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Detection of Shiga-like toxin producing *Escherichia coli* from raw milk cheeses produced in Wallonia

Jacques Vivegnis, Mohamed El Lioui, Alexandre Leclercq, Bernard Lambert, Jacques Decallonne

Unité de Microbiologie. Faculté des Sciences agronomiques. Université catholique de Louvain. Place Croix du Sud, 2/12. B–1348 Louvain-la-Neuve (Belgique). E-mail : vivegnis@mbla.ucl.ac.be

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Shiga-like toxin *Escherichia coli* (STEC) implicated in aqueous diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome, has become a serious health problem in various countries. In Belgium, all cases are sporadic and no outbreak has been detected so far. Cattle are thought to be a reservoir for *E. coli* O157:H7, and many foodborne diseases have been associated with the consumption of minced beef, beefburgers and raw milk. Recently, foodborne outbreaks were concerned with different unusual foods such as acidic products. Although some data suggest that STEC are not prevalent within dairy products, the aim of this work was to assess the prevalence of *E. coli* O157 and non-O157 STEC in raw milk cheeses produced in the Southern part of Belgium (Wallonia). For this purpose, 153 frozen samples of soft and semi-soft cheeses made with raw cow, ewe and goat milk were analysed for the presence of *E. coli* O157 and STEC. By using a dynabeads immunomagnetic separation technique (Dynabeads® anti-*E. coli* O157, Dynal) followed by streaking onto sorbitol MacConckey agar, no sample was found contaminated by *E. coli* O157 serotype. By using polymerase chain reaction achieved from a loopful of confluent bacterial material growing onto MacConckey agar, the use of consensus primers detected *stx* genes in 11.1% of the samples but Shiga-like toxin producing strains could be isolated only in five of them (3.3%). The isolation rate seems to be optimum for samples with a thermotolerant coliform count arround or below 10^2 cfu g^{-1}. The five Shiga-like toxin isolates were identified as belonging to the species *Hafnia alvei* or *Enterobacter amnigenius* without any accessory virulence factors needed to cause illness. Nevertheless, because of the ability of STEC to survive adverse conditions and the possibility for commensal non-pathogenic enteric bacteria to become pathogenic, raw milk cheeses are to be considered at risk for foodborne STEC contamination.
Keywords. Raw milk cheese, *Escherichia coli*, O157, Shiga-like toxin, STEC, Wallonia (Belgium).

Détection d'*Escherichia coli* producteurs de Shiga-toxines dans des fromages artisanaux fabriqués au lait cru en Région Wallonne. Depuis leur première description comme agent responsable de toxi-infection alimentaire (1982), les souches d'*Escherichia coli* O157:H7 et autres *E. coli* entérohémorragiques sont connues pour être les principaux agents infectieux responsables de diarrhées hémorragiques qui peuvent s'accompagner de complications sévères atteignant le système rénal. Ces *E. coli* produisent une ou plusieurs Shiga-toxines (Stx) ou Véro cytotoxines (STEC). Les bovins et autres ruminants représentent le réservoir principal des STEC et de nombreuses épidémies ont été associées principalement à la consommation de viande de boeuf et de lait cru, mais également à du cidre de pomme et divers produits laitiers. En ce qui concerne plus particulièrement les fromages produits au lait cru les données disponibles concernant la prévalence de ces germes sont peu nombreuses. Dans cette optique, l'objectif du travail visait à évaluer la présence d'*E. coli* O157 et de STEC dans des fromages artisanaux produits en Région Wallonne (Belgique). L'étude a porté sur l'analyse de 153 échantillons de fromages à pâte molle et demi-dure produits au lait cru, conservés sous forme congelée à -20 °C. La mise en évidence d'*E. coli* O157 a été réalisée après concentration immunomagnétique (Dynabeads® anti-*E. coli* O157, Dynal) suivie d'un isolement sur gélose MacConckey au sorbitol. L'identification des souches sorbitol négatives a été réalisée à l'aide de galeries biochimiques (API 20E) et de tests complémentaires (production d'indole, absence de ß-glucuronidase et test de Klieger). Aucun échantillon n'était contaminé par *E. coli* O157. La recherche de STEC a été effectuée par amplification génomique (PCR) à partir d'un écouvillonnage de colonies cultivées sur agar MacConckey. Elle a été réalisée à l'aide d'amorces dégénérées amplifiant les gènes *stx*. Ces gènes ont été retrouvés dans 11,1 % des échantillons mais les souches productrices de Shiga-toxines ont pu être isolées pour cinq échantillons seulement (3,3 %). Le taux d'isolement semble être optimum pour les échantillons ayant une charge en coliformes thermotolérants proche ou inférieure à 10^2 cfu g^{-1}. Les cinq souches Stx

