Surveillance of **shigatoxin-producing *E. coli*** (VTEC) in refrigerated fresh minced beef on the French market in 2015

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Abstract

Shigatoxin-producing *Escherichia coli* (VTEC) are considered as major pathogens causing severe and sometimes lethal infections in humans. Although more than 200 serotypes have been reported, only seven of them have been consistently associated with severe cases. Transmission of VTEC to humans occurs mainly through consumption of undercooked minced beef contaminated by animal faeces. Although there are no statutory criteria, meat containing one of these strains is considered as harmful to health. Thus, the surveillance plan conducted in 2015 aimed to assess, for fresh minced beef on the French market, the rate of contamination by VTEC identified as a higher risk in order to assess consumer exposure.

The results obtained confirm that the contamination rate for meat was low (0.3%; 95CI [0.01-1.9]) and similar to those obtained previously, suggesting that the risk of human exposure *via* the consumption of minced beef in France remains limited. The only strain isolated was an O103:H2 VTEC strain showing genetic markers of greater virulence.

The Directorate General for Food will continue to monitor VTEC contamination in beef collected on the market in 2016.

Keywords

VTEC, EHEC, Surveillance, Minced beef, 2015, France

Résumé

Surveillance des* E. coli *producteurs de shigatoxines (VTEC) dans les viandes hachées de bœuf réfrigérées mises sur le marché en 2015.

Les Escherichia coli *producteurs de shigatoxines (VTEC) sont des agents pathogènes majeurs, responsables d'affections parfois mortelles. Bien que plus de 200 sérotypes aient été rapportés, seuls sept sont responsables de la majorité des épidémies et affections sévères recensées. La viande hachée de bœuf contaminée par le contenu digestif des animaux porteurs et insuffisamment cuite reste une des principales sources de contamination de l'Homme. Bien qu'il n'existe aucun critère réglementaire, une viande contenant une de ces souches est considérée comme « dangereuse ». Aussi, le plan de surveillance 2015 visait à établir le taux de contamination des viandes hachées de bœuf réfrigérées mises sur le marché en France par les souches VTEC identifiées comme les plus à risque et, par conséquent à apprécier l'exposition du consommateur à ce danger.*

Les résultats obtenus confirment que le taux de contamination des viandes hachées de bœuf réfrigérées est faible (0,3% ; IC95 [0,01-1,9]) et du même ordre de grandeur que ceux obtenus précédemment, ce qui suggère que le risque d'exposition de l'Homme via la consommation de viande hachée de bœuf en France reste limité. L'unique souche isolée est une souche VTEC O103:H2 possédant des marqueurs génétiques de virulence accrue.

En 2016, la direction générale de l'Alimentation poursuivra la surveillance de la contamination des viandes hachées de bœuf par ces agents pathogènes au stade de la distribution.

Mots-clés

VTEC, EHEC, surveillance, viandes hachées de bœuf, 2015, France

Pathogenic shigatoxin-producing *Escherichia coli* (VTEC) are considered a major public health concern in several regions of the world due to the extreme severity of the symptoms they cause (AFSSA, 2003). Indeed, pathogenic VTEC are responsible for sporadic cases and outbreaks of haemorrhagic colitis as well as rare life-threatening infections affecting children in particular, such as haemolytic uremic syndrome (HUS). HUS is the main cause of acute renal failure in children under three years of age. The mortality rate varies from 3% to 5% and more than a third of patients suffer long-term kidney damage (AFSSA, 2003).

Although more than 200 serotypes of pathogenic VTEC strains have been involved in human infections, only a few have been consistently associated with severe outbreaks and infections. In France, VTEC strains belonging to one of the five O26:H11, O103:H2, O111:H8, O145:H28 and O157:H7 serotypes are associated with 70% to 80% of reported cases and have been defined as highly pathogenic (AFSSA, 2010; Brugère *et al.*, 2012). In the United States, VTEC strains of the same serotypes as well as the VTEC O45 and O121 strains are considered as presenting the greatest risk.

