Maisons-Alfort, 7 July 2011

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on the current state of scientific knowledge and information available for making recommendations, following the onset of several cases of haemolytic-uraemic syndrome (HUS) observed in France in June 2011 and suspected of being related to the consumption of sprouts

ANSES conducts independent and pluralistic scientific expertise.
ANSES's public health mission involves ensuring environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also helps to protect the health and well-being of animals and plant health and assesses the nutritional properties of food.

It provides the competent authorities with the necessary information concerning these risks as well as the requisite expertise and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its Opinions are published.

1. REVIEW OF THE REQUEST

On 29 June 2011 the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) issued a formal internal request for an emergency Opinion on the state of scientific knowledge and information available for making recommendations, following the onset of several cases of haemolytic-uraemic syndrome (HUS) reported in France in June 2011, suspected of being linked to the consumption of sprouts.

2. BACKGROUND AND PURPOSE OF THE REQUEST

On 22 June 2011, cases of haemorrhagic diarrhoea and HUS were reported in adults, mainly women, in the Bordeaux region. The first investigations of these cases incriminated a strain of *Escherichia coli* (*E. coli*) belonging to serotype O104:H4, a bacterium genetically related to that responsible for the outbreak reported in Germany in May 2011.

A European traceability survey, coordinated by the European Food Safety Authority (EFSA), with the support of the health authorities of Member States of the European Union was undertaken to try to identify a possible common source. Following the publication of the EFSA report of 5 July 2011 on this traceability study which established a link between the *E. coli* 0104:H4 intoxication and seeds imported from Egypt, the European Commission decided, that same day, to remove all of the batches of fenugreek seeds imported into Europe by an Egyptian exporter between 2009 and 2011 from the market, then analyse and destroy them. This decision also includes suspending

importation of Egyptian seeds and beans intended for sprouting[1] until 31 October (including seeds, fruits and spores for sowing, leguminous vegetables, shelled or unshelled, fresh or chilled, fenugreek, dried leguminous vegetables, shelled, whether unshelled or broken, soybeans, seeds and nuts, even broken).

ANSES, along with experts from its laboratories and experts from the French National Reference Laboratory (Vet-AgroSup, Lyon), the French National Centre of Reference (*Institut Pasteur*, Paris) and its affiliated laboratory (CHU Robert Debré, Paris), the French Institute for Public Health Surveillance (InVS), the French National Institute for Agricultural Research (INRA, Avignon and Angers) and the group for the study and control of varieties and seeds (GEVES) as well as the French National Institute of Health and Medical research (INSERM) unit 1043 of the National Veterinary College of Toulouse (ENV), set up a Working Group to share experience and draw on all scientific and technical knowledge that may facilitate understanding of the situation and the detection of *Escherichia coli* O104 in sprouted or dormant Fenugreek seeds, suspected of being the cause of both outbreaks.

It is difficult to detect the pathogenic bacterium in these seeds and only a very small number of pathogenic bacteria are necessary to cause the disease. This paper was thus written:

- to highlight the main characteristics of the pathogen involved and the related epidemiological data;
- to highlight the critical steps to be considered when producing these sprouts and possible sources of contamination at different stages in the production process;
- to review the latest information on the outbreak in progress;
- to list critical points for special attention by the investigators;
- to make recommendations for consumers and operators.

3. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with the French standard NFX50-110 "Quality in Expert Appraisal Activities – General Requirements of Competence for Expert Appraisals (May 2003)".

The collective expertise was provided by an emergency collective expert assessment group (GECU) on "*Escherichia coli* O104:H4 / sprouts" between 29 June and 5 July 2011.

This group of experts met on 30 June 2011 via a teleconference and issued the following Opinion, which was validated on 6 July 2011 by electronic transmission.

4. ANALYSIS AND CONCLUSION OF THE GECU

The acronym STEC (for Shiga toxin-producing *Escherichia coli*) or VTEC (Verotoxin-producing *Escherichia coli*), which are rigourously equivalent, includes all strains of *E. coli* with *stx* genes encoding specific toxins, which are termed Shiga toxins (Stx) or verotoxins. Thus, this is a genetic definition. However, not all STEC strains are pathogenic for humans. Only a few of them cause diarrhoea, or bloody diarrhoea and a severe complication, HUS, which is the leading cause of acute kidney failure in children less than three years old. STEC strains, implicated in the onset of these symptoms in human beings, are called enterohaemorrhagic. They are then referred to as EHEC (for enterohaemorrhagic *E. coli)*. This is a clinical definition, based on symptoms observed in humans.

The distinction drawn between the two definitions (genetic and clinical) reflects the fact that the complete list of virulence genes involved in the pathogenicity of the EHECs is not known at this time. Thus, all EHECs are capable of producing at least one Stx toxin (Stx1 and/or Stx2) and have at least one of the *stx* genes (*stx1* and/or *stx2*). All EHECs (including the strain O104:H4) are thus STECs but not all STECs are EHECs (STEC strains are not all pathogenic for humans).

[1] http://europa.eu/rapid/pressReleasesAction.do?reference=IP/11/831&format=HTML&aged=0&language=FR&guiLanguage=en, consulted on 5 July 2011

From an epidemiological standpoint, it is essential to distinguish between isolates of *E. coli.* Thus, each strain of *E. coli* (pathogenic or not) can be classified according to its surface antigens:
- somatic O antigens, polysaccharide in nature, form part of the outer membrane of the bacterial wall. Determined using antiserum, this antigen is used to classify *E. coli* by serogroups (O157, O104, etc.);
- flagellar H antigens, consisting of flagellin and placed on the surface of the wall, allow bacterial motility.

