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Editorial

This issue of EUROREFERENCE focuses specifically on the hepatitis E virus (HEV), with three articles looking more closely at this topic.

A Point of view article on the challenges that changing food systems, from farm to fork, pose to reference activities and health surveillance provides another perspective.

There is also a focus article on H2020 that aims to clarify the H2020 programme's philosophy and application procedures for calls for proposals that are likely to be of interest to research and reference activities. The article follows a practical, step-by-step approach that we hope you will find useful!

We wish you a good read

The editorial team

Summary

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Useful websites and links to InVS and EFSA websites

Here are a few websites and links where you can find interesting information on the theme developed in this edition.

French Institute for Public Health Surveillance (InVS) website

- Foodborne viruses: http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Risques-infectieux-d-origine-alimentaire/Gastro-enterites-aigues-virales/Aide-memoire
- Foodborne viral hepatitis: http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Hepatites-virales/Hepatites-virales-Generalites
- Hepatitis A: http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Hepatites-virales/Hepatite-A
- Hepatitis E: http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Hepatites-virales/Hepatite-E

EFSA website: recent publications on foodborne viruses (July 2014 - January 2015):

- Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella*, Yersinia, *Shigella* and Norovirus in bulb and stem vegetables, and carrots): http://www.efsa.europa.eu/en/efsajournal/doc/3937.pdf
- Flavivirus - tick-borne encephalitis virus (TBEV) in raw drinking milk: http://www.efsa.europa.eu/en/efsajournal/doc/3940.pdf
- Risk of transmission of Ebola virus (EBOV) via the food chain: http://www.efsa.europa.eu/en/efsajournal/doc/3884.pdf
- Risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in tomatoes): http://www.efsa.europa.eu/en/efsajournal/doc/3832.pdf
- Tracing of food items in connection to the multinational hepatitis A virus outbreak in Europe: http://www.efsa.europa.eu/en/efsajournal/doc/3821.pdf
- The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012 (Calicivirus et rotavirus): http://www.efsa.europa.eu/en/efsajournal/doc/3547.pdf
- The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013: http://www.efsa.europa.eu/en/efsajournal/pub/3991.htm or http://ecdc.europa.eu/en/publications/Publications/EU-summary-report-trends-sources-zoonoses-2013.pdf

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Inter-laboratoty proficiency tests agenda-EU Reference Laboratories

EURL mandate	Projects 2015		
	ILPT	Workshops	Trainings
Coagulase-positive staphylococci, including (S. aureus)	- Detection of staphylococcal enterotoxins (April) - CPS counts (November)	28-29 May	- 1 session on detection of staphylococcal enterotoxins - 2 sessions on quantification of staphylococcal enterotoxins - 1 session on characterisation of CPS by PCR or typing of CPS by PFGE or spa-typing
Listeria monocytogenes	- *Listeria monocytogenes* counts - *Listeria monocytogenes* typing by PFGE	25-27 March	- 1 session on typing by PFGE (Maisons-Alfort) + 2 training sessions on site (NRL) - 1 session on growth tests and study of aging for *Listeria monocytogenes*
Milk and dairy products	- Counting of somatic cells (2nd quarter) - Activity of alkaline phosphatase (November)	October	- 1 session on counting somatic cells
Brucellosis	- Brucellosis milk ELISA	15-16 October	- 1 session on bacteriology and molecular biology (1st half year) - 1 session on serology (2nd half year)
Residues of certain substances mentioned in Annex VII, Section I, Point 12 b), of Regulation (EC) No 882/2004	- Control of group A6 banned substances, nitrofurans, in foods of animal origin (1st half year) - Control of group B1 authorised antimicrobials in meat: at the screening step and confirmatory step (2nd half year)	State-of-the-art in microbiological control of antibiotic inhibitory residues in various foods of animal origin and relevant issues behind this type of control including use of rapid testing methods" (October)	
Rabies/rabies serology	-Diagnostic (4 techniques) (June)	27-28 May Zagreb (Croatia)	Based on results of previous ILTs (autumn)
Equine diseases (other than African horse sickness)	- Dourine: detection of *Trypanosoma equiperdum* antibodies in serum through complement fixation tests (spring) - Contagious equine metritis: detection of *Taylorella equigenitalis* by culture method or PCR (spring)	Dourine and contagious equine metritis (early October)	
Bee health	- Quantification of CBPV by quantitative real-time PCR (January-February) - Identification of *Nosema* species by PCR (May-June)	September	- Epilobee (June) - 1 session on diagnosis of bee diseases (September)

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Inter-laboratory proficiency testing in France

In order to ensure the reliability of the analyses carried out by the network of laboratories that it coordinates, a reference laboratory organises training sessions on new methods and undertakes inter-laboratory proficiency testing to assess the effectiveness of official analyses.
These proficiency tests are organized at intervals determined by the reference laboratory depending on the difficulty of the methods used and the number of years since the network was set up. Testing is performed at a frequency of one to four tests per two-year period.
In practical terms, the reference laboratory prepares samples and sends them to the accredited laboratories for testing. The contents of the samples are, however, known only to the reference laboratory. The accredited laboratories then apply the official method and communicate their results to the reference laboratory. Any non-compliant results are discussed with the laboratories in order to identify changes that need to be made.
Inter-laboratory proficiency testing is organized in the fields of animal health, food safety, and plant health, for all National Reference Laboratory (NRL) mandates, including non-ANSES mandates.

Animal Heath	> 2015 provisional calendar (pdf)
Plant Health	> 2015 provisional calendar (pdf)
Food safety - microbiology	> 2015 provisional calendar (pdf)
Food safety – chemical contaminants	> 2015 provisional calendar (pdf)

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Lab news

How and where to find European funding for research activities

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What is Horizon 2020?

Horizon 2020, also known as H2020 is the 8th Framework programme for research-development and innovation funded by the European Union for the period 2014 to 2020. Participating in Horizon 2020 is an excellent opportunity to extend one's network, improve visibility among European and international research teams, and find alternative sources of funding to further research activities carried out with State funding.

What is the philosophy behind H2020?

Unlike previous European Framework Programmes For Research, Horizon 2020 is more heavily focused on innovation, a key concept in the area of research and development over the last few years, to help bring the European economy to an internationally competitive level by creating more added value on the basis of innovative results from research. To this end, Horizon 2020 is structured in such a way, that it aims to stimulate and facilitate the transition from completed research to development and marketing of new products. This involves bridging what is known in research-development as the «valley of death»: the interval in the value chain when sufficient resources need to be mobilised to finance the risk incurred by developing and marketing a product, when it is not yet known whether it will be a success and whether there will be sufficient return on investment and future profits.

To improve the chances of success when applying for H2020 funding, it is essential that researchers think about their projects in terms of innovation and in a global context, i.e. where results of research activity must help to create leverage enabling European society to be more present and competitive on the international market. Concerning reference and research activities aimed at detecting pathogens and contaminants, using cutting-edge techniques such as next-generation sequencing (NGS) or high-throughput PCR makes it possible to save time, money, and human resources. For these reasons, research projects based on these technologies are far more likely to receive funding than those that are more conventional.

How does H2020 work?

H2020 is made up of a vast number of calls for proposals that are either generic, known as open calls, or thematic. Concerning thematic calls, the work programmes are established for two years and are revised half-way. They are based on proven comitology, drawing on all players in research, from public or private research laboratories, to the European Commission, and ministries of the Member States and H2020 associated countries (Norway, Turkey, and Israel, among others).

The definition of research subjects within the various H2020 work programmes is based on the legal framework of H2020 (http://ec.europa.eu/research/participants/data/ref/h2020/legal_basis/sp/h2020-sp_en.pdf), and on the definition of a matrix bringing together strategic orientations, cross-functional focus areas and specific calls. These strategic orientations, focus areas and calls are redefined for each new two-year work programme.

H2020 also aims to be highly innovative, even concerning its organisation, since all aspects of the programme are now paper-free and go through the participant portal (http://ec.europa.eu/research/participants/portal/desktop/en/home.html), which provides all the information on the work programmes and closed, open and forthcoming calls for proposals, as well as all tools needed to submit and manage a research project.

Procedure to follow to access the various work programmes of the H2020 calls for proposals:

- 1. Click on the following link: *H2020 Calls for Proposals*;
- 2. In the main panel, listing all the H2020 sub-programmes, select the relevant sub-programme from the six main topics within H2020 (note that the final topic "Euratom" is an independent sub-programme within H2020);
- 3. Based on the filter applied, open calls are then displayed for the selected sub-programme;
- 4. Then click on the call you are interested in, and on the "Call documents" tab, where you will find the work programme document with a title following this form: "WP H2020 – call reference".

In terms of monitoring calls for proposals, there is one call per year for each sub-programme. However, the opening date and deadline for calls in the various sub-programmes are not synchronised. In addition, they are not published on the same date from one year to the next. It is therefore important to visit the participant portal regularly and to note open or forthcoming calls for proposals. Alerts for forthcoming calls are nonetheless published on the participant portal at least three months before opening. **Monitoring every three months** should thus be sufficient to detect future programmes.

It is also possible to sign up to the RSS feed on H2020 calls for proposals by clicking on the RSS logo on the left of the calls for proposals page (e.g. next to «Call Updates»), or by clicking on the calendar icon next to the RSS feed logo to download in MS-Outlook, the H2020 calls for proposals calendar, which enables you to set reminders for opening dates and deadlines for the calls that you have chosen.

On the left of the Calls for Proposals page, there is a search function for closed, open, or forthcoming calls, enabling you to search for relevant calls using key words. To search calls, click on "Search Topics".

With the aim of helping project holders to save time and to avoid unnecessary investment of resources, **the two-step application procedure** has now become standard in H2020, with a first short step requiring minimum investment by the applicant. If the first step is successful, a second more detailed step is opened in which the full project is described (consortium, detailed activities, associated budget, and deliverables).

