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Characteristics of the Shiga-toxin-producing enteroaggregative *Escherichia coli* O104:H4 German outbreak strain and of STEC strains isolated in Spain

Azucena Mora,[1] Alexandra Herrera,[1] Cecilia López,[1] Ghizlane Dahbi,[1] Rosalia Mamani,[1] Julia M. Pita,[2] María P. Alonso,[2] José Llovo,[3] María I. Bernárdez,[1,3] Jesús E. Blanco,[1] Miguel Blanco,[1] Jorge Blanco[1]*

[1]*E. coli* Reference Laboratory (LREC), Department of Microbiology and Parasitology, Faculty of Veterinary Science, University of Santiago de Compostela (USC), Lugo, Spain. [2]Unit of Microbiology, Lucus Augusti Hospital, Lugo, Spain. [3]Service of Microbiology, University Hospital Complex of Santiago de Compostela (CHUS), Santiago de Compostela, Spain

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Summary. A Shiga-toxin-producing *Escherichia coli* (STEC) strain belonging to serotype O104:H4, phylogenetic group B1 and sequence type ST678, with virulence features common to the enteroaggregative *E. coli* (EAEC) pathotype, was reported as the cause of the recent 2011 outbreak in Germany. The outbreak strain was determined to carry several virulence factors of extraintestinal pathogenic *E. coli* (ExPEC) and to be resistant to a wide range of antibiotics. There are only a few reports of serotype O104:H4, which is very rare in humans and has never been detected in animals or food. Several research groups obtained the complete genome sequence of isolates of the German outbreak strain as well as the genome sequences of EAEC of serotype O104:H4 strains from Africa. Those findings suggested that horizontal genetic transfer allowed the emergence of the highly virulent Shiga-toxin-producing enteroaggregative *E. coli* (STEAEC) O104:H4 strain responsible for the outbreak in Germany. Epidemiologic investigations supported a linkage between the outbreaks in Germany and France and traced their origin to fenugreek seeds imported from Africa. However, there has been no isolation of the causative strain O104:H4 from any of the samples of fenugreek seeds analyzed. Following the German outbreak, we conducted a large sampling to analyze the presence of STEC, EAEC, and other types of diarrheagenic *E. coli* strains in Spanish vegetables. During June and July 2011, 200 vegetable samples from different origins were analyzed. All were negative for the virulent serotype O104:H4 and only one lettuce sample (0.6%) was positive for a STEC strain of serotype O146:H21 (*stx*1, *stx*2), considered of low virulence. Despite the single positive case, the hygienic and sanitary quality of Spanish vegetables proved to be quite good. In 195 of the 200 samples (98%), <10 colony-forming units (cfu) of *E. coli* per gram were detected, and the microbiological levels of all samples were satisfactory (<100 cfu/g). The samples were also negative for other pathotypes of diarrheagenic *E. coli* (EAEC, ETEC, tEPEC, and EIEC). Consistent with data from other countries, STEC belonging to serotype O157:H7 and other serotypes have been isolated from beef, milk, cheese, and domestic (cattle, sheep, goats) and wild (deer, boar, fox) animals in Spain. Nevertheless, STEC outbreaks in Spain are rare. [**Int Microbiol** 2011; 14(3):121-141]

Keywords: enterohemorrhagic *Escherichia coli* · enteroaggregative *E. coli* · Shiga toxin · serotypes O104:H4, O157:H7, O146:H21

***Corresponding author:** J. Blanco
Laboratorio de Referencia de *E. coli* (LREC)
Departamento de Microbiología y Parasitología
Facultad de Veterinaria
Universidad de Santiago de Compostela
27002 Lugo, Spain
Tel. +34-982822108
E-mail: jorge.blanco@usc.es

A brief review of STEC

Escherichia coli was first discovered in the gut in 1885 by the German bacteriologist-pediatrician Theodore von Escherich, who called it *Bacterium coli commune*. Most *Escherichia coli* strains are nonpathogenic members of the

intestinal microbiota of humans and other animals, but some strains have acquired virulence factors that enable them to cause important intestinal and extraintestinal diseases, such as diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), urinary tract infections (UTI), septicemia, and neonatal meningitis.

Categories of pathogenic *Escherichia coli* strains, phylogenetic groups, and typing. Five main categories or pathogroups of diarrheagenic *E. coli* are recognized: typical enteropathogenic *E. coli* (tEPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin (verotoxin)-producing *E. coli* (STEC/VTEC), and enteroaggregative *E. coli* (EAEC). In addition, the adherent-invasive *E. coli* (AIEC) pathovar has been increasingly implicated in the etiopathogenesis of Crohn's disease. Strains causing extraintestinal infections are included in the pathogroup of extraintestinal pathogenic *E. coli* (ExPEC) [4,13,14,37,45,65,66,81].

Phylogenetic analysis has shown that *E. coli* is composed of four main phylogenetic groups (A, B1, B2, and D). ExPEC mainly belong to phylogroups B2 and D, and AIEC to phylogroup B2; diarrheagenic *E. coli* normally belong to phylogroups A, B1, and D, and the non-pathogenic commensal strains to phylogroups A and B1 [15,18,45,61].

Pathogenic *E. coli* strains are detected and characterized using phenotypic (biotyping and serotyping) and genetic (PCR, MLST, and PFGE) methods. Identification of the pathogenic potential of a strain is of great importance in determining its O:H serotype and virulence genes. A limited number of *E. coli* reference laboratories have made available all O (O1 to O185) and H (H1 to H56) antisera necessary for complete serotyping, which is an essential basis for differentiating pathogenic strains and is often the starting point in their characterization. Furthermore, outbreaks can be preliminarily identified by the detection of an increased number of isolates within a particular serotype. Pulsed-field-gel-electrophoresis (PFGE) is a band-based molecular technique with high discriminatory power. It allows the identification of clusters of epidemiologically related isolates within O:H serotypes and is intended for tracing outbreaks in a limited time period. Multi-locus-sequence typing (MLST) is a sequence-based method targeting seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*). It has generally less discriminatory power than PFGE but is the most reliable method to determine the genetic relatedness of epidemiologically unrelated isolates. *E. coli* strains are assigned by MLST to different sequence types (STs), and within each ST diverse clusters can be observed by PFGE. As of August 2011, the *E. coli* MLST database consisted of 3942 isolates belonging to 2383 STs [4,9,15,18,37,54,60].

An *E. coli* genome contains between 4200 and 5500 genes, with <2000 genes conserved among all strains of the species (the core genome). The bacterium's pan-genome (genetic repertoire of a given species) consists of almost 20,000 genes [50]. Continuous gene flux occurs during *E. coli* divergence, mainly as a result of horizontal gene transfers and deletions. This genetic plasticity accelerates the adaptation of *E. coli* to varied environments and lifestyles, as it allows multiple gene combinations that result in phenotypic diversification and the emergence of new hypervirulent (STEC and EAEC O104:H4-B1-ST678) and successful (ExPEC O25b:H4-B2-ST131) strains that combine both resistance and virulence genes, which in classical pathogenic *E. coli* strains traditionally have been mutually exclusive [14, 15,52,56,68,76].

STEC/VTEC: pathogenic potential, serotypes, prevalence in clinical samples, reservoirs, and routes of transmission. Shiga-toxin-producing *E. coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), are important emerging pathogens that cause food-borne infections and severe and potentially fatal illnesses in humans, such as HC and HUS [4,6,9,37,42,65,75]. STEC/VTEC strains produce two powerful cytotoxins, called Shiga toxins or verotoxins (Stx1/VT1 and Stx2/VT2), which are encoded in the genome of prophages. There are three subtypes of Stx1 (a, c, and d) and seven of Stx2 (a, b, c, d, e, f, and g). Stx2/VT2 is the most potent toxin, and producing strains are usually associated with more serious infections [37,59].

STEC have other, additional virulence factors, the most important being a protein called intimin, which is responsible for both the intimate adhesion of bacteria to the intestinal epithelium and the attaching and effacing lesion. Intimin is encoded by the gene *eae*, which is part of the chromosomal pathogenicity island LEE (locus for enterocyte effacement). Intimin binds to the cell receptor Tir and the complex is translocated by bacteria to the enterocyte via a type III secretion system (TTSS) [32]. Analysis of the variable C-terminal encoding sequence of *eae* defines at least 29 distinct intimin types (α1, α2, β1, β2, β3, γ1, θ1, κ, δ, ε1, ε2, ε3, ε4, ε5, ζ1, ζ3, η1, η2, ι1-A, ι1-B, ι1-C, ι2, λ, μ, υ, ο, π, ρ, σ) [12,33,55] that have been associated with tissue tropism. Severe diseases in humans are usually associated with *eae*-positive strains of enterohemorrhagic serotypes (O157:H7; O26:H11;

O103:H2; O111:H8, H– ; O145:H–). However, it has also been shown that intimin is not essential for the virulence of certain STEC strains. In fact, STEC O104:H21 and O113:H21 strains lacking *eae* were responsible for an outbreak and a cluster of three HUS cases in the USA and in Australia, respectively [4,9,41].

STEC strains that cause human infections belong to a large number of O:H serotypes (a total of 472 serotypes are listed on the authors' website [http://www.usc.es/ecoli/SEROTIPOSHUM.htm]. Most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serotype O157:H7. Given the importance of serotype O157:H7 in human disease, it is common to consider STEC serotypes in two major categories, O157 and non-O157 [4,8,37,42].

STEC strains have been classified into five seropathotypes (A to E), according to incidence and association with HUS and outbreaks. Seropathotype A includes strains of the highly virulent serotypes O157:H7 and O157:H–, involved in numerous outbreaks in many countries and frequently associated with HUS cases and HC. Seropathotype B comprises non-O157 serotypes causing occasional outbreaks but it is relatively common in sporadic cases associated with HUS and HC (O26:H11; O103:H2, O111:H8, H–, O121:H19, O145:H–).The non-O157 serotypes of seropathotype C are associated only with sporadic cases, including those of HUS and HC (O5:H–, O91:H21, O104:H21, O113:H21, O121:H–, O165:H25, and others). In seropathotype D are the serotypes associated with diarrhea without severe symptoms, i.e., not linked to outbreaks and HUS sporadic cases (O7:H4, O69:H11, O103:H25, O113:H4, O117:H7, O119:H25, O132:H–, O146:H21, O171:H2, O172:H–, O174:H8, and others). Seropathotype E is composed of many STEC serotypes of strains isolated from animals, foods, and environmental samples but not implicated in disease in humans [37,41].