E. coli can be classified by serotype according to the association of O and H antigens, within each serogroup (for example, O104:H4). These are epidemiological markers as the lipopolysaccharide, which forms the bacterial wall is not in itself a virulence factor responsible for actual clinical symptoms.
Consequently, other genetic markers are screened for in a bacterium or in a food matrix, outside a clinical setting, such as genes encoding for somatic and flagellar antigens (for example, the specific gene *wzx*O104 of serogroup O104).
So-called typical EHECs have a characteristic adhesion factor, intimin, encoded by the *eae* gene (AFSSA 2010) and are capable of producing Shiga toxins. Moreover, EHECs belonging to the serotype O157:H7 are the most commonly isolated strains and the source of several EHEC outbreaks in the world.

■ Review of available information related to the outbreak

• Epidemiological situation in France

On 22 June 2011, two hospitals in Bordeaux reported the first six cases of bloody diarrhoea and two cases of HUS to the Regional Epidemiological Centre (CIRE) of the Aquitaine region. An epidemiological investigation was promptly undertaken to identify the source of this episode of clustered cases (Gault G 2011).
In all, 7 HUS cases, 4 cases of bloody diarrhoea and one case of simple diarrhoea were identified on 5 July 2011. These 12 people had all attended an “open house” day in a Recreation Centre for young children (CLPE) in Bègles on 8 June. The 12 people included seven women and five men, from 6 to 64 years of age. All reported having eaten sprouts that day. The twelve people began having symptoms between 13 and 20 June. An EHEC O104:H4 infection was confirmed for eight of the twelve. The microbiological diagnosis of the other cases is pending.
A cohort study of all those who attended the CLPE “open house” day, began on 29 June for the purpose of documenting this outbreak epidemiologically and clinically. Two cases of person-to-person transmission with one family were reported.

Following the publication of the EFSA report on a Europe-wide traceability study which showed a link between cases of intoxication and seeds imported from Egypt, the European Commission decided to remove all of the batches of fenugreek seed imported by an Egyptian exporter between 2009 and 2011 form the market, then analyse and destroy them. This decision also included suspending importation of Egyptian seeds and beans intended for sprouting until 31 October.”

• Traceability investigations

The seeds consumed at the CLPE, during the festivities on 8 June 2011 were organic fenugreek, rocket and mustard seeds purchased at Jardiland and sprouted at the CLPE in individual empty jam pots with no specific substrate.
Jardiland’s supplier is the C company (UK), which markets these seeds in packets after repackaging them, itself. Their sources are as follows:
- for organic fenugreek (consumed at the CPLE): the A company (primary wholesale importer), located in Germany and supplied from Egypt;
- for conventional fenugreek, rocket and mustard: the D company, located in Italy and supplied from India.

The A company also supplied the farm involved in the German outbreak with organic fenugreek from Egypt, which therefore constitutes a common factor in these two episodes.
Traceability investigations are conducted internationally as part of a Task Force involving the Netherlands, United Kingdom, Italy, Germany and France since 27 June 2011 under the auspices

of EFSA. A report, published on 5 July 2011, reviewed the information collected and the findings of the traceability investigation, conducted for the purposes of determining and documenting each stage in the chain, from production to distribution, for the products suspected of being the cause of the outbreaks observed in Germany and France in May-June 2011. This survey found that batch # 48088 imported from Egypt in November 2009 and received in Germany by a primary wholesale importer A in December 2009 was sold to a German wholesale distributor B in December 2009 and a wholesale distributor C in Great Britain in January 2010. The German wholesale distributor B supplied the German producer of the sprouts in February 2011 and the wholesale distributor C in Great Britain supplied the French distributor in January 2011. Thus, a single initial batch probably led to both the German and French outbreaks. This batch was distributed in total to 70 companies in 12 European countries.

The trace back investigation concerning distribution of the suspect batch of organic fenugreek in France indicated that Company C supplied four brand names including Jardiland.

- Characteristics of the strain involved in the outbreak

E. coli O104:H4 strains, isolated in five reported cases of HUS in June 2011, in the Bordeaux region and associated with the consumption of sprouts, have been characterised (Gault G 2011). These isolates are similar to those identified and characterised following the outbreak observed in May-June in Germany of this year (Bielaszewska, Mellmann *et al.* 2011). They do indeed have indistinguishable molecular profiles, obtained by pulse field gel electrophoresis using *Xba*I and *Not*I enzymes (total bacterial DNA restriction profile analysis) (Gault G 2011). This means that these strains are related, from an epidemiological point of view, because they are genetically similar, if not identical (which can only be confirmed by a complete sequencing of the strains, which is in progress). This homology of isolates taken from French patients and those of German patients, combined with the rarity of the strain causing these outbreaks, suggests that these two events are related to a common source.

This epidemic strain belongs to the serotype O104:H4, and has the *stx2* gene (*stx*2a variant) which encodes the STX2 toxin. It does not have the *eae* gene (encoding intimin), *hlyA* (encoding haemolysin A) and *astA* (encoding the EAST1 toxin) but hosts the *aggR* gene, encoding a factor regulating the expression of fimbriae responsible for very strong adhesion to the intestinal mucosa. In addition, this strain has a profile of multi-antimicrobial resistance (resistance to the following compounds: ampicillin, cefotaxime, ceftazidime, streptomycin, sulphamethoxazole, trimethoprim, cotrimoxazole, tetracycline and nalidixic acid). PCR analyses have identified the presence of the *bla*CTX-M-15 gene encoding an extended spectrum betalactamase (ESBL) and that of the *bla*TEM gene encoding a penicillinase.

