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REVIEW

Raw milk and raw milk cheeses as vehicles for infection by Verocytotoxin-producing *Escherichia coli*

CHRISTOPHER L BAYLIS*

Campden BRI, Microbiology Department, Chipping Campden, Gloucestershire GL55 6LD, UK

Raw milk and raw milk cheeses can be a source of food-borne pathogens, including Verocytotoxin (Shiga toxin)-producing Escherichia coli *(VTEC/STEC). Outbreaks of VTEC O157: H7 infections have been attributed to the consumption of raw milk and associated dairy products. Although the general prevalence of VTEC O157 in raw milk and raw milk cheeses is low, it can be higher for non-O157 VTEC. The clinical significance of many of these VTEC is unclear, although some are associated with disease. Studies show that* E. coli *O157 strains can survive the various stages of the cheesemaking process and that raw milk and raw milk cheeses remain a potential vehicle for VTEC infections.*

Keywords *Escherchia coli*, Raw cheese, Raw milk, Verocytotoxin.

INTRODUCTION

Many of the common enteric pathogens such as *Salmonella*, *Escherichia coli* O157: H7 and *Campylobacter* are carried in the intestinal tract of ruminants, including domestic animals used in milk production, e.g. cows, sheep and goats. Preventing faecal material contaminating the milk is an important step in reducing the prevalence of pathogens entering raw milk. Effective cleaning procedures, including removing faecal material from udders prior to milking, can reduce the risk, although heat treatment of raw milk is the most important process used to eliminate the risk from viable vegetative pathogenic bacteria and provide a safe product.

Raw milk can be a significant source of food-borne pathogens, and there have been numerous food-poisoning outbreaks associated with direct consumption of raw milk, milk that has been inadequately heat-treated, or milk that has been re-contaminated after heat treatment. The presence of pathogens in milk is likely to arise from contamination by faecal material during the milking process. Good hygiene, including the removal of faecal material from udders and ensuring a clean environment, is therefore important. Contaminated milking parlour equipment and floors can facilitate the spread of these pathogens to the udders; subsequently, milking equipment including teat cups, pipelines, filters and bulk storage vessels can become colonized (O'Loughlin and Upton 2001). Failure to implement effective cleaning and disinfection procedures can result in contamination of subsequent batches of product.

Besides the risks associated with consuming raw milk there are concerns over the safety of cheeses made from raw milk. Although cheese can be made safely with raw milk, there have been food-poisoning outbreaks linked to raw milk cheeses caused by *Salmonella*, *Campylobacter*, *Staphylococcus aureus* and *E. coli* O157: H7 (Zottola and Smith 1991; De Buyser *et al.* 2001). Nevertheless, raw milk remains popular for cheesemaking because of enhanced organoleptic properties, notably the flavour it imparts to the final product. In Europe, cheese manufacture is controlled by the Food Hygiene Regulations (European Commission 2004a, 2004b) and cheeses are generally considered to be one of the safest foods consumed. However, dairy products including cheese can occasionally contain pathogenic bacteria. The most dangerous of the vegetative pathogens associated with raw milk and raw milk cheeses are the Verocytotoxin-producing *E. coli* (VTEC), especially VTEC O157: H7, which is the topic of this review.

Verocytotoxin-producing *E. coli*

Escherichia coli is a common Gram-negative, nonspore-forming bacterium belonging to the family Enterobacteriaceae. It is found in the gastrointestinal tract of man and other animals, although it can be found in water, soil and food, often as a result of faecal contamination. Because of this association, *E. coli* is used as an indicator of potential faecal contamination of food and water (Baylis and Petitt 1997). Most *E. coli* strains are harmless commensal organisms; however, pathogenic strains have evolved which are responsible for distinct types of clinical disease in man.

There are two recognized groups of pathogenic *E. coli*. Extraintestinal pathogenic *E. coli* (ExPEC) represent *E. coli* associated with urinary tract infections and newborn meningitis, whilst the intestinal pathogenic *E. choli* (IPEC) are responsible for a

*Author for correspondence.
E-mail: c.baylis@campden.co.uk

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range of diarrhoeal diseases. Within the IPEC there are currently six distinct groups of *E. coli* that are associated with food-borne disease: VTEC, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC) and the diffusely adherent *E. coli* (DAEC). These diarrhoeagenic *E. coli*, the associated virulence mechanisms and their role in disease are described elsewhere (Sussman 1997; Nataro and Kaper 1998; Donnenberg 2002; Baylis *et al.* 2006).

