection of EAEC is based on the amplification of the *aggR* gene (transcriptional regulator located in a plasmid) and the chromosomal *aaiC* gene which forms part of a type VI secretion system; EIEC is detected by the amplification of the plasmid gene *ipaH* and ETEC by the amplification of the genes which encode the LT and ST toxins (http://old.iss.it/vtec/). Although the European Union Reference Laboratory does not offer any specific protocol for the detection of EPEC, the strains which are carriers of the *eae* gene and do not amplify the genes of the Stx would fall under the definition of this pathotype. Therefore, the available protocol could be used for STEC.

Considering that several of these pathotypes are of human origin and therefore transmitted via the faecal-oral route, they may be found in almost any type of food susceptible to faecal contamination or incorrect handling; however, a large number of cases of the disease have been linked to vegetable products, especially leafy vegetables, which include lettuce, spinach, ruccula and, in general, various mixed vegetables, whose involvement in the transmission of pathogenic agents has significantly increased in recent years in nearby countries (Callejón et al., 2015) (Yang et al., 2017). Therefore, the category of ready-to-eat chopped vegetables might be considered to be the most appropriate for preparing a plan for the monitoring of pathotypes of *E. coli* in food. Some studies conducted on samples of ready-to-eat salads reveal the regular presence of certain pathotypes of *E. coli*; a study performed in Mexico on 130 samples of salads purchased in restaurants detected the presence of *E. coli* in 85 % of the samples and of these 7 % were positive for certain pathotypes of *E. coli*: ETEC, EIEC and also non-STEC O157 (Castro-Rosas et al., 2012) and another similar study, in this case on samples of salads containing cooked vegetables, revealed a percentage of contamination with *E. coli* of 72.3 % and 4.1 % of samples were carriers of the STEC, EPEC and ETEC pathotypes (Bautista-De León et al., 2013).

2.2.3.4 Future steps

The gastrointestinal diseases produced by the different pathotypes of *E. coli* already pose a significant problem for health and, as diagnosis methods become standardised and applied in a routine manner, it is expected that they will increase. The transmission of these pathotypes often occurs via the faecal-oral route, and their presence in water used for the production of vegetables may increase their incidence in foods of plant origin, as suggested by the rising trend observed in recent years (Callejón et al., 2015). The studies consulted reveal the regular presence of pathotypes of *E. coli* in samples of ready-to-eat salads and other vegetable products; European legislation already considers the monitoring of *E. coli* as an indicator of faecal contamination in this food category, and this could serve as a base for monitoring pathogenic strains.

The pathotypes of *E. coli* of most interest for prospective studies have already been mentioned in this document; likewise, it is important to consider new species or pathotypes which may be defined. In this respect, the species *Escherichia albertii* is an emerging pathogen, carrier of the *eae* gene, involved in the attaching and effacing lesions produced by EPEC and EHEC. It has been linked with outbreaks of foodborne disease and isolated in some food, including chicken carcasses. However, standardised diagnosis techniques for this microorganism are still to be defined (Nimri, 2013) (Lindsey et al., 2015).

One last aspect of future interest related with *E. coli* is the growing concern about the presence of strains carrying antimicrobial resistance genes in the food chain, such as the extended spectrum β-lactamase (ESBL) producers. Although its presence has been mainly studied in foods of animal origin, resistant strains, some of them with pathogenic characteristics, have also been detected in plant products, which may therefore be a vehicle for the transmission of bacteria carrying resistance genes (van Hoek et al., 2015) (Skočková et al., 2015).

2.2.4 Clostridium difficile in fresh meat

C. difficile is an anaerobic gram-positive spore-forming microorganism, which is commonly found in the intestinal tract of humans and other animals. It may cause a spectrum of diseases known as "*Clostridium difficile* infection" (CDI) ranging from mild symptoms of diarrhoea to pseudomembranous colitis and, on occasions, sepsis, and is usually associated with the administration of antibiotics (González-García et al., 2005) (Asensio and Monge, 2012).

2.2.4.1 Hazard identification

Clostridium difficile is an anaerobic spore-forming bacterium which was initially described as a regular component of the gut microbiota of new-born infants, although at the same time its capacity to produce toxins which are highly lethal for mice was observed (Bartlett, 2008). At the end of the 70s, its association with the development of pseudomembranous colitis associated with the use of antibiotics was confirmed, and it is currently considered the primary cause of nosocomial diarrhoea in developed countries; it forms part of the normal faecal microbiota of 1-3 % of residents in the community and more than 20 % of hospitalised adults, whether symptomatic or asymptomatic. Its spread in hospitals, an environment contaminated by spores, is facilitated, and the risk increases in proportion to the length of the hospitalisation (Rodríguez-Pardo et al., 2013); in addition, in recent years infections have been detected in patients who have not had recent contact with the health care system (González-García et al., 2005) (Asensio and Monge, 2012). Its taxonomic reclassification was recently proposed, including it in a new genus *Clostridioides*. However, this does not suppose any change to its pathogenic characteristics (Lawson et al., 2016).

Exposure to *C. difficile* is assumed to be due to the intake of spores, given their capacity to survive in the environment and be directly transmitted, due to contact with symptomatic or asymptomatic carriers, or indirectly due to contaminated objects (Asensio and Monge, 2012). Foods may be a potential source of exposure, as it has been isolated from several types of production animal, mainly pigs, and also foods of meat origin, vegetables and fish products (Álvarez-Pérez et al., 2013) (National Food Institute, 2016) (Rodriguez et al., 2016) (EFSA, 2017) (Brown and Wilson, 2018) (Candel-Pérez et al., 2018), although no cases of foodborne disease have been recorded. The average prevalence of *C. difficile* in meat and meat derivatives ranges between 0 and 15 %. In studies carried out in the United States and Canada, prevalence values of between 20 and 63 % were obtained, while in Europe the values were much lower (0 to 6.3 %). However, it is not clear whether this is due to seasonal differences as the detection rate was more frequent in the samples taken in winter, or to geographical differences (Rodríguez et al., 2016) (Crobach et al., 2018). In the case of fish products, in Italy isolation rates of 53 % were reported in bivalve molluscs, although not all the isolated strains were toxigenic; in all the cases they corresponded to production zones with high contamination (Pasquale et al., 2012). In ready-to-eat vegetables *C. difficile* was also found with a low prevalence (2.9 %) in France; its presence in this type of food may be due to contamination at source or may occur during processing (Eckert et al., 2013).