Specifically, this epidemic strain hosts a plasmid, called IncI1/pST31/CTX-M-15, which has a pST31 profile as determined by pMLST (for plasmid Multi Locus Sequence Typing). This plasmid contains the *bla*CTX-M-15 gene, often found in bacteria isolated in humans, particularly for the bacterial genus *Salmonella,* in strains isolated from patients in Africa (Weill, Perrier-Gros-Claude *et al.* 2004; Blomberg, Jureen *et al.* 2005; Touati, Benallaoua *et al.* 2008; Usha, Chunderika *et al.* 2008). Moreover, strains isolated in animals have also been described with this pST31 – CTX-M-15 association for isolates from cattle in the United Kingdom (Kirchner, Wearing *et al.* 2011) and the pST31 plasmid subtype has already been described for *Salmonella* isolated from cattle in France (Madec, Doublet *et al.* 2011).

Concerning the serotype of the epidemic strain (O104), it has only been described rarely in the literature as being associated with severe cases of human infections. Some strains of EHEC O104:H21 have been identified in the world but only for cases of lesser importance in terms of seriousness and frequency. An outbreak was reported with a strain of EHEC O104:H21 in the state of Montana in 1994 (Feng, Weagant *et al.* 2001). An O104:H4 strain type MLST 678 (like the one identified in the German outbreak in 2011) had been isolated in Germany in a case of HUS in 2001 (Mellmann, Bielaszewska *et al.* 2008). A case of human infection with O104:H4 was identified in South Korea in 2005 (Bae, Lee *et al.* 2006). It involved a 29 year old woman with an HUS case but for which there was no evidence that she had consumed any contaminated food. This Korean strain

is genetically different from the strain responsible for the current German and French outbreaks. In France, two strains had previously been isolated from patients with HUS (2004 and 2009) and three O104:H4 strains had also been described in Finland and Georgia (2009 and 2010) (Scheutz, Moller Nielsen et al.2011). Finally, very few strains of EHEC, belonging to the serogroup O104, have been identified in the strains described to date and the associated epidemiological characteristics have usually been different from the strains identified in the current epidemic.
The epidemic strain of serotype O104:H4 is thus quite particular. It does not have the *eae* gene but has other adhesion factors that are characteristic of enteroaggregative *E. coli* (EAEC) strains giving them the capacity to form biofilms (Scheutz, Moller Nielsen et al. 2011). These EAEC strains are known to adhere strongly to the intestinal mucosa. Thus adhesion is much stronger than that observed for EHECs, which are usually eliminated from the intestinal mucosa in 8 to 10 days. Patients affected by this epidemic strain are probably subjected to exposure to the Stx 2 toxin over a long period of time, which may explain the severity of the cases observed in Germany and France and the unusually high mortality rate (48 deaths for the German outbreak, plus two other deaths reported in Sweden and the United States, as of 1 July 2011).

This epidemic strain is not an EHEC on the genetic level but an EAEC strain which acquired an *stx2* gene. The genome sequence of the epidemic strain in fact strongly resembles that of an African strain of EAEC O104:H4 (strain 55989), previously identified in an HIV-positive patient who suffered from persistent diarrhoea (Bernier, Gounon *et al.* 2002). This O104:H4 strain is very different genetically from other strains of EHEC O104 (specifically O104:H21) which up to now have been isolated during outbreaks. Another enteroaggregative Stx toxin-producing strain had previously been described during a French outbreak that occurred in 1994, but it belonged to another serotype (O111:H2) (Morabito, Karch et al. 1998).
These EAEC strains are rare in Europe but are known to cause aqueous diarrhoea (travellers' sickness) in other countries where sanitary conditions and difficulties in procuring drinking water are implicated during the onset of these cases of diarrhoea (Scheutz, Moller Nielsen et al. 2011).

The *E. coli* intestinal pathogens that are virulent to humans are usually not as resistant to antibiotics. In particular, they host extended spectrum beta-lactamases only very rarely. The current outbreak involves a serious epidemic, a rare serotype (O104:H4) and antimicrobial resistance of great concern to human medicine (ESBL).

- Methods for screening bacteria in food and methodological obstacles
  - Update on the specificity of the physiological state of bacteria in the seeds

The seeds carry highly diverse microflora whose load varies depending on the production, post-harvest processing and storage conditions and on the nature of the plant species itself.

The seeds are a particularly stressful environment for microorganisms, as there is very little water activity (optimum a_w for preservation of seeds = 0.33, O. Leprince, personal communication). Contamination of a seed is spatially heterogeneous. The seed coats can be contaminated on their outer but also their inner surfaces and some bacteria also colonise the surfaces of the embryo.

Seed disinfection operations thus have a generally limited effectiveness related to the difficulty in reaching the bacteria situated in the innermost sites or simply in dislodging them from the tegumentary folds in which they have settled and that are protected by a biofilm. This difficulty with decontamination is of course also related to the concomitant wish to maintain good quality seed germination. The metabolic activity of bacteria associated with seeds is extremely low, or zero. Phytopathogenic bacteria in particular survive in this environment, some for many years (over 15 years for some phytopathogenic bacteria on beans) (Maude 1996). It is highly likely that some quickly move into a viable but nonculturable state after undergoing hydric stress. There is not much literature available on this aspect. Similarly, the survival of *E. coli* on seeds for long periods remains largely undocumented (Beuchat and Scouten 2002).

  - Update on methods of detecting and identifying bacteria in seeds

A formal, standardised method for screening STEC O104:H4 strains in foods was disseminated by the European Union Reference Laboratory[2] and adopted by the French National Reference Laboratory[3].

Briefly, the protocol to be used is shown in the diagram in Figure 1. When the matrix to be analysed is composed of seeds for sprouting, the following should be taken into account:

1- Seeds are usually contaminated at a very low level (0.1 to 1.8 cfu/g, as for *Salmonella* (Liao and Fett 2003).
2- Seeds are generally dry. Consequently, any STEC strains present are stressed *a priori*. Nevertheless, germination is a favourable step for the revival of the bacteria because it takes place in conditions characterised by moisture, heat and potential release of nutrients.
3- Any bacteria present can be found both on the surface as well as inside the seeds. In the latter case, contamination of the seeds occurs during plant growth and seed formation (primary contamination of crops). The surface contamination of seeds, in turn, may occur during all stages of preparation, storage and handling of the seeds (secondary contamination).