The five pathogenic categories (seropathotypes A to E) of STEC will surely be expanded by a special category made up of serotype O104:H4, recently emerged in Germany, since never to date has a strain caused so many severe cases of HUS (852 only in Germany by July 26, 2011). Note that strain O104:H4, unlike most strains of categories A and B, does not have the LEE pathogenicity island containing the *eae* gene. The lack of an adhesin (intimin) encoded by *eae* is compensated by AAF/I (aggregative adherence fimbria I) and the enteroaggregative character of the strain [68,76].

According to published data, non-O157 STEC were first described as the possible cause of sporadic cases of HUS in 1975 in France, where the hospital historically reported the presence of STEC of serotype O103 in some patients. The first reported outbreak caused by serotype O145:H– occurred in 1984, but the vehicle of infection could not be determined. Also in 1975, STEC O157:H7 was first identified as a potential human pathogen in a Californian patient with bloody diarrhea; in 1982, it was associated with a food-based (beef) outbreak [42].

In 2007, the incidence of O157 and non-O157 infections in the USA was 1.19 and 0.59 per 100,000 habitants, respectively. In the European Union (EU), the incidence of STEC infections in 2006 and 2007 was 1.1 and 0.6 per 100,000 habitants, respectively. Over 70% of the cases of human STEC infections in the USA and 50% of those in the EU were attributed to serotype O157:H7. Therefore, among the STEC, *E. coli* serotype O157:H7 is the most notorious agent, involved in approximately 73,500 cases of infection in the USA each year. The Centers for Disease Control and Prevention (CDC) estimates that approximately 37,000 infections are annually due to non-O157, but with fewer cases of HUS than produced by O157:H7 [42]. Over 500 non-O157 serotypes have been involved in human infections as agents of diarrhea, HUS, and HC, although the true prevalence of non-O157 VTEC infections has been probably underestimated because the standard methods of clinical routine in many laboratories do not include the detection of this group. Recent recommendations from the CDC for the diagnosis of STEC are that laboratories perform both a culture for the specific detection of O157:H7 and an assay for Shiga toxins [35]. A review of laboratory practices in the USA up to the year 2000 reported that while 95% of 388 laboratories tested human stool for *E. coli* O157:H7 by culture, only 3% used a Shiga toxin immunoassay or a PCR test capable of identifying non-O157:H7 serotypes.

Data from 2008 showed that 35% of laboratories participating in a proficiency testing program used a test for Shiga toxins. In Spain and many other European countries, the current situation is similar to that of the USA in the year 2000. During the period 2005-2009, 16,263 confirmed human STEC cases were registered in EU member states [23]. In 2009, a total of 3573 confirmed cases were reported from 18 member states (Austria, 91 cases; Belgium, 96; Denmark, 160; Estonia, 4; Finland, 29; France, 93; Germany, 878; Hungary, 1; Ireland, 237; Italy, 51; Luxembourg, 5; Malta, 8; Netherlands, 313; Slovakia, 14; Slovenia, 12; Spain, 14; Sweden, 228; United Kingdom, 1339). In addition, Iceland, Norway, and Switzerland reported 8, 108, and 42 such cases, respectively. In 2009, the EU notification rate was 0.75 per 100,000 population. Only two to six deaths due STEC infections were reported annually from 2006 to 2009 [23].

Data from different countries indicate that sporadic cases of non-O157 STEC greatly outnumber outbreak cases. This is also true for STEC O157. From 1984 to 2009, 80 important outbreaks ascribed to non-O157, and from 1982 to 2006, 207 outbreaks ascribed to O157 were reported. Comparing data of outbreaks caused by O157:H7 and non-O157, non-O157 STEC strains are much less often associated with meat, water, and vegetables as outbreak vehicles and much more often attributed to person-person contact or unknown vehicles. These differences might be due, in part, to the better analytical methods available for STEC O157:H7. In addition, STEC O157:H7 is more virulent than some non-O157 strains and thus outbreaks are recognized and investigated in depth much faster [23,42].

Ruminants have been identified as the main reservoir for STEC O157:H7 and non-O157 [1,2,8,29,37]. STEC have been isolated from cattle, sheep, goats, and deer and occasionally from other wild and domestic animals; however, it is believed that in most cases STEC are transiently present in these animals, acquired through food or water contaminated by the feces of ruminants. In any case, these accidental hosts can be vehicles of infection for humans.

Cattle is the most important source of human infections (beef, dairy products, bovine fecal contamination). Data on the prevalence of STEC O157 and non-O157 vary widely for both dairy cattle (0.4–74%) and meat cattle (2.1–70.1%) among different countries. Cattle often carry multiple serotypes, some of which do not seem to be of high risk for humans because they do not express any of the most important virulence factors. STEC are not considered pathogenic to ruminants, except when infections occur in young animals before weaning (involved in neonatal diarrhea) [8,23,37]. A study in Germany found a positive association between infections caused by different STEC serotypes and the density of cattle in an area. From data on over 3000 STEC cases, analyses indicated that risk for infection increased by 68% per 100 additional cattle/km^2 [29].

A probable source of infection for cattle is food and drink contaminated with feces from infected animals. Studies of these microorganisms document that STEC O26 can survive for long periods in manure: up to three months in manure pits and liquid manure, and one year in fields fertilized with manure, depending on temperature and soil type. The persistence of these pathogens in manure-contaminated environments has health implications for their transmission, not only in farm production but also in other settings, such as county fairs and farm schools, in which children are exposed [23,37,42].

Fecal material may contaminate meat during slaughter, may be washed into lakes or drinking water sources, or may be deposited in fruits and vegetables when manure is used for fertilization or sewage-contaminated water for irrigation. Humans, therefore, may become infected directly, through contact with an infected person or animal carrier, or indirectly, through the environment, food, drinking water, or surface water contaminated with fecal material containing STEC from human or animal origin [23,37,42].

Shiga-toxin-producing enteroaggregative *Escherichia coli* O104:H4. An emerging serotype

On 26 May 2011, Germany reported a nationwide outbreak of HUS caused by STEC of serotype O104:H4. The German outbreak was declared officially over by the Robert Koch Institute (RKI) of Berlin on July 26, 2011, 3 weeks after the last date (July 4) of onset for a case with an epidemiological link [70]. Between May 1 and July 4, 2011, Germany reported 852 HUS cases and 3469 cases with diarrhea (and/or with HC), of which 50 patients died (including 32 HUS patients). According to the European Centre for Disease Prevention and Control (ECDC), 49 HUS cases and 76 cases with diarrhea were reported in 13 other European countries, including eight HUS cases from the French outbreak and two sporadic cases from Spain. Additional cases related to the outbreak were reported from the USA and Canada [23,27,70].

Several groups concluded that the outbreak was caused by a STEC strain belonging to serotype O104:H4, with virulence features common to the enteroaggregative *E. coli* (EAEC) pathotype [5,23,76]. This combination is very rare and was previously described in strains of serotype O111:H2 involved in a small outbreak of HUS in children in France [57]. In addition, the German outbreak strain was found to possess several virulence factors of extraintestinal pathogenic *E. coli* (ExPEC) and to have acquired resistance to numerous antibiotics, including third-generation cephalosporins, owing to several plasmid-borne genes encoding TEM-1 and CTX-M-15 β-lactamases [20,23]. Table 1 shows the main virulence factors and characteristics of the O104:H4 outbreak strain. Serotype O104:H4 is very rare and has been diagnosed and reported in humans in a few cases only (Table 2); it has never been reported in animals and food. However, there are a few known animal strains of serogroup O104 with H antigens different from H4 (Table 3) [23].

Table 1. Properties of the STEAEC O104:H4 outbreak strain
Serotype O104:H4
Phylogroup B1
MLST sequence type ST678 (*adk* 6, *fumC* 6, *gyrB* 5, *icd* 136, *mdh* 9, *purA* 7, *recA* 7)

Gene	Location	Virulence factor	Presence
Virulence genes of STEC/VTEC/EHEC			
stx1/*vtx1*	Prophage	Shigatoxin 1	negative
stx2a/*vtx2a*	Prophage	Shigatoxin 2 (variant 2a)	positive
eae	LEE pathogenicity island (chromosome)	Intimin (adhesin)	negative
E-hlyA	Plasmid	Enterohemolysin	negative
$lpfA_{O26}$	Chromosome	Structural subunit of long polar fimbriae (LPF) of STEC O26	positive
saa	Plasmid	Saa (STEC autoagglutinating adhesin)	negative
ter	Chromosome	Tellurite resistance	positive
Virulence genes of EAEC			
aggA	Plasmid (pAA)	Subunit of aggregative adherence fimbria AAF/I	positive
aggR	Plasmid (pAA)	Master regulator of a package of EAEC plasmid (pAA) virulence genes, including AAF/I adherence factor	positive
aatP	Plasmid (pAA)	ABC protein responsible for transporting the dispersin protein out of the outer membrane	positive
aap	Plasmid (pAA)	Secreted protein named dispersin, and is responsible for dispersing EAEC across the intestinal mucosa	positive
sepA	Plasmid (pAA)	SepA. *Shigella* extracellular protein. May induce mucosal atrophy and tissue inflammation in *S. flexneri*	positive
sigA	Chromosome	SigA protein, an IgA protease-like homologue	positive
pic	Chromosome	Pic protein with mucinase activity involved in the intestinal colonization	positive
astA	Plasmid	EAEC heat-stable enterotoxin 1 (EAST1)	negative
Virulence genes of ExPEC			
irp2 and fyuA[a]	Chromosome	Component of iron uptake system (siderophore yersiniabactin) on high pathogenecity island (HPI)	positive
iha	Chromosome	IrgA homologue adhesin (*Iha*)	positive
aer	Chromosome or plasmid	Aerobactin siderophore	positive
hlyA	Chromosome or plasmid	Alpha-hemolysin	negative

Microbiological properties:

Lactose fermentation (+), sorbitol fermentation (+) and β-glucuronidase (+).
Indole (+), citrate (–) and H_2S production (–).
Excellent growth in cefixime telurite sorbitol MacConkey (CT-SMAC) agar.