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Lastly, H2020 was designed to simplify the process for researchers and research project managers. Unlike previous framework programmes in which recipients had to make their way through an immense regulatory jungle where each sub-programme had specific rules and procedures, all programmes within H2020 work using the same rules. There are a few very rare exceptions but they are mostly justified and acceptable to recipients.

H2020 has three pillars:

• Pillar 1 called “Excellent Science”. For relevant funding of research in reference activities, it covers:
- initial and continuing education by research, through Marie Sklodowska-Curie Actions, for the funding of Master or Doctorate courses, but also mobility of researchers as part of lifelong continuing education;
- research infrastructures (the “Infrastructure” programme);
- and aligning the research capabilities of less advanced Member States with those of the most advanced (the “Spreading excellence and widening participation” programme).

The calls for proposals in the various programmes of the first pillar are open calls that do not cover a specific topic, meaning that a project can be proposed on any topic. However, these funding programmes are extremely competitive, making it necessary to present applications of very high quality. This requires considerable investment from project holders during their preparation.
Note that on the participant portal, calls for proposals of the first pillar are mainly registered under “Excellent Science”, but also under “Spreading excellence and widening participation” and “Science with and for Society”.

• Pillar 2 called “Industrial Leadership”. Its purpose is to fund the development and marketing of products using the results of research. This pillar is mainly directed at private industries and is of little interest for research in reference activities in animal health, plant health, and food safety.
There is however an exception in this second pillar, the LEIT programme (Leadership in Enabling and Industrial Technologies), which covers funding for research in biotechnologies. Calls in this sub-programme are thematic. Part of funding for research in biotechnologies may include aspects of metagenomics and bioinformatics for processing all «omics» data, which is of interest upstream of research and reference activities.

• Pillar 3 called “Societal Challenges”. This pillar focuses on seven major societal challenges, subjects that are dealt with in a global way as part of collaborative research between at least three distinct partners from at least three different Member States or associated countries. As such, like in previous framework programmes, calls for proposals in the third pillar of H2020 are thematic. However, instead of focusing on a pathogen or group of specific pathogens in an animal or plant species or group of animal and plant species for one or more particular health or economic reasons, and based on prioritisation of problems following careful assessment by experts upstream, calls in H2020 are more likely to attempt to respond to questions concerning, for instance, the outlook for food production in the European Union. In this topic, the place, role, and efficiency of animal production are issues that include a significant health aspect and in which scientists working in research and reference activities will have an important role to play.
Calls for proposals in the third pillar therefore have a much broader scope than calls for collaborative research in the previous framework programmes. The consortiums established to respond to the calls are, as a result, much bigger, and consortiums with more than 20 partners are common in H2020. This was rare in previous programmes. The projects funded are supposed to help respond to major societal issues. The project outcomes should help to create leverage to improve European competitiveness in the international arena, thus having a positive effect on European economic growth, and ultimately, a positive impact on the quality of life of Europeans.
In this third pillar, two societal challenges are particularly relevant for research and reference activities:
- challenge 1 on human health which deals specifically with issues of public health related to exposure to chemical contaminants through the environment, and antimicrobial resistance;
- and challenge 2 on food security, sustainable agriculture and forestry, and marine and maritime research, which deals with all the issues related to animal health and welfare, plant health, and public health in relation to food.

In both these challenges, the research actions undertaken could specifically involve methodological research for detection and control of pathogens and other chemical contaminants.

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Point of view

Adapting to changes in food systems: scientific challenges ahead

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Given the rapid and constant changes that are occurring in all the phases of food systems, i.e. production, processing, distribution, and consumption, public- and private-sector players in the area of food safety need to have suitable analytical tools to ensure improved prevention and control of risks to consumers.

Food systems, including all players, institutions, and technical tools that form a link between primary production of biological materials intended for human consumption and consumers of products, are undergoing regular and rapid changes. These changes have an impact on the quality of food products on offer to consumers but also on the type and characteristics of hazards, such as biological or chemical agents, that may contaminate food. Consumers are also changing as society evolves (population aging, immunodepressed sub-populations, shifts in meal habits) and these transformations are leading consumers to change their consumption practices.

Constantly changing risk factors

Agricultural production methods have moved from market gardening models to modern intensive agriculture over the past half century. From nearby production with local and rapid consumption, we have moved to mass production with widespread, deferred consumption. As a result, a production incident will potentially have more serious consequences given the size of the consumer population that will be affected. An example is the food poisoning outbreak of 2001 in the United States related to melons contaminated by the bacterium *Listeria monocytogenes*. This foodborne illness outbreak led to 13 deaths and more than 60 other cases. Epidemiological surveys found a single source of contamination on a farm in Colorado but the impact was without precedent given the mass distribution to more than 17 different states.

Processing methods have also seen significant changes. New processes and automation for instance mean that we have to adjust our food safety systems. Implementation of high-temperature cooking processes for certain foods, such as chips, potato crisps, or breakfast cereals, can generate compounds like acrylamide, a substance recognised by the International Agency for Research on Cancer (IARC) as a known carcinogen for animals and possibly humans. In 2013, a study on contamination of certain foods by acrylamide was carried out in France by the Directorate General for Competition, Consumer Affairs and Fraud Control (DGCCRF). This study showed that several product types had an acrylamide content greater than recommended values. In 2008, China faced a large-scale scandal concerning milk products and infant formula contaminated by melamine. Although this episode was caused more by fraud than accident, this processing method led to illness in more than 94,000 people. Lastly, intensive use of cleaning agents and disinfectants in the agro-food industry and generalised refrigeration in food processes have contributed to selection of more resistant microbiological agents. Psychrotrophic bacteria or those resistant to high temperatures, as well as antibiotic-resistant bacteria and cleaning product-resistant bacteria, known as persisters, are of concern in the food production sector. Over the last 10 years in France, the National Reference Laboratory (NRL) for *Salmonella* at ANSES has found a significant increase in the number of multi-resistant *Salmonella* Kentucky isolates from the food chain, specifically with resistance to fluoroquinolones, antibiotics that are therapeutically important in human medicine.

World Trade Organization agreements and the European single market today enable free circulation of food products. EU food imports and exports have doubled since 2005 and the globalisation of trade in raw materials and food products has had enormous consequences on food safety. The 2013 horse meat scandal whereby horse meat was sold as beef shows the complexity of supply, transport, and processing networks which are known to be risk factors given the differences in legislation and regulations between states. Likewise, in 2012, sale of strawberries from China to school canteens in Germany resulted in infection of more than 11,000 school children by norovirus.

Rapid changes to food production and processing methods and globalisation of trade have significantly altered food consumption habits. Changes in eating habits concern both ready-to-eat food preparation processes and the development of collective catering and/or fast food. A clear example is the exponential growth in the consumption of raw food products such as sushi, carpaccio or raw vegetables. These new consumption models are not without safety risks since cooking is a method of controlling and eliminating microbial contamination. This is particularly true for raw fish in which the prevalence of *Anisakis* contamination, a specific parasite of fishery products, ranges from 7 to 75% depending on the species. Back in 2003, the French Institute for Public Health Surveillance estimated the incidence of anisakiasis to be eight cases per year in France on the basis of data from 1985 to 1987. Considering the increased consumption of raw fish in France and although it is a rare parasitosis, the incidence of anisakiasis is very likely to increase and must be monitored.

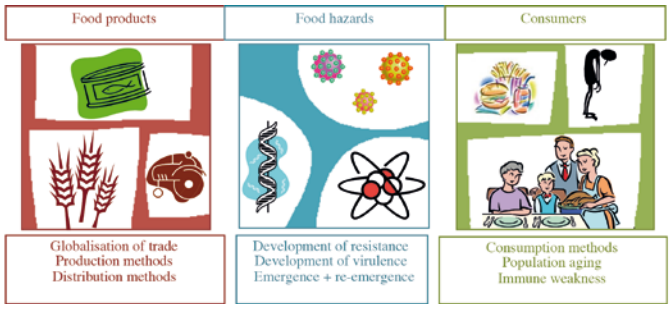

Figure 1: Typology of changing risk factors in food safety

Point of view

All these changes in risk factors (summarised in Figure 1) can be clearly illustrated through one of the most significant food safety crises in Europe in the last few years. In 2011, Germany faced a serious foodborne illness epidemic that involved the infection of nearly 4000 people, with more than 50 deaths. This crisis had wide-ranging health impacts but also an economic impact with the loss of more than 1 billion Euros for the fruit and vegetable sector which was wrongly incriminated in the outbreak. The epidemic was mainly due to:

- fenugreek seeds produced in Egypt, exported to Europe, and sold mainly in Germany (12 cases were also identified in France following consumption of these seeds).
- contamination of seeds by a new strain of *Escherichia coli* O104:H4 which was originally a non-virulent strain (entero-adherent *E. coli*) but that acquired virulence and resistance factors (enterohaemorrhagic *E. coli*).
- development of a new pattern of consumption of germinated raw seeds that mainly affected young adult female consumers who were sensitive to this type of consumption.

Given these changes, how should we adjust the tools we use?

What would be the best approach to deal with hazards that are increasingly virulent or resistant, use of inputs in agricultural production that are more and more complex, higher diversity of sources and quality of raw materials for processed food products, and changes in consumption practices that disrupt our food safety management and control systems?