It would appear that, unlike other foodborne diseases caused by species of the *Clostridium* genus, such as *C. perfringens*, the growth of *C. difficile* in the food is not necessary and the infection

is produced by the intake of spores which germinate in the gastrointestinal tract, stimulated by the presence of bile salts (Warriner et al., 2017). The disease production method starts with intestinal colonisation, facilitated by the disruption of the microbiota by antibiotic treatment or other patient factors, such as advanced age or the use of gastric acid inhibitors (Freeman et al., 2010) (Asensio and Monge, 2012). This dysfunction of the gut microbiota enables the germination of the spores, as it prevents the microbial transformation of the primary bile acids, activators of *C. difficile* spore germination, into secondary acids, able to inhibit it (Brown and Wilson, 2018) (Crobach et al., 2018). After intestinal colonisation the plant cells produce two toxins: A (TcdA, enterotoxic) and B (TcdB, cytotoxic), which are responsible for the majority of the symptoms of the disease, producing intestinal mucosal lesions and the build-up of fluids. The majority of the strains isolated from clinical cases produce both toxins, although there is a small percentage of clinical strains which only produce toxin B (Voth and Ballard, 2005) (National Food Institute, 2016) (Brown and Wilson, 2018). Some strains of *C. difficile* produce a binary toxin, called CDT, which is related to serious clinical symptoms, but whose role in the development of the disease has not been explained and it is not known with certainty whether or not it really contributes to the virulence or if it is a molecular virulence marker (National Food Institute, 2016) (Brown and Wilson, 2018).

Various types of strains are differentiated, according to their virulence and molecular characteristics. The main type is the 027 ribotype, which has been involved in outbreaks in North America and in Spain, and is assuming growing importance; this ribotype has a mutation in the toxin-producing regulating genes which makes it produce more than other strains. Ribotype 078 is also considered highly virulent and is extremely prevalent in Europe, as well as infecting the youngest patients who are not always undergoing antibiotic treatment (Freeman et al., 2010) (National Food Institute, 2016) (Brown and Wilson, 2018). The data available on circulating ribotypes in Spain is limited; in a study conducted in 2009 in Barcelona, 147 strains were typified from 17 hospitals and 48 different ribotypes were observed. The most common of these were the 241 (26 %), the 126 (18 %) and the 078 (7 %), and no examples of the 027 were found (Rodríguez-Pardo et al., 2013).

2.2.4.2 Detection and monitoring

No standardised protocol exists at present for the detection of *C. difficile* from food samples. Nor is the clinical diagnosis standardised as the laboratory tests for detection *C. difficile* do not differentiate between asymptomatic colonisation and clinical infection and microbiological evidence (diarrhoea, paralytic ileus or toxic megacolon) must be accumulated (Alcalá-Hernández et al., 2015). The clinical laboratory diagnosis is usually based on the antigen detection of the A and/or B toxins or other antigens of *C. difficile* which are available commercially. Techniques have also been developed based on nucleic acid amplification, especially of the toxin B (*tcdB*) gene. As regards culture techniques, selective media are used for isolation from samples of faeces, including CCFA agar (cycloserine-cefoxitin-fructose agar) or CCEY (cycloserine-cefoxitin and egg yolk agar); non-selective media enriched with blood and with taurocholate and lysozyme may also be used, to facilitate recovery by increasing the spore germination (Sorg and Sonenshein, 2008). However, the culture does not permit differentiation between toxigenic and non-toxigenic strains, and therefore

it is necessary to perform additional toxin detection tests, making the diagnosis slower (Alcalá-Hernández et al., 2015).

The different studies which address the detection of *C. difficile* in foods use different methodologies recently revised by Candel-Pérez et al. (2018), making it difficult to establish comparisons between them. Heat treatment or alcohol is frequently used to inactivate contaminating vegetative cells, as are enrichment broths supplemented with agents which stimulate spore germination; lastly, isolation in solid media normally uses antibiotic agents such as cycloserine and cefoxitin, together with culture in anaerobic conditions (Alcalá-Hernández et al., 2015) (Candel-Pérez et al., 2018). In the absence of a standardised method, some researchers have proposed a consensus method for use in the routine analysis of meat in the United States. This method consists in an enrichment of 10 g of sample in 90 ml of BHI broth (in the case of whole pieces, such as chicken breasts or pork chops, a buffered peptone water rinse is prepared which is then used to inoculate the BHI broth), incubated at 35-37 ºC for 3-5 days; an aliquot of 1 ml is treated with 1 ml of 95 % ethanol for 1 hour, centrifuged at 3 800 g/10 minutes, the supernatant is decanted and the sediment re-suspended in the remaining liquid. Finally, the anaerobic blood agar (anaBAP) and CCFA media are inoculated with taurocholate (CCFA-ST) with a drop of the sediment obtained after the treatment with alcohol, incubating in anaerobic conditions at 35 ºC for 96 hours (Limbago et al., 2012). This protocol was developed in three different laboratories on samples of minced meat experimentally inoculated with an approximate concentration of 100 spores/g and then this was used to analyse 1 755 samples of meat, with negative results in all the samples, although other species of the *Clostridium* genus were detected. This would seem to indicate that *C. difficile* is not a regular contaminant present in the meat in the United States (Limbago et al., 2012), although it may also be that the number of spores present is below the detection limit (which has not been established in the Limbago et al. (2012) method), as other studies carried out in the United States on meat and meat products indicate a fairly high prevalence (of more than 40 % in some cases) (Songer et al., 2009).