The natural reservoir for pathogenic VTEC is the digestive tract of ruminants. The consumption of raw or undercooked contaminated minced beef has been identified as one of the main routes of contamination during investigations of HUS cases identifying a responsible food (AFSSA, 2003).

In accordance with Directive 2003/99/EC, European Union Member States are required to set up a surveillance system for zoonoses and zoonotic agents. VTEC are included on the list of agents to be monitored, featured in Annex I (A) of this directive. In addition to the control pressure exerted on production sectors, the implementation of surveillance plans for VTEC contamination in at-risk matrices (mainly minced beef and raw-milk cheeses) is one of the actions taken for the protection of public health. These plans provide estimates of food contamination levels in various stages of the food chain. The data obtained also make it possible to make assumptions about the risk factors. Mitigation measures can be then established. Surveillance plan results are communicated to risk assessment agencies: i) ANSES in France, and ii) the European Food Safety Authority (EFSA) in Europe, for summarising with the data of other Member States.

There are currently no statutory microbiological criteria for VTEC in minced beef. However, the French authorities consider a beef burger containing a highly pathogenic VTEC strain to be "unsafe" as defined in Article 14 of Regulation (EC) No 178/2002, since it is injurious to health, given the severity of the related infections and the French habit of consuming this food undercooked[1].

The aim of the VTEC surveillance plan conducted in 2015 was to collect data for assessing the rate of contamination in refrigerated minced beef on the French market and therefore to evaluate the consumer exposure to this hazard.

Materials and methods

Sampling protocol

The surveillance plan should include 306 samples from different one-unit (n=1) batches of refrigerated minced beef in the distribution stage. All sample were taken in retail outlets such as supermarkets, hypermarkets and discount stores, which account for 95% of purchases of butcher's meat in France.

These 306 samples were planned in mainland France, in proportion to the number of inhabitants per region (Figure 1), in order to be as representative as possible of consumer exposure. The samples should be spread out throughout the year 2015.

Each sample should correspond to at least 100g of minced beef, prepacked in its original packaging (shrink-wrapped, vacuum-packed or packaged in a protective atmosphere) and labelled. The expiration date should be valid until the date of the analysis.

Nature of the tested contaminants

The following pathogenic bacteria were detected:

- VTEC strains considered in France as highly pathogenic to humans (AFSSA, 2010), i.e. strains owning the *stx* (*stx1* and/or *stx2*) and *eae* virulence genes and belonging to one of the five O157:H7, O26:H11, O145:H28, O103:H2 and O111:H8 serotypes,
- VTEC strains considered as pathogenic (AFSSA, 2010), harbouring the *stx* (*stx1* and/or *stx2*) and *eae* virulence genes, and belonging either to the O45 serogroup or to the O121 serogroup, targeted by the American regulations.

Analytical method used

In order to take into account potential differences in the contamination of minced beef, 100g of meat were collected in various places in minced beef in order to establish a sample. After homogenisation, the test portion per sample was 25g.

The detection of target VTECs was realised in accordance with the official authorised methods[2], adapted from the ISO TS 13136 method[3], recommended by EFSA (EFSA, 2009), and the official American method MLG 5B(4)(4) (Figure 2):

- a first step of enrichment of the investigated food allows the potential pathogenic strains to multiply and reach detectable levels,
- a second real-time PCR detection step uses nucleic acids extracted from this polymicrobial enrichment broth. The main markers of the target VTEC strains are detected: *stx* genes (Perelle *et al.*, 2004), *eae* genes (Nielsen *et al.*, 2003), and genes associated with the seven serogroups of interest (Perelle *et al.*, 2004 and MLG 5B method),
- a third bacterial isolation step implemented only if the results obtained previously are positive, i.e. if the *stx* gene AND *eae*

Box.

Objective
This surveillance plan aimed to assess contamination by VTEC strains in refrigerated minced beef on the French market and therefore evaluate consumer exposure.

Programming framework
- Directive 2003/99/EC.
- EFSA Opinion of 30 October 2009.
- AFSSA Opinion of 27 May 2010.