Antibiotic resistance:

TEM-1 and CTX-M-15 β-lactamases

Resistance to: ampicillin, amoxicillin/clavulamic acid, piperacillin/sulbactam, piperacillin/tazobactam, cefuroxim, cefuroxin-axetil, cefoxitin, cefotaxim, ceftazidim, cefpodoxim, streptomycin, nalidixic acid, tetracylin, and trimethoprim/sulfamethoxazol.

Sensitive to: imipenem, meropenem, amikacin, kanamycin, gentamicin, tobramycin, ciprofloxacin, norfloxacin, nitrofurantoin, chloramphenicol, and fosfomycin.

[a]The *irp2* and *fyuA* genes were detected in more of 90% of EAEC and 80% of septicemic ExPEC.
Information obtained from: Robert Koch Institut [70], National Reference Laboratory for *E. coli* (BfR), Bielaszewska et al. [5] and Rasko et al. [68].

Table 2. Overview of reported human STEC/VTEC and/or EAEC O104:H4 strains

	Year	Shiga-toxins	HUS	Reference
STEC/VTEC and EAEC strains				
Germany (2 cases)	2001	*stx2a*	yes	[46]
Georgia (2 cases)	2009	*stx2a*	yes	[76]
Italy	2009	*stx2a*	yes	[85]
Finland	2010	*stx2a*	no	[76]
Germany (outbreak)	2011	*stx2a*	yes	[5,76]
France (outbreak)	2011	*stx2a*	yes	[34,76]
STEC/VTEC confirmed and EAEC strains? (unknown)				
France	2004	unknown	unknown	[76]
STEC/VTEC strains				
Korea	2005	*stx1* and *stx2*	yes	[3,84]
EAEC strains				
Central African Republic	1995 or 1996	negative	no	[5]
Spain (3 cases)	1996	negative	no	This study
Denmark	2000	negative	no	[76]
Mali (6 cases)	2009	negative	no	[76]

EAEC is a pathotype of diarrheagenic *E. coli* defined as *E. coli* that do not secrete the heat-stable (ST) or heat-labile (LT) toxins of ETEC, and with a characteristic aggregative pattern (AA) of adherence to HEp2-cells in culture. This property is usually due to the presence of AAF/I to AAF/IV, whose expression is regulated by the *aggR* gene, located on the large EAEC virulence plasmid pAA [18,66,76]. EAEC infections are usually associated with prolonged watery diarrhea, particularly among children and in travelers to developing countries [48,61,66]. However, EAEC is also a significant cause of diarrhea in Europe, including Spain [12,18].

Table 3. STEC/VTEC O104 strains of animal and food origin

Serotype	Origin	Country	Year	Reference
O104:H21	Cattle	Spain	1993-1997	[10]
O104:H12	Cattle	Austria	2009	[23]
O104:H21	Cattle	Austria	2009	[23]
O104	Ground meat	Germany	2005	[23]
O104:H7	Ovine	Spain	1997	[7]
O104:H7	Lamb meat	India	2001-2002	[23]
O104:H7	Calf	Argentina	1999/2000	[23]
O104	Bovine carcasses	USA	1999	[23]
O104:H7/HNM	Ovine meat	New Zealand	1998	[23]

Source: Technical report of ECDC and EFSA, June 2011, completed with data from LREC.

The fact that EAEC has been rarely identified in animals suggests that they are not zoonotic but rather exclusively human pathogens.

But what is the origin of the outbreak strain of serotype O104:H4? Mellmann et al. [47] characterized the complete genome of the outbreak isolate LB226692 and a historic STEC/EAEC O104:H4 HUS isolate from 2001 (01-09591) by a rapid next-generation sequencing technology. Phylogenetic analyses of 1144 core *E. coli* genes indicated that the HUS STEC/EAEC strains are closely related to the previously published sequence of the EAEC strain 55989 isolated in the late 1990s in Africa, but only distantly related to common EHEC serotypes. Despite this close relationship, the outbreak strain differs from the 2001 strain in plasmid content, fimbrial genes, and antibiotic resistance genes. Mellmann et al. [47] proposed a model in which EAEC 55989 and EHEC O104:H4 strains evolved from a common EHEC O104:H4 progenitor; they suggested that, by stepwise gain and loss of chromosomal and plasmid-encoded virulence factors, a highly pathogenic hybrid of EAEC and EHEC emerged as the current outbreak clone (Fig. 1). Due to its hybrid pathogenicity characteristics, Brzuszkiewicz et al. [16] designated the new pathotype "Entero-Aggregative-Hemorrhagic *E. coli* (EAHEC)."

Rasko et al. [68] used third-generation, single-molecule, real-time DNA sequencing to determine the complete genome sequence of the German outbreak strain, as well as the genome sequences of seven diarrhea-associated EAEC of serotype O104:H4 strains from Africa (isolated in Mali in 2009) and of four EAEC reference strains belonging to other serotypes. Genome-wide comparisons were performed on the basis of these EAEC genomes as well as those of 40 previously sequenced STEC, ETEC, EPEC, and ExPEC isolates belonging to different serotypes. The EAEC O104:H4 strains were closely related and formed a distinct clade among *E. coli* and enteroaggregative *E. coli* strains. However, the genome of the German outbreak strain could be distinguished from that of the other O104:H4 strains because it contains a prophage-encoding Shiga toxin 2 and a distinct set of additional virulence and antibiotic-resistance factors. The findings of Rasko et al. [68] suggest that horizontal genetic exchange allowed the emergence of the highly virulent Shiga-toxin-producing enteroaggregative *E. coli* (STEAEC) O104:H4 strain that caused the German outbreak (Fig. 1). More broadly, these findings highlight the way in which the plasticity of bacterial genomes facilitates the emergence of new pathogens [50]. The main lesson from this outbreak is that we should be aware of the capacity of *E. coli* species to accommodate new combinations of genes,

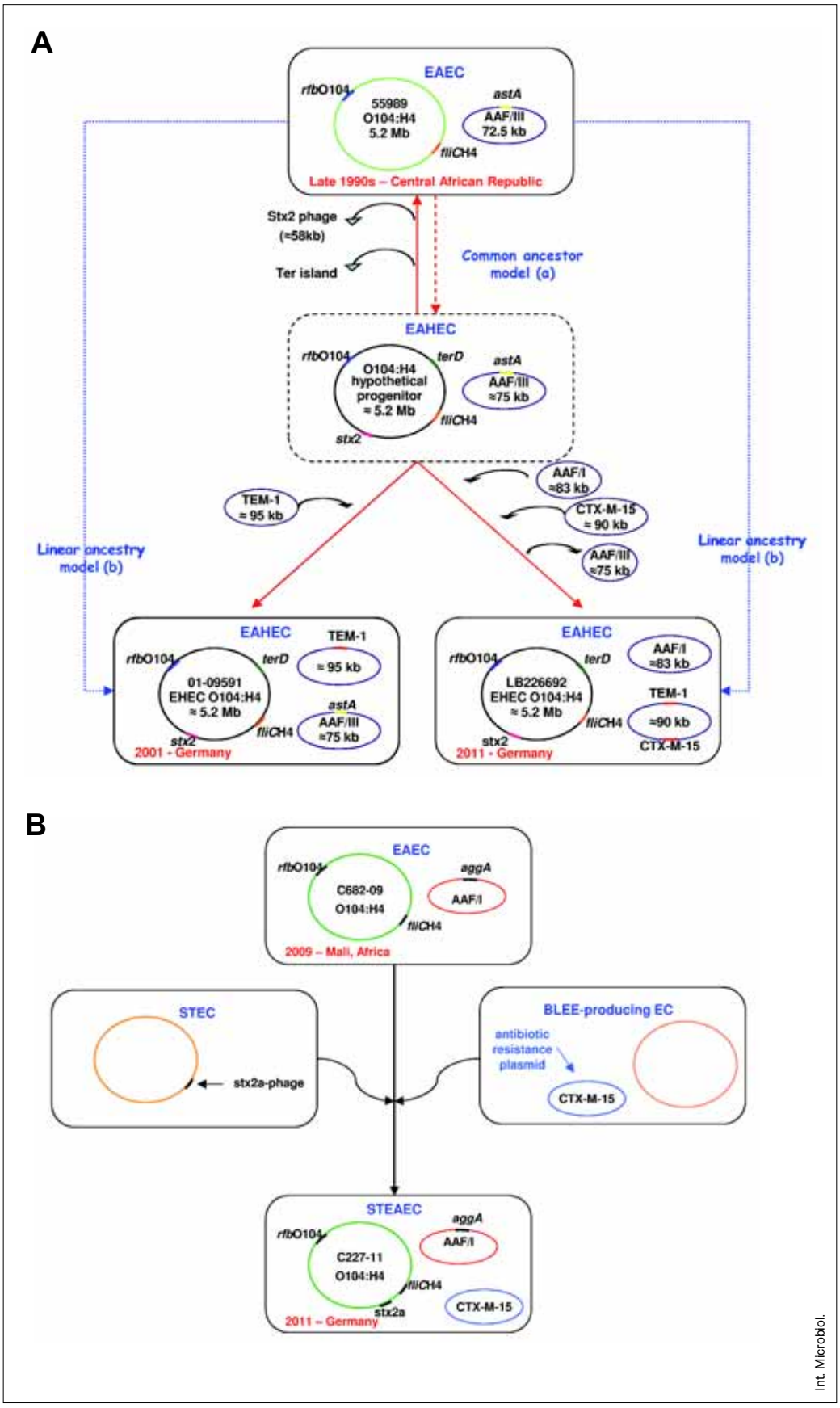

Fig. 1. Evolutionary models of the origin of the German STEAEC O104:H4 outbreak strain. Top (**A**): common ancestor model and linear ancestry model proposed by Mellmann et al. [47]. Bottom (**B**): model proposed by Rasko et al. [68], similar to that proposed by Rohde et al. [71] and Brzuszkiewicz et al. [16]. These studies revealed that the outbreak strain belonged to an EAEC lineage that has acquired genes encoding the Stx2a toxin and antibiotic resistance.

leading to the emergence of highly aggressive strains. Furthermore, antibiotic pressure in human and veterinary medicine should be kept as low as possible, as it will select these strains once they become resistant [20].