To minimise the impact of these changes on the effectiveness of our risk factor analysis systems, it is particularly important to adjust our monitoring systems. Although early detection of alert signals and effective traceability are a good starting point, these systems must move beyond the national perimeter to integrate into international systems for rapid exchange of information and contact between countries. The type of information generated and exchanged must also evolve to better describe the characteristics of hazards and epidemiological situations. This information, whether it comes from monitoring of the environment, animals, human illnesses, adverse effects (toxicovigilance, nutrivigilance, etc.), foodborne illness outbreaks, or foodstuffs (French Observatory of Food Quality (OQALI), monitoring and control plans), must be brought together and meta-analysed to extract all relevant knowledge to help in controlling health risks.

Analytical technologies provide us with tremendous perspectives. High-throughput genomic approaches and high-resolution mass spectrometry provide us with a vast amount of information, down to the molecular or atomic level, and in biology, this mass of data enriches monitoring of changes in pathogenic strains, their virulence factors, as well as chemical substances and their toxic potential.

The “omic” sciences, referring to genomics, transcriptomics, proteomics, and metabolomics (see box), can be used to closely analyse the effects of a xenobiotic substance of infectious or toxic origin on the body. In particular, these techniques help to study the response of the genome to exposure to these toxic agents (toxicogenomics) but have also proven to be excellent diagnostic methods.

Genomics involves all the analyses of the structure of genomes, i.e. sequencing and identification of genes.
Transcriptomics and proteomics are focused on the functioning of the genome, particularly transcription and protein production.
Metabolomics studies metabolites (amino acids, carbohydrates, fatty acids, etc.) found in biological fluids such as blood and urine or in body tissues.

Spectral analysis using NMR spectroscopy or high-resolution mass spectrometry provides increasingly specific information on the presence of a toxic compound in a foodstuff or human sample. These studies can be carried out in a targeted manner when the xenobiotic substance is known, or in a non-targeted way when the xenobiotic compound is not known. They also provide an overall view of the metabolome of a biological sample and of the changes to it caused by an exogenic contaminant.

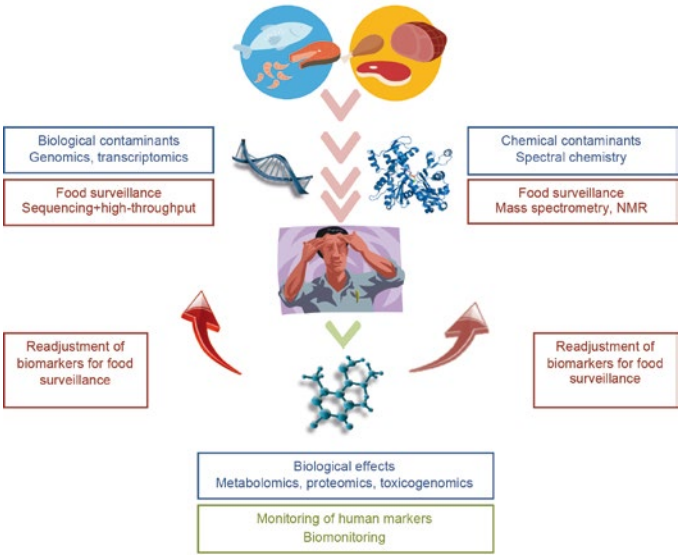

Figure 2: Contribution of analytical tools to monitoring

Identification and accurate quantification of a pathogen, chemical substance, products of gene expression for a cell or tissue, and metabolites, help to develop a veritable «infectious or toxicological footprint” of a food substance or food contaminant through screening of the relevant biomarkers.

Data are obtained more and more rapidly and in ever greater quantities, but they must go hand in hand with understanding and interpretation of the information they provide for the specific objective: product testing, risk assessment, or surveillance of emerging risks. The area of bioinformatics has the new challenge of simultaneously analysing a very large amount of data, “big data”, originating from different sources. Interpretation of these data requires the use of powerful bioinformatics methods that are now available and that make it possible to characterise

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biological systems in great detail. Progress is also expected to build expert systems that can explain the relationships between a genetic or molecular footprint, a biomarker, and a toxic effect. Data communication, exchange and analysis technologies have made constant progress over the last few years. The rate of development of these technologies has been a revolution for the area of risk factors. However, we have found that the systems implemented nationally, in Europe, or internationally are still very siloed, informational, and not very interactive.

There are of course information exchange systems, such as RASFF (Rapid Alert System for Food and Feed) at the European level, or INFOSAN (the International Food Safety Authorities Network) internationally, that can be used to rapidly communicate information on the presence of hazards in exported food products or on the emergence of new risks for the consumer, but they focus mainly on regulated hazards that are known and detectable. In the case of the example concerning *Escherichia coli* O104:H4, these systems are not particularly effective in dispelling doubts about a hazard that is difficult to characterise.

A number of initiatives have recently been launched in Europe to better exchange and analyse the information needed to prevent food safety risks. Under the impetus of the European Commission, the European Food Safety Authority (EFSA) is considering setting up genotype and phenotype characterisation databases for bacterial strains found in foods in Europe. The first databases will be for *Salmonella*, *Listeria monocytogenes* and enterohaemorrhagic *E. coli*. These databases will be connected to those of the European Centre for Disease prevention and Control (ECDC) in order to establish relationships between human clinical strains and food strains. Hopefully this tool will be able to prevent diffusion of emerging virulent bacterial clones, to implement suitable control tools, and to deal with the source of the pathogen as rapidly as possible.

Another important initiative is that managed by the emerging risks unit of EFSA, known as EMRISK, which set up as of 2010 an exchange network on emerging risks with partner organisations in the Member States and non-EU countries. EMRISK is tasked with evaluating and developing tools to detect emerging risks in human food and animal feed. It is developing a computer-based collection and analysis tool for metadata available on the internet. This holistic approach relies on information concerning patient reports and food contamination, but also from other areas and disciplines such as economics, international trade, climate change, and human factors, or specific knowledge about supply chains, distribution zones, and production lines, and knowledge on livestock farming and plants. Preliminary analyses carried out with this tool have shown that it is able to detect signals very early on, but its development must be pursued to cover additional media, geographic areas, and "expert" databases.

In a context of changes to food systems, public- and private-sector players, managers and scientists have a broad range of innovative and powerful tools. "Omic" sciences, spectral analysis, and meta-analysis of increasingly large amounts of specific data are making it possible to adapt our surveillance tools and to adjust our management and control systems in the area of food safety. Technical tools and related expertise are being implemented gradually but, much like in the area of globalisation of trade, the efficiency of surveillance systems will only be optimised if there is greater sharing of information and data, paving the way for future collaborative projects.

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Hepatitis E virus: a foodborne zoonotic virus threatening the immunocompromised patient

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Hepatitis E is an acute form of liver disease caused by HEV, an RNA virus infecting humans and a number of animal species among which swine may represent a main reservoir. Large outbreaks occur sporadically in developing areas due to waterborne genotype 1 HEV, but zoonotic and foodborne transmission of genotype 3 strains is increasingly reported in developed countries, where acute hepatitis presents mild symptoms and low mortality. Recently, g3 HEV has emerged as a significant threat for immunocompromised subjects, including solid organ transplanted patients, exiting into chronic liver disease, cirrhosis and high mortality. Transmission in these patients remains unknown.

The virus and its epidemiology

The hepatitis E virus (HEV) is a single-stranded RNA virus of the Family *Hepeviridae* (Meng, 2010; Smith *et al.,* 2014), and presents an unenveloped capsid made of a single protein encoded by the Open Reading Frame 2 (ORF2). The ORF1 codes for the proteins of the viral replication complex, which are cleaved post-translationally, and the ORF3 protein is still orphan of a definite function.

As other RNA viruses, the high mutation rate during viral replication is the main cause of the broad genome diversity of HEV, based on which field strains can be distinguished in at least 7 established genotypes. Four of these, namely genotypes g1 through g4, infect humans, and g3 and g4 strains are also known to infect several animal species, including swine, wild boar, deer, rabbits, and other wild species. These viral genotypes have been recently proposed to form the novel Genus *Orthohepevirus A* (Smith *et al.,* 2014). Based on the deduced ORF2 amino acid sequence and the high cross-reactivity in laboratory testing, it is assumed that despite genetic differences all g1-4 HEV strains belong to a single serotype. However, the lack of an *in vitro* seroneutralization test has hampered fully conclusive antigenic characterization of HEV strains.

In animals such as swine, HEV causes an asymptomatic or sub-clinical infection of the liver, although focal lesions in the hepatic tissue can be shown by histology and immunohistochemistry. Differently, in man infection may result in acute hepatitis with a wide range of severity, although in most cases disease is resolved in a few weeks without permanent liver damage (Aggarwal, 2011). Whereas the lethality rate of human hepatitis E is between 0.5 – 3%, in pregnant women the rate of fatal outcome may approach 30% of cases, which is apparently restricted to HEV genotypes g1 and g2. This severe form has been reported in endemic countries with low health standards and sanitation of Africa, Asia and Central America, where large waterborne epidemic outbreaks due to g1 and g2 HEV involving hundreds to many thousands of cases have been repeatedly shown to occur (Aggarwal, 2011).

No major outbreaks of foodborne HEV infection in man have been reported this far, although g3 HEV infection is widespread among farmed swine globally, and the presence of viral genomic RNA in liver and other pork products has been reported in several studies. Nonetheless, foodborne transmission has been implicated in sporadic cases and small outbreaks of hepatitis E, which are for the most part associated with g3 HEV strains genetically related to the strains infecting pigs. The reported co-clustering of nucleotide sequences of animal and human origins from a same geographical area further supports the zoonotic transmission of these viruses. Besides the implication of swine-derived food, specific recent investigations have shown a higher risk of acquiring acute hepatitis E among coastal populations of UK and other countries, which highlight shellfish as a possible additional risk factor via foodborne transmission, although higher recreational use of seawater might also be involved.