Protocol
- **Target bacteria**
  - VTEC strains highly pathogenic to humans. These are strains owning the stx and eae virulence genes and belonging to one of the five O157:H7, O26:H11, O145:H28, O103:H2 and O111:H8 serotypes.
  - Pathogenic VTEC strains, i.e. strains harbouring the *stx* and *eae* virulence genes and belonging to the O45 or O121 serogroup.
- **Affected products:** viandes de bœuf hachées réfrigérées (toutes origines).
- **Stage of the food chain:** distribution.
- **Definition of a "case':**
  Non-compliance if isolation of one of the targeted strains.
- **Number of samples and sampling method**
  Three-hundred and six samples were taken in mainland France between February and December 2015, broken down by region in proportion to the number of inhabitants.
  Each sample was collected in its original packaging in the refrigerated self-service section of supermarkets and hypermarkets.
- **Sampling strategy:** random.
- **Analytical method, nature of sampling**
  The test portion (25g) was analysed in accordance with the official methods adapted from Technical Specification ISO 13136: 2012.

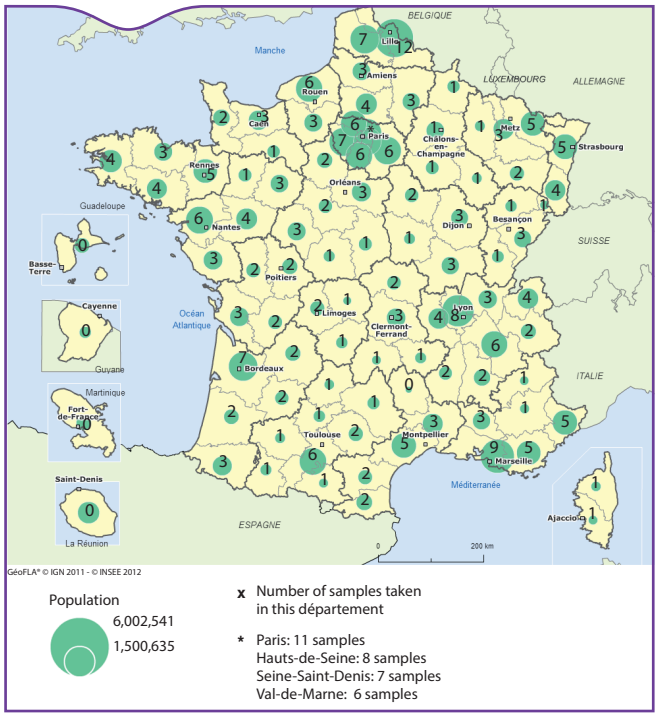


Figure 1. Departmental breakdown of the number of samples planned by number of inhabitants (http://www.statistiques-locales.insee.fr and 2012 INSEE data)

1. http://agriculture.gouv.fr/sites/minagri/files/documents/pdf/_Guide_Gestion_Alerte_Revision_2_jlt_2009_COMPLETEE_VDef__cle09fc34.pdf.
2. The official authorised methods are listed in Guidance Note DGAL/ SDSSA/ SDPRAT/N2013-8179 and are available online at the following address: http://agriculture.gouv.fr/laboratoires-agrees-methodes-officielles-alimentation-568.
3. Technical Specification ISO TS 13136:2012 "Microbiology of food and animal feed – Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens – Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (VTEC) and the determination of O157, O111, O26, O103 and O145 serogroups".
4. Official American method MLG 5B.05 "Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (VTEC) from Meat Products and Carcass and Environmental Sponges" available at the following address: http://www.fsis.usda.gov/wps/wcm/connect/7ffc02b5-3d33-4a79-b50c-81f208893204/MLG-5B.pdf?MOD=AJPERES.

gene AND one of the specific genes of the targeted serogroups are detected concomitantly in the enrichment broth. This specific isolation step for bacteria belonging to the serogroup detected from the enrichment broth uses both immunomagnetic separation (IMS) techniques and direct isolation,