Epidemiologic investigations initially supported the hypothesis that the outbreaks in Germany [27] and France [34] were linked and that they were due to fenugreek seeds imported from Egypt [26]. However, there has been no isolation of the causative strain O104:H4 from any of the fenugreek seeds analyzed. In the scientific literature, associations between outbreaks of STEC infection and both vegetable products, particularly contaminated sprouting seeds, and green leafy salad vegetables are increasingly recognized [25,26,28,79,82]. The largest STEC O157:H7 outbreak to date was in 1996 in Sakai City (Osaka, Japan) and was linked to the consumption of white-radish sprouts [49]. It remains unclear why the German STEAEC outbreak strain was so virulent. As noted, a novel suite of adhesion factors might provide an explanation. Alternatively, perhaps this strain is able to exploit more efficient mechanisms for toxin release. It is worth remembering that strains of EAEC have caused large sprout-associated outbreaks before, including one outbreak, caused by serotype ONT:H10**,** that affected more than 2000 people in Japan in 1993 [39]. Thus, there is clearly an urgent need to understand how the German outbreak strain and other strains of EAEC adhere to and colonize seeds and seedlings. Also, outbreaks of *Salmonella* infection associated with the consumption of raw seed sprouts are not rare. Thus, sprouts must be acknowledged as high-risk foods in which contamination usually starts with contaminated seeds, as the high humidity required to trigger sprouting provides ideal conditions for bacterial multiplication.

STEC/VTEC in Spain

STEC in patients with diarrhea and HC. From 1992 to 2011, the LREC-USC, in collaboration with the Microbiology Unit of Hospital Complex Xeral-Calde of Lugo (Lucus Augusti Hospital), processed 13,962 stool cultures for the presence of STEC O157:H7 and non-O157 (Table 4). Samples were isolated from inpatients and outpatients of all ages who mostly had diarrhea or gastroenteritis. STEC were detected in 393 (2.8%) of the stool cultures tested. In total, STEC O157:H7 accounted for 63 (0.5%) cases of infection and non-O157 STEC for 251 (1.8%). Serotype O26:H11 was the most frequently detected among the non-O157 VTEC strains ([9] and unpublished data).

Table 4. STEC/VTEC prevalence in patients from the Lucus Augusti Hospital of Lugo

	No. of stool samples from adult and children with diarrhea						
		STEC/VTEC					
Year	Total assayed	O157:H7 isolated		Non-O157 isolated		Total Detected	
1992–1999	5054	24	0.5%	87	1.7%	126	2.5%
2003–2005	3970	12	0.3%	75	1.9%	144	3.6%
2006–2010	4692	27	0.6%	85	1.8%	119	2.5%
May–July 2011	246	0	0%	4	1.6%	4	1.6%
TOTAL	13,962	63	0.5%	251	1.8%	393	2.8%

Results obtained from 1992 to 1999 are already published data [9], whereas results from 2003 to 2011 are unpublished data.

From 2005 to 2011, the LREC-USC also processed, in collaboration with the Microbiology Service of University Hospital Complex of Santiago de Compostela (CHUS), a total of 1479 stool cultures for the presence of STEC O157:H7 and non-O157 (Table 5). Samples were isolated from inpatients and outpatients of all ages, most of whom had bloody diarrhea. STEC were detected in 32 (2.2%) of the stool cultures tested. In total, 13 (0.9%) cases of infection were associated with STEC O157:H7 and 16 (1.1%) with non-O157. Serotype O146:H21 was the most frequently isolated among the non-O157 STEC (unpublished data).

Table 5. STEC/VTEC prevalence in patients from the University Hospital Complex of Santiago de Compostela (CHUS)*

	No. of stool samples						
		STEC/VTEC					
Year	Total assayed	O157:H7 isolated		Non-O157 isolated		Total detected	
2005	93	3	3.2%	1	1.1%	4	4.3%
2006	114	1	0.9%	0	0%	1	0.9%
2007	283	3	1.1%	6	2.1%	9	3.2%
2008	329	3	0.9%	3	0.9%	6	1.8%
2009	233	0	0%	3	1.3%	4	1.7%
2010	276	3	1.1%	2	0.7%	6	2.2%
2011	151	0	0%	1	0.6%	2	1.3%
2005–2011	1479	13	0.9%	16	1.1%	32	2.2%

*Unpublished data.

Table 6. STEC/VTEC serotypes most frequently detected among human strains isolated in Lugo (2003–2011)*

Serotype	Phylogenetic group	ST	Seropathotype	Intimin type
O5:H–	A	ST342	C	β1
O26:H11	B1	ST21	B	β1
O103:H2	B1	ST17	B	ε1
O111:H8	B1	ST16	B	θ
O113:H21	B1	ST56	C	–
O118:H16	B1	ST21	C	β1
O145:H–	D	ST32	B	γ1
O146:H21	B1	ST442	D	–
O157:H7	D	ST11	A	γ1

*Unpublished data.

None of the STEC strains isolated in Galicia from human patients between 1992 and 2011 belonged to serotype O104:H4, the serotype implicated in the German outbreak. Table 6 shows the most frequent STEC serotypes found among strains isolated from humans in Spain, together with the phylogenetic group, ST, seropathotype, and intimin-type ([9] and unpublished data).

Figure 2 shows a dendrogram of the *Xba*I macrorestric-

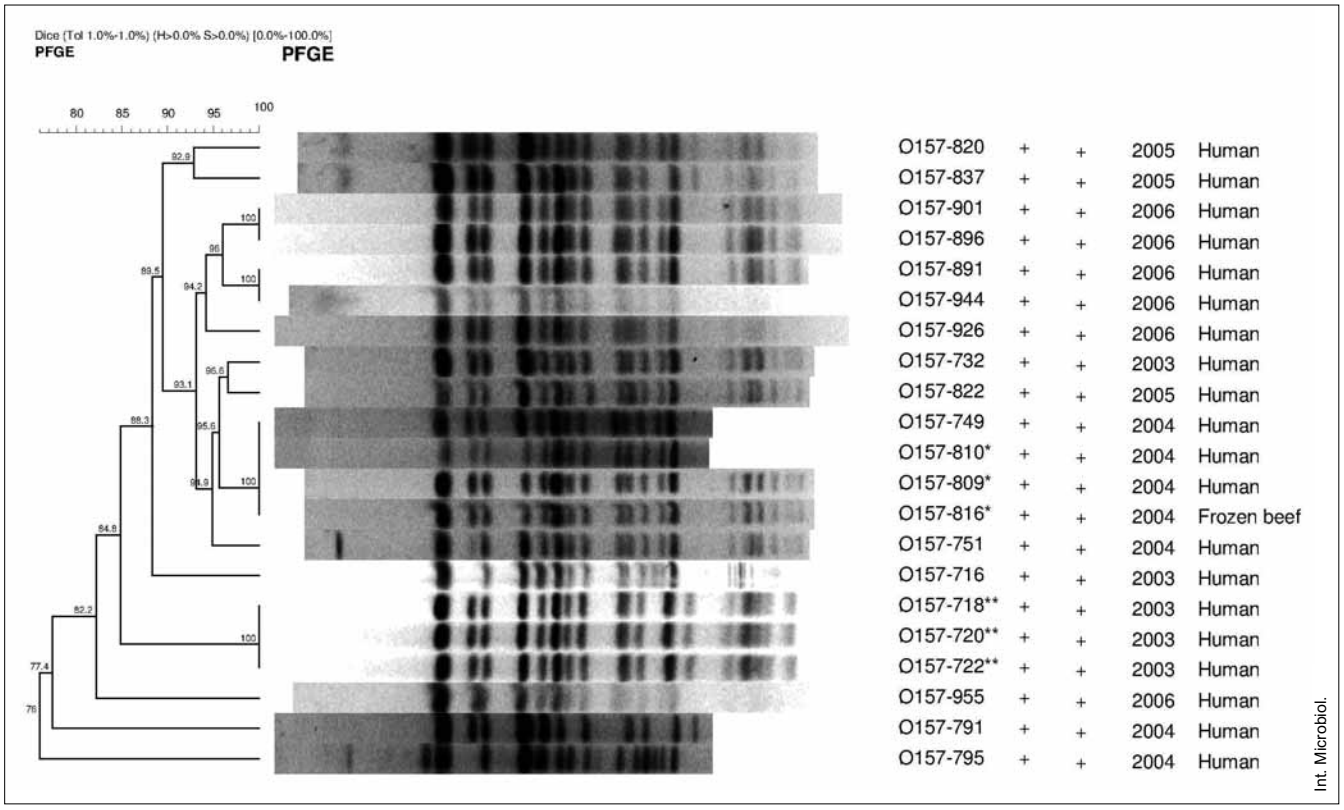

Fig. 2. Dendrogram of the *Xba*I macrorestriction profiles of 21 STEC strains of serotype O157:H7 isolated in Galicia, northwestern Spain. Strain code, *stx*1, *stx*2, year of isolation and origin, are shown on the right side of the dendrogram.*O157-809 strain isolated from a patient with HC, O157-816 strain isolated from a sample of frozen beef consumed by the patient, and O157-810 strain isolated from an asymptomatic relative . **O157-718, O157-720 and O157-722 strains isolated from two patients and an asymptomatic carrier of the same family, respectively. Unpublished data.

