Shiga toxin-producing *Escherichia coli* in meat and vegetable products in Emilia Romagna Region, years 2012-2013

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Abstract

In 2012-2013 Emilia-Romagna Region introduced a monitoring plan for Shiga toxin-producing *Escherichia coli* (STEC) in foodstuff. Six hundred eighty-nine meat samples and 273 fruit and vegetable products were analyzed according to ISOTS13136. Pre-enriched samples were tested by multiplex real time PCR targeting the virulence genes *eae*, *stx1* and *stx2*. *Stx2* positive samples were investigated for the presence of serogroup O104 associated gene. O103, O111, O145, O157, O26 associated genes were tested on samples positive for *stx* in association with *eae* gene. Isolation of *E. coli* strains was attempted from samples positive for serogroup-associated genes. Thirty-four meat products (4.9%) resulted positive for *stx*1 and/or *stx*2 genes and 46 (6.7%) for *stx*1 and/or *stx*2 genes in association with *eae* gene. Forty-five (6.5%) samples resulted positive at least at one serogroup. Serogroup O103, O104, O111, O145, O157 and *O26* genes were detected respectively in 1.3, 0.3, 0.1, 3.9, 2.9 and 2.5% samples; 0.6% samples resulted positive for STEC isolation (2 *E. coli* O103 and 2 *E. coli* O157). It is worth noting that STEC virulence genes were detected at high frequency (19%) in fresh pork meat sausages. Four (1.5%) vegetable samples were positive for *stx*1 and/or *stx*2 genes and 1 (0.4%) for *stx*1 and/or *stx*2 genes in association with *eae* gene; none resulted positive for the tested serogroups.

Only a low number of samples positive by molecular methods were confirmed by cultural isolation. It is therefore of the uttermost importance for appropriate risk management, to be fully aware of the meaning of the analytical result.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are a group of highly pathogenic *E. coli* strains producing two phage-encoded cytotoxins, called Shiga toxins (Stx1 and Stx2), which are the common feature and main virulence factors of STEC and are directly correlated with human pathogenicity (Lindgren *et al*., 1993). In addition, some STEC strains are also able to attach to intestinal epithelial cells and form *attaching and effacing* lesions through an adhesin called intimin, which is encoded by the *eae* gene. The lower intestinal tract of ruminants is considered to be the main reservoir of STEC. STEC comprises serologically different strains; *E. coli* O157:H7 is the serotype that has been linked to most of the outbreaks of food-borne disease and has led to the largest number of hemolytic uremic syndrome (HUS) cases in humans. However, there is increasing evidence that also other serotypes can cause serious illness in humans (Caprioli *et al*., 1997, 2005). In most human infections, transmission occurs primarily by ingestion of contaminated food: most often inadequately cooked meat, especially minced beef, raw milk and raw milk products and ready to eat (RTE) products as fresh fruit and vegetables. Following *E. coli* O104:H4 outbreak occurred in Germany in May 2011, Emilia Romagna Region instituted in 2012 a testing program for O157, O26, O111, O103, O145 and O104:H4 STEC, both at production and at retail level, for foodstuff considered at risk of contamination.

The aim of this study is to report on STEC detection in Emilia Romagna Region during the 2012-2013 monitoring plan, the extent of contamination in each food category analyzed is also considered.

Materials and Methods

A total of 689 meat and 273 vegetable samples were collected in Emilia Romagna Region from retail outlets, large retailers and processing plants. Meat samples comprised fresh meat pork sausages, minced meat and processed meat products (as skewer, breaded cutlet, roast meat, *etc*.) made of meat from different species here grouped in poultry meat and meat from species other than poultry. Vegetable samples comprised pre-cut fruits and vegetables, seeds and sprouted seeds. All meat samples were to be eaten after cooking while vegetable and fruit samples were all RTE. The sampling procedure was not uniform since the 689 meat samples were composed by 553 samples constituted of 1 sample unit and 136 samples constituted of 5 sample units. Similarly, the 273 vegetable and fruit samples were composed by 143 samples constituted of 1 sample unit and 130 samples constituted of 5 sample units. These samples were analyzed according to ISO TS 13136 (draft version).