positives ont été identifiées à *Hafnia alvei* ou *Enterobacter amnigenius*. Elles ne possédaient pas les facteurs de virulence accessoires qui leur conféreraient un caractère pathogène. Néanmoins, étant donné d'une part la capacité des STEC à survivre dans des conditions défavorables (pH acide, température de réfrigération, faible activité en eau, etc.) et d'autre part la possibilité pour des souches commensales d'acquérir un pouvoir pathogène, les fromages fabriqués au lait cru sont à considérer comme un risque potentiel de toxi-infection alimentaire par des *E. coli* producteurs de Shiga-toxines.
Mots-clés. Fromages au lait cru, *Escherichia coli*, O157, Shiga-toxine, STEC, Wallonie (Belgique).

1. INTRODUCTION

Shiga-like toxin producing *Escherichia coli* (STEC) has recently been recognised as a new pathogen for man, causing diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). There is an increasing number of cases and outbreaks of *E. coli* serotype O157:H7 infection, many of which are foodborne associated. Food implicated are of bovine origin, in particular minced beef, beefburgers and raw milk. However, several recent foodborne outbreaks were concerned with different unusual foods as acidic foods (e.g. fermented dairy products, apple cider), cantaloupe and salad vegetables (Feng, 1995; Reilly, 1998).

Although the incidence of *E. coli* O157:H7 with cheese-associated outbreaks seems to be very low in the United States (Altekruse *et al.*, 1998), raw milk cheeses have been associated to some food poisoning in Europe (Ammon, 1997). In the French outbreak occurring in 1992–1993 (Deschênes *et al.*, 1996), the serotype responsible was a non-O157 (O103:H2) and the case control study showed that the occurrence of HUS was linked to the consumption of cheese made with unpasteurised mixed cow and goat milk.

In contrast to North America, the United Kingdom and Germany, the epidemiological Belgian data indicate that only one fourth of STEC strains isolated in hospitals belong to serogroup O157 (Pierard *et al.*, 1994). In a Belgian study (Acheson, Keutsch, 1996), 1% among the 10,241 stool specimens were positive for STEC. In that positive subset, 38% were O157:H7 but 62% were non-O157 serotypes. One patient in the non-O157 group presented haemorrhagic colitis and another one TTP. STEC outbreaks were reported outside Europe. For instance, an outbreak due to an O111 strain led to 23 cases of HUS and one death in Australia. In Japan, O111 and O145 serogroups have caused outbreaks (Acheson, Keutsch, 1996). Therefore it seems careful to assume that any food contaminated with Shiga-like toxin *E. coli* and which possess accessory virulent factors could be at risk for public health.

In a previous study (Vivegnis *et al.*, 1998), we evaluated the bacteriological quality of raw milk cheeses produced in the Southern part of Belgium (Wallonia). Results from bacteriological analysis of 153 cheese samples were compared to food microbiological criteria. The pathogens of concern were *Salmonella* and *Listeria monocytogenes*. About 37% of the samples studied showed *Enterobacteriaceae* count exceeding 10^5 cfu g^{-1}, and 13% exhibited thermotolerant coliforms counts over 10^5 cfu g^{-1}. The aim of the present study was to assess the prevalence of *E. coli* O157 and non-O157 STEC serotypes in raw milk cheeses produced in Wallonia.

2. MATERIALS AND METHODS

2.1. Sample collection

One hundred fifty-three samples of soft and semi-soft cheeses made with raw cow, ewe and goat milk were analysed for the presence of *E. coli* O157 and STEC. Samples had previously been assayed for general microbiology quality (Vivegnis *et al.*, 1998) and then frozen at -20°C during a minimum of six months to a maximum of one year. The day before analysis, samples were thawed overnight at 4°C.