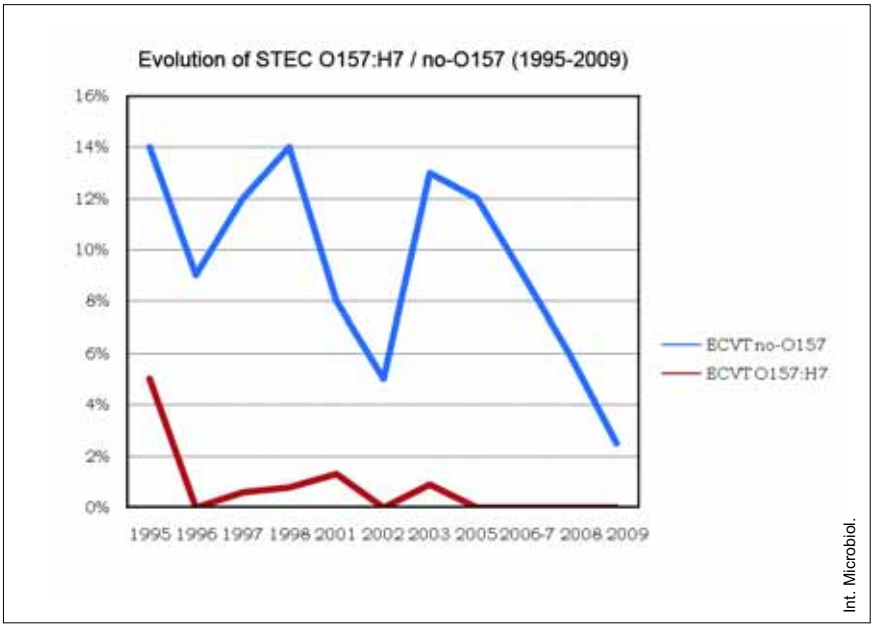

Fig. 3. Prevalence of STEC in beef sampled in the city of Lugo.

tion profiles of 20 STEC strains of serotype O157:H7 isolated from 2003 to 2006 in various hospitals of Galicia and of one O157:H7 strain isolated from beef consumed by a patient. All 21 belonged to phagetype 8 and showed high homogeneity, with 15 strains included in a cluster of similarity >85%. Strain O157-809 was isolated from a patient with HC, strain O157-816 from a sample of beef consumed by the patient, and strain O157-810 from an asymptomatic relative. Strains O157-718, O157-720, and O157-722 were isolated from two patients and an asymptomatic carrier of the same family, thus representing a small outbreak ([9] and unpublished data). STEC is the third most frequently identified enteric pathogen in stool cultures at the Lucus Augusti Hospital, after *Salmonella* and *Campylobacter*, thus accounting for a significant number of infections in our area [9,12]. Data from Galicia suggest that, per year in Spain, STEC O157:H7 is responsible for more than 500 cases of infection, and non-O157 for more than 2000. Therefore, STEC likely produces 5.0 to 6.0 cases per 100,000 habitants.

STEC in beef. In recent years, we have detected a significant decrease in the prevalence of STEC in beef sold in the city of Lugo. Especially important is the absence of positivity for STEC O157:H7 in the period 2005–2009 (Fig. 3). STEC O157:H7 was detected in eight cases (0.6%), and non-O157 in 146 (10%), among 1445 samples analyzed between the years 1995 and 2009. None of the STEC strains isolated from beef in Lugo belonged to serotype O104:H4, implicated in the German outbreak. However, the level of samples contaminated with non-O157 VTEC is still too high, pointing out the need to improve hygiene, control, and surveillance throughout the food chain ([53] and unpublished data).

STEC in chicken and pork. We have also conducted studies on the STEC prevalence in chicken and pork meat. During 2009 and 2010, 200 samples of chicken breast were analyzed. All samples were negative for STEC O157 but two (1%) were positive for non-O157. Among the 110 samples of pork analyzed in 2011, none was positive for STEC O157 but six (5.5%) were positive for non-O157.

Although chicken showed a lower prevalence of STEC than beef and pork, it had higher levels of *E. coli* contamination since only 45% of chicken samples had <10 cfu/g compared to 65% of beef and 71% of pork samples. Clearly, also in these meats it is necessary to decrease the levels of *E. coli* contamination (unpublished data).

STEC in dairy products. Rey et al. [69] examined 502 dairy products from 64 different ovine and caprine flocks and from six dairy plants in Extremadura. Sampling was conducted monthly between March 2003 and June 2004 and yielded 360 samples from unpasteurized milk obtained from the bulk tank, 103 samples from fresh cheese curds, and 39 from cheese. STEC strains were detected in 39 (11%) of the samples from the bulk tank, 4 (4%) of those from fresh cheese curds, and 2 (5%) of those from cheese; O157:H7 serotype was isolated from one (0.3%) bulk tank sample. A total of nine STEC strains (O27:H18, O45:H38, O76:H19, O91:H28,

O157:H7, ONT:H7, ONT:H9 and ONT:H21) were identified in this study.

In another study, Caro et al. [17] characterized 13 STEC strains isolated from sheep dairy products in Castilla y León. Eight strains isolated from milk belonged to serotype O157:H7. Three STEC strains (two of serogroup O14 and one ONT) were detected in two samples (2.4%) of "Castellano" cheese, one with a 2.5- and the other one with a 12-month ripening period.

STEC in vegetables. In 2008, 100 lettuces from various commercial establishments in the city of Lugo were analyzed by the LREC-USC for the presence of STEC. An acceptable microbiological quality was determined, since 99% of the lettuces tested had <10 cfu *E. coli*/g. In addition, the 100 lettuces were negative for the presence of STEC and other types of diarrheagenic *E. coli* (unpublished data).

The so-called cucumber crisis, 2011. On 31 May 2011, our LREC-USC received samples from the organic farm involved in the so-called cucumber crisis and suspected by the German Health authorities. The samples submitted by the Junta de Andalucía consisted of 70 cucumbers organically produced; 13 plates of coliform chromogenic medium (37°C/24 h) obtained by filtration of 100 ml of water for irrigation; and four plates of tryptone bile X-glucuronide medium (TBX) obtained from two samples of soil (incubation in duplicate at 37 and 44°C, 24 h, respectively) previously enriched in Mossel EE broth (37°C, 24 h). The type of analysis requested for all samples was the detection and characterization of STEC and EAEC strains.

The 17 plates (13 from water samples and four from soils) were sub-plated onto lactose-MacConkey (LMAC) agar and cefixime tellurite sorbitol-MacConkey (CT-SMAC) agar and then incubated at 37°C for 24 h. The 70 cucumbers were analyzed by dividing them into seven pools of ten units each. Additionally, three units of three random pools were analyzed individually. Thus, 10 samples were globally tested (seven pools and three units). Enrichment cultures were established by adding a 25-g test portion of cucumber skin aseptically cut with individual scalpel blades to 225 ml of buffered peptone water (BPW), followed by incubation for 18–24 h at 37°C. The cultures of LMAC, CT-SMAC, and BPW were then analyzed by conventional PCR, aimed at the detection of the *stx1* and *stx2* genes, typical of STEC, and of *pAA*, typical of EAEC. All samples were negative by PCR for the three genes tested; therefore, STEC and EAEC were not detected in any of the water, soil, or cucumber samples analyzed. The primers and conditions used are described in the section "Analytical methods for STEC detection in foods."

We also analyzed the most probable number of *E. coli* per gram of the cucumber samples. In all cases, counts were <10 cfu

Table 7. STEC/VTEC prevalence in vegetables sampled between June and July of 2011*

		Most Probable Number of *Escherichia coli*				
Vegetable	No. samples	<10	10–99	>99	STEC/VTEC	EAEC, ETEC, tEPEC and EIEC
Cucumbers	32	32 (100%)	0	0	0	0
Tomatoes	36	36 (100%)	0	0	0	0
Lettuces	54	50 (93%)	4 (7.4%)	0	1 (1.9%)	0
Bean sprouts	6	6 (100%)	0	0	0	0
Endives	2	2 (100%)	0	0	0	0
Broccoli	7	7 (100%)	0	0	0	0
Leeks	2	2 (100%)	0	0	0	0
Peppers	6	6 (100%)	0	0	0	0
Carrots	1	1 (100%)	0	0	0	0
Onions	1	1 (100%)	0	0	0	0
Packaged salads	53	52 (98%)	1 (1.9%)	0	0	0
TOTAL	200	195 (98%)	5 (2.5%)	0	1 (0.5%)	0

*Unpublished data.

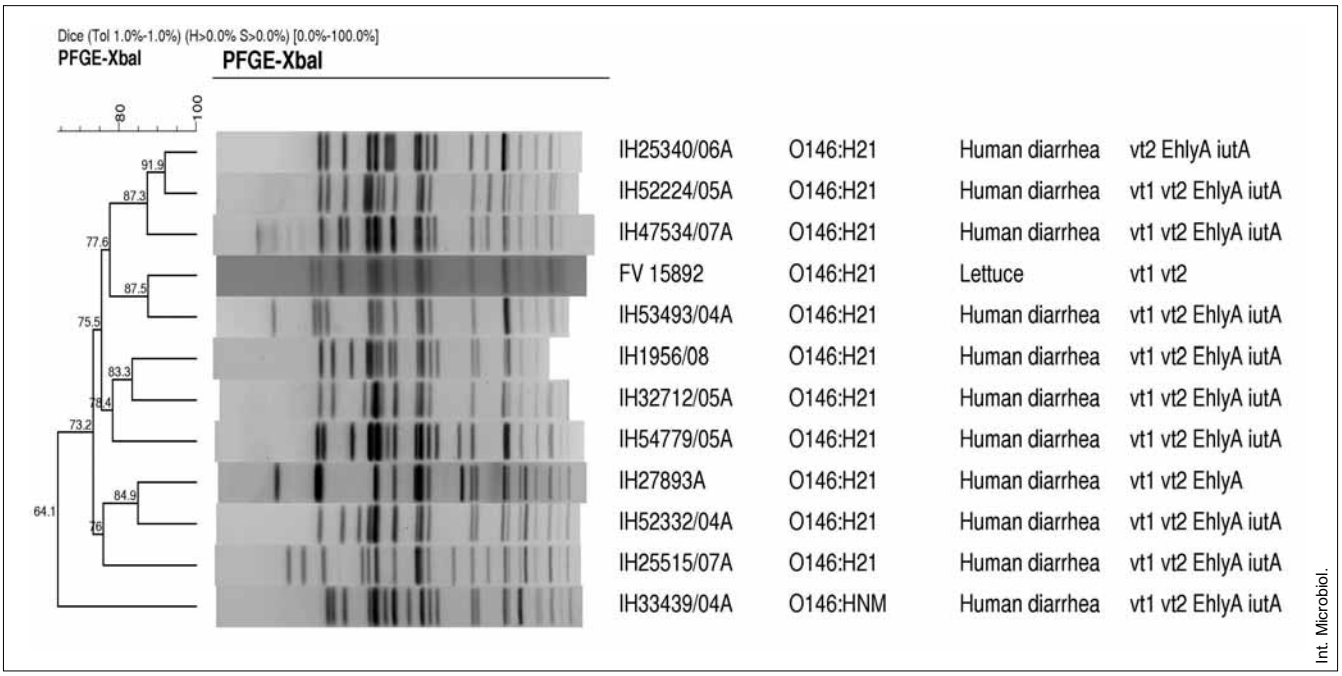

Fig. 4. Comparison of STEC strains of serotype O146:H21. Dendrogram of the *Xba*I macrorestriction profiles. Strain code, serotype, origin, and virulence genes are shown at the right side of the dendrogram. Unpublished data.

per gram of sample, which implied that no *E. coli* colony grew on the Petrifilm Select plates (unpublished data). The analysis report for the samples was closed on June 2, 2011.