Enrichment broths of seeds to be sprouted may contain inhibitors of the PCR used for the detection of *E. coli*, which is why internal controls are integrated in the methods used by the NRL (DNA polymerase inhibitors).

Thus, to increase the performance of the general method, for analysing the seeds for sprouting, and as recommended by the **EU reference laboratory for *Escherichia coli*, including Verotoxigenic *E. coli* (VTEC)**, these steps must be followed:

1- The test sample of sprouting seeds must be **50 g** (instead of the 25 g required for other matrices) to increase the sensitivity of the protocol.
2- The sprouting seeds **must be ground** in a sterile container (e.g., a Stomacher bag) with a mortar and pestle or other similar tool (subject to sterilising beforehand) before adding the enrichment broth. Please note that this grinding step is particularly tricky (there is a risk of perforating the stomacher bag, therefore the bags should be tripled or even quadrupled).
3- Add **450 ml of buffered peptone water (BPW)**. Be aware of the risk of perforating the bags; do not use a Stomacher blender; mix manually.
4- Incubate the ground seeds for **24 h at 37°C +/- 1°C** (with or without stirring).
5- Take **5 ml enrichment broth with some ground seed debris**.
6- **Vortex** to loosen any bacteria adhering to the seed debris.
7- **Centrifuge** 1min at 500 x g to sediment the debris.
8- Recover **1ml of the supernatant** and use it for extracting the DNA.
9- **The DNA prepared in this way will be diluted to 1:10 before use**. If internal controls do not amplify, dilute the DNA to 1:30.

This standardised method was implemented by the French NRL. It should be noted that the required test sample is 50g (Loukiadis, personal communication).

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[2] Laboratory procedure for detection and identification of Verocytotoxin-producing *Escherichia coli* (VTEC) O104:H4 in food by Real-Time PCR. V2- EU reference laboratory for E. coli. Department of veterinary Public Health and Food Safety; Unit of Foodborne Zoonoses *Istituto Superiore di Sanita*. June 2011. Downloadable from the site: http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3

[3] *Détection des Escherichia coli producteurs de shigatoxines (STEC) O104:H4 épidémiques dans les aliments (y compris végétaux) par PCR en temps réel, version 2/2, LNR français STEC/LMAP* [Detection of Shiga toxin-producing *Eschericia coli* (STEC) 0104:H4 outbreak in food (including vegetables) by real-time PCR, Version 2/2 French NRL STEC/LMAP (Laboratory for studies of pathogenic food microorganisms). Veterinary campus of Lyon. Vet-AgroSup June 2011. Downloadable from the site: http://www.vetagro-sup.fr/services/espace-entreprises/%C3%A9quipements-scientifiques/plateaux-techniques/lmap