The diagnosis of infection

Limited replication of HEV in *in vitro* systems has been reported on either conventional or three-dimensional cultures, but adaptation of field viral strains towards efficient progeny virus production has not been fully achieved yet, and the infant piglet is still the only reliable albeit problematical animal model of HEV propagation *in vivo*. Diagnosis of infection in either man or animals is usually performed serologically by search of either specific IgM or IgG serum antibodies using commercial tests based on recombinant g1 or g3 viral capsid antigens. Tests using recombinant viral proteins can be easily adapted to analysis in different animal species and are largely independent of the infecting HEV strain due to broad inter-genotype antigenic cross-reactivity. RT-PCR and, particularly, RT-qPCR assays are also largely used for rapid virus detection in both research laboratories and hospital practice, although the limited duration of the viremic phase in both humans and animals and the large nucleotide variation displayed by HEV strains represent a challenge for molecular diagnosis. Viral shedding with stools has been largely documented in swine, and more than 30% of pig feces are commonly found to be HEV-positive at any time in most swine farms throughout Europe. However, HEV shedding has no prognostic significance in swine, and is hardly useful as an indicator of risk for either professional exposure or the food chain. Although hepatitis E still seems to have a low prevalence in humans within industrialized countries, the more sensitive serodiagnostic assays recently made available commercially have detected much higher seroprevalence in the Dutch, French and other western country populations, between 30 to 50% of normal blood donors (Slot *et al.,* 2013). This leads to hypothesize that silent infection or subclinical forms of disease may in fact be largely present in “non-endemic” areas of the world, which would fit with the high risk of foodborne transmission expected on the basis of g3 HEV infection prevalence in swine but underscored using human clinical indicators.

Further optimization of highly sensitive methods for detection of HEV contamination in either foodstuff eaten raw, including shellfish, vegetables and berry fruit, in addition to pork and

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game, or surface water is desirable in order to collect data for risk assessment. Similarly, surveillance systems of acute hepatitis should be implemented to include search for HEV markers on a regular basis, as is for other hepatitis viruses.

The disease in the normal patient

In the developed areas of the world including European countries, clinically forms of genotype 1 HEV infection are still predominant and occur as sporadic cases, mostly in subjects with recent history of travelling to endemic countries in other continents, but an increasing number of cases of acute g3 HEV infection have been reported in recent years. These latter are considered to be authochtonous, and a possible association with food at risk, particularly swine derivatives, has been discussed in several instances.

Although pediatric hepatitis E patients are known, the disease and infections particularly involve adult males, which is more evident among the populations of industrialized countries, suggesting the risk factors for infection do not normally invest children. The reasons for the higher frequency of disease among males are not known, but both professional and/or living behavior and sex-related physiological factors might have a role.

Similar to the g1 infection in the Western populations, human hepatitis caused by g3 strains is usually a self-limiting illness that lasts from a few days to weeks in the immunocompetent patient (Kamar *et al.,* 2012). The incubation time is normally 2-6 weeks and main symptoms range from nausea and fever to vomiting, abdominal pain, malaise, up to hepatomegaly, asthenia and jaundice that affect between 40 and 75% of patients. The alanine aminotransferase (ALT) level ranges broadly, but is more frequently between 1,000 and 3,000 IU/ml of blood, and no particular ALT change seems to depend on the infecting viral genotype.

On a clinical basis, hepatitis E can be misdiagnosed as a drug-induced liver injury, particularly at older ages, and attention must be paid to possible concomitance between the onset of symptoms and the administration of poly-pharmacological therapy.

Bioptic samples are not normally made available, given the benign evolution of human disease in most cases, and a detailed description of the pathological damage and pathogenetic mechanisms is still unavailable. Details on the progression of infection are mainly derived from the pig model of HEV experimental infection in a few animal studies conducted in the last decade, although their significance is somewhat limited by the absence of clinical symptoms in the pig.

In pregnant women, infection with g1 or g2 HEV strains is particularly aggressive, and for these otherwise normal subjects fulminant liver failure is a major cause of death, which is particularly high during the last trimester, together with obstetric complication. The reasons for such a high rate of negative outcome are still unclear, but it may be related to the status of immune tolerance against the fetus, which is associated with reduced T-cell activity and cytokine production during large part of pregnancy, and the down-regulation of antigen presentation, involving significant changes in the hormone profile, in particular progesterone and estrogen and chorionic gonadotropin (Kamar *et al.,* 2014).

Hepatitis E in the immunocompromised patient

Fatal and fulminant cases of hepatitis E are more frequent in subjects with underlying chronic liver disease, or in patients with active HIV infection. During the past few years, HEV infection has also been shown to possibly evolve into chronic hepatitis and cirrhosis in subjects with compromised health conditions, in particular organ transplant (kidney, heart, liver, kidney-pancreas, bone-marrow) recipients, hematological patients receiving chemotherapy, and patients co-infected with HIV (Kamar *et al.,* 2012). Under similar circumstances, g3 HEV infection can lead to an excess of mortality throughout acute or sub-acute liver failure, affecting up to 10% of cases. In all these cases, a significant reduction in the immune status parameters can be appreciated, due to either pathogenetic mechanism of the co-infecting agent or to the pharmacologically induced immunosuppression. In fact, chronic hepatitis E is rare among AIDS patients under cART treatment, most likely because therapy allows maintain the anti-HEV immune response at an effective level.

Remarkably, chronic infections have never been reported in association with HEV genotypes other than g3.

The transmission mechanisms of HEV in patients subjected to hematological or organ transplantation have not been completely elucidated, although fecal-oral transmission through consumption of food at high risk of HEV contamination, particularly raw and undercooked pork meat or products, seems to be as important as in case of acute hepatitis E among the normal population (Legrand-Abravanel *et al.,* 2010). Although food at higher risk of HEV contamination, such as undercooked pork or contaminated water, is unlikely to be part of the transplant patient diet, the new information gathered on the long shedding period of HEV, the probable protracted infectious status of normal subjects, and the apparent large circulation of HEV among asymptomatic subjects may altogether support a pre-infection with HEV as the cause of symptomatic acute hepatitis E and its chronic evolution in this part of the population.

On the light of recently demonstrated high seroprevalence among blood-donors, the possible role of blood transfusion and blood derivative administration in transmitting HEV may not be excluded, although a clear demonstration of this transmission route has not be provided yet. Chronic HEV infection, defined as the persistence of viral RNA in the serum or feces of the patient for at least 6 months (i.e. for > 3 months after infection) in transplanted subjects, evolves into chronic liver disease and cirrhosis in approximately 60% and 10% of cases, respectively, usually within 3 to 5 years after primary infection with HEV. Therefore, occurrence of acute hepatitis E in organ transplanted patients is being considered a major risk factor for sever liver disease that needs to be carefully considered, calling for the identification of effective prevention and control measures in this category of patients.

In a future, HEV vaccination might become an important pre-requisite for a more favorable prognosis of organ transplantation or for use in controlling hepatitis E in patients with concurrent liver or immunological disorders. Given the presently low prevalence of acute symptomatic disease in the normal population, it is more difficult to think that a large use of vaccination against HEV would be accepted. Phase III vaccine trials using a recombinant HEV vaccine have been conducted in China, showing both high safety and efficacy, and this vaccine was

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eventually licensed for human use, although at present limited to that country. Anti-viral drugs might also be important tools to be used during immunosuppressive treatment of transplant recipients or in case of acute hepatitis E onset. Noteworthy, a limited number of studies have reported that drugs such as ribavirin and microphenolic acid may be efficacious to contain HEV infection and its possible chronic evolution, indicating that implementation of efforts in development of antiviral chemotherapeutical protocols should be recommended.

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Autochthonous cases of hepatitis E: where does the virus come from? Impact of pig slurry treatment on reduction of the viral load and prevalence of the virus in food substrates

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Abstract
In recent years, several autochthonous hepatitis E cases and a high seroprevalence have been reported. A potential source of contamination is the consumption of pork products or food contaminated by an environmental source. The objective of this study was to evaluate the prevalence of hepatitis E virus (HEV) in food samples, not only pork products, and to evaluate pig slurry as a potential source of environmental contamination.
A large prevalence study was conducted on 440 food samples collected in international food companies in 2011 as part of assessment of viral risks in their Hazard Analysis & Critical Control Points (HACCP) plan, including pork liver sausages, shellfish, fruits, vegetables, herbs and spices, process water, and ready-to-eat foods. The kit hepatitisE@ceeramTools™ was used for real time RT-PCR detection. Samples were also tested for norovirus GI, norovirus GII, and hepatitis A virus (HAV). A study was also conducted on pig slurry collected from 3 pig breeding farms positive for HEV to evaluate the persistence of HEV after various slurry treatments.
The results obtained for HEV demonstrate a prevalence of 0.9% with positive samples including pork liver sausage, pepper and bay leaf powder. On the 440 samples tested, the prevalence levels for norovirus GI, GII and HAV were 2.95%, 8.6% and 0.45%, respectively.
Concerning untreated pig slurry, 67% was positive for HEV. After treatment, 27% of pig slurry was still positive for HEV. Among this, 30% of treated pig slurry was positive for HEV after composting, 50% after dehydration, and only 5.6% of the pig slurry treated by anaerobic digestion was positive for HEV.
To our knowledge, this is the first large-scale study conducted on HEV prevalence in food samples to try to understand the origin of autochthonous hepatitis E cases and the potential origin of contamination in food samples. Our results demonstrate that HEV prevalence in food samples is in the same range as HAV. Spreading of pig slurry does not appear to be an agricultural practice at risk for HEV. These results demonstrate that types of foods other than pork liver products do not seem to be a potential source of contamination. This study could be helpful to evaluate the origin of human hepatitis E cases and to better prevent autochthonous HEV cases.