2.2. *E. coli* O157 detection

A 10-g portion of cheese sample was added to 90 ml of modified trypticase soya broth (mTSB; Oxoïd) containing novobiocin (20 mg l^{-1}) and thoroughly homogenized in a stomacher (Lab-Blender 400, Seward UAC House). The homogenates were incubated at 42°C for 6 h without shaking.

Immunomagnetic separation (IMS) was used to concentrate O157 serotype (Dynabeads® anti-*E. coli* O157; Dynal™). The IMS procedure was performed according to the manufacturer's instructions using 1 ml of the 6 h enrichment culture added to 20 µl of Dynabeads. Avolume of 50 µl resuspended Dynabeads was used to streak the sorbitol MacConckey agar (Oxoïd) containing potassium tellurite 2.5 mg l^{-1} and cefixim 0.05 mg l^{-1} (TC-SMAC). Plates were further incubated at 37°C for 24 h. Sorbitol non-fermenting colonies were purified and then biochemically identified using API 20E (bioMérieux) and complementary tests (indole production, Klieger fermentation, ß-glucuronidase activity). The final confirmation was performed by detecting virulence factors by genetic amplification. When applied to raw milk cheese, the detection rate of this method is about 20 cfu 25 g^{-1} (Vernozy-Rozand, 1997).

2.3. Detection of STEC strains by polymerase chain reaction (PCR)

The protocol used was described by Pierard and co-workers (1997). The enrichment medium (1:10 dilution from 10 g cheese) was MacConckey broth (Oxoïd). After blending the sample in a stomacher, the incubation was conducted at 37°C during 24 h. After subculture onto MacConckey agar, a loopful of confluent bacterial material was suspended in sterile water and heated at 100°C during 10 min to release DNA. PCR was directly performed using consensus primers amplifying the Shiga-like toxin *stx* genes (Karch, Meyer, 1989). For each PCR-positive sample, a maximum of 20 colonies obtained on the MacConckey agar plate was tested separately in order to isolate STEC strains. Positive *stx* consensus PCR isolated colonies were subsequently identified through biochemical tests (API 20E, bioMérieux) and complementary tests (indole production, Klieger test, ß-glucuronidase activity). Virulence factors (*stx*1, *stx*2 gene) were finally detected in cheese isolates by the PCR procedure of Pollard *et al.* (1990); *eaeA* gene was identified by Gannon PCR (Gannon *et al.*, 1993).

2.4. Numeration of thermotolerant coliforms and *E. coli*

In order to study the relationships within the enteric flora, the numeration of thermotolerant coliforms and ß-glucuronidase positive *E. coli* was carried out throughout the non incubated MacConckey homogenate. It was performed by using Petrifilm™ *E. coli* (3M Products) incubated during 24 h at 44°C. Moreover, in order to analyse the effect of long freezing storage on the survival of STEC, the specific numeration of thermotolerant coliform group was achieved and compared with results obtained from a previous study (Vivegnis *et al.*, 1998).

3. RESULTS AND DISCUSSION

3.1. Freezing influence

The effect of freezing/thawing on the number of thermotolerant coliforms gives some information about the survival of STEC strains in frozen cheese samples. Generally, the counts after freezing were significantly lower than those before freezing. In some instances, the decrease exceeded five log units.

These observations can partly be explained by the fact that the two numeration procedures are not totally equivalent. Both methods use colony count with violet red bile lactose agar incubated at 44°C, but by using Petrifilm™ we detect lactose fermenting colonies which produce acid and gas, the former technique (V 08-060; AFNOR, 1996) detecting acid producing colonies with or without gas production.

Another factor that can explain the underestimation after freezing is the lack of a ressuscitation step before inoculation. It is well know that when microorganisms are subjected to environmental stresses such as freezing, many of the individual cells undergo metabolic injuries, resulting in their inability to form colonies on selective media that uninjuried cells can tolerate. In a few instances, the number of viable cells found would be lower than the actual number by a factor of three log units (Jay, 1996).