In fact, the quantification of the number of spores of *C. difficile* present in food is an additional problem, as there is no recommendation regarding the number of spores which would be acceptable in a food product, nor have precise quantitative methods been developed. The few studies which address the enumeration of *C. difficile* in meat observe maximum values of 20-33 spores/g, although the clinical relevance of these results has not been determined and, in any case, it is necessary to consider the high resistance the spores of *C. difficile* have to the treatments used in the preparation of the foods (Weese et al., 2009) (Knight et al., 2016).

From the epidemiological point of view, the typification of isolated microorganisms is also important. Various molecular techniques are available for this purpose, where PCR-ribotyping is the most widespread technique in Europe, while pulsed field electrophoresis (PFGE) is more widely used in the United States. The use of two different methods of molecular typification result in different methods of referring to the strains, making the exchange of data between laboratories more complicated (Knetsch et al., 2013). In order to resolve this situation, a standard protocol has been proposed, based on PCR-ribotyping with resolution using capillary electrophoresis ("CE-PCR ribotyping"). The main disadvantage for universal adoption is the equipment required for the genetic analysis,

although this type of equipment is already widely used in the majority of epidemiological research centres (Fawley et al., 2015).

2.2.4.3 Future steps

In spite of the disparity of data found in the scientific literature, it would appear that the contamination of food with *C. difficile* is fairly common, although their concentration is low (Songer et al., 2009) (Limbago et al., 2012). In addition, no data is available about cases of human disease directly linked to foodborne transmission, although the presence of the same ribotypes in isolated strains of cases of human disease, food and production animals suggests that food may be a potential path of infection (National Food Institute, 2016) (Warriner et al., 2017). It would be important to define the infective dose of spores which may trigger the colonisation, as no indications are available in this respect; in the case of infant botulism, an infection caused by the intake of spores of *C. botulinum*, studies with animal models indicate that the intake of 170 spores (between 80 and 360) produces infection in 50 % of the animals used (Sugiyama et al., 1978). It would also be necessary to consider which populations are the most susceptible to infection. These would include individuals over the age of 65 years old and patients receiving antibiotic treatment. But there are also other risk factors such as treatment with gastric acid inhibitors (proton-pump inhibitors), immunosuppressive therapies or patients with inflammatory bowel disease. An increase in the rate of infection in women in the period around the time of childbirth has also been observed (Asensio and Monge, 2012).

The control of the presence of *C. difficile* in food is difficult to address, given the high resistance of spores to culinary treatment. Consequently, strategies should be based on reducing contamination along all the food production stages. In this respect, the use of vaccinations on animals has been suggested, to reduce the number of carrier animals and limit the use of antibiotics which may affect the prevalence of *C. difficile* in animals for slaughter (National Food Institute, 2016) (Warriner et al., 2017). The introduction of adequate hygiene measures among health workers and food handlers is also of great importance. This includes the frequent washing of hands with soap and water, instead of using alcohol-based disinfectants which are ineffective against the spores of *C. difficile* (Rodríguez-Palacios et al., 2013) (National Food Institute, 2016). Heat treatments of at least 85 ºC for 10-15 minutes is important in the preparation of food. However, it should be noted that this is not a totally effective treatment and cooked foods must be allowed to cool and be kept refrigerated, as recommended for other similar pathogens (Rodriguez-Palacios et al., 2013) (National Food Institute, 2016) (Candel-Pérez et al., 2018).

2.3 Parasites

2.3.1 Protozoa: *Toxoplasma*

2.3.1.1 General characteristics and impact

Toxoplasma gondii is the protozoan parasite distributed worldwide which causes toxoplasmosis, a zoonotic infection of medical significance in immunodepressed patients, pregnant women and congenitally infected new-born infants. Rapid and accurate diagnosis of the disease and, consequently, the correct and effective treatment are essential.

An estimated 30 % of the global population are seropositive (Scallan et al., 2011) and food contributes to between 42 and 61 % of all the cases, depending on the geographical zone (Belluco et al., 2017). The principal path of transmission for *T. gondii* is orally, and the most important source of infection is raw or undercooked meat and fourth range vegetables. Milk is considered to be a potential source of infection, as the infectious parasite in its tachyzoite form can be transmitted through animal fluids. Whatever the food source, consumer habits play a critical role, as *T. gondii* can be inactivated through cooking, freezing and curing depending on the characteristics of the product.

Humans, and almost all warm-blooded animals, including mammals and birds, can be infected by this intracellular parasite. Although almost all warm-blooded animals can act as intermediate hosts, the parasite's life cycle is only completed in cats and other felines (including the lynx in Europe), the only definitive host able to facilitate the sexual propagation of the parasite. The felines may spread millions of oocysts during primary infection, causing huge environmental contamination as, after sporulation, the oocysts are infectious for the accidental hosts when they ingest contaminated products.

Primary infection with *T. gondii* in individuals with a healthy immune system normally appears with mild flu-like symptoms, whereas in individuals with a depressed immune system, it may cause potentially mortal infections. The impact of *T. gondii* on healthy individuals is not clear, but it is known that the parasite may form cysts in the tissues with a potential for reactivation during periods of immunodepression. In addition, the transplacental transmission of *T. gondii*, when the primary infection is caught during pregnancy, may cause serious problems such as miscarriage, stillbirth or severe foetal malformation. Recently, toxoplasmosis has been considered a risk factor for neurological and psychiatric disorders. As a result, diagnosis of toxoplasmosis is critical in four groups of individuals; immunodepressed patients such as HIV infected patients or patients with organ transplants, pregnant women who acquire the infection during pregnancy, congenitally infected foetuses and new-born infants, and those with retinochoroiditis (Rostami et al., 2018).