Introduction
The hepatitis E virus (HEV) causes acute hepatitis outbreaks with enteric transmission in humans that are fairly similar to hepatitis A epidemics though generally more severe (Emerson and Purcell, 2003). Although most hepatitis cases resolve spontaneously, fatal outcomes are reported (1-2% of cases). The risk of fulminant hepatitis in pregnant women can reach 25%, even though these cases have to date been reported only in emerging countries (Smith, 2001). Chronic hepatitis is also more and more frequently reported, especially in immunodepressed patients (Bihl and Negro, 2009; Gerolami *et al.,* 2008; Kamar *et al.,* 2008). Recently, many sporadic cases of hepatitis E unrelated to travel to endemic areas have been reported in developed countries. In France, the National Reference Centre (NRC) for entero-transmissible hepatitis has described a significant increase in the number of human cases of hepatitis E between 2002 (9 cases, creation of the NRC) and 2011 (249 cases), partly related to better diagnosis of this pathogen (Nicand *et al.,* 2011; Roque-Afonso, 2011).
In 1997, Meng *et al.* demonstrated genetic similarities between a new porcine virus (porcine HEV) and a strain of human HEV (Meng *et al.,* 1997). This discovery pointed to the potential role of porcine HEV strains in autochthonous human cases. As a result, many studies were carried out in various animal populations and showed that HEV is able to infect many animal species, including pigs, its primary reservoir (Cooper *et al.,* 2005; de Deus *et al.,* 2008; Meng *et al.,* 1997). A direct link between consumption of infected products and cases of autochthonous human hepatitis was reported following ingestion of raw deer meat (Tei *et al.,* 2003), raw wild boar meat (Tamada *et al.,* 2004), and raw liver sausages called figatelli (Colson *et al.,* 2010).
Bivalve molluscs can concentrate viral particles during the filtration process involved in their method of nutrition. The hepatitis E virus has been detected in shellfish collected in various regions of Europe and Asia (Crossan *et al.,* 2012; Donia *et al.,* 2012; Li *et al.,* 2007). Maunula *et al.* (2013), described the presence of HEV in raspberries.
Pigs infected by HEV shed the virus for 3 to 4 weeks in large amounts. Pig farming practices therefore result in high-dose exposure of animals to HEV (Kasorndorkbua *et al.,* 2005). The HEV status in pig slurry stored in installations such as concrete and earth basins remains to be studied, along with the impact of slurry treatment on elimination of the hepatitis virus.
The study objectives were as follows:
- to evaluate the prevalence of HEV compared to prevalence rates observed for noroviruses and hepatitis A in various food substrates sampled at manufacturers as part of HACCP plans, including a limited number of products containing pig liver;
- to determine whether spreading of products following treatment of pig slurry constitutes an at-risk practice that could lead to contamination of crops.
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Materials and methods
Hepatitis E virus, mengovirus and samples
Development and validation of the detection method for hepatitis E virus were carried out using the available international WHO standard for this virus. This standard corresponds to HEV-positive plasma measured in international units and containing 250,000 IU/mL. HEV-positive pig faeces provided by ANSES (Dr Nicolas Rose, Swine epidemiology and welfare unit, Ploufragan, France) were used to supplement this validation.
Mengovirus vMC0, used as a process control, was obtained from the CeeramTools® Mengo Extraction Control kit (Ceeram,

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La Chapelle-sur-Erdre, France). In compliance with ISO/TS 15216, a yield of 1% mengovirus validates the process.
Detection of the foodborne viruses norovirus (NV), hepatitis A (HAV), and hepatitis E (HEV) by real-time RT-PCR was performed on 441 samples of various food substrates (**Table 1**) undergoing viral analysis as part of HACCP plans in agro-food businesses operating in Europe. This enabled determination of prevalence data.
The following food substrates were evaluated in this study: 230 samples of various herbs and spices, 77 fruits, 62 process waters, 36 shellfish (oysters and mussels), 20 pork-free ready-to-eat meals, 12 vegetables, and 4 figatelli.
The analysed substrates were mainly from Europe but some were from Asia or Africa, for example the spices. Specific information on the origin of samples was however difficult to obtain and these particulars are not detailed in this article.
122 faeces and pig slurry samples and 44 samples of compost from a field study were collected to evaluate the impact of various treatment methods for pig slurry (spreading, composting, anaerobic digestion, and dehydration). Two collection networks were used: a private network of veterinary laboratories in the Morbihan department and ANSES (Dr Nicolas Rose, Swine epidemiology and welfare unit, Ploufragan, France). Samples were taken at various stages of slurry treatment in swine farms located in Brittany. The treatment process was evaluated through sampling at different stages depending on the type of treatment: faecal matter, compost, raw and treated water, and semi-liquid muddy water from lagooning.

Elution / concentration of viral particles in food substrates and extraction of viral RNA

For the samples of shellfish, fruits and vegetables, and herbs and spices, the method outlined in Standard ISO/TS 15216 was applied. In short, after adding mengovirus vMC0, 2 g of digestive tissue of shellfish were treated with proteinase K by incubation for 1 h at 37°C, then 15 min at 60°C. After centrifugation at 3000 g at room temperature for 5 min, the supernatant was collected. Viral capsid lysis was performed on 500 µL of supernatant using NucliSens® lysis buffer (bioMérieux) by incubation at 56°C for 30 min. RNA was then extracted and purified using NucliSens® reagents (bioMérieux) as per the supplier's recommendations.
To 25 g of fruit or vegetable samples, and to 5 g of dried herbs and spices, 40 mL of TGBE buffer (tris-glycine-beef extract buffer, pH 9.5) were added, together with the mengovirus vMC0 control extract. Bags were then agitated constantly for 20 min at room temperature. The supernatant obtained was centrifuged for 20 min at 10,000 g at 4°C. pH was adjusted to 7.2 +/- 0.2. Viral particles were precipitated with polyethylene glycol (PEG) (1/4 Vol) under agitation for 1 h at 4°C, then centrifuged for 30 min at 10,000 g at 4°C. The pellet was then re-suspended in 500 µL of phosphate buffered saline (PBS1X) then clarified using chloroform/butanol. After 15 min of centrifugation at 13,500 g at 4°C, the upper aqueous phase was retained for lysis. Nucleic acids were extracted as described previously.
For the samples of process waters, mainly loaded with particles, an alternative method that was more suitable than Standard ISO/TS 15216 was applied. One litre was concentrated by cross-flow filtration using a filter cartridge (Sartorius) after adding mengovirus vMC0. After rinsing the cartridge with 20 mL of glycine buffer, a 40 mL concentrate was obtained. Secondary concentration was then performed by incubation in 50% PEG for 1 h at 4°C, followed by centrifugation for 20 min at 11,000 g at 4°C. The pellet was then suspended in 1 mL of PBS1X and clarified using chloroform/butanol. After 15 min of centrifugation at 13,500 g at 4°C, the upper aqueous phase was collected and the viruses lysed, and nucleic acids extracted as described previously.
Concerning figatelli, the process developed by the Maisons-Alfort Laboratory for Food Safety, Enteric viruses unit (Martin-Latil *et al.* 2014 *EuroReference*) was applied. In short, 30 mL of distilled water were added to 3 g of the substrates ground in a Stomacher bag (2 min, 260 rpm). The elution was performed at room temperature under agitation for 10 min after addition of mengovirus vMC0. The homogenate was clarified by centrifugation for 15 min at 8000 g at 4°C, and viral particles were then precipitated with PEG (1/4 Vol) for 2 h at 4°C and concentrated by centrifugation for 30 min at 8000 g. The eluate was recovered for lysis.

Elution / concentration of viral particles in pig faeces/slurry and composts and extraction of viral RNA

A 10% to 50% suspension of faeces or slurry was prepared in PBS. The suspension was then clarified by centrifugation for 30 min at 3000 g at 4°C. The supernatant was collected then clarified for a second centrifugation for 15 min at 10,000 g at 4°C. If the resulting supernatant was not clear, the second centrifugation step was repeated. Lysis and extraction of RNA was performed using 500 µL of suspension with NucliSens® reagents (bioMérieux) as described previously.
For "solid" samples (for example sawdust compost), 5 g of sample were taken and transferred to a filter bag containing 40 mL of TGBE buffer (tris-glycine-beef extract, pH 9.5). The bags were agitated constantly for 20 min at room temperature. Through the filter, the supernatant was recovered then centrifuged for 20 min at 10,000 g at 4°C. The pH of the supernatant obtained was adjusted to 7.2 +/- 0.2. Ten milliliters of PEG-NaCl 5X were then added to 40 mL of supernatant and agitated for 1 h at 4°C then centrifuged for 30 min at 11,000 g at 4°C. The pellet was then suspended in 1 mL of PBS1X and clarified using chloroform/butanol. After 15 min of centrifugation at 13,500 g at 4°C, the upper aqueous phase was collected and the viruses lysed and nucleic acids extracted.
For "semi-liquid" samples, for example samples from settling basins or lagooning basins, a protocol similar to the one used for the extraction of HEV in faeces or slurry was applied. Three millilitres of sample were taken to apply the protocol described previously.

Quantitative RT-PCR

The nucleic acid extracts obtained were tested using the real-time RT-PCR kit hepatitisE@ceeramTools™, following the supplier's recommendations and with SDS7300 or SDS7500 systems (Applied Biosystems). RNA extracted from the food substrates was also tested for NoVGI, NoVGII, and HAV using the real-time RT-PCR kits norovirusGI@ceeramTools™, norovirusGII@ceeramTools™, and hepatitisA@ceeramTools™ (Ceeram, La Chapelle-sur-Erdre, France). Positive controls containing RNA extracted from virus suspensions and a negative control containing all the reagents except the RNA extract were included in each set of experiments. The internal amplification control (IAC) contained in the hepatitisE@ceeramTools™ kit made it possible to validate each reaction. In addition, each RNA extract was tested undiluted and diluted

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to 1/10th in duplicate. All the samples were characterised by a cycle threshold (Ct). A standard curve for each viral target was produced using serial dilutions of viral suspensions. Mengovirus extraction yields were calculated for each sample based on the corresponding standard curve.