Vegetable sampling, 2011. As a consequence of the German outbreak, we decided to conduct a large sampling to determine the presence of STEC, EAEC, and other types of diarreagenic *E. coli* in the vegetables sold in Spain. Between June and July of 2011, 200 vegetables from different geographic origins were analyzed at the LREC-USC (Table 7) (unpublished data). The 200 vegetable samples were negative for the virulent serotype O104:H4 and only one sample (0.6%) was positive for a STEC strain of serotype O146:H21 (*stx*1 *stx*2), considered of low virulence (category D). Despite the positive case, the hygienic and sanitary quality of the vegetables offered for sale in the city of Lugo proved to be quite good, since in 195 (98%) of the samples <10 cfu of *E. coli* per gram were detected. Of concern is the fact that a sample of packaged salad showed a most probable number of *E. coli* per gram of 10–99, since this product is sold ready to eat. Special vigilance should be paid to food consumed directly without any previous care. We believe that the 200 samples analyzed were representative of the Spanish situation since 116 of 200 came from superstore centers that distribute throughout the country. These 116 samples were negative for STEC.

The *Xba*I-PFGE macrorestriction profile of the STEC O146:H21 strain isolated from lettuce was compared with the profiles of 11 STEC strains belonging to the same serotype and isolated from patients (Fig. 4) (unpublished data). The vegetable strain showed a cluster of 87.5% identity with a human strain isolated in 2004. Although serotype O146:H21 is currently included in category D, based on a lack of association to date with outbreaks or severe cases (HUS), we highly believe it should be monitored because some of the patients from whom we isolated O146:H21 developed HC. In addition, strains of serotype O146:H21 have been isolated in Spain from beef, cattle, sheep, goats, deer, wild boars, and foxes.

STEC in cattle. In an analysis of the role of cattle as a reservoir of STEC (1993–1995), we found that one third of the calves and cows of Galicia were carriers. Furthermore, 12% of the calves and 22% of the farms sampled were positive for highly virulent STEC serotype O157:H7. In 1998, we conducted a study in Navarra in two slaughterhouses and five feedlots. The number of STEC O157:H7 carriers detected was very high: 10% at slaughterhouse A, 19% at slaughterhouse B, 23% at feedlot 1, 22% at feedlot 2, 8% at feedlot 3, but 0% in feedlots 4 and 5. These data obtained in Spain are in agreement with those reported in other countries, and confirm that around 10% of cattle are colonized by STEC O157:H7 [8,10,11,44].

Table 8. STEC/VTEC serotypes most frequently found in Spain

Origin	Year, locality [Reference]	Number of strains	*eae*+ strains	Serotypes most frequently found (number of strains)
Human	1992–1999 Lugo [9]	126	56%	O26:H11, H– (14); O91:H21, H– (4); O111:H8, H– (5); O113:H21 (3); O145:H8, H– (3); O146:H21 (3); O157:H7 (24)
Human	2003–2011 Lugo (UD)[a]	213	68%	O5:H– (3); O26:H11, H– (43); O103:H2 (7); O111:H8, H– (13); O113:H21 (4); O118:H16 (6); O145:H– (4); O146:H21 (13); O157:H7 (41)
Bovine	1993–1999 Galicia [10]	514	29%	O2:H27 (7); O2:H29 (5); O4:H4 (11); O8:H2 (9); O20:H19 (18); O22:H8 (25); O26:H11, H– (26); O64:H– (4); O77:H41 (21); O82:H8 (7); O91:H21 (8); O103:H2, H– (8); O105:H18 (15); O113:H4 (8); O113:H21 (33); O116:H21, H– (10); O118:H16, H– (4); O126:H20 (4); O128:H– (4); O141:H8 (4); O156:H– (9); O157:H7 (82); O162:H21 (4); O171:H2 (20); O171:H25 (4); O174:H– (5); O174:H2 (8); O174:H21 (6); O177:H11, H– (10); ONT:H19 (16)
Ovine	1997 Extremadura [7]	384	6%	O5:H– (19); O6:H10 (25); O6:H– (3); O52:H45 (3); O91:H– (64); O104:H7 (9); O110:H– (7); O112:H– (7); O117:H– (16); O123:H– (3); O128:H– (46); O128:H2 (14); O136:H20 (11); O146:H8 (14); O146:H21 (27); O156:H– (13); O157:H7 (5); O166:H28 (11); O176:H4 (9); ONT:H21 (17)
Ovine	Madrid [62]	63	3%	O5:H– (13); O6:H10 (3); O26:H11 (2); O91:H– (6); O128:H– (6); O146:H21 (8); O166:H28 (4)
Caprine	2003 Murcia [19]	106	0%	O5:H– (7); O76:H19 (30); O91:H14 (3); O126:H8 (6), O128:H2,H– (4); O146:H21,H– (10); O166:H28 (3); ONT:H4 (3); ONT:H21 (18)
Caprine	Madrid [62]	41	2%	O5:H– (3); O81:H21, H– (11); O128:H2, H– (2); O146:H21 (2); O166:H28 (8)
Wild ruminants	2004–2005 Extremadura [72]	65	0%	O2 (7); O8 (5); O128 (5); O146 (25); O166 (4); O174 (6)
Wild boars	2007–2008 Extremadura [74]	17	35%	O6:H10 (1); O23:H21 (2); O109:H– (1); O127:H2 (1); O142 (1); O146:H21 (1); O157:H7 (5); O157:H21 (2); ONT (3)
Beef	1995–2003 Lugo [53]	96	26%	O1:H10 (2); O8:H21 (4); O22:H8 (4); O26:H11, H– (4); O64:H5 (3); O75:H8 (2); O77:H41 (2); O103:H2, H– (3); O111:H– (2); O113:H21 (2); O157:H7 (8)
Beef	2005–2009 Lugo (UD)	53	30%	O5:H– (2); O26:H11, H– (9); O6:H10 (2); O146:H21 (5); O174:H21 (2)
Milk	2003–2004 Extremadura [69]	9	11%	Ovine: O45:H38 (1); ONT:H7 (2); ONT:H9 (1) Caprine: O27:H18 (1); O76:H19 (1); O91:H28 (1); O157:H7 (1); ONT:H21 (1)
Ovine dairy products	1999 Castilla y León [17]	13	61%	Milk: O71 (2), O157:H7 (8) Cheese: O14 (2), ONT (2)
Vegetables	2011. This study	1	0%	O146:H21 (1)

[a]UD: unpublished data.

The serotypes and virulence genes of 514 bovine STEC strains isolated in Spain were also determined. Although they belonged to a wide number of serotypes (66 serogroups and 113 serotypes), 52% of the strains belonged to only 10 serotypes (O4:H4, O20:H19, O22:H8, O26:H11, O77:H41, O105:H18, O113:H21, O157:H7, O171:H2 and ONT:H19). Moreover, the seropathotypes of many bovine strains were those previously found among STEC causing infections in humans (Table 8) [4,9,10,40,44,83]. None of the STEC bovine strains belonged to serotype O104:H4; however, we did find two O104:H21 *stx1 stx2* ST672 strains (Fig. 5).

STEC in sheep. In 1997, we conducted a study in collaboration with the Faculty of Veterinary Sciences of Cáceres.