The clinical symptoms of infection by *T. gondii* are unspecific; traditionally, diagnosis of toxoplasmosis has been based on laboratory tests, in particular serological tests and biotests. But the variability in the sensitivity and specificity of these tests, the time required and the impossibility of differentiating between the strains of parasites has led to the development and use of new serological, molecular and imagery tests for the rapid and precise diagnosis of the disease. Enzyme-linked immunoadsorption assay (ELISA) was the principal method used last century. Methods based on improved ELISA such as chemiluminescence (CLIA), enzyme-linked fluorescence assay (ELFA), immunochromatographic test (ICT), plasma IgG avidity test and immunosorbent agglutination assays (ISAGA) have demonstrated high sensitivity and specificity. Recent studies using recombinant or chimeric antigens and the multi-epitope peptide method have demonstrated very promising results in the development of new strategies able to discriminate recently acquired infections of a chronic infection. The real-time polymerase chain reaction (PCR) and the loop-mediated isothermal amplification (LAMP) are two recently developed PCR techniques with high levels of sensitivity and specificity that could be useful for the early diagnosis of the infection. Imagery techniques such as computed tomography (CT), magnetic resonance imaging (MRI), nuclear imaging and ultrasound

(US) are useful in the diagnosis of cerebral and ocular toxoplasmosis, although independently their results would not be specific (Rostami et al., 2018).

The methods developed recently with greater sensitivity for the diagnosis of toxoplasmosis such as serology, molecular and imaging techniques have their advantages and limitations but used in combination may achieve precise and effective results. To this purpose, IgG/IgM ELISA in combination with RT-PCR/LAMP in amniotic fluid and prenatal ultrasound are recommended for the detection of acute infection in pregnant women and also as a preventive measure against congenital transmission. Serological detection (by ELISA using *Toxoplasma* lysate antigen (TLA), recombinant or chimeric antigens) is the perfect standard in new-born infants, although RT-PCR/LAMP on umbilical cord blood or whole blood may be useful for the definitive diagnosis. In addition, the combination of ELISA (using TLA, recombinant or chimeric antigens), RT-PCR/LAMP on cerebrospinal fluid (CSF) and MRI or CT scans may produce a definitive diagnosis in immunocompromised patients (Rostami et al., 2018).

In the United States, *T. gondii* is included among the 31 pathogenic agents causing 9.4 million episodes of foodborne disease, responsible for 8 % of the main causes of hospitalisation and 24 % of the deaths (Scallan et al., 2011).

Notification of outbreaks in food of human toxoplasmosis is obligatory in accordance with the Zoonoses Directive 2003/99/EC (EU, 2003) (transposed by Royal Decree 1940/2004). Although congenital toxoplasmosis is reported to the ECDC, the national monitoring systems for this infection vary from country to country, and only three member states (the Czech Republic, France and Slovakia) have an active system for monitoring congenital toxoplasmosis covering the whole population. In 18 Member States and Iceland, the implementation of a monitoring system is obligatory, the United Kingdom has a voluntary system and Spain has another unspecified system. Therefore, it is not possible to make a good estimate of the prevalence of congenital toxoplasmosis in the EU.

Although congenital toxoplasmosis continues to be a rare disease in the EU, 47 cases were reported in 19 Member States in 2016. Excluding the figures from France, the number of cases reported is comparable to the annual number of cases (an average of 40 cases/year) and a notification rate of 1.48 cases per 100 000 inhabitants in 2012-2015. France reported the highest notification rates in 2012-2015, with a significant increase of 142.2 % rising from 12.7 to 30.8 cases per 100 000 inhabitants. In 2016, the highest notification rates were in Poland, Slovenia and Slovakia with 5.42, 4.84 and 3.60 cases per 100 000 inhabitants, respectively (EFSA, 2017).

2.3.1.2 Prevalence in food data

Recently, the WHO reported that foodborne toxoplasmosis has spread due to raw or undercooked meat and fresh products, causing up to 20 % of the total of foodborne diseases in the EU and affecting more than one million people in Europe each year (WHO, 2015).

In a systematic review and meta-analysis of controlled case studies conducted by Belluco et al. (2017), with limitations due to the low number of cases, the risk of acute *T. gondii* infection is indicated for three different food matrices: the consumption of raw or undercooked meat (without specifying the animal source) *Odds ratio* (OR) 3.44 (1.11-9.16), the consumption of raw or undercooked

beef, OR 2.22 (1.57-3.12), and the consumption of raw or undercooked sheep, OR 3.85 (1.85-8.00). However, the consumption of raw or undercooked pork, raw eggs and unpasteurised milk were not found to be significant risk factors.

According to information from the EU Member States in 2016, the majority of livestock species are infected by *Toxoplasma*. However, it is not possible to draw epidemiological conclusions to assess the risks for human beings due to the reduced size of the samples. The total number of sheep and goat samples tested (clinical and monitoring/supervision samples) only represents a small fraction of the sheep (85.5 million) and goat (12.5 million) populations in the EU, respectively (Eurostat, 2016).

The detection of specific antibodies of *Toxoplasma*, and the detection of the parasite's DNA, does not imply a direct risk for consumers; in fact, there is no direct correlation between the presence of antibodies and/or DNA and the infectivity of the parasite. In addition, as the tissue cysts are not uniformly distributed in the edible tissues, a negative result, obtained using a direct detection method in a serologically positive animal, may not exclude the presence of infectious cysts in other edible parts.