- a fourth step for the phenotypic (API20E) and genotypic characterisation of the *E. coli* strains isolated in the previous stage. More specifically, somatic and flagellar antigens are analysed by PCR to confirm the serotype of the *E. coli* strains isolated (Perelle *et al.*, 2004; Auvray *et al.*, 2008; Madic *et al.*, 2010). The *stx1, stx2*

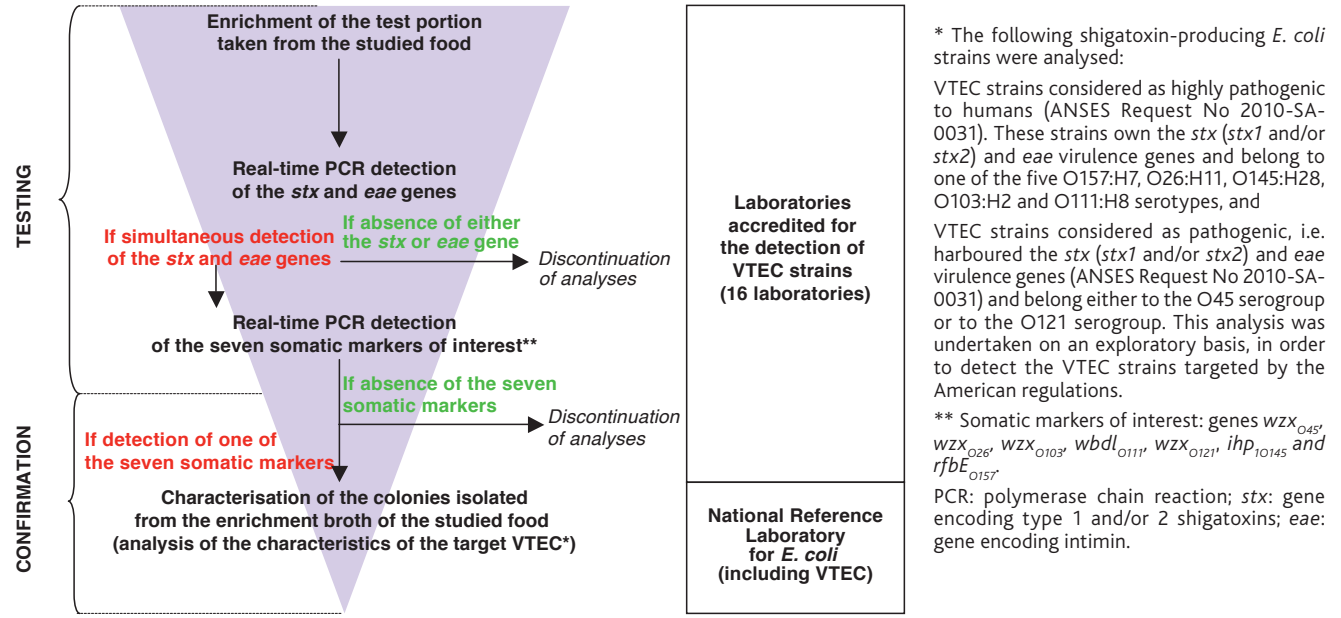
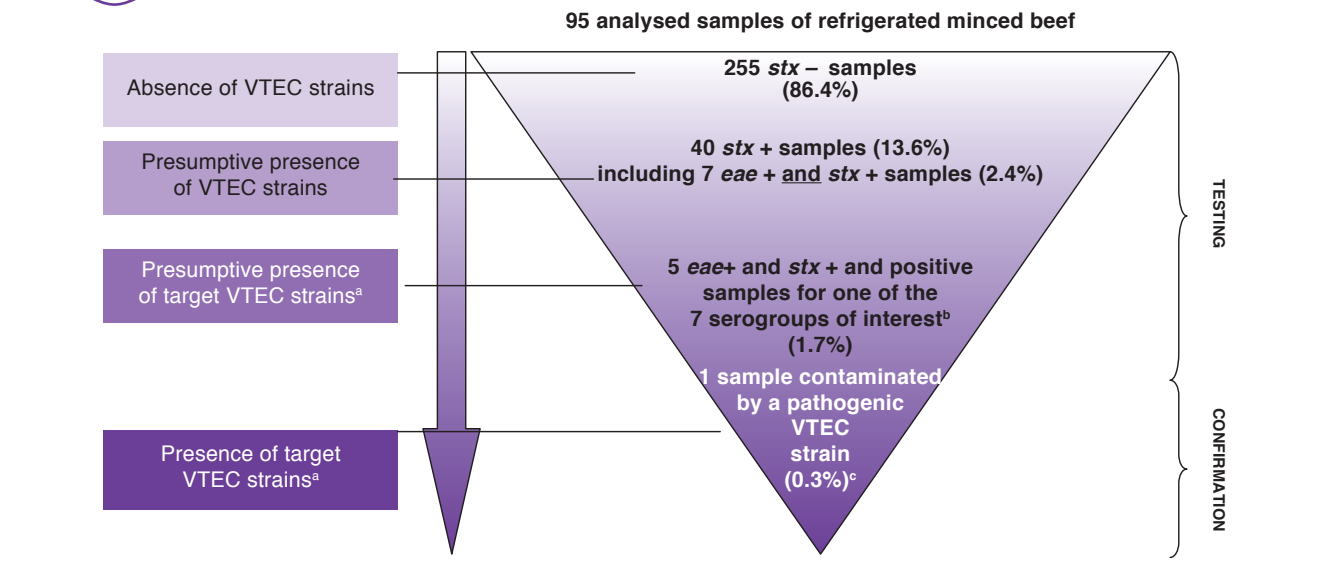