Results

Evaluation of HEV prevalence in food

The sampling plan for the prevalence study included all the at-risk substrates described in ISO/TS 15216 and in the Directive on the application of general food hygiene practices to control of viruses in food. The samples were analysed as part of self-monitoring to address the viral risk in an HACCP plan. The number of samples per type of food substrate was however dependent on the production activities of the food processor businesses involved in this study.

For the 441 samples analysed, 7 types of substrate were represented. Analyses were carried out in 2011. For all these samples, a minimum yield of 1% mengovirus vMCO was obtained, thus validating the test results.

Among the 441 samples analysed, the presence of HEV genomes was found in 2 figatelli out of 4 and in 2 herbs and spices out of 230, indicating prevalence of about 0.9% of HEV contaminated food. The prevalence for all analysed samples not containing pork was 0.46% and 0.9% only for the samples of herbs and spices, versus 50% for samples of figatelli containing pig liver. The obtained Ct values for figatelli samples were 31.23, corresponding to an amount of 4775 genome copies/gram and 30.18, corresponding to 9603 genome copies/gram. The Ct values for samples of pepper and bay leaf powder were 36.4 and 37.2, respectively. The viral load in these samples was not quantifiable; it was below 500 genome copies/test sample, corresponding to the limit of quantification of the method.

Genotyping of the identified positive samples was not carried out since the very low viral load did not enable recovery of sufficient material to obtain a workable result.

On the same samples, the prevalence rates for norovirus GI, norovirus GII, and HAV were 2.95%, 8.6% and 0.45%, respectively. The number of positive samples and the prevalence by substrate analysed is given in **Table 1**.

For the food substrate most represented in this study, herbs and spices, the prevalence rate for HEV, HAV, and NoVGII was about the same at less than 1%. For NoVGI, 8 samples were found to be positive of out 230 analysed, corresponding to a higher prevalence than for the other viruses at 3.5%.

Evaluation of pig slurry treatment on reduction of HEV viral load

Of the 20 initially selected farms, three (A, B and C) were found to be positive with HEV levels sufficiently high to carry out the study. A total of 123 raw slurry samples taken from basins or directly from animals in different housing areas were analysed. The presence of HEV nucleic acids was found in 82 samples, i.e. 67% positive samples. The results obtained for slurry on the various farms are presented in **Table 2**.

Table 2. Samples of slurry and treated slurry analysed by farm

	Number of samples	Number of slurry samples	Number of positives	Number of treated samples	Number of positive treated samples
Farm A	58	48	26	10	3
Farm B	70	54	43	16	11
Farm C	38	20	13	18	1
Total	**166**	**122**	**82**	**44**	**12**

Among the positive samples, the viral concentrations were variable. For *farm A*, the observed contamination levels in untreated slurry ranged from absence of detection to 1.46×10^6 genome copies/g, with a lower mean for the whole farm at 2.26×10^4 genome copies/g. Concerning *farm B*, contamination levels in untreated slurry ranged from absence of detection to 3.97×10^5 genome copies/g, with a mean of 2.53×10^4 genome copies/g for the whole farm. On *farm C*, contamination levels in untreated slurry ranged from absence of detection to 7.74×10^3 genome copies/g, with a mean of 1.5×10^3 genome copies/g for positive samples for the whole farm.

Since each farm has its own treatment system, three types of treatments were evaluated.

Farm A used sawdust composting to treat slurry.

Farm B used a slurry dehydration treatment plant leading to three types of products that can be exploited: fermented compost, settling basin supernatant, and lagooning water.

Farm C also used an anaerobic digestion treatment plant for slurry, leading to three types of products that can be exploited: raw slurry, treated slurry, and lagooning water. The results obtained for the different farms are shown in **Table 3**.

Of the 166 samples analysed, 122 were slurry samples and 44 were samples from slurry treatment. Of these 122 samples, 82 (67%) were identified as HEV-positive, with contamination levels ranging from 118 genome copies/g to 1.46×10^6 genome copies/g.

Table 1. Prevalence data for analysed food substrates

Type of substrate	Number of samples analysed	Number of HEV positives	Prevalence of HEV (%)	Other food viruses Number of positive samples (prevalence in %)		
				NoVGI	NoVGII	VHA
Herbs and spices	230	2	0.9	8(3.50)	1 (0.45)	1 (0.45)
Fruits	77	0	0	0 (0)	2 (2.60)	0 (0)
Process waters	62	0	0	0 (0)	3 (4.85)	0 (0)
Shellfish (oysters, mussels)	36	0	0	5 (13.9)	32 (88.9)	0 (0)
Prepared meals	20	0	0	0 (0)	0 (0)	0 (0)
Vegetables	12	0	0	0 (0)	0 (0)	1 (8,3)
Figatelli	4	2	50	0 (0)	0 (0)	0 (0)
Total	**441**	**4**	**0.9**	**13 (2.95)**	**38 (8.6)**	**2 (0.45)**

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For the treated samples, the presence of HEV was identified in 12 samples, i.e. 27% of treated samples, with concentration levels ranging from 85 genome copies/g to 3.34×10^4 genome copies/g.
On *farm A*, which treats slurry by sawdust composting, of the 10 analysed composts, 3 were found to be HEV-positive, with low contamination levels of 17 to 740 genome copies/g.
On *farm B*, which uses a slurry treatment plant, of the 6 samples of compost tested, 3 were found to be negative. For the 3 positive samples, contamination levels ranging from 100 to 6680 genome copies/g were detected. Four samples from settling basins were tested: all were HEV-positive with levels ranging from 80 to 600,000 genome copies/mL. For the lagooning basin test sample, 1030 genome copies/mL were detected.
On *farm C*, of the 6 samples of raw slurry sampled at the plant, a single sample was found to be positive with 416 genome copies/g. For the 12 other samples taken at the plant, corresponding to treated slurry or lagooning basin samples, none were identified as positive.
On average, the quantity of HEV detected in treated slurry samples was lower than in untreated slurry samples.
More detailed data on HEV reduction levels are given in **Table 3**.
On *farm A*, treating slurry by sawdust composting, a logarithmic reduction in viral load of 1.88 was observed.
On *farm B*, using a treatment plant, the mean reduction for all treated samples was 0.76. Reduction was 0.79 in fermented compost, 0.1 in supernatant from a settling basin, and 1.39 in the lagooning basin.
On *farm C*, a mean reduction of 3.29 was calculated. The reduction was 0.56 in raw slurry and 3.17 in both treated slurry and in the lagooning basin.

Discussion

The first part of this study aimed to evaluate the prevalence of HEV in various food substrates and not only in pork products identified as presenting a risk. This large study on 441 samples showed HEV prevalence of 0.9% in all the food substrates, a prevalence rate lower than that observed for noroviruses in the same samples. However, it is similar to rates found for hepatitis A virus. In the study performed by Maunula *et al.* (2013), an HEV prevalence of 0.98% was found in raspberries. These data for raspberries are similar to the overall prevalence rate found in this study.
The prevalence of HEV in the most represented substrates in this study, i.e. 230 samples of herbs and spices, was identical to the prevalence rate found for the samples overall. Moreover, the prevalence rate for HAV and NoVGII of 0.45% is comparable to data found for HEV, *versus* 3.5% for NoVGI. The herbs and spices are mainly produced in tropical areas of Africa, South America, and Asia, mostly using traditional methods. They are exposed to many sources of contamination, particularly microbiological: irrigation with water of insufficient sanitary quality, contact with soil and with untreated biological soil improvers, as well as handling by farmers or harvesters who are potentially vectors of contamination. The zoonotic properties of HEV also appear to suggest animal contamination, unlike HAV and noroviruses which do not have animal reservoirs. Assessment of bibliographic data on the microbial quality of these raw materials shows that the samples present highly diversified contamination, with the presence of enteric bacteria and yeasts and moulds in high quantities, particularly in untreated products (McKee *et al.,* 1995; Garcia *et al.,* 2001; Omafuvbe *et al.,* 2004; Hara-Kudo *et al.,* 2006; Choo *et al.,* 2007). The data obtained in this study confirm the potential risk related to these substrates through the presence of enteric viruses. The data must however be interpreted with caution since the viral load in these samples was very low and must be considered in relation to the infectious dose in humans.
Concerning the other substrates, Serracca *et al.* (2012) did not demonstrate the presence of HEV in ready-to-eat meals (110 samples). These results confirm those in our study on the same type of food substrate. None of the mollusc samples tested were found to be HEV positive. However, of 153 samples of molluscs tested, Diez-Valcarce *et al.* (2012) found an HEV-positive rate of 3%. These data suggest that bivalve molluscs may be a substrate with greater risk for HEV and for noroviruses GI and GII, as well as hepatitis A virus, due to their filtering activity that could concentrate the viruses present in a contaminated environment. In our study, 2 of the samples of raw pig liver products (figatelli) out of 4 presented contamination with hepatitis E virus. These findings are consistent with those reported by Martin-Latil *et al.* (*EuroReference*, 2014) indicating that 1 in 3 pig liver-containing products was contaminated by HEV. The data in this study demonstrated an HEV prevalence rate equivalent to that of HAV, and variability of the presence of the virus depending on the analysed substrates.
The second part of this study involved evaluation of pig slurry as a potential source of contamination of the environment and of food. Three swine farms were identified as positive for the virus and were interesting in that they used three different systems of

Table 3. Impact of treatment

	Raw slurry initial viral load		Treatment			Logarithmic reduction in viral load (initial load-load after treatment)
			Type of treatment	Viral load after treatment		
	Copies/g	Log10		Copies/g or mL	Log10	
Farm A	2.26×10^4	4.35	composting	294	2.47	1.88
Farm B	2.53×10^4	4.4	fermented composting	4117	3.61	0.79
			settling basin supernatant	2×10^4	4.3	0.1
			lagooning basin	1030	3.01	1.39
Farm C	1.5×10^3	3.17	raw slurry after anaerobic treatment	416	2.62	0.56
			treated slurry	0	0	3.17
			lagooning basin	0	0	3.17

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slurry treatment. The mean viral RNA levels for hepatitis E found for the various farms were relatively constant (104 genome copies/gram of slurry), with the exception of farm C where a lower viral load was detected (103 genome copies/gram of slurry). The loads found in final products following treatment were quite low or even very low for farm C, where the viral load was initially lower. These findings are consistent with those in published studies (Kasorndorkbua *et al.,* 2005; McCreary *et al.,* 2008; Garcia *et al.,* 2013).