Twenty-five g of each sample were diluted ten-fold (w/v) in 225 mL of the recommended enrichment broth and incubated at 37±1°C for 21±3 h. Bacterial DNA was extracted from 1 mL of enriched broth using Gen elute™ bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) as described by the manufacturer. All primers and probes used in this study are reported in ISO 13136:2012 and published previously (ISO, 2012; Perelle *et al*., 2004; Nielsen and Andersen, 2003).

Multiplex real time PCR targeting the virulence genes *eae*, *stx1* and *stx2* was conducted in a 25 µL reaction volume using the following reaction mixture: 1 X Taqman®Universal PCR Master mix (Applied Biosystems, Carlsbad, CA, USA), 450 nM each of the forward and reverse primers, 100 nM of each labeled probe and 4 µL DNA template. A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems) was included in each PCR reaction. Real time-PCR thermal cycling was conducted using a StepOne Plus system (Applied Biosystems). The cycling parameters were: 95°C hold for 10 min for initial denaturation of the DNA and activation of the hot-start Taq polymerase, followed by 40 cycles of amplification of 95°C for 15 s, and 60°C for 60 s.

Samples resulted positive for the presence of *stx*2 gene were tested for *E. coli* O104 serogroup-associated gene. Sample positive for the presence of *stx*1 and/or *stx*2 in association with *eae* gene were tested for the detection of *E. coli* O103, O111, O145, O157, O26, serogroup-associated genes. Serogroup specific PCR reactions were conducted in a 25 µL

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reaction volume using the following reaction mixture: 1 X Taqman®Universal PCR Master mix (Applied Biosystems), 900nM each of the forward and reverse primers, 250 nM of the labeled probe and 4 µL DNA template. The PCR instrument and program were the same used for the previous reaction.

When serogroup-associated genes were detected, the isolation of the strain from the enrichment sample broth was attempted. Enriched samples were plated on Tryptone Bile X-Glucuronide (TBX) agar and incubated for 18-24 h at 37±1°C. Up to 50 colonies with *E. coli* morphology were picked up and point-inoculated on Nutrient agar (NA). Pools of 10 colonies were tested by real time PCR for the presence of virulence genes *eae*, *stx1* and *stx2,* afterward colonies from positive pools were tested singularly in order to identify STEC strain. In the case of positive molecular detection of virulence genes, and lack of cultural isolation of the strain, the sample is suspected of containing a STEC (presumptive positive).

In the subsequent data analysis, samples constituted by 5 sample units were counted as one sample. If all the sample units resulted negative, the sample was classified as negative, if at least one sample unit resulted positive the sample was classified as positive.

Results

Eighty (11.6%) meat samples resulted presumptively positive for STEC; among them 34 (4.9%) resulted positive for *stx*1 and/or *stx*2 genes and 46 (6.7%) for *stx*1 and/or *stx*2 genes in association with *eae* gene. Fourty-five (6.5%) samples resulted positive at least at one serogroup (Table 1).

Serogroup O103, O104, O111, O145, O157 and O26 associated genes were detected respectively in 9 (1.3%), 2 (0.3%), 1 (0.1%), 27 (3.9%), 20 (2.9%) and 17 (2.5%) meat samples (Table 2). It is worth noting that STEC virulence genes were detected in 41 out of 213 (19%) fresh sausages from pork meat. STEC were isolated from 4 (0.6%) meat samples: one strain of *E. coli* O103 *eae* + *stx1*+ was isolated from fresh meat pork sausage, one strain of *E. coli* O157 *eae*+ *sxt1*+, *stx2*+ was isolated from bovine minced meat, one *E. coli* O157 *eae*+ *stx2*+ and one *E. coli* O103 *eae* + *stx1*+ were isolated from bovine hamburgers.

Five (1.8%) vegetable samples resulted presumptively positive for STEC; among them 4 (1.5%) samples (2 samples of salad and 2 samples of soya beans) were positive for *stx*1 and/or *stx*2 genes and 1 (0.4%) sample of salad resulted positive for *stx*2 in association with *eae* gene; no serogroup-associated genes were detected (Table 3).