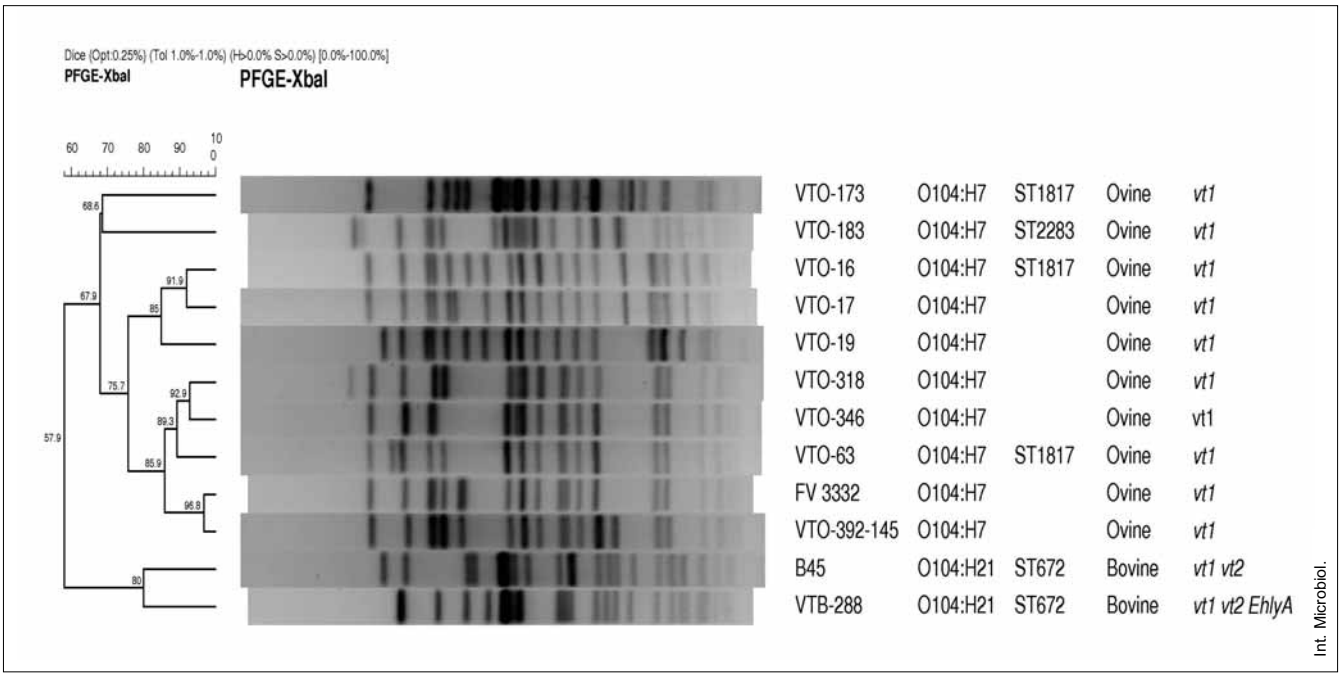

Fig. 5. Dendrogram of the *Xba*I macrorestriction profiles of 12 animal STEC strains of serotypes O104:H7 and O104:H21 isolated in Spain. Strain code, serotype, ST, origin, and virulence genes are shown on the right side of the dendrogram. Unpublished data.º

An analysis of 1300 samples from 93 farms in Extremadura showed that 0.4% of the lambs were colonized by STEC O157:H7 and 36% by non-O157 [7]. Among 384 ovine strains, 35 serogroups and 64 serotypes were established, with 72% of the strains belonging to one of the following 12 serotypes: O5:H–, O6:H10, O91:H–, O117:H–, O128:H–, O136:H20, O146:H8, O146:H21, O156:H–, O166:H28, and ONT:H21. The STEC seropathotypes of ovine strains differed from those of bovine strains, but many had also been detected in STEC strains of human origin (Table 8). Importantly, the *eae* gene was present in only 6% of the ovine strains, compared to 29% of those from cattle and 56% of those from humans. Although none of the ovine STEC strains belonged to serotype O104:H4, ten strains with serotype O104:H7 *stx1* (mostly ST1817) were identified (Fig. 5).

STEC in goats. In 2003, we conducted a study in collaboration with the Faculty of Veterinary Sciences of Madrid and Murcia [19]. Fecal samples from 222 healthy dairy goats (adults and kids) on 12 farms in Spain were screened for the presence of STEC. Non-O157 STEC strains were isolated in 48% of the animals, more frequently from adults and replacement animals than from kids. STEC O157 was not detected. Among 106 STEC caprine strains, 25 serotypes were determined, with the most common being serotypes O5:H–, O76:H19, O126:H8, O146:H21, ONT:H–, and ONT:H21. None of the 106 strains carried *eae*. However, 16% of the caprine STEC strains belonged to serotypes involved in HUS (Table 8). Serotype O104:H4 was not identified among any of the caprine STEC strains.

A longitudinal study was conducted on two dairy farms to investigate the pattern of shedding of STEC in goat herds in the Murcia region [63]. Fecal samples were taken from 20 goat kids once weekly during the first 4 weeks of life and then once every month for the next 5 months of life, and from 18 replacement animals and 15 adults once every month for 12 months. The proportion of samples containing STEC was higher for replacement animals and adults (86 and 79%, respectively) than for kids (25%). About 90% of the STEC colonies isolated from healthy goats belonged to five serogroups (O33, O76, O126, O146, and O166) but the most frequent serogroups of these isolates, except one, were different in the two herds studied. STEC O157:H7 was found in three kids on only one occasion. None of the STEC isolates, except the three O157:H7 isolates, was *eae*-positive. The patterns of STEC shedding in goat kids were variable whereas most of the replacement animals and adults were persistent STEC shedders. The results showed that isolates of STEC O33, O76, O126, O146, and O166 are adapted for colonizing the goat intestine but infection with STEC O157:H7 in goats seems to be transient [63].

STEC in wild animals. In the study of Sánchez et al. [72] fecal samples were collected from 243 wild ruminants, including *Cervus elaphus*, *Capreolus capreolus*, *Dama dama*, and *Ovis musimon*, and examined for STEC using both phenotypic (Vero cells) and genotypic (PCR and PFGE) methods. Fecal samples were collected from animals killed by hunters during 2004 and 2005 in the Extremadura. STEC were isolated from 58 (24%) of the samples and a total of 65 isolates were characterized. The *ehxA* gene was detected in 37 (57%) of the isolates but none contained the *eae* gene. The isolates comprised 12 O serogroups, although 80% were restricted to O2, O8, O128, O146, O166, and O174. The most commonly isolated STEC bacteria, from the O146 serogroup, exhibited a high degree of polymorphism as indicated by PFGE. STEC isolates of serogroups O20, O25, O166, O171, O174, and O176 had not previously been found in wild ruminants. This was the first study to confirm that wild ruminants in Spain are a reservoir of STEC and are thus a potential source of human infection [72].

In another study of Sánchez et al. [74], fecal samples from 212 wild boars were collected (Extremadura, Spain, 2007 and 2008) and examined. STEC O157:H7 and non-O157 were isolated from 7 (3%) and 11 (5%) animals, respectively. *E. coli* O157:H7 isolates belonged to phage types associated with severe human illness: PT14, PT34, and PT54. Indistinguishable PFGE types were found in *E. coli* O157:H7 isolates recovered from a wild boar and from a human patient with diarrhea living in the same geographic area.

Recently, in the LREC-USC, we studied the presence of STEC in populations of deer, boar, and fox (Galicia, 2009–2010). Of the 179 deer that were sampled, STEC were isolated from 97 (54%) stool samples, while 25 (9.5%) of the 262 boars tested were STEC-positive. Among the 260 fecal samples from foxes, STEC were isolated from six animals (2.3%) (unpublished data).

None of the STEC strains isolated from Galician wildlife belonged to serotype O104:H4, nor had the STEC been isolated in previous studies carried out in collaboration with the Faculty of Veterinary Science of Cáceres [72,74].

STEC in aquatic environments. The study of García-Aljaro et al. [31] included a phenotypic and genotypic characterization of 144 STEC strains isolated from urban sewage and animal wastewaters in Barcelona, using a *stx*2-specific DNA colony hybridization method. The STEC strains isolated belonged to 34 different serotypes. Only one O157:H7 strain was positive for the intimin gene *eae*. Forty-one different seropathotypes were determined. On the basis of the occurrence of virulence genes, most non-O157 STEC strains are assumed to be low-virulence serotypes.

Shiga-toxin phages. Shiga-toxin phages were detected by TaqMan qPCR in all the beef samples and in 69% of the salad samples obtained from the city of Barcelona by Imamovic and Muniesa [38]. Stx phages from the samples were propagated in *E. coli* C600, *E. coli* O157:H7, and *Shigella* strains and further quantified. The results showed that 50% of the samples carried infectious Stx phages, isolated from plaques generated by lysis. However, despite the apparent abundance of Stx phages in these samples, they showed acceptable microbiological levels for human consumption based on European and US regulations. The origin of the Stx phages found in the food samples could not be determined. However, high densities of Stx phages have been detected in human and animal environments, pointing to an environmental origin. The significance of this study is that infectious free Stx phages are present in commercial food samples. The presence of Stx phages in food can cause *stx* transduction to the bacterial flora present in the matrices, generating new STEC strains in the samples, perhaps during storage. Similarly, the ingestion of free Stx phages present in food can lead to the conversion of commensal gut bacteria. However there are no reports indicating that *stx* transduction in food is of relevant concern.

Phage typing of STEC O157:H7 strains. Phage typing was used for the epidemiological subtyping of a collection of STEC O157:H7 strains isolated in Spain between 1980 and 1999 [51]. Phage typing was performed using the method of Khakria et al. [43] at the National Center for Microbiology (Madrid, Spain), with the phages provided by The National Laboratory for Enteric Pathogens, Laboratory for Disease Control, Ottawa, Ontario, Canada. The 16 different phages used were capable of identifying 88 phage types. Phage typing distinguished 18 phage types among 171 strains isolated from different sources (67 human, 82 bovine, 12 ovine, and 10 beef-product samples). However, five phage types, phage type 2 (PT2; 42 strains), PT8 (33 strains), PT14 (14 strains), PT21/28 (11 strains), and PT54 (16 strains), accounted for 68% of the study isolates. PT2 and PT8 were the most frequently occurring among human (51%) and bovine (46%) strains. Interestingly, there was a significant association between PT2 and PT14 and the presence of acute pathologies [51].

In another study [73], 46 *E. coli* O157:H7 isolates obtained from the feces of different healthy ruminants (sheep, beef cattle, and red deer) and from unpasteurized goat milk over a period of 11 years (1997–2008) were characterized. All of them

Table 9. Outbreaks of STEC/VTEC in Spain

Place	Year	Type	Seropathotype	No. Afected
Ibiza	1986	British tourists	O157:H7 *stx2*	3 (+ 3 asymptomatic)
Balearic Islands	1994	British tourists	O157:H7 *stx2* PT2	
Álava	1995	Children in countryside	O111:H- *stx1*	13
Fuerteventura	1997	European tourists in four hotels	O157:H7 *stx2* PT2	14 (3 with HUS)
Guipúzcoa	1999	Children in nursery	O157:H7	8 (1 with HUS + 6 asymptomatic)
Guipúzcoa	1999		O157:H7	2 (1 with HUS + 2 asymptomatic)
Barcelona	2000	Children from three schools	O157:H7 *stx2* PT2	175 (6 with HUS)
Lugo	2003	Family outbreak	O157:H7 *stx1 stx2* PT8	3
Lugo	2003	Family outbreak	O26:H11 *vt*	4
Cáceres	2007		O157:H7 *stx2* PT14	3

http://www.usc.es/ecoli/BROTES.html

originated from the Extremadura. An atypical *E. coli* O157:H7 strain (sorbitol-fermenting and β-glucuronidase positive) originating from deer feces was detected. Genes encoding Shiga toxins were present in 69.6% of the isolates, all of which carried only the *stx2* gene. The isolates were from nine different phage types, although 67.4% were restricted to only three: PT14, PT34, and PT54. PT54 was the most prevalent phage type and was detected in isolates from cattle, sheep, and deer. The majority of the isolates were from phage types previously found in strains associated with human infection.