The treatment by composting used on farm A appears to be quite effective since a very low viral RNA concentration was found in the final product. Garcia *et al.* (2013) demonstrated that following composting, the final product did not present HEV contamination, suggesting it is safe to use as an agricultural fertiliser. The treatment used on farm C appears to be effective since very low contamination was found in a single final product. On farm B, contamination levels in the various treatment products appear to present a greater risk. Results found for settling basin supernatant and the lagooning basin were consistent with the study carried out by Kasorndorkbua *et al.* (2005). In their study, the authors showed that HEV found in basins and lagoons was infectious after inoculation in pigs. Use of supernatant from settling basins in agriculture such as production of fruit and vegetables could possibly lead to contamination of these foods. This contamination could be a potential risk for humans in the event of consumption. The question of the infectious dose in humans remains nonetheless open.

In conclusion, our results show that the prevalence of HEV in food samples is similar to that for HAV in substrates such as herbs and spices. The origin of contamination by HEV could not be determined. Human activities, whether direct or indirect through contaminated water, or animal sources through spreading or wildlife, could be the source of contamination. Evaluation of pig slurry and of products resulting from slurry treatment shows, however, that spreading treated pig slurry does not appear to constitute a practice with an HEV risk.

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HEV detection in raw pig liver products using a quantitative RT-PCR method

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Hepatitis E virus (HEV) is responsible for acute hepatitis outbreaks in humans in countries with poor sanitation. More recently, many sporadic cases of acute hepatitis E, not linked with travel to endemic regions, have been reported in developed countries. In France, foodstuffs containing raw pig liver were suspected on several occasions to be the cause of autochthonous cases of HEV infection occurring between 2007 and 2009. Within the framework of a national official surveillance plan (DGAI/SDSSA/N2010-8330) for HEV contamination of food containing raw pig liver conducted in 2011, a quantitative detection method was developed, validated and used for HEV genome quantification on 70 food samples. This method consists of three steps: 1) elution-concentration of virus, 2) viral RNA extraction, and 3) a one-step duplex RT-qPCR for detecting HEV and the murine norovirus (MNV-1). MNV-1 was used as a process control for monitoring the quality of the whole extraction procedure. The results showed that about one third of figatelli and pig liver sausages tested positive for HEV genome and that the viral load was more than 103 genome copies of HEV per gram in 55% of samples.

Introduction

Hepatitis E virus (HEV) is transmitted mainly through the gastrointestinal tract after ingestion of contaminated food or water. HEV is recognised as the main causative agent of acute hepatitis in countries with poor sanitation, where it follows an endemo-epidemic pattern. Genotypes 1 and 2 are found in humans in endemic regions while genotype 3, and less frequently genotype 4, are related to sporadic cases of acute hepatitis in developed countries. In 2009, the French Food Safety Agency (AFSSA) issued two reports (Requests 2009-SA-0101 and 2009-SA-0146) pointing to the risk of HEV infection following ingestion of figatelli (raw sausage made from pig liver). The recommendations made in these reports and incrimination of products containing pig liver in autochthonous cases of hepatitis E highlighted the importance of having a sensitive and reliable method for the detection of HEV in foods.

Implementation of detection methods in the area of viral diagnosis in food safety is based on detection of viral RNA using sensitive and specific RT-qPCR methods. The two main difficulties related to detection involve the low concentration of viruses in foods and the presence of substances in the sample that inhibit the PCR reaction. In 2013, technical specifications concerning the detection of norovirus and hepatitis A virus in foods were published(ISO/TS_15216-1:2013; ISO/TS_15216-2:2013). Hepatitis E virus, still considered an emerging virus, was not identified as a priority when work began to standardise detection methods in food virology. However, the general recommendations of the European Committee for Standardization (CEN/TC275/WG6/TAG4) concerning viral detection in food safety provide for a series of controls, including a negative extraction control, virus extraction process control, positive and negative RT-PCR control, and inhibition of RT-PCR control. Adding a process control for each test sample is essential because it helps to determine the effectiveness of processing and to assess the presence of inhibitors for PCR amplification reactions. Determining the mean yield obtained for the process control in a given substrate also enables determination of the acceptable level of yield to validate analysis of the sample.

As part of the surveillance plan carried out in 2011, the objective of this study was to develop and validate a method to extract and detect the HEV viral genome by quantitative RT-PCR in food substrates containing raw pig liver (figatelli and liver sausages) and to analyse 70 samples from the four categories of food potentially posing a risk for the consumer (figatelli, liver sausages, salted dried pig livers, quenelles).

Materials and methods

Hepatitis E virus, murine norovirus, and samples from the surveillance plan

To develop and validate the detection method for the HEV viral genome, artificially contaminated samples were prepared using a viral suspension of HEV (genotype 3f; Genbank accession number: JF718793) obtained from a faecal extract from an infected pig, provided by the Laboratory for Animal Health (ANSES, Maisons-Alfort). The faecal sample was suspended in a 10 mM PBS buffer at pH 7.4 (10% final (w/v)) and centrifuged at 4000 g for 20 minutes at 4°C. The supernatant containing HEV viral particles was aliquoted and stored at -80°C. The number of copies of HEV RNA in the viral suspension was determined by RT-qPCR using a standard curve obtained with HEV RNA transcribed *in vitro*.

Murine norovirus MNV-1 (CW1) was amplified then titred in RAW 264.7 cells (murine macrophage line, ATCC TIB-71).

As part of the surveillance plan for HEV contamination of delicatessen products containing raw pig liver (DGAL/SDSSA/N2010-8330) covering 400 samples (Pavio *et al.,* 2013), 70 samples were selected in order to maintain the initial distribution of the 400 samples in terms of geographic origin and type of product (figatelli, liver sausages, salted dried liver, quenelle paste) then analysed by quantitative RT-PCR.

Quantitative HEV detection method in food samples containing raw pig liver

Elution / concentration of viral particles and extraction of viral RNA

The food sample (3 g) was cut into pieces using a scalpel and placed into a Stomacher bag. After adding the MNV-1 used as

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a process control (50,000 TCID50), 30 mL of distilled water was added and the sample was ground in the Stomacher bag (2 min, 260 rpm). Elution was performed at room temperature under agitation for 10 minutes. The homogenate was clarified by centrifugation (8000 g, 15 min, 4°C) and viral particles were then precipitated with 0.25 volumes of polyethylene glycol (PEG) for 2 h at 4°C and concentrated by centrifugation at 8000 g for 30 min. Viral particles were lysed directly to extract viral RNA using an automated extractor (NucliSens® easyMAG™).

Quantitative RT-PCR

The molecular model for detection of HEV is based on the model described by (Jothikumar *et al.,* 2006), targeting the ORF2/ORF3 region. The molecular model for detection of MNV-1 targeting ORF1 was determined using Beacon Designer software (Bio-Rad, Marnes-la-Coquette, France). Taqman probes for HEV and MNV-1 detection were labelled respectively with ROX or 6-FAM in 5', and BHQ2 or BHQ1 in 3'.

For HEV detection, the sequences used for TaqMan probes and primers were:
HEV-5260-F: 5'-CGGTGGTTTCTGGGGTGAC-3',
HEV-5330-R: 5'-AGGGGTTGGTTGGATGAATATAG-3'
HEV-5280-T: 5'-ROX-GGGTTGATTCTCAGCCCTTCGC-BHQ2-3'.

For MNV-1 detection, the sequences used for TaqMan probes and primers were:
MNV-3193-F: 5'-CCGCCATGGTCCTGGAGAATG-3',
MNV-3308-R: 5'-GCACAACGGCACTACCAATCTTG-3'
MNV-3227-T: 5'-FAM–CGTCGTCGCCTCGGTCCTTGTCAA-BHQ1-3'.

All quantitative RT-PCR runs were performed using the CFX96™ system (Bio-Rad, Marnes-la-Coquette, France). Reactions were carried out using the RNA UltraSense™ One-Step Quantitative RT-PCR System (Fisher Bioblock Scientific, Illkirch, France). Positive controls containing RNA extracted from virus suspensions and negative controls containing all the reagents except the RNA extract were included in each set of experiments. The thermo cycler programme for the one-step RT-qPCR was 60 min at 55°C for reverse transcription of viral RNA; a denaturation step of 15 min at 95°C, then 40 cycles of PCR (15 s at 95°C, 1 min at 60°C and 1 min at 65°C). Each RNA extract was tested undiluted and ten-fold diluted in duplicate. All the samples were characterised by a cycle threshold (Ct). Negative samples did not have a Ct value. A standard curve for each viral target was produced using serial dilutions of the viral suspension. The slopes (S) of the regression lines were used to calculate the amplification efficiency (E) of the RT-qPCR reactions, using the formula: $E = 10^{|-1/s|} - 1$. The extraction yields for HEV and MNV-1 were calculated for each sample on the basis of the corresponding standard curve.