Data in the scientific literature indicates a high incidence of toxoplasmosis due to contact between the parasite and the animal (sheep, pigs, goats, horses, small mammals including rodents, pets and wild animals), suggesting that the control of this parasite is extremely difficult and is only possible in livestock bred under strict conditions of stabling. Recent studies on pigs reveal a significant decline in recent years of the prevalence of *Toxoplasma* in commercialised pigs; however, a rise in prevalence is expected due to the increase in the number of animals bred using extensive farming methods (Dubey, 2009) (Papini et al., 2017). The high incident rate of *Toxoplasma* in sheep (21 %) and goats (9 %) may partly be explained by their feeding habits. Sheep may be more susceptible to *Toxoplasma* in comparison with other livestock, although the high positivity of specific *Toxoplasma* antibodies using indirect testing would appear to be the result of vaccinations.

Certain risk factors are associated with a higher risk of transmission from animals to humans such as the presence of farm cats and open air and on farm breeding systems (Opsteegh et al., 2016).

Limited information is available on the contamination of vegetables, fruit and drinking water by *Toxoplasma* oocysts in the EU. In 2016, none of the Member States reported on research on these matrices.

Lastly, direct and indirect tests for detecting *Toxoplasma* did not provide evidence for distinguishing between an infection due to the intake of meat infected with tissue cysts and the intake of food contaminated by cat oocysts. An understanding of the paths of transmission would improve the risk assessment of this food parasite and facilitate the identification of the control measures.

2.3.1.3 Methodologies available

In the EU there is no regulation for the surveillance and monitoring of *Toxoplasma* in animals, therefore the available information is determined by the national legislation of each Member State. Consequently, the detection of *Toxoplasma* in animals varies with the country, according to the diagnosis methods used, and the different matrices analysed. It should also be remembered that both the age of the animals and the production systems at farm level affect the prevalence of *Toxoplasma.*

The main animal species tested include small ruminants (goat and sheep), bovine and porcine animals and pets (cats and dogs) using animal abortion samples (ruminants) and clinical investigations. In 2016, the indirect diagnosis methods (serological) used for the detection of *Toxoplasma* in animals were the latex agglutination test (LAT), ELISA, immunofluorescence (IFA) and the complement fixation test (CFT), and the direct methods included histology, immunohistochemistry, PCR and real-time PCR. The first are used for the detection of specific antibodies of *Toxoplasma* in serum or samples of meat juice while the second are applied to organs or tissues. A higher prevalence was detected using indirect diagnostic tests (20 %) than direct (6.1 %). The prevalence was higher in small ruminants (ovine and caprine animals; around 9 %) followed by bovine animals (around 3 %) and pigs (around 2 %).

The majority of the Member States use indirect methods for detecting *Toxoplasma*. Opsteegh et al. (2016) made a comparison between the direct and indirect methods and concluded that detection based on indirect methods is not recommended for detecting viable *T. gondii* in bovine and equine animals, and that in these species direct detection methods are recommended. For pigs, poultry and small ruminants, serological methods may be used for the detection of high-risk animals/flocks, but not as an indicator for infection in an individual animal. In addition, direct methods must be applied on matrices taken post mortem as it has been shown that the parasite load in different skeletal muscles in sheep and pigs does not change much, and the parasite is mainly found in the brain, heart and lung tissue.

It should be remembered that the direct methods are expensive and can only be applied to a small quantity of tissue. Therefore, they can only be used on a very limited number of samples which may not be representative of the target population, making it difficult to obtain reliable epidemiological data. In addition to the direct detection methods, it is important to identify the strains as their pathogenicity and virulence for humans varies between the genotypes and atypical strains.

2.3.1.4 Future steps

The consumption or raw or undercooked sheep or beef is a major source of transmission of *T. gondii* to human beings as the epidemiological samples show. It is important to consider this risk, especially in persons of risk due to the severe effects of toxoplasmosis in special circumstances. In general, adequate cooking of the meat of all species is necessary, and caution should be applied when eating undercooked meat due to the unknown effect of the chronic formation of *T. gondii* bradyzoite cysts (Belluco et al., 2017).

A harmonised protocol for the collection of samples and the detection of *T. gondii* would improve studies on the prevalence of the different livestock species in the EU.

To obtain suitable epidemiological data on the circulation of this pathogen among the different livestock species in Member States, a standardised protocol for the collection of samples and detection of the parasite is required. In addition, when a sampling scheme is established, it must consider important epidemiological variables such as: species, age of the production sector, breeding conditions (free-range game, animals bred outside or inside), contact with cats or rodents if the animals are bred inside, information about the water supply and type of feed, which may be

contaminated by cat faeces and consequently by *Toxoplasma* oocysts, if these are not correctly controlled or treated.

Risk factor studies must be based on data obtained using direct methods rather than indirect methods. To control the risk of *Toxoplasma* and recommend intervention strategies in the livestock (for example, vaccination), it is important to collect and analyse information obtained from epidemiological investigation and surveys which standardise the sample matrix (brain, heart, lungs), the test method (preferably direct methods) and the target population (species and risk categories).

2.3.2 Protozoa: *Cryptosporidium*

2.3.2.1 General characteristics

Cryptosporidium is a genus of protozoan parasites belonging to the *subphylum* Apicomplexa. Although the location of the *Cryptosporidium* genus is disputed, the most recent classification considers it as a separate group within this taxon (Adl et al., 2012). At present, the genus consists of 25 species, many of which are relatively host-specific, and approximately 50 % of the species have some degree of zoonotic potential, implying that they have the potential to infect humans and domestic animal species.