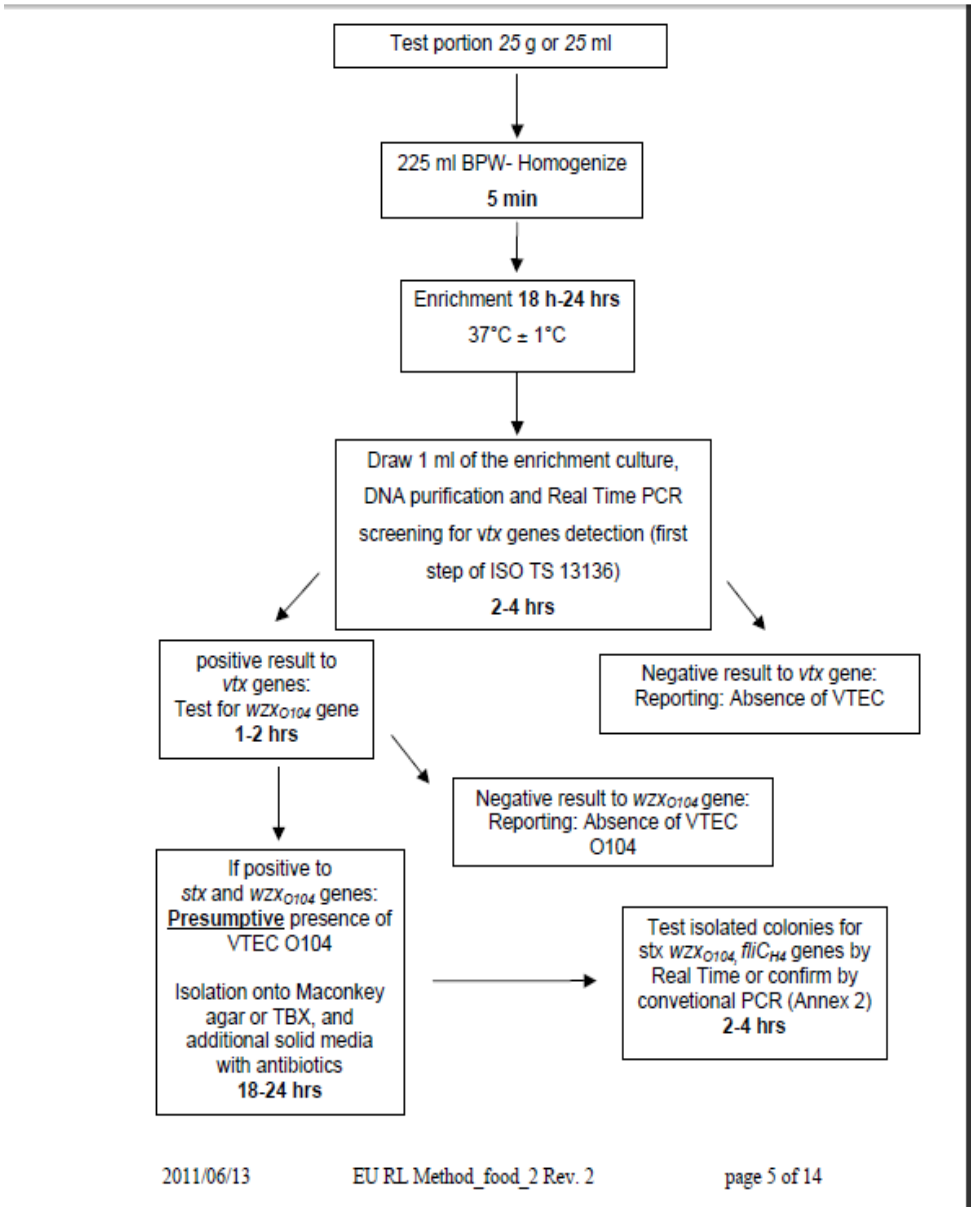

Figure 1: Analysis protocol for the detection and identification of STEC O104:H4 in foods by real-time PCR (source: EU reference laboratory for *Escherichia coli*, including Verotoxigenic *E. coli* (VTEC)).

Another unofficial method, involving a seed germination stage, has also been developed by the German NRL (Beutin L, personal communication at the French NRL). Considering that germination causes the multiplication of *E. coli* O104:H4 responsible for the two outbreaks, it is likely that the sprouting process causes relief of stress for the bacteria. The germination process exploits conditions of moisture, temperature and the release of nutrients that may facilitate a resumption of bacterial growth and the detection of *E.coli* O104:H4.

However, the German NRL's method requires a significantly longer time (up to 10 days for onion and garlic seeds) and a minimum test sample of 100g of seeds.

This more optimal method was implemented concurrently and in addition to the official method by the French NRL when the number of samples received for analysis allowed for it (Loukiadis, personal communication).

Other regrowth methods used by plant pathologists to detect plant pathogenic bacteria in seeds could also be assessed subject to the availability of sufficient samples of the fenugreek batch:

Official methods	Numbers	Sub-samples	Volume	Extraction of target bacteria	Methods
Large-seed legumes (beans-peas-soy)	Minimum 5000 seeds	5 X 1000	Sterile demineralised water volume = 2.5 X weight in sub-samples of 1000 seeds e.g.: 1L water for 200g of seeds	Maceration at 4°C 18-24h while stirring	Isolation in semi-specific media[4][5][6]
Cruciferae (cabbage)	Minimum 30000 seeds	3 X 10000	100 mL saline solution with tween for 10000 seeds	Maceration while stirring 2h to 2h30 at room temperature	Isolation in semi-specific media[7]

Sprouting causes significant proliferation of the bacterial flora associated with seed (Darrasse, Darsonval *et al.* 2010). Depending on the prevailing temperature conditions during germination, the relative proportions of different members in the community may vary and thus cause problems during qPCR-based detection tests, and the target DNA/ non-target DNA ratio can prove quite unsatisfactory.
The problem with great proliferation of bacterial (or fungal) flora associated with seeds also poses problems with microbiological methods such as isolation on media or BIO-PCR (enrichment on solid media followed by PCR).
Enrichment in liquid media has been tested on some plant matrices but has never been validated for routine use with seeds.

It is essential to have a standard European method in order to at least be able to compare the results. The supply of sufficient samples of the suspect batch of fenugreek (there are not enough of them available in France to date for systematically testing several regrowth methods) by a number of European laboratories could allow a standard comparison of several methods.

- Samples taken and tests conducted

Sampling of seeds was carried out initially at the recreation centre and the garden store, and then supplemented by samples from another store (see table below). With reference to the Germans' observations (isolation of O104: H4 in the rinse water of a packet that had contained sprouts and retrieved from the dustbin of a patient), the NRL has also conducted research on pathogens in rinse water and on seed packets.

In terms of the representativeness of the samples, the number of samples taken at the recreation centre (opened packets) and sent to the NRL was insufficient (less than 50g); there was no remaining open packet of organic fenugreek sprouts at the recreation centre.

[4] Bean seeds, detection of *Pseudomonas savastanoi pv. phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli* detection by isolation in nutrient media. Method **BHs/99/02** b.

[5] Pea seeds, detection of *Pseudomonas syringae pv. pisi* by isolation in nutrient media. Method **BHs/99/03** b.

[6] Soy seeds, detection of *Pseudomonas syringae pv glycinae* by isolation in nutrient media. Method **BHs/99/04** b.

[7] Seeds of *cruciferae*, detection of *Xanthomonas campestris* pv. *campestris* by isolation in nutrient media. Method **BHs/99/05** b.

As of 5 July 2011, no positive results had been obtained on the first products sampled (seeds and packaging) by the official method and the German method.