Figure 2. Diagram of the main stages of the method used for the analysis of VTEC strains, and stakeholders responsible for its implementation as part of the surveillance plan conducted in 2015 (adapted from Loukiadis *et al.*, 2012)



a. The following shigatoxin-producing *E. coli* strains were analysed:
(1) VTEC strains considered as highly pathogenic to humans (ANSES Request No 2010-SA-0031). Such strains own the *stx* (*stx1* and/or *stx2*) and *eae* virulence genes and belong to one of the five O157:H7, O26:H11, O145:H28, O103:H2 and O111:H8 serotypes, and
(2) VTEC strains considered as pathogenic, i.e. owning the *stx* (*stx1* and/or *stx2*) and *eae* virulence genes (ANSES Request No 2010-SA0031) and belonging either to the O45 serogroup or to the O121 serogroup. This analysis was undertaken on an exploratory basis, in order to detect the VTEC strains targeted by the American regulations.

b. Analysed somatic markers of interest: genes *wzxO45, wzxO26, wzxO103, wbdlO111, wzxO121, ihp1O145* and *rfbEO157.*

c. The isolated highly pathogenic VTEC strain was an O103:H2 strain having all the phenotypic and genotypic characteristics of the major typical EHEC strains (ANSES Request No 2010-SA-0031) (combination of O103:H2, *eae* e and presence of the gene). This strain also has the *ehx* gene (encoding enterohaemolysin) and the entire OI-122 island.

+: gene detected by PCR; -: gene not detected by PCR.

eae: gene encoding intimin; OI: O island; PCR: polymerase chain reaction; *stx*: gene encoding type 1 and/or 2 shigatoxins.

Figure 3. Summary of the results obtained from the surveillance plan for contamination by VTEC strains in refrigerated minced beef on the French market in 2015

and *eae* virulence factors in the collected isolates were analysed by PCR (Perelle *et al.*, 2004; Nielsen *et al.*, 2003). Genotypic characterisations in addition to those proposed by Technical Specification ISO/WD TS 13136:2012 were also undertaken: PCR analysis of the *ehx* gene (Tzschoppe *et al.*, 2012), *eae* gene variants (Nielsen *et al.*, 2003), *stx* gene variants (Scheutz *et al.*, 2012) and the presence of OI-122 (Karmali *et al.*, 2003).