As far as serotype O157:H7 is concerned, a good survival rate has been found either in ground beef (Doyle, Schoeni, 1984), or in raw milk (Ansay, Kaspar, 1997) during storage at -20°C. In the latter study, raw milk inoculated with 10 cfu ml^{-1} and further kept at -20°C displayed detectable *E. coli* O157:H7 after 63 days of storage. The ability of STEC strains to survive adverse conditions, including low pH, low water activity, refrigerated storage seems to be higher than for non-STEC strains (Guraya *et al.*, 1998; Rigsbee *et al.*, 1997).

3.2. *E. coli* O157 detection

About 35 % of cheese samples showed thermotolerant coliform contamination while 23% contained ß-glucuronidase positive *E. coli* (**Figure 1**). In some cases, *E. coli* level was quite high, exceeding 10^5 cfu g^{-1}. Retail surveys on soft and semi-soft cheese have demonstrated *E. coli* for 34 % of the samples (Ansay, Kaspar, 1997). It may therefore be assumed that *E. coli* represents a natural flora of dairy products and that some contamination with *E. coli* is likely to happen during the production and/or processing steps of the cheese.

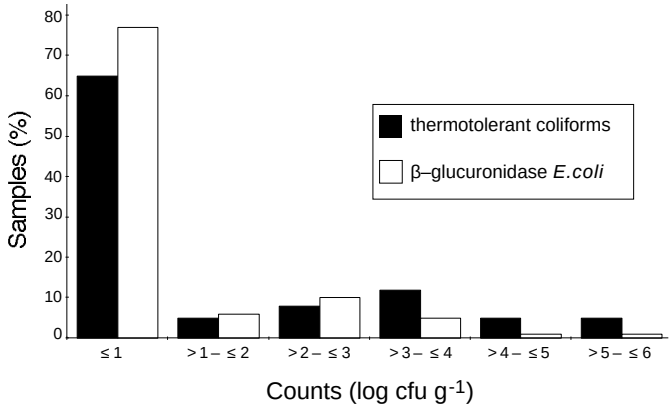

Figure 1. Thermotolerant coliforms and *E. coli* distribution in the samples— *Distribution des coliformes thermotolérants et des* E. coli *dans les échantillons*.

None of the 153 cheese samples was contamined by *E. coli* O157 by using IMS, although one sample gave a typical non-sorbitol feature on TC-SMAC but this isolate was further identified as *Hafnia alvei*. The used IMS procedure was also found somewhat difficult to carry out on cheese samples due to the fatty matrix interfering with the settling of the beads during the washing steps. To compare detection level of two *E. coli* O157 immunologic methods, Vernozy-Rozand and co-workers (1997) proved that IMS was less sensitive than VIDAS™ technique when applied to raw milk cheeses.

In the same way, Ansay and Kaspar (1997) did not isolate *E. coli* O157:H7 from any of the 69 soft and semi-soft cheeses dealing with Blue, Camembert, Brie, Muenster, Colby, Havarty and Monterey Jack. Unfortunately, the nature of milk treatment was not mentioned in the study, but it may be assumed that some of the cheeses tested were made from pasteurized milk. Furthermore, the lack of data on the prevalence of O157:H7 within dairy processing led them to analyse 1,104 environment samples (e.g. equipment surfaces, floors, utensils, aprons, shoes and worker's hand, etc.). All those samples tested were found negative for *E. coli* O157:H7 indicating that this serotype was not widely disseminated in dairy ingredients and processing environments and that it poorly survives, or is readily eliminated by sanitation practices.

In a risk assessment study, Reitsma and Henning (1996) followed the survival of *E. coli* O157:H7 during the production of Cheddar cheese. Cheeses spiked with 10^3 cfu ml^{-1} of milk showed viable *E. coli* O157:H7 in 25 g of food after 158 days of storage at 2°C. At an initial contamination level of 1 cfu ml^{-1} milk, viable *E. coli* O157:H7 was still detected after 60 days. They concluded that this organism can survive and even grow during Cheddar cheese-manufacturing process. On pasteurized process sliced cheese, a storage at elevated temperature (30°C) can also support the survival, but not the growth, of this serotype (Glass *et al.*, 1998) since O157:H7 populations decreased on an average by 2.1 log cfu ml^{-1} during 36 h, then remained unchanged through 96 h. For cottage cheese (Guraya *et al.*, 1998), Camembert and Feta cheeses (Ramsaran *et al.*, 1998), the same conclusions could be observed. The results of these studies suggest that salt, pH, temperature and storage time would interact to increase the inhibition of *E. coli* O157:H7 although some data tend to prove that *E. coli* O157:H7 may persist in dairy products until the time of consumption.