Product	**Sampling sites**	**Quantities analysed**	**Results**
Red cabbage sprout seeds	Recreation centre	1 unopened packet (9.3 g)	Negative (official method) in the sample analysed (9.3 g)
Adzuki sprout	Recreation centre	1 opened packet (21.4 g)	Negative (official method) in the sample analysed (21.4 g)
Onion sprout seeds	Recreation centre	1 opened packet (30.3 g)	Negative (official method) in the sample analysed (30.3 g)
Rocket sprout seeds	Recreation centre	1 opened packet (39.7 g)	Negative (official method) in the sample analysed (39.7 g)
	Another Jardiland garden store (same batch as that of the recreation centre)	2 packets of 50 g each	1/2 Negative (TS ISO 13136 official method) in the samples analysed (50 g) 1/2 Negative (German NRL method) in the samples analysed (seeds and sprouting media)
	Jardiland (a different batch from that of the recreation centre)	5 packets of 50 g each	3/5 Negative (TS ISO 13136 official method) in the samples analysed (50 g) 2/5 Negative (German NRL method) in the samples analysed (seeds and sprouting media)
Organic fenugreek sprout seeds	Jardiland (place of purchase)	6 packets of 50 g each	5/6 Negative (official method) in the samples analysed (50 g) 1/6 Negative (German NRL method). (seeds and sprouting media)
	Another Jardiland (a different batch from the first)	6 packets (1 batch packets of 50 g each)	3/6 Negative (TS ISO 13136 official method) in the samples analysed (3x50 g) 3/6 Negative (German NRL method) in the samples analysed (seeds and sprouting media)
White mustard seeds for sprouting	Another Jardiland	6 packets (2 batches, 3 packets of 50 g each)	2/6 Negative (TS ISO 13136 official method) in the samples analysed (3x50 g) 4/6 Negative (German NRL method) in the samples analysed (seeds and sprouting media)
Water	Recreation centre	3 L	3/3 Negative (TS ISO 13136 official method) in the samples analysed (3 L)
Gaspacho	Recreation centre (unopened factory-sealed block)	4 L	4/4 Negative (TS ISO 13136 official method) in the samples analysed (4 X 25 mL)

As of 5 July 2011, the bacterium *E. coli* O104:H4 could not be found in any sprouted seeds or seeds for sprouting, that were analysed in Germany (several hundred analyses were performed) or in France. Analyses are still continuing and will continue subject to the availability of fenugreek samples from the potentially incriminated batch. To date, the bacterium was recovered in Germany not only in the packet that contained the sprouts, taken from the dustbin of a patient, but also in various foods found in the patients' refrigerators, suggesting contamination by the patients themselves.

- Production processes of sprouted seeds and seeds for sprouting

Since a very small amount of pathogenic STECs can cause human infection, any survival or growth in the environmental medium can have important consequences in terms of public health. The consumption of raw vegetables has already been widely described as one mode of human

contamination by STECs (NACMCF 1999). Alfalfa sprouts and even white radish sprouts have been implicated in particular (Taormina, Beuchat *et al.* 1999; Breuer, Benkel *et al.* 2001).

Sprouts have been identified as a particular problem because of the growth potential of pathogens during the germination process. If pathogens are present on or in the seeds, sprouting conditions may favour their proliferation. Therefore, it is essential that this hazard be taken into account at each stage of the food chain, from the producer of the seeds for sprouting and/or sprouts to the distributor that sells to the consumer in order to reduce the risk of human contamination to an acceptable level.

Sprouts can be contaminated during germination by several sources and particularly by low quality irrigation water, by bacteria found in the soil, and by contact with contaminated organic fertiliser. *E. coli* can in fact persist for a very long time in the environment.

Previous studies have shown that *E. coli* O157:H7 can survive in drinking and or bathing water for several weeks, and more specifically, at cold temperatures. In addition, after three months in this environment, these bacteria can develop into viable nonculturable forms. Outbreaks of O157:H7 involving possible contamination by a water source (surface water, ground water or even river water) have been described (AFSSA 2003).
In addition, *E. coli* can persist up to several months on or in soil, depending on their nature[8], as well as in organic effluents (manure, liquid manure, sewage sludge, wastewater, etc.) (Maule 2000; AFSSA 2003; Islam, Doyle *et al.* 2004; Loukiadis, Kerouredan *et al.* 2006).

Seeds are usually contaminated in their seed coats and/or on the surface of the embryo. The potential difficulty of detaching bacteria from the surface of the seeds should be emphasised, once these have been contaminated, particularly because a biofilm may develop on them. Biofilm rods and *cocci* have indeed been found on alfalfa seeds (Matos, Garland *et al.* 2002), and are also common on bean seeds contaminated by plant pathogens. The possible presence of *E. coli* in these biofilms that potentially protect the bacteria from environmental stress has not yet been documented. The possibility of contamination of deep plant tissue is suspected. However, this has only been demonstrated experimentally (Solomon, Yaron et al. 2002; FAO/WHO 2008).

There are various routes of seed contamination (floral route, vascular route, contact with fruit tissue, with contaminated debris during harvesting and threshing, etc.). Contamination during harvesting or hulling is possible as well as contamination during packaging.

Cleaning/disinfection measures of seeds prior to germination will have the dual effect of reducing the overall burden of contamination and lower the risk of cross-contamination between seeds or debris and dust from seeds and pods.

However, after this washing stage, a minute residual amount of *E. coli* O157:H7 on seeds can result in spreading during transport and storage, and strong contamination of the final product, posing a risk to human health (Barak,Whitehand *et al.* 2002). Consequently, the US Food and Drug Administration (FDA) specifically recommended in 1999, that sprout producers decontaminate seeds before germination and screen for *Salmonella* and *E. coli* O157:H7 in irrigation water after 48 hours of germination (Anonymous 1999).

Concerning the hygiene of industrial work surfaces, the data in the literature indicate that *E. coli* O157 is sensitive and does not persist on treated surfaces. The adhesion of EAEC and their survival on inert surfaces has not yet been documented.

In 1997, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) published a review of knowledge about epidemics related to the consumption of sprouts and

[8] University thesis of B. Frémaux, viva on 14 décembre 2007, Université Claude bernard Lyon 1 « *Ecologie des* Escherichia coli *producteurs de shiga-toxines (STEC) dans les effluents d'élevage bovins et le sol* » [Ecology of shiga toxin-producing Escherichia coli (STEC) in manure from cattle and ground]

identified the primary pathogens to be considered and the production practices posing the greatest risk (NACMCF 1999). In its conclusions, the committee recommended supplementing the training of all stakeholders with respect to the microbiological safety of sprouts. The committee also emphasised the systematic implementation of good farming practices to reduce the risk of microbial contamination of seeds for sprouting as well as seed cleaning, storing and handling practices that minimise the risk of contamination.