The detection of the *stx* and *eae* genes and markers associated with any of the seven analysed serogroups was performed by the network of laboratories accredited for undertaking official analyses for the detection of VTEC, spread out across France[5][5]. Additional and confirmatory analyses were undertaken by the National Reference Laboratory (NRL) for *E. coli* including VTEC[6] (Figure 2).

Statistical analyses

In order to take into account uncertainties related to sampling fluctuations, the confidence interval with a 95% probability of containing the actual contamination rate was calculated with the R software (version 3.0.1, R Core Team., 2013) (error risk a set at 5%). The obtained rates were compared using Fisher's test (significance with a p-value £ 0.05) after verification of data normality.

Results

A total of 306 samples were collected: this corresponds to a 100% sampling rate in relation to the initial specification. However, only 295 of these 306 collected samples (96.4%) were analysed since eleven samples did not comply with the plan's instructions. The analysed minced beef was primarily of French origin (97.3%, 99% and 100% of the samples were collected from animals born, raised and slaughtered in France respectively). Such beef mainly intended to be consumed cooked (286/295, i.e. 96.9%) and usually had a fat content of 5% (135/295, i.e. 45%) or 15% (136/295, i.e. 45%).

Figure 3 summarises the sampling results. Of the 295 analysed samples, 290 were found to be negative. More precisely:

- 235 samples (79.7%) showed negative results for both the *eae* and *stx* genes,
- 20 samples (6.8%) had a positive PCR result for the *eae* gene only,
- 33 samples (11.2%) showed a positive PCR result for *stx* (*stx*1 and/or *stx*2) genes only,
- 2 samples (0.7%) had a positive PCR result for both the *stx* and *eae* genes but a negative result for all the seven analysed serogroup markers.

Only five samples (5/295, i.e. 1.7% of the analysed samples) had positive PCR results for the *stx* and *eae* genes and a positive signal for at least one of the seven tested serogroups. Such samples were considered as presumptive positive samples. None of the markers associated with serogroups O157, O111, O45 and O121 were detected. The detected serogroup markers corresponded to serogroups O103 (*wzxO103*, three samples), O145 (*ihp1O145*, two samples) and O26 (*wzxO26*, two samples). Two samples showed a positive signal for two serogroups simultaneously.

Only one presumptive positive sample out of 5 was confirmed as containing a VTEC strain considered as highly pathogenic (1/295, i.e. 0.3% of the analysed samples; 95CI [0.01- 1.9])) (Figure 3). This sample was a beef burger with a fat content of 15% that was intended to be consumed cooked. The meat was of French origin (animals born, raised and slaughtered in France).

The phenotypic and genotypic characteristics of the isolated strains are shown in Table 1. The highly pathogenic VTEC strain isolated belongs to the O103:H2 serotype and has all the phenotypic and genotypic characteristics of the major typical EHEC strains as defined in the AFSSA Opinion of 2010 (Request No 2010-SA-0031) (combination of serotypes, *eae* gene variants and presence of the genes encoding one of the types of shigatoxins). This strain also contains the *ehx* gene (encoding enterohaemolysin) and the entire OI-122 island, suggesting that it may have increased pathogenicity (AFSSA, 2008). Indeed, OI-122 contains genes encoding Nle effectors (non-LEE encoded effectors, whose role in the pathogenicity of strains remains unclear, even though they are not found in non-pathogenic strains). In general, the more complete this island (e.g. presence of one, two, three or four of the analysed OI-122 genes), the more the disease associated with these strains is severe (HUS) (AFSSA, 2008).

Discussion

The data from the VTEC surveillance plans show, irrespective of the surveillance stage and therefore irrespective of the biases inherent in the programme, low and similar contamination rates in minced beef over the past few years. The contamination rate observed in 2015 in refrigerated minced beef on the market was not significantly different from the results of previous surveillance plans (Loukiadis *et al.*, 2012). In fact, during the 2009 and 2010 surveillance plans on VTEC contamination in refrigerated minced beef collected during distribution, 0.1% (95CI [0.0-0.5]) and 0.2% (95CI [0.1-0.5]) of the analysed samples were respectively confirmed as being contaminated by a highly pathogenic VTEC strain. These results underline that consumer exposure to this hazard *via* the consumption of minced beef poses a low risk.