Outbreaks caused by STEC in Spain. Although the situation in Spain seems worrisome, since many animals are carriers of STEC strains that could contaminate food, human STEC outbreaks in Spain are rare (Table 9) [6,58,64,67]. In fact, the last major outbreak was in 2000 in a school in Barcelona. It was caused by serotype O157:H7 [58].

Analytical methods for STEC detection in foods

STEC O157:H7 can usually be readily identified in the laboratory based on its inability to ferment sorbitol or cleave the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide within 24 h, which distinguishes it from other *E. coli*. Some atypical STEC O157:H– can ferment sorbitol but these are rare, except in Germany where they are quite frequent and highly virulent. The detection and identification of non-O157 STEC are, however, more difficult and time-consuming, since these strains do not show special characteristics allowing their ready identification. The best medium for isolating the most virulent STEC strains (including O104:H4, O157:H7, and serotypes belonging to seropathotype B) is cefixime tellurite sorbitol MacConkey agar (CT-SMAC).

Once the serotype of the German outbreak strain became known, molecular methods were developed for its detection based on conventional and real-time PCR [5,24,76]. The protocol used by the LREC-USC was immediately improved, including newly designed primers for the specific detection of serotype O104:H4. Our protocol comprises two methods (A and B) (Tables 10 and 11). Method A is specific for the detection of STEC/VTEC O157:H7 and method B for the detection and isolation of any type of STEC/VTEC (O157:H7 and non-O157, including O104:H4 and others such as O26, O103, O111, O145, and O146). Method B also detects other diarrheagenic groups of *E. coli*. The protocol is based on a PCR using specific primers for the detection of the genes *stx*1 and *stx*2, *rfb*(O104), *rfb*(O157), *fliC*(H7), *fliC*(H4) of STEC/VTEC, and other virulence genes specific for other categories of diarrheagenic *E. coli*.

General recommendations to consumers and conclusions

It is necessary to inform the population of the risk associated with improper food handling and preparation. Specifically,

Table 10. Protocol used at the LREC-USC for detection of STEC/VTEC in food samples

Method A Detection of STEC/VTEC O157:H7	Method B Detection of STEC/VTEC O157:H7 and non-O157 (including O104:H4), EAEC, EPEC, ETEC, EIEC
25 g of food sample 225 ml of buffered peptone water with vancomicine, cefixime and cefsulodin (vccAPT) 37ºC/6 h	25 g of food sample 225 ml of buffered peptone water 37ºC/6 h
Inmunomagnetic separation (IMS) Dynabeads anti-*E. coli* O157	1 ml to 9 ml of MacConkey broth 37ºC/18–24 h and 44ºC/18 h (duplicate)
Isolation from IMS onto: Cefixime Tellurite Sorbitol MacConkey agar (CT-SMAC) 37ºC/18–24 h Sorbitol MacConkey agar (SMAC) 37ºC/18–24 h	Isolation onto: Lactose MacConkey agar (LMAC) 37ºC/18–24 h CT-SMAC agar 37ºC/18–24 h LMAC agar 44ºC/18–24 h CT-SMAC agar 44ºC/18-24h
Detection by PCR of genes encoding shiga-toxins (verotoxins) *stx1*/vtx1 and *stx2/vtx2, rfb*(O157), *fliC*(H7), from the confluence growth of agar plates	Detection by PCR of genes encoding shiga-toxins (verotoxins) *stx1*/vtx1 and *stx2/vtx2, rfb*(O104), *fliC*(H4) and specific virulence genes of EAEC, EPEC, ETEC and EIEC from the confluence growth of agar plates
In case of positive PCR for any of the cited genes, selection of 10 colonies sorbitol negative and analysis by PCR for genes encoding shiga-toxins (verotoxins) *stx1*/vtx1 and *stx2/vtx2*	In case of positive PCR for any of the cited genes, selection of 50 colonies. PCR in pools of 10 colonies, and afterwards individually in case of positive pools
In case of positive PCR for individual colony, confirmation of O157 and H7 antigens by serotyping and PCR.	Determination of O:H serotype of all STEC/VTEC, EAEC, EPEC, ETEC and EIEC detected and isolated

meat, and especially ground meat, must be sufficiently cooked. It is important to avoid the cross-contamination of foods to be eaten raw with those (meat) that have to be cooked; this is best accomplished by separating the two areas of preparation in the kitchen. Food handlers should wash their hands thoroughly following any contact with meats. The proper distribution of food in the refrigerator is important so as to avoid juices dripping from food to be cooked (meat and fish) onto others that will be consumed without heating (salads). Vegetables to be eaten raw in salads should be thoroughly washed. Only milk subjected to a minimum pasteurization heat treatment should be considered safe for consumption.

The discussion in this review allows us to draw the following conclusions:

- To date, the recent (2011) EC outbreak in Germany was the largest reported worldwide in terms of the number of HUS cases.
- The serotype of the outbreak strain, O104:H4, is very rare and only a few human cases have been reported. Furthermore, this serotype has never been detected in animal/food.
- The outbreak strain shows a combination of virulence factors from STEC/VTEC and enteroaggregative *E. coli* (EAEC).
- Comparison studies of the complete genome sequence of various isolates of the German outbreak strain and of African EAEC isolates of serotype O104:H4 suggest that the outbreak strain belongs to an EAEC lineage that acquired genes encoding the Stx2a toxin and antibiotic resistance.
- Our recent study (from June to July 2001) showed that the microbiological quality of Spanish vegetables is quite good. However, one sample (0.6%) was positive for a STEC strain of serotype O146:H21.
- Consistent with data from other countries, STEC belonging to serotype O157:H7 and other serotypes were isolated from beef, milk, cheese, and domestic and wild animals in Spain.
- Although the situation may seem worrisome, human STEC outbreaks in Spain are rare. In fact, the last major outbreak was in 2000, in a school in Barcelona. However, our data suggest that, in Spain, STEC O157:H7 is annually responsible for more than 500 sporadic cases of infection, and non-O157 for more than 2000.

Table 11. Oligonucleotide primers used for PCR detection of STEC/VTEC and EAEC genes

Genes	Primers	Oligonucleotide sequence (5′-3′)	Fragment Size (bp)	Anneling temperature	Reference
STEC/VTEC *stx*$_1$/ *vtx*$_1$	VT1-F VT1-R	TCGCTGAATGTCATTCGCTCTGC TCAGCAGTCATTACATAAGAAC	539	55ºC	This study
STEC/VTEC *stx*$_2$/ *vtx*$_2$	VT2-F1 VT2-F2 VT2-R	TTTCTTCGGTATCCTATTCCC TGTCTTCAGCATCTTATGCAG CTGCTGTCCGTTGTCATGGAA	358	55ºC	This study
STEC/VTEC[a] *stx*$_1$/ *vtx*$_1$ *stx*$_2$/ *vtx*$_2$	VT-F1 VTf-F VT-R-VT1 VT-R1-VT2 VT-R-VT2f	TTGAACAAAATAATTTATATGT TGGAACGGAATAACTTATATGT GCTTCAGCTGTCACAGTAACAA GCTTCTGCTGTGACAGTGACAA GCTTCTGCTATCACTGTGACAA	292	52ºC	This study
wzx-O104	O104-F O104-R	CGTTTAGCCGGAAATGAGAA TGAAACGACACCACTTATTGC	630	58ºC	This study
fliC-H4	H4-F H4-R	GCAGCGTATTCGTGAACTGA GCTGGATAATCTGCGCTTTC	713	66ºC	This study
O157 *rfb*E	O157-AF O157-AR	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	497	55ºC	[21]
*fliC*h7	H7-F H7-R	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625	55ºC	[30]
wzx-wzy O26	*wzx-wzy* O26F *wzx-wzy* O26R	AAATTAGAAGCGCGTTCATC CCCAGCAAGCCAATTATGACT	532	60ºC	[22]
fliC-H11	*fli*CRH11-1 *fli*CRH11-2	ACTGTTAACGTAGATAGC TCAATTTCTGCAGAATATAC	248	54ºC	[22]
STEC/VTEC EPEC *eae*[b]	EAE-V3F EAE-MBR	CATTGATCAGGATTTTTCTGGT TCCAGAATAATATTGTTATTACG	510	55ºC	This study
eae-â1	B1F B1R	CACAATTAATGCACCGGGT GCTTGATACACCTGATGACT	241	55ºC	[12]
eae-ã1	EAE-FB EAE-C1	AAAACCGCGGAGATGACTTC AGAACGCTGCTCACTAGATGTC	804	55ºC	[12]
EAEC pCDV432	pCVD432/start pCVD432/stop	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	60ºC	[77]
Typical EPEC *bfpA*	EP1 EP2	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	326	60ºC	[36]
EIEC *ipaH*	EI1 EI2	GCTGGAAAAACTCAGTGCCT CCAGTCCGTAAATTCATTCT	424	55ºC	[80]
ETEC LT-I *eltA*	LT-A-1 LT-A-2	GGCGACAGATTATACCGTGC CCGAATTCTGTTATATATGTC	696	55ºC	[78]
ETEC STa *est*	STA-1 STA-2	ATTTTTATTTCTGTATTGTCTTT GGATTACAACACAGTTCACAGCAGT	176	48ºC	[12]

[a]Primers for detection of all Shiga-toxin subtypes.

[b]Primers for detection of all *eae* alleles.

- The recent outbreak of STEAEC O104:H4 in Germany had international dimensions and illustrated more than ever the urgent need for a National Reference Laboratory in each of the involved countries as a national focal point for the dissemination and sharing of information and methodology.
- The epidemiologic surveillance of STEC must be reinforced, focusing efforts on the detection of this new hypervirulent strain O104:H4.

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