A guide for good hygienic practices and HACCP principles "*Végétaux crus prêts à l'emploi*" [Raw vegetables ready for use], written by French industry professionals and undergoing expert assessment at ANSES, should cover all the critical points previously outlined and listed in the reference documents.

- **Feedback from the outbreak – discussions of hypothetical contamination scenarios**

The source of the epidemic strain is not known at this time. However, the characteristics of this strain suggest that it resulted from the horizontal transfer of a phage carrying the *stx2* gene, encoding for the Stx2 toxin, from a donor bacterium (probably an EHEC or a STEC) to a recipient bacterium belonging to the EAEC pathovar, which has thus become more pathogenic (Brzuszkiewicz, Thurmer *et al*. 2011).
The acquisition of this phage by this EAEC may have occurred under different conditions: probably in a human or animal digestive tract, but also in the outside environment (sewage, wastewater, manure, liquid manure, etc.). It is not possibly at the current time to confirm or refute these different hypotheses even if this event is more likely to occur in an environment where bacteria can be in a physiological state conducive to cell activity (in the presence of nutrients, moisture and heat) and facilitating genetic exchange. Conversely, the possibility of horizontal transfers appears to be unlikely in the sprouts themselves.

More generally, it is reasonable to consider the emergence of a particularly virulent clone, similar to the development of EHEC in the 1980s. Indeed, to date, it is not possible to know whether the outbreak is an epiphenomenon or if a particularly virulent new pathotype has emerged.
Therefore, it appears to be necessary to verify whether a new reservoir (human, animal, environmental or food) is emerging. To this end, two avenues could be pursued:
- Study the encounter between the virulence genes of the strain and these enteroaggregative factors: particularly via PCR screening of different genetic markers;
- Learn more about the genesis of *E. Coli* pathovars, in order to develop tools to detect them.

To date, irrespective of the genesis of this strain, several hypotheses can be advanced to explain the occurrence of this French outbreak in relation to the previous German outbreak.
The sprouts may have become contaminated at different stages:
- during production of seeds for sprouting;
- during packaging/repackaging;
- during germination
- when harvesting the sprouts and/or preparing them for consumption.

During the German outbreak, several contamination hypotheses were issued initially: contamination of sprouts occurred via a healthy carrier and or via water used, on the German farm located in Basse – Saxe; or else there was pre-existing contamination on imported seeds for sprouting (contamination of raw materials).
Given the geographic location of exposure, confined to the north of Germany, the hypothesis of local contamination at the farm might be more likely, particularly since the peak of the outbreak was at a distinct point in time.

Since the onset of the Bordeaux episode, involving this same rare strain over a very short period of time, the hypothesis of contamination of raw materials appears to be more likely. However, the lack of reported cases in France before the outbreak in Bordeaux, between the date of distribution of the suspect batch on French territory (200 retail sites) and now makes this hypothesis less likely.

The existence of a potential human factor (a person exposed in Germany, a healthy carrier who would have contaminated sprouts at the CLPE in Bègles, before, during and/or after sprouting) was a possible, albeit improbable hypothesis, which was ruled out by the epidemiological investigation.

Moreover, the occurrence in 2011 of outbreaks in connection with batches of seeds for sprouting that had been harvested over a year before, assumes that these pathogenic bacteria can survive for a very long time, in a dry environment. There is little published work describing the survival of *E. coli* on seeds. For example, the population of *Escherichia coli* O157:H7*,* artificially inoculated in alfalfa seeds intended for sprout production, was divided by a factor of about 100 after six months at 25°C for a water activity of 0.15. There was greater decline of bacteria at higher temperatures or with more intense water activities (Beuchat and Scouten 2002). The presence of *E. coli* in seeds stored for over a year remains hypothetical and would likely be influenced by the initial contamination level. However, the survival abilities of the O104:H4 strain considered under seed storage conditions are not known at this time. Other pathogenic enterobacteria such as *Salmonella* can survive for a year on alfalfa seeds with a viability loss of only a factor of 10 (Beuchat and Scouten 2002).

It should be noted that contamination via the package can be ruled out, since the packaging material has been analysed by the French NRL.

Given the current state of scientific knowledge available, the most likely route of introduction of *E. coli* O104:H4 in the food chain seems to be contamination of organic fenugreek seeds for sprouting during their production or packaging in Egypt. However, investigations would be needed to refine the various possible scenarios of seed contamination before sprouting and of the sprouts themselves:

- The hypothesis of contamination in Egypt, during production:

The epidemic strain has a genetic base in EAEC, which does, *a priori,* circulate in this region of the world. An estimate of the frequency of asymptomatic carriage of these serotype O104:H4 EAECs in Egypt would be very useful.
This contamination scenario assumes the survival of pathogenic bacteria on a low a_w (water activity) matrix for an extended period of time. This survival may be facilitated by a protective biofilm on the surface of the seeds. The persistence could also be much longer if the initial contamination burden were high.
The first results of the European tracing study have not yet explained the lack of another outbreak in Europe.
Additional information from the Egyptian authorities, to assist in evaluating the manufacturing conditions of batches of seeds for sprouting Egypt, would be helpful.
Sampling and analyses carried out during the production stage (irrigation water, finished products, healthy carriers, soil, organic manure), at the operators identified in the European tracing study, for the purpose of bacteriological analyses, could contribute valuable information. However, it would occur more than 20 months after importation of the batch in Europe. The collaboration of European scientists, who could help local operators and managers analyse the situation as well as with sampling and testing, could be offered.