The highly pathogenic VTEC strain isolated in 2015 belongs to the O103:H2 serotype. It is thus potentially capable of causing characteristic attaching and effacing lesions of the intestinal mucosa in humans, responsible for diarrhoea symptoms, and of isproducing shigatoxin type 1, variant a. This toxin, involved in the destruction of the capillary endothelial cells of the colon, kidneys and brain, which can cause haemorrhagic colitis, HUS or even coma (AFSSA, 2003). This serotype of VTEC strains has been isolated in minced beef in previous surveillance plans. However, it is generally less prevalent than O26:H11 and O157:H7 VTEC in such foodstuff (Loukiadis *et al.*, 2012). VTEC O103:H2 strains were responsible for 2% of the 114 HUS cases identified in children under the age of fifteen in France in 2014 and 1.4% of the 698 cases identified over the 2010-2014 period (http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Risques-infectieux-d-origine-alimentaire/Syndrome-

5. A total of sixteen laboratories were accredited for VTEC detection for the implementation of the 2015 plan (the list is available at the following address: http://agriculture.gouv.fr/sites/minagri/files/e._coli_VTEC_dans_le_cadre_des_pspc_-_liste_des_ laboratoires_agrees_v13.pdf).
6. National Reference Laboratory (NRL) for *E. coli* including VTEC – Research laboratory for pathogenic microorganisms in food (LMAP) – VetAgro Sup Veterinary Campus in Lyon (formerly ENV Lyon).

Table 1. Phenotypic and genotypic characteristics of the highly pathogenic VTEC strain isolated in refrigerated minced beef collected in the distribution stage in the framework of the 2015 surveillance plan

Strain	Phenotypic characteristics		Genotypic characteristics*							
	API 20E identification profile	Serotype**	*eae* (variant)	*stx1* (variant)	*stx2* (variant)	*ehx*	OI122			
							papC21	*sen 26*	*efa132*	*efa133*
85-93	5 144 572	O103:H2	+ (ε)	+ (1a)	-	+	+	+	+	+

* determined by PCR (ISO TS 13136:2012 and other references cited in the Materials and methods section).
** determined by PCR (the target genes for the determination of serotypes are given in AFSSA Opinion No 2008-SA-0122 and were analysed by PCR according to ISO TS 13136:2012 for somatic markers and according to Madic et al., 2010 for flagellar markers)

hemolytique-et-uremique) but have never been involved in episodes of clustered food-borne cases in France (Loukiadis *et al*., 2012).

Note that no VTEC O45 or O121 strains tested in beef in the United States have ever been found in France.

In all cases, when VTEC strains are detected, operators must withdraw the product from the market, search for possible sources of contamination, and take suitable control measures to reduce risks of contamination. These mitigation measures apply in accordance with the instructions of the DGAL[7].

All of the obtained results highlight the significance of measures taken upstream by professionals to control this hazard. Health control plans help reduce the risk of marketing contaminated products, at the slaughterhouse by taking into account the cleanliness of animals and controlling certain at-risk stages (oesophageal ligation, bagging the rectum, stripping the hide and evisceration in particular (ANSES, 2014)) and then during processing by complying with good hygiene practices and verifying the effectiveness of mitigation measures through self-inspections at critical points (including the inspection of raw materials in the production stage). Moreover, raising the awareness of consumers as to observance of the cooking instructions indicated on product labels, for minced beef in particular (see "Set of recommended good hygiene practices for consumers"[8]), is also a way to reduce the risk of human contamination downstream.

The obtained results were published in the "summary" note for the French authorities and were communicated to EFSA for publication in its "zoonoses" report (available at the following address: http://www.efsa.europa.eu).

In 2016, the DGAL continued to monitor contamination by VTEC strains in minced beef (refrigerated and frozen) by implementing a surveillance plan in the distribution stage.

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