Discussion

This study reports on a two year STEC monitoring plan carried out in Emilia Romagna Region. Reported results show that 80 of 689 (11.6%) meat samples and 5 of 273 (1.8%) vegetable samples were presumptively contaminated by STEC. Cultural method allowed isolation of only 4 STEC strains, out of 689 meat samples tested.

In case of STEC, since the indirect evidence of the virulence genes presumptively determines the presence of the bacteria, the isolation of the strain is needed to confirm the presence of *stx* genes in addition to relevant virulence factor in the same live cell whilst excluding the presence of free DNA or free *stx* phages in the enrichment culture (EFSA, 2013). This step can delay identification because of difficulties in developing culture media specifically or differentially allowing the growth of STEC (EFSA, 2013), but is needed as molecular methods could overestimate the real STEC

Table 1. Detection of virulence genes in meat and processed meat products according to the type of foodstuff analyzed.

Foodstuff		Number of tested samples	Positive for *stx*1 and/or *stx2*	Positive for *stx1* and/or *stx2* with *eae*	Positive for one or more serogroups
Fresh meat pork sausages		213	15	26	27
Minced meat and processed meat products	Poultry	208	1	3	3
	Other animal species	268	18	17	15
Total (%)		689	34 (4.93)	46 (6.68)	45 (6.53)

The number of samples resulted positive at least at one serogroup is also reported.

Table 2. Detection of serogroup-associated genes in meat and processed meat products according to the type of foodstuff analyzed.

Foodstuff		O103	O104	O111	O145	O157	O26
Fresh meat pork sausages		6	2	-	16	12	5
Minced meat and processed meat products	Poultry	-	-	1	2	2	-
	Other animal species	3	-	-	9	6	12
Total (%)		9 (1.31)	2 (0.29)	1 (0.15)	27 (3.92)	20 (2.90)	17 (2.47)

Table 3. Detection of virulence genes in fruit and vegetable products according to the type of foodstuff analyzed.

Foodstuff	Number of tested samples	Positive for *stx1* and/or *stx2*	Positive for *stx1* and/or *stx2* with *eae*	Positive for one or more serogroups
RTE fruits and pre-cut vegetables	228	2	1	-
Seeds and sprouted seeds	45	2	0	-
Total (%)	273	4 (1.5)	1 (0.4)	-

RTE, ready-to-eat. The number of samples resulted positive at least at one serogroup is also reported.

contamination.

The level of contamination observed in this study is in line with what has been previously found in other countries (Fantelli and Stephan, 2001; Pradel *et al*., 2000) and with EU data reported by EFSA (EFSA, 2011). On the contrary, Rantsiou *et al*. (2012) recently reported 70% presumptive positivity and 27% STEC prevalence in meat samples from Piedmont Region. This discrepancy could be due to different types of meat samples included in the analysis.

Even if cattle are considered the major reservoir of STEC, what emerges from our data is that pig could also have a role. Indeed, STEC virulence genes were detected at high frequency (19%) in fresh sausage made of pork meat and one *E. coli* O103 strain was also isolated.

Vegetable samples showed low prevalence of presumptive STEC, strain isolation was not attempted since serogroup-associated genes were not detected. Nevertheless, as reported by EFSA, any RTE product contaminated with an isolate of STEC of serogroup O157, O26, O103, O111, O104 in combination with *stx* and *eae* genes should be considered as presenting a potentially high risk for diarrhea and HUS, but for any other serogroups in combination with the same genes, the potential risk is regarded as high for diarrohea and currently unknown for HUS (EFSA, 2013). Our results show that only a sample of salad on 273 might be presumptively included in the latter category.

Conclusions

Since different types of food are characterized by different risk of being a STEC vehicle to humans (*i.e.*, food to be cooked before consumption *vs* RTE), the evaluation of risk assessment associated with a positive test result should always take into account the type of food involved.

The potential contribution in the epidemiology of human STEC infection related to possible reservoir other than bovine should be further investigated, as highlighted by the high frequency of virulence genes detection and the isolation of one verocytotoxic *E. coli* O103 strain from pork meat.

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