Increased controls of each batch are recommended, to screen for *E. coli* O104:H4, when seeds are imported from countries where the incidence of EAEC is assumed or known to be high, until the situation has been fully understood.

- The hypothesis of contamination in Germany, during repackaging:

This hypothesis is less likely than the previous one but possible unpacking/repackaging by the wholesale importer should be investigated to assess the risks of cross-contamination during these operations.

- The hypothesis of contamination of seeds during germination

or of their preparation at the CLPE in Bègles by an asymptomatic carrier who had travelled in northern Germany:

To date, this scenario is the least likely. Investigations conducted among staff who handled the seeds found no evidence to support this hypothesis.

- **Recommendations and areas for further research.**
  - Concerning the strain implicated

It would be useful to:

- generate data on its ability to survive in seeds, and its adhesion on surfaces (depending on the environment and the nature of the material to be considered). In particular, investigate the possibility that there are biofilms on the surface of the seeds and their role in the survival of *E. coli*;
- generate data on the impact of cleaning/disinfection processes on this specific strain, especially on sprouts or seeds for sprouting, taking into consideration the constraints for organic farm products;
- standardise detection methods in Europe and draw the users' attention to the importance of having accurate information on the primers and probes used in commercial detection kits to ensure their compliance with EU-RL recommendations;
- Investigate methods of soaking seeds prior to the microbiological analysis itself, used by plant pathologists for regrowing pathogenic bacteria;
- generate data on the virulence of antimicrobial-resistant *E. coli* (of the type ESBL) of human, animal, food or environmental origin.
- generate data on the antimicrobial resistance of strains carrying virulence genes of human, animal, food or environmental origin;
- obtain data on the prevalence of these enteroaggregative Shiga toxin-producing strains, in various reservoirs and countries;
- conduct studies in order to characterise the forms and mechanisms of infection of an EAEC strain by an *stx* phage.

  - Concerning the investigation of the Bordeaux episode

It would be useful to:

- have results from analyses of the environment in which the seeds were produced;
- obtain information on the conditions of fertilisation at the seed production sites, on the bagging, hulling, harvesting, transporting (import/export, types and sizes of bags), storage (rodents present?) and repackaging conditions;
- have tests performed on samples, taken in sufficient quantity (see the "methods" section), from suspect batches.

  - Concerning the specificity of human populations affected during these outbreaks

It would be useful to:

- get explanations or hypotheses for explaining the specificity of the affected population (largely adult females): genetic factors, variable dietary exposure (exploitation/acquisition of data).

  - Concerning sprouting practices under industrial conditions

It would be useful to:

- assess the impact of germination processes implemented, and especially the effect of temperature, on the survival and proliferation of pathogenic STECs, during germination under industrial conditions;
- assess the effectiveness of cleaning and disinfection measures applied to surfaces and raw materials, with respect to these enteroaggregative and/or Shiga toxin-producing strains, with regard to the items outlined in this Opinion;
- Develop methods for detecting pathogenic STECs found in seeds, or during the germination process to optimise batch monitoring.

- Concerning the consumption of sprouts

In view of the conclusions of the traceability survey coordinated by EFSA and the results of the epidemiological investigation, it is important at this stage to advise consumers not to grow sprouts for their own consumption. Nor should they eat seeds or sprouts without cooking them at high temperatures (70°C, 2 min.) beforehand. Indeed, seeds sold to be eaten as sprouts are often packaged as seed mixtures and cross-contamination cannot be excluded at this time. This recommendation may be reviewed at the end of the outbreak (i.e., approximately 30 days after the onset of the last case), on the basis of the knowledge acquired.

Personal and collective hygiene is still the basis of prevention. In particular, it must include an emphasis on scrupulous hand washing after using toilets, but also before preparing and eating meals. These general hygiene recommendations are essential for avoiding so-called 'secondary' cases, resulting from direct or indirect contact between patients and their families. Faecal excretion of these pathogenic bacteria by sick people can continue after the cessation of symptoms. Detailed recommendations for preventing the transmission of infection with Shiga toxin-producing E. coli are available on website[9] of the French Institute for Public Health Surveillance and that of the European Centre for Disease Prevention and Control (ECDC)[10].

It would also be advisable, in the longer term, to examine health risks related to the consumption of sprouts when sprouting is done by individuals, particularly by assessing the growth rates of pathogenic *E. coli* potentially present on/in the seeds to be sprouted, depending on the different procedures specified on the seed packets or more generally under reasonably foreseeable conditions.

It would also be advisable to draw the consumer's attention to the importance of adopting hygiene measures when sprouting seeds at home (thorough cleaning/disinfection of seed germinators in particular, and careful hand washing before and after handling seeds and sprouts).

5. THE AGENCY'S CONCLUSION AND RECOMMENDATIONS

These are the points of analysis that the French Agency for Food, Environmental and Occupational Health & Safety is able to provide, following the onset of several cases of haemolytic and uraemic syndrome (HUS) observed in France (in the Bordeaux region) in June 2011, suspected of being related to the consumption of sprouts.

[9] http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Risques-infectieux-d-origine-alimentaire/Syndrome-hemolytique-et-uremique/Actualites/Cas-groupes-de-syndrome-hemolytique-et-uremique-SHU-Nord-juin-2011-Point-au-5-juillet-2011, consulted on 6 July 2011.

[10] http://ecdc.europa.eu/en/healthtopics/escherichia_coli/prevention_measures/Pages/prevention_measures.aspx , consulted on 6 July 2011.

The Director General

Marc MORTUREUX

KEY WORDS

Key words:
Pathogenic E. coli, Shigatoxins, haemolytic and uraemic syndrome (HUS), seeds for sprouting, sprouts, seeds.

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