Statistical analysis

Statistical analyses were performed using MATLAB software (version 6.5.1).
To test method validation, the impact / effect of adding the MNV-1 process control was first evaluated based on extraction yields for HEV using a univariate analysis of variance (ANOVA). A multivariate ANOVA was then performed to evaluate the effect of four experimental factors on the extraction yields for HEV: sample dilution, type of food (figatelli, liver sausages), quantity of HEV and inter-assay variability. Concerning extraction yields obtained for MNV-1, three variables were tested, i.e. sample dilution, type of food (figatelli, liver sausages) and inter-test variability.

Results

Validation of the HEV detection method in figatelli and liver sausages

The HEV detection method was validated for figatelli and liver sausages that were artificially contaminated since they accounted for more than 75% of the samples collected during the surveillance plan. The limit of detection (LOD) for HEV and mean extraction yields for HEV and the MNV-1 process control obtained from four replicate experiments are shown in **Table 1**.

Table 1 : Mean extraction yields for HEV and MNV-1
Four experiments were performed and each sample was analysed by RT-qPCR in duplicate and the number of positive tests is indicated in brackets. The LOD_{100} corresponding to the detection of HEV in the four replicate experiments is highlighted in grey. nd: not detected.

Inocula / 3g		Mean extraction yields in figatelli (%) (positive Ct values / 8)		Mean extraction yields in sausages (%) (positive Ct values / 8)	
HEV	MNV-1	HEV	MNV-1	HEV	MNV-1
0	0	nd	nd	nd	nd
875	0	43.9 ± 26.9 (2/8)	nd	nd	nd
1,750	0	22.8 ± 11.0 (4/8)	nd	9.8 (1/8)	nd
8,750	0	14.7 ± 11.3 (7/8)	nd	3.2 ± 2.1 (2/8)	nd
17,500	0	9.6 ± 6.5 (7/8)	nd	4.9 ± 2.5 (6/8)	nd
87,500	0	8.7 ± 2.5 (8/8)	nd	2.7 ± 2.1 (8/8)	nd
0	50,000	nd (0/8)	11.6 ± 7.0	nd	1.6 ± 0.9
875	50,000	28.6 ± 9.4 (3/8)	13.0 ± 6.6	nd	2.0 ± 1.3
1,750	50,000	35.7 ± 33.4 (6/8)	13.4 ± 7.1	nd	1.2 ± 1.1
8,750	50,000	7.6 ± 5.6 (5/8)	12.1 ± 4.1	3.0 ± 1.7 (2/8)	3.1 ± 1.2
17,500	50,000	5.6 ± 5.7 (6/8)	15.8 ± 7.7	2.9 ± 2.1 (2/8)	3.2 ± 0.4
87,500	50,000	6.6 ± 2.0 (8/8)	12.9 ± 3.8	1.1 ± 1.0 (7/8)	6.5 ± 6.5
Mean extraction yields (%)		**18.4**	**13.1**	**3.9**	**2.9**

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Table 2: Results of analyses on 70 samples in the PS-VHE plan, broken down by type of food
The number of positive analyses for HEV and the obtained extraction yields for the MNV-1 process control are shown.

Types of products	Total number of analyses	Number of HEV-negative analyses	Number of HEV-positive analyses	Extraction yields for MNV-1 (%)			
				< 0.1%	0.1-1%	1-10%	>10%
Figatelli	33	21	12	0	6	20	5
Liver sausages (dried or fresh)	27	17	10	1	4	22	2
Salted dried liver	5	5	0	2	3	0	0
Quenelle and quenelle paste	5	5	0	3	2	0	0
Total	70	48	22	6	15	42	7

The presence of the MNV-1 process control did not affect extraction yields for HEV (univariate ANOVA; p-value = 0.10). Mean extraction yields obtained from figatelli were 13.1% for MNV-1 and 18.4% for HEV. In liver sausages, mean extraction yields were 2.9% for MNV-1 and 3.9% for HEV. Extraction yields for HEV and MNV-1 were not improved after ten-fold dilution of RNA samples (results not shown).
Results were analysed using a statistical test of multivariate ANOVA which showed that (i) extraction yields were higher in figatelli than in sausages, both for HEV and the MNV-1 process control; and (ii) virus extraction from these two types of food samples did not generate significant inhibition of amplification of the two viral genomes by PCR.

Quantitative detection of HEV in 70 samples in the surveillance plan (DGAl/SDSSA/N2010-8330)

The results of analyses on 70 samples containing raw pig liver are given in **Table 2**. Presence of HEV genomes was found in 12 figatelli out of 33, and in 10 liver sausages out of 27, indicating prevalence of about 36% for HEV contaminated food. Quantitative data for the HEV-positive samples are provided in **Table 3**. The HEV viral load in figatelli and liver sausages was between 4 x 10^1 and 2 x 10^6 genome copies of HEV / g. The quantity of HEV found was >10^2 genome copies / g in 95% of positive samples, > 10^3 genome copies / g in 55% of positive samples, and > 10^4 genome copies / g in 27% of positive samples.
The MNV-1 process control was used to check all the steps of the analysis from viral extraction from the food substrate to quantitative detection by RT-qPCR. The extraction yield ranges for MNV-1 obtained for the 70 samples containing raw pig liver are presented in **Table 2**.
The extraction yield of the MNV-1 process control obtained from most of the figatelli and liver sausages analysed was > 1%. However, extraction yields obtained for MNV-1 from the other two food categories, i.e. salted dried liver and quenelles, were all < 1%. For 5 samples out of 10, the extraction yield obtained for MNV-1 was even lower than 0.1%.

Table 3: Quantity (genome copies) of HEV detected in positive samples in the PS-VHE plan

Ct values	Copies of HEV genome / 3 g	Number of Figatelli	Number of liver sausages
< 30	> 2E+05	3	1
30 - 36	1,6E+03 - 2E+05	7	2
36,1 - 40	1,2E+02 - 1,6E+03	2	7

Discussion

As part of the surveillance plan on hepatitis E virus contamination of delicatessen products containing raw pig liver at the production stage, this study was intended to develop and validate a method for quantitative detection of the hepatitis E (HEV) viral genome in at-risk food substrates in order to quantitatively assess 70 samples.
The preliminary tests carried out during development of the method for HEV detection highlighted the importance of including an elution / concentration step for viral particles by polyethylene glycol (PEG) before extraction of viral RNA (results not shown). The virus concentration step is generally essential since contamination levels of food by enteric viruses are low.
As a result, concentration of enteric viruses (enterovirus, hepatitis A virus, norovirus, adenovirus, and astrovirus) from a wide variety of food products such as pasta salads, meat, whipped cream, and shellfish, is carried out most commonly by viral precipitation in PEG (Stals *et al.,* 2012). This was also the approach adopted by the European working group CEN/TC275/WG6/TAG4 to detect norovirus and hepatitis A virus in vegetables and mixed berries (ISO/TS_15216-1, 2013; ISO/TS_15216-2, 2013).
Validation experiments for the HEV detection method carried out on artificially contaminated figatelli and liver sausages showed that the mean extraction yields for HEV from figatelli (18.4%) are statistically higher than those obtained from liver sausages (3.9%). These extraction yields are consistent with those described for the detection of hepatitis A virus and norovirus from plants and fruits (Blaise-Boisseau *et al.,* 2010; Martin-Latil *et al.,* 2012a).
To validate viral diagnosis in food safety, it is essential to use a process control virus as described in many studies and review articles (Baert *et al.,* 2011; Coudray *et al.,* 2013; Martin-Latil *et al.,* 2012b; Stals *et al.,* 2012). The process control is added in a defined amount to the test sample before viral extraction and technical specifications (ISO/TS_15216-1, 2013; ISO/TS_15216-2, 2013) propose that viral diagnosis should be validated if process control yields ≥ 1%. The virus selected as a process control must be cultivable, non-enveloped, positive polarity ssRNA (single stranded), and of similar size to the target virus in order to provide a suitable morphological and physico-chemical model. It must also show persistence in the environment similar to the target viruses, and should not be found naturally in foods under normal circumstances. For this study, murine norovirus (MNV-1) was selected as the process control virus, like in many previous studies (Coudray *et al.,* 2013; Martin-Latil *et al.,* 2012a, b; Stals *et al.,* 2009). Extraction yields

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of the process control (MNV-1) higher than 1% were obtained in 80% of the figatelli and in 83% of the liver sausages analysed. For quenelles (5 samples) and dried salted liver (5 samples), extraction yields of the MNV-1 process control obtained were lower, at < 1%. These results confirm the need to use a process control virus to validate the detection method for HEV in each type of food and suggest that improved detection of HEV in quenelles and salted dried liver should be pursued in future studies.

The high prevalence, with a third of tested products showing positive for HEV, associated with high levels of contamination (55% of positive samples had a contamination level greater than 103 genome copies per gram), support the possibility of HEV transmission to humans via these foods. Although the validated detection method is based on determination of viral genomes and does therefore not provide information on the presence of infectious HEV particles in the analysed samples, the infectivity of HEV from figatelli has been demonstrated in cell models (Berto *et al.,* 2013).

In conclusion, development of routine methods showing the infectivity of HEV is required to obtain a better understanding of the viral risk. Nonetheless, it appears warranted to monitor animal reservoirs of HEV, to issue recommendations to avoid entry of HEV into foods, and to emphasise good cooking practices to limit the risk of human contamination. HEV diagnostic methods should be validated based on the type of food in order to determine the prevalence of HEV in all at-risk substrates in a reliable manner. Lastly, additional studies to determine the probability of infection depending on the quantity of HEV genome copies present in a sample may be needed to better assess the viral risk.

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