3.3. STEC detection

Among the most important virulence characteristics of STEC strains is their ability to produce one or more Shiga-like toxins (Stx). Shiga-like toxin *E. coli* strains have been shown to produce alone, or in combination, either Stx1, or Stx2 and Stx2v variants toxins. Production of Shiga-like toxins is not sufficient to cause disease since other factors are thought to contribute to the virulence of enterohaemorrhagic *E. coli* including a 60 MDa virulence plasmid and a pathogenicity island called LEE (locus of enterocyte effacement) that encodes proteins, such as intimin (encoded by *eaeA*), involved in attaching and effacement (Feng *et al.*, 1998). The 60 MDa plasmid encodes a enterohaemolysin that, when associated with specialised transport systems, may allow STEC to use blood released into the intestine as a source of iron (Mead, Griffin, 1998). Since those toxins are the main virulence factors of STEC, the use of PCR consensus primers amplifying genes *stx1, stx2* and its variants (*stx2v*) may be considered as an efficient screening method.

Stx genes were detected in 17 cheese samples (11.1%), but Shiga-like toxin producing strains could be isolated only from five of them (samples 02, 10, 49, 87 and 89). According to Pierard and co-workers (1997), this low isolation level can probably be related to the loss of *stx* genes in vitro by some STEC strains or to an unfavourable proportion of STEC *versus* other *E. coli* strains. In our study, it is very likely that thermotolerant coliforms were detected and not only *E. coli* flora (**Figure 2**). Except for samples 74 and 90, a thermotolerant coliform count arround or below 10^2 cfu ml^{-1} resulted into Shiga-like toxin producing strain isolation. The absence of ß-glucuronidase positive *E. coli* is not fully correlated with isolation rate (e.g. samples 74, 90, 92, 96, 118, 132, 144). As the *Enterobacteriaceae* count was high for all of the five samples which allowed isolation, this global flora does not seem to have any direct influence on the isolation rate. To overcome this difficulty, colony blot or DNA/DNA hybridisation assay can be use to detect and isolate STEC (Padhye, Doyle, 1992).

Biochemical identification of the five Shiga-like toxin producing isolates resulted either in *Hafnia alvei* (samples 02, 10, 87 and 89), or into *Enterobacter amnigenius* (sample 49). None of these five strains involved *stx2* genes, and by amplification of *stx1* a fragment located above the characteristic band was produced. Detection of *eaeA* gene gave a lower aspecific fragment. Several cases of non-*E. coli* Shiga toxin-producing infections are now documented (Acheson, Keusch, 1996). In one case reported in 1995, a patient in Australia with severe diarrhoea symptoms that led to HUS was infected with a strain of Stx2 producing *Enterobacter cloacae*. Another outbreak in Germany involved a strain of *Citrobacter freundii* that could express Stx2, and some patients infected with this organism developed HUS. Sandwiches prepared with green butter added with