The VTEC which are also commonly referred to as Shiga-toxin producing *E. coli* (STEC), comprise over 400 serotypes of *E. coli* (Scheutz and Strockbine 2005). The common feature which defines this group is their ability to produce a distinct range of toxins termed Verocytotoxins (VT) which show potent cytotoxicity against Vero cells (Konowalchuk *et al.* 1977). Owing to the similarity in structure and biological activity of these toxins and the toxin produced by *Shigella dysenteriae* type 1 (O'Brien and Holmes 1987), they are also termed Shiga toxins (Stx). Two antigenically distinct types of VT (VT1 and VT2) were initially identified (Scotland *et al.* 1985), although there are now at least three subtypes of VT1 (VT1, VT1c and VT1d) and six subtypes of VT2 (VT2a, VT2c, VT2d, VT2e, VT2f and VT2g) recognized (EFSA 2007). The genes for these toxins (*vtx/stx*) are generally encoded by prophages of the lambda (λ) family, and VTEC strains may produce either VT1 or VT2 alone or together. The most common combination of toxin types associated with severe human disease are VT2 and VT2c (Friedrich *et al.* 2002; Persson *et al.* 2007). In contrast, VT1c and other toxin types (e.g. VT2e, VT2f and VT2g) are commonly associated with milder disease or are rarely associated with human disease (Friedrich *et al.* 2003; Beutin *et al.* 2007).

Clinical manifestations of infection with VTEC range from asymptomatic carriage through mild diarrhoea to life-threatening conditions such as haemorrhagic colitis (HC) and the severe complications of haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP), which can result in kidney damage, renal failure and even death (Tarr *et al.* 2005). Some VTEC, notably those belonging to serotype O157: H7, are associated with severe disease. Besides toxin production, some VTEC have the ability to cause attaching and effacing (A/E) lesions on epithelial cells using similar mechanisms to those found in EPEC. The process of A/E is mediated by a pathogenicity island termed the locus of enterocyte effacement (LEE) which is an important cluster of genes in the bacterial chromosome involved in this process. The LEE encodes a type III secretion system and the *eaeA* gene codes for the outer membrane protein intimin, which mediates binding of the bacterium to the host cell surface. Strains carrying *eaeA* are often associated with more severe forms of disease. Additional virulence factors can be carried on large plasmids found in some VTEC. In VTEC O157, a large (*ca* 92 kb) plasmid termed pO157 carries the gene *hly/ehxA* which encodes for enterohaemolysin, *katP* for catalase-peroxidase and *espP* which codes for an extracellular serine protease (Burland *et al.* 1998). These and other genes have all been associated with pathogenicity, although their exact role in disease is not yet fully understood. Some VTEC strains lacking *eae* and *ehxA* genes have, on occasion, been shown to cause human illness, so these genes may not necessarily be essential for a VTEC to cause disease (Neill 1997).

Not all VTEC are associated with severe human disease, however, there are many VTEC serotypes that have been isolated from animals and foods which have rarely or never been associated with cases of human infection. Therefore, the clinical significance of these VTEC serotypes or the potential risk they pose has not yet been fully elucidated. Some studies have shown differences between VTEC from dairy products, including cheese, and those commonly isolated from humans. In a study by Pradel *et al.* (2008), VTEC strains from dairy food were predominantly carrying *vtx1* and few were harbouring the *eae*, *espP* and *katP*, which are commonly associated with disease-causing strains isolated from patients.

Dairy animals as a source of VTEC

Cattle have long been identified as a major reservoir for VTEC, including VTEC O157: H7 (Chapman *et al.* 1993a; Hancock *et al.* 1994). Cattle are known to shed a range of VTEC, including VTEC O157: H7, and others are associated with human disease. In a study of 514 VTEC isolates from diarrhoeic and healthy cattle in Spain, 20% carried *vtx1*, 54% carried *vtx2* and 26% carried both toxin genes (Blanco *et al.* 2004). In addition, enterohaemolysin and intimin genes were detected in 63% and 29% of the isolates, respectively. Overall, 85% of the cattle isolates were identified as those associated with human disease and 54% were serotypes associated with human HUS.