C. hominis is associated almost exclusively with infection in humans, and the majority of the infections in human beings are due to infection with this species or with a less host-specific species, *C. parvum*. Other important species as human pathogens include *C. meleagridis*, which is mainly associated with infection in turkeys, but has also been identified with relative frequency in children in South America, and *C. cuniculus*, associated with infection in rabbits, but also an aetiological agent in an outbreak of waterborne cryptosporidiosis. Some species of *Cryptosporidium* have only been associated with sporadic cases of human infection (for example, *C. ubiquitum*, which is more usually associated with infections in sheep and cervids) or particularly associated with infections in immunocompromised patients (for example, infections by *C. suis*, which is generally diagnosed in infections in pigs, and infections by *C. felis*, more commonly associated with infections in cats).

The *Cryptosporidium* life cycle is direct (without an intermediate host) but, nevertheless, it is quite complex and contains both a sexual cycle and an asexual cycle. The transmission stage is the oocyst. These are the only exogenous stage and are excreted in the faeces of the infected hosts. They are spherical, approximately 3-5.5 µm in diameter, and have a 50 nm thick resistant wall with three or four layers, with an internal layer of glycoprotein which appears to provide strength and flexibility (Jenkins et al., 2010). Each oocyst contains four naked sporozoites, which are developed and very infectious after excretion (unlike other genus of Apicomplexa such as *Eimeria* spp., *Cystoisospora* spp. or *Toxoplasma gondii*, where the sporozoites are encased and require a period of development in the environment after excretion). Infection with *Cryptosporidium* starts when a susceptible host ingests a viable oocyst. This may be direct faecal-oral ingestion, or through a vehicle such as water or contaminated food. In theory, the infective dose is only one oocyst; however, not all species of *Cryptosporidium* and not all strains or subtypes of a species are equally infectious for humans. Different studies have established infective doses for *C. hominis* and *C. parvum* in the range of 10 to

100 oocysts. However, the dose-response analysis must not be limited to the considerations of the parasite as the host variations may also be of relevance (Teunis et al., 2002a, b).

When the viable oocysts are ingested by a susceptible host, the sporozoites are generally released in the small intestine where they invade the epithelial cells and are located epicellularly and develop to the trophozoite stage. The repeated asexual reproduction cycles result in the production of large quantities of meronts, which divide to form merozoites, and each mature merozoite leaves the meront to infect another host cells, with the resultant destruction of the initial host cell. This asexual cycle results in a huge multiplication of the parasites. The sexual cycle involves he development of microgametes and macrogamonts, with microgametes produced from the microgamonts which fertilise the macrogamont, and finally result in the production of oocysts. The oocysts sporulate while they are inside the host and may divide inside the same host, resulting in the re-invasion of the epithelial cells and the continuation of the infection. Alternatively, the oocysts are excreted in the faeces and are immediately infectious for the next host. Due to the asexual cycle, which results in the large multiplication of the parasite, thousands of oocysts are excreted from an infected host; more than 1 x 10^5 oocysts in healthy and immunocompetent adults (Chappell et al., 1996), or even more than 10^9 oocysts in patients with AIDS with symptomatic cryptosporidiosis (Goodgame et al., 1993).

Cryptosporidiosis is an enteric disease. Symptoms usually start around 1 week after the infection, but incubation periods ranging from 1 day to 2 weeks have also been described. It is usually self-limited in immunocompetent individuals, but a high relapse rate has been described (Hunter et al., 2004a). Symptoms are characterised by aqueous diarrhoea, often voluminous and sometimes mucoid, but rarely bloody, abdominal pain, nausea and vomiting. The diarrhoea is normally acute, but may also be persistent. Nevertheless, in some individuals, the infection may largely be asymptomatic. Other extraintestinal symptoms (joint pain, pain in eyes and headaches) generally associated with infection by *C. hominis* have also been described (Hunter et al., 2004). In addition, *Cryptosporidium* is one of the four main aetiological agents associated with severe infant diarrhoea in developing countries (Kotloff et al., 2013). Symptoms are often more severe in immunodepressed patients and the infection may become chronic, debilitating and potentially mortal, with large volumes of diarrhoea, the possibility of the spread of infection beyond the primary site and weight loss.

Cryptosporidium oocysts are extremely robust. They are able to survive for prolonged periods in the damp cold conditions in which fresh products are usually stored, and can also survive in more adverse conditions, such as in contact with chlorine. However, they are susceptible to drying, heating and freezing-defrosting, and some types of irradiation, including ultraviolet, and some chemical treatments. This means that for food products intended to be consumed with a minimum of processing (without cooking) to conserve their sensory qualities, the original contamination should be avoided. The most effective means of controlling this contamination in fresh products is the application of Good Agricultural Practices (GAPs) during primary production, Good Manufacturing Practices (GMPs) during processing and Good Hygiene Practices (GHP) before consumption (Dawson, 2005). GAPs include the use of non-faecal water for irrigation, the application of fertilisers and pesticides, and preventing domestic animals from grazing or contaminating vegetable plots, and en-

suring that wild animals do not have access to these crop areas. The preliminary treatment of wastewater before use in irrigation may reduce the quantity of viable pathogens, including the oocysts of *Cryptosporidium*, which would otherwise contaminate the fresh products. Suitable treatments include UV irradiation and ozone treatment (Kalisvaart, 2004) (Orta de Velásquez et al., 2006). Ultrafiltration membrane filtration with a submerged hollow fibre system has also been shown to be adequate (Lonigro et al., 2006).

Potable water must be used during food processing (Sutthikornchai et al., 2005) and if the washing water is reused, the disinfection procedures should be effective in order to inactivate the *Cryptosporidium* oocysts. It should also be remembered that the washing procedures as such may be ineffective for eliminating the oocysts from fresh products. If it is not possible to avoid contamination and elimination by washing or other procedures is ineffective, inactivation procedures must be applied to guarantee the microbiological safety of the food.