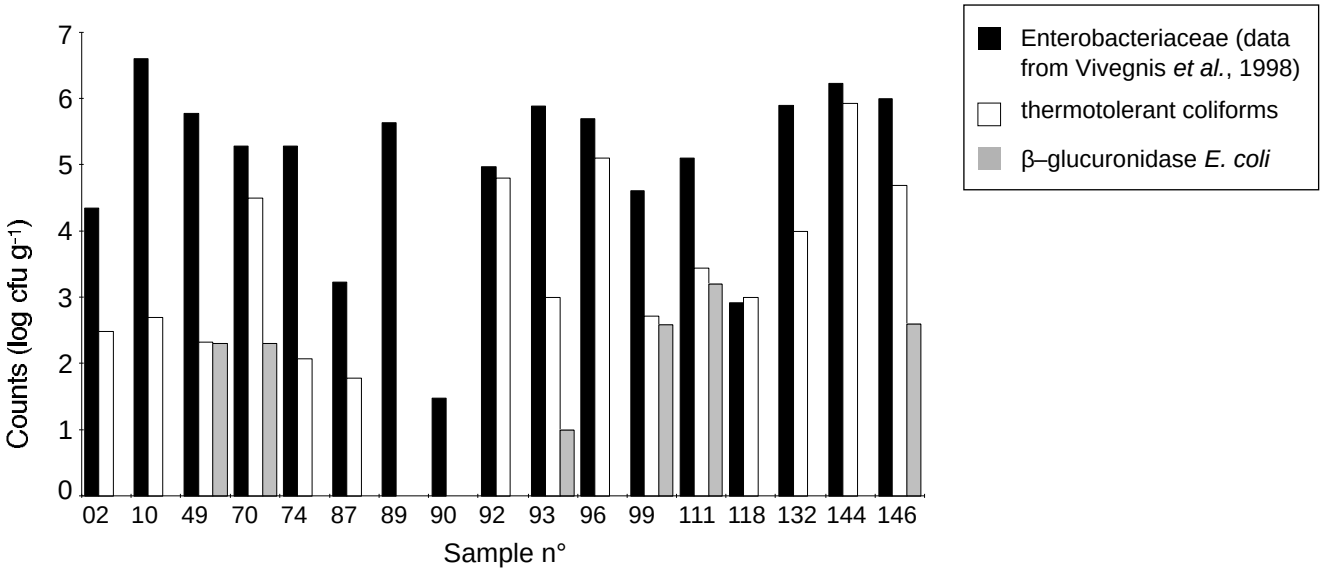

Figure 2. Positive samples by consensus PCR — *Échantillons positifs par PCR*.

Shiga-like toxin isolates are originating from samples 02, 10, 49, 87, 89 — *Les souches productrices de Shiga-toxines ont été isolées des échantillons 02, 10, 49, 87, 89.*

contaminated parsley were suspected to be the infection vehicle.

C. freundii is frequently reported as an environmental species and as a nosocomial agent with a broad range of virulence factors including Shiga-like toxins (Tschäpe *et al.*, 1995). *C. freundii* is closely related to *E. coli* and *C. freundii* strains may be considered as good recipients for horizontal gene transfer. In the same way, it could also be possible that some other species belonging to *Enterobacteriaceae* family (e.g. *H. alvei* or *E. amnigenius*), can partly display the same proprieties as the German *C. freundii* strain. However, the characterization of the virulence factors on the former isolated strains tends to prove that they possess variant toxins with a lower pathogenicity, since most patients developing HUS are infected with strains harbouring the *stx2* type gene (Caprioli *et al.*, 1995).

These results are in agreement with those of Quinto and Cepeda (1997) who analysed soft cheeses made with raw (n=221) and pasteurized (n=75) cow milk for toxigenic *E. coli*. Detection was based on cytotoxicity tests performed on Vero cells. One raw milk sample was positive for STEC. The serogroup was O2 which has already been reported as being responsible for several HUS cases.

4. CONCLUSIONS

Bovine products have been mostly implicated in foodborne infections with *E. coli* serotype O157:H7. However, recent outbreaks indicate that other food types may also be considered as vehicles of transmission for this pathogen. It can be underlined that acidic food that were once thought to be of low risk can no longer be considered safe because of the acid-tolerant proprieties of this pathogen. This survey was unable to demonstrate the presence of either *E. Coli* O157, or potentially pathogen STEC in raw cheese samples. Nevertheless, because of the high adaptability level of commensal non-pathogenic enteric bacteria to acquire virulence factors or to express silent genes (Germani, 1996), we must keep in mind that raw milk cheese may represent a hazard of enterohaemorrhagic food poisoning. Therefore the initial level of contamination is critical in determining product safety, and the dairy industry, as the public health authorities must ensure that all safety measures to prevent entry and multiplication of such a pathogen are applied in cheese-manufactering plants. Advisable rules for prevention and control must be based on good hygienic practices and will be best carried out through the implementation of the HACCPprocedure.

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