Although raw meat and undercooked meat products have been commonly associated with outbreaks of VTEC O157: H7 infection, raw milk and dairy products have also been implicated in cases of human infection, including HUS. The prevalence of VTEC in dairy cattle and their products has been reviewed by Hussein and Sakuma (2005). In this review the prevalence of VTEC in faeces of dairy cattle was reported to be between 0.2% and 48.8% and 0.4% and 74.0% for *E. coli* O157 and non-O157 VTEC, respectively. Verocytotoxin-producing *E. coli* serotypes isolated from dairy cattle

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and associated with disease include O2: H29, O22: H8, O26: H11, O103: H2/H$^-$, O111: H8/H$^-$, O113: H21 and O145: H$^-$ (Hussein and Sakuma 2005).

Besides cattle, other domestic animals used to produce milk, such as goats and sheep, also harbour these bacteria and shed them in their faeces. Compared with cattle, higher prevalence of *E. coli* O157 and other VTEC in sheep and goats has been reported (Beutin *et al.* 1993; Sidjabat-Tambunan and Bensink 1997; Fagan *et al.* 1999). In Germany, reports indicate prevalence rates for non-O157 VTEC in sheep, goats and cattle of 67%, 56% and 21%, respectively (Beutin *et al.* 1993). Similar results were found during another German study which reported VTEC prevalence rates of 32%, 75% and 18%, respectively in sheep, goats and cows (Zschock *et al.* 2000).

In addition to the high prevalence of VTEC in sheep and goats, these animals appear to harbour a broad range of VTEC serotypes, including VTEC associated with human infection. In a study by Rey *et al.* (2003), comparison of 253 VTEC strains isolated from sheep in Spain revealed 43% to be carrying *stx* (*vtx* 1) gene, 4% carried *vtx* 2 and 53% carried both toxin genes. In addition, the virulence-associated genes *ehxA* and *eae* were present in 47% and 4% of strains, respectively. The majority of these VTEC strains belonged to six serogroups (O6, O91, O117, O128, O146 and O166) and 71% belonged to nine serotypes (O6: H10, O91: H$^-$, O117: H$^-$, O128: H2, O128: H$^-$, O146: H21, O166: H28, O76: H19 and O157: H7). Rey *et al.* (2003) also reported the discovery of 10 new VTEC O: H serotypes, not previously described.

In dairy herds, faecal contamination of udders poses a risk of pathogens entering the raw milk. Preventing faecal material from contaminating the milk is therefore an important step in reducing the prevalence of VTEC and other pathogens in raw milk. The implementation of effective cleaning procedures to remove faecal material from udders prior to milking can reduce the risk but not eliminate it entirely. The risk of transferring pathogens, including VTEC, to milk can be further reduced by hygiene precautions during milking.

Good animal husbandry on the farm reduces udder infections, which can allow pathogenic bacteria to be introduced into the milk. Bovine mastitis can be caused by *E. coli* (Jones 1990) but there is little if any published evidence that mastitis can be caused by *E. coli* O157. Turutoglu and Madul (2002) reported the isolation of *E. coli* from 50 (13%) of 382 mastitic milk samples in the Burdur province of Turkey, but none of these were identified as belonging to serogroup O157. There is also little published information to indicate that VTEC strains are responsible for mastitis, although they could potentially gain access from the udder to the milk in mastitic cows (Stephan and Kühn 1999). Both VTEC and other *E. coli* come from the same faecal origin and they can readily be isolated from the cow's environment, so there is no reason to assume that VTEC cannot enter milk by the same routes used by other enteric bacteria.

Pasteurization is an effective treatment for removing vegetative pathogens, including VTEC, from milk. Without this step there is always the risk of pathogenic bacteria being present in the milk. Raw milk and dairy products produced using unpasteurized milk, such as cheeses, have been responsible for outbreaks of food-borne illness (Djuretic *et al.* 1997). With the increased popularity of products such as cheeses made from unpasteurized milk, strict hygiene, good quality and process control during their manufacture are essential.

OUTBREAKS ASSOCIATED WITH RAW MILK AND ASSOCIATED CHEESE

Raw milk

Raw milk consumption is considered potentially hazardous and has been associated with several types of infection including brucellosis, cryptosporidiosis, campylobacteriosis, *E. coli* O157 infection, listeriosis, salmonellosis, yersiniosis, tuberculosis and staphylococcal enterotoxin poisoning (Potter *et al.* 1984). Despite the apparent dangers, consumption of raw milk does still occur. In England and Wales the most common vehicle of infection associated with milk-borne outbreaks between 1992 and 2000 was unpasteurized milk (52%) (Gillespie *et al.* 2003). The second most common vehicle reported by Gillespie *et al.* (2003) was milk sold as pasteurized (37%), with most of the outbreaks (67%) being linked to farms. The most common pathogens detected in these outbreaks were *Salmonella* (37%), VTEC O157 (33%) and *Campylobacter* (26%).