The most commonly used disinfectant is chlorine. The effectiveness of standard chlorination for inactivating the oocysts of *Cryptosporidium* in fresh products depends on a variety of factors, including the contact time. In particular, for certain more delicate products, such as soft fruit (for example strawberries and raspberries), the method of treatment may provide an insufficient contact time for its effective inactivation. On the other hand, the use of sodium dichloroisocyanurate (NaDCC) in the inactivation of various intestinal protozoa, including the oocysts of *Cryptosporidium* in fresh vegetable products and raw fruits, may be suitable for use in domestic and restaurant environments, and in the catering industry (El Zawawy et al., 2010). Other disinfectants which may be appropriate for inactivating the oocysts of *Cryptosporidium* in fresh produce include gaseous chloride dioxide (Ortega et al., 2008), and a combination of levulinic acid and sodium dodecyl sulphate (SDS) (Ortega et al., 2011). Research on the latest disinfection technique is based in the reduction of more than 6 log of the bacterial pathogens (*Salmonella* and *E. coli* O157) in lettuce (Zhao et al., 2009). Other technologies which may be of use in the fresh produce industry for inactivating the oocysts of *Cryptosporidium* include irradiation (UV, cobalt-60), treatment with high hydrostatic pressure and ozonisation. It should be noted that the use of sequential inactivation treatments may optimise the existing treatments by means of synergistic effects (Erickson and Ortega, 2006).

Cryptosporidium does not thrive well in carbonated drinks (Friedman et al., 1997), and a loss of viability has also been observed in beer (including alcohol free beer), associated with a low pH. Furthermore, high temperature-short time pasteurisation (HTST) has been found to successfully inactivate the oocysts of *Cryptosporidium* (Harp et al., 1996). In addition, viable oocysts in milk are not inactivated in the fermentation process used for the manufacture of yoghurt; however, the mixing and freezing process used in the making of ice-creams apparently leads to the complete loss of viability (Deng and Cliver, 1999). In addition, different inactivation techniques have been tried including ultrahigh pressures (Slifko et al., 2000), HTST pasteurisation (Deng and Cliver, 2001), UV irradiation (Hanes et al., 2002) and organic acids combined with hydrogen peroxide are effective on cider (Kniel et al., 2003).

2.3.2.2 Distribution and prevalence

Cryptosporidium spp. is distributed globally and human infections have been found in more than 100 countries (Fayer, 2008). Although the estimations of prevalence vary between countries, age groups and studies, it is clear that children in developing countries are the most affected (Shirley et al., 2012); it has been suggested that cryptosporidiosis is responsible for around 20 % of the cases of infant diarrhoea in developing countries (Mosier and Oberst, 2000). In 2004, the common link between cryptosporidiosis and poverty resulted in its inclusion in the WHO "Neglected Disease Initiative" (Savioli et al., 2006). In Europe, the notification ratio for cryptosporidiosis in humans is 3.1 cases per 100 000 inhabitants (ECDC, 2018), but there is clear heterogeneity in the prevalence results. While several countries such as Ireland, the United Kingdom and Sweden have a prevalence of more than 5 cases per 100 000 inhabitants, the majority of the remaining countries which send information to the ECDC report fewer than 90 cases, and several countries do not even report any information about this infection (ECDC, 2018). However, the highest confirmed rate of cases is also found in the 0 to 4-year-old age group (12.6 cases per 100 000 inhabitants) (ECDC, 2018).

2.3.2.3 Methodologies available

On the whole, the diagnosis of cryptosporidiosis is associated with the finding of oocysts (or, less frequently, its antigens or DNA) in faecal samples. Although antibody-based detection in serum, saliva or faeces is also possible for demonstrating exposure to *Cryptosporidium*, it is only of proper diagnostic benefit if seroconversion can be demonstrated, as otherwise a positive result can also indicate past exposure.

For faecal samples, a concentration technique such as formol-ether (ethyl acetate) or flotation (often sucrose or sodium chloride) is usually used prior to microscopy. As the oocysts are very small, the use of a staining technique, particularly using antibodies labelled with a fluorochrome and screening with fluorescence microscopy (immunofluorescence antibody testing, IFAT), is recommended. IFAT is considered to be a gold standard, although other techniques such as modified Ziehl-Neelsen (mZN) or auramine phenol staining may also be used. However, some oocyst staining methods do not perform well on oocysts that have been preserved in polyvinyl alcohol fixatives. Furthermore, when mZN staining was used, a lack of sensitivity and specificity was described and this has led to the overestimation of the prevalence of the infection (Chang'a et al., 2011). Immunological techniques, mainly ELISA, have been developed, and immunochromatographic tests are commonly used as rapid tests. The main disadvantage of such rapid assays, in addition to the relatively high cost, is the low sensitivity and specificity (Johnston et al., 2003) (Robertson et al., 2006). However, the tests are very simple to use and provide results in minutes. Molecular methods, and mainly the specific detection of the DNA of the parasite in faecal samples using real-time PCR will probably become the method of choice, especially if multiple PCR systems are used which permit the simultaneous detection of different enteric pathogens (Stensvold et al., 2011). A recent review of the current methods for the detection and identification of oocysts in faecal samples has been carried out by Ahmed and Karanis (2018a).

The detection of food or water contaminated by oocysts of *Cryptosporidium* is based on isolating and identifying the oocysts, or their DNA. Nevertheless, the concentration of oocysts of *Cryptospo-*

ridium in a faecal sample of an infected individual is significantly higher and therefore the detection in food or water is much more difficult. Therefore, the diagnosis of infection by *Cryptosporidium* using the identification of oocysts in faecal samples and the detection of contamination through the identification of oocysts of *Cryptosporidium* in food samples are not entirely comparable.

The detection of oocysts of *Cryptosporidium* in drinking water involves the concentration of particles which are approximately the size of oocysts of *Cryptosporidium* (or larger) from a relatively large sample of water (minimum 10 l) by filtration (flocculation and sedimentation may also be used, but less commonly, or continuous flow centrifugation may also be used), centrifugation often eluting these particles from the filter in a smaller volume, and then isolating the oocysts before detection. The detection of the oocysts is generally carried out by drying the final concentrate of around 50 µl on a microscope slide and examining it with IFAT. The fluorescent monoclonal antibody stain used is normally fluorescein isothiocyanate (FITC) and, in addition the staining of the sporozoite nuclei with 4',6 diamidino-2-phenylindole (DAPI) is also used to improve detection providing additional visual markers for identification.

In principle, the approximation for testing the contamination of food with oocysts of *Cryptosporidium* is similar to that used for water. However, it is not possible to filter a large volume (nor is the filtration of colloidal fluids, such as milk, practical) and instead, some type of elution technique must be used. This means that only a relatively small amount of the product can be tested. In addition, variations in the biochemical and physical characteristics of the different food products make a horizontal strategy infeasible, as a technique which is appropriate for one food type may result in recovery efficiencies which are suboptimal in others. Ahmed and Karanis (2018b) summarise the different methods used for the recovery of oocysts of *Crytosporidium* based on each food matrix, in addition to revising the advantages and disadvantages of the different methods for their recovery and the factors which affect efficiency. The IFAT continues to be the method of choice for detection and is the designated method in the ISO 18744:2016 standard for testing vegetables and berries for *Cryptosporidium* (ISO, 2016). It is surprising that the IFAT (which is essentially a microscopy-based detection system) has not been replaced by a technique such as real-time PCR or LAMP, particularly when the equipment required for IFAT, a fluorescence microscope, is expensive, whereas the price of PCR equipment is constantly becoming more competitive and, in addition, offers the opportunity to simultaneously identify different species. However, the presence of different types of PCR inhibitors and the inability to detect non-nucleated parasites means that these techniques are not fully established in food laboratories.

2.3.2.4 Future steps

Cryptosporidium is a parasite recognised as one of the main causes of waterborne diarrhoeal disease in human beings all over the world, with long-term consequences such as malnutrition, delayed growth and cognitive deficits in small children in environments with limited resources, and which cause opportunist infection in immunodepressed hosts. In spite of the global load of the cryptosporidiosis, the options for treatment are limited to support therapy and one medicine, nitazoxanide, which inhibits the pyruvate ferredoxin oxidoreductase (PFOR) enzyme interrupting the metabolism

of the parasite, but which is of limited effectiveness in malnourished children and is ineffective in immunodepressed individuals. The search for new pharmacological strategies is much hindered by the notorious unreadability of the parasite and the limitation of technical tools available for its study. Although significant progress has been made in the development of new drugs for the treatment of cryptosporidiosis, some candidates do not eliminate the detachment of oocysts, moderately reducing the severity or duration of the diarrhoea (or both), or their toxicity has not been assessed or they have not been assessed in preclinical trials. To date, none of the medicines has reached the clinical trial in humans' stage. Nevertheless, new analogues with increased efficiency and lower toxicity levels for human cells may be developed (Bhalchandra et al., 2018).

This waterborne and foodborne intestinal parasite is a threat to human health even in industrialised countries. Diagnostic tests have limitations regarding performance, cost, time, availability, etc. The choice of the most suitable and precise method for the detection of *Cryptosporidium* is a difficult decision. There are a large variety of methods, ranging from simple to highly complex, for the detection and identification of *Cryptosporidium* in faeces, where the most sensitive are direct immunofluorescence and molecular diagnosis (PCR, real-time PCR, LAMP). Faeces are the easiest material to process and visual confirmation of oocysts under a microscope dispels any doubt of infection, although it is of limited value for identifying species of oocysts and assessing the patient's condition. The standardisation and validation of procedures in the different laboratories is recommended.

However, there are no standard methods of detection for the oocysts of *Cryptosporidium* in food and expert personnel are required to ensure both their control and detection. Therefore, complementary methods should be used with consideration for their limitations. The majority of methods available, adapted from those used for water, include conventional or molecular strategy detection, the latter being far more sensitive, quick and easy to perform than microscopic techniques, with the possibility of differentiating species and genotypes. The techniques for certain "high risk" foods (especially ready-to-eat salads and freshly prepared food) have been improved. The Bacteriological Analytic Manual (BAM) of the FDA (Food and Drug Administration) provides a protocol designed to detect the oocysts of *Cryptosporidium* in washes of fresh products and in filterable samples such as juices (Ahmed and Karanis, 2018b).

The nature of the different food matrices (size, consistency, shape and surface area) make their processing for the detection and identification of oocysts more difficult as many preliminary steps are required (elution, concentration, purification and isolation). Therefore, the need for a standard, reliable and tested method which adapts to the different food matrices is essential for guaranteeing the quality of the test and establishing food safety monitoring.

Conclusions of the Scientific Committee

A review has been made of certain biological hazards for which no specific regulation exists and which may suppose an emerging risk for health. The list of hazards addressed in this report is not intended to be exhaustive, as it does not consider all the possible new biological hazards. Its approach is to serve as starting point for the possible performance of prospective studies. For this reason, special attention is given to indicating the foods which may be of special importance in

the transmission of the hazards considered and describing the methodologies available for their detection in food samples.

At the same time shortcomings are identified in the knowledge of these hazards which may be the starting point for promoting research aimed at improving knowledge about them.

Lastly, information has been included about the possibilities for controlling the transmission of microorganisms along the food chain, which may serve to improve knowledge about these microorganisms among the consumers and other sectors involved.

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