Direct consumption of raw milk is much less common than consumption of pasteurized milk, although it is still permitted in some countries. In UK, the Animal Health Dairy Hygiene Inspectorate (formerly the Dairy Hygiene Inspectorate) has estimated consumption of raw cows' drinking milk to be of the order of 0.01% of cows' milk consumption (FSA 2005). In Scotland, the sale of raw milk has been banned since 1983 following a number of milk-related outbreaks and more recently the sale of raw milk and cream from any species was banned. In England and Wales, the sale of raw milk is still permitted but only directly to the consumer by registered production holdings such as at the farm gate or in a farmhouse catering operation or through milk roundsmen. Although there are no known sales in Northern Ireland, similar controls

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to those in England and Wales currently apply. In England and Wales the sale of raw milk from sheep, goats or buffaloes is not subject to the same restrictions imposed on raw cows' drinking milk, which has tight marketing controls, although other requirements and restrictions are imposed on these.

In UK and USA there have been several outbreaks associated with milk and dairy products. In the USA between 1973 and 1992, raw milk accounted for 46 outbreaks reported to the Centers for Disease Control involving *Campylobacter* (57%), *Salmonella* (26%), *Staphylococci* (2%) and *E. coli* O157: H7 (2%) and 13% which could not be linked to a particular agent (Headrick *et al.* 1998). In UK, 20 general food-poisoning outbreaks associated with consumption of milk and dairy products were reported to the PHLS Communicable Disease Surveillance Centre between 1992 and 1996 (Djuretic *et al.* 1997). The pathogens involved were *Salmonella* (55%), *Campylobacter* (25%), VTEC O157 (15%) and *Cryptosporidium parvum* (5%). Milk was implicated in 16 outbreaks, of which 10 were associated with unpasteurized milk. Interestingly, of the three *E. coli* O157 outbreaks reported, only one was caused by unpasteurized milk. The other two outbreaks were caused by pasteurized milk, where post-pasteurization contamination and failure in pasteurization respectively, were identified as key factors. In 1994 an outbreak caused by VTEC O104: H21 in Montana, which resulted in four cases of HC, was also attributed to post-pasteurization contamination of milk (Centers for Disease Control 1995).

Two cases of HUS associated with consumption of raw milk provided early evidence that raw milk can be a vehicle for the transmission of *E. coli* O157 to humans (Martin *et al.* 1986). In Sheffield, UK, *E. coli* O157 was linked to untreated milk (Chapman *et al.* 1993b). Since then there have been several documented cases or outbreaks of *E. coli* O157 infection caused by consumption of raw milk. These include the consumption of raw goats' milk in Austria in 2001 (Allerberger *et al.* 2001) and British Columbia in 2001 (McIntyre *et al.* 2002) and raw cows' milk in the USA in 2005 (Denny *et al.* 2008) and in 2006 (Centers for Disease Control 2008). In Austria, unpasteurized cows' milk has been associated with two cases of HUS caused by VTEC O26 (Allerberger *et al.* 2003). In the event of an *E. coli* O157 outbreak associated with raw milk, failure to restrict the distribution of contaminated product and prevent further consumption can lead to prolonged outbreaks that are intermittent and unpredictable (Keene *et al.* 1997).

Besides direct consumption of raw milk, defective pasteurization and post-process contamination can both yield milk-containing pathogens, leading to outbreaks of infection. One example of this was the Red House Dairy outbreak in West Lothian, Scotland, in 1994, which resulted in over 100 people becoming infected by *E. coli* O157: H7 phage type (PT) 2 VT2 (Upton and Coia 1994). This outbreak resulted in nine children developing HUS and one elderly woman developing TTP. This remains the largest reported milk-borne outbreak of *E. coli* O157 infection and at the time it was the first involving a heat-treated milk supply. Since then there have been others; for example in North Cumbria, UK, an outbreak involving 60 cases of infection from *E. coli* O157 PT 21/28, VT2 and VT2c in 1999 was caused by defective pasteurization (CDSC 1999b, 1999c, 1999d).

Cheese

Several outbreaks involving pathogenic *E. coli* have been caused by the consumption of contaminated cheese. In USA, imported French cheese was associated with outbreaks involving pathogenic *E. coli* in soft ripened Camembert cheese in 1971 (Marier *et al.* 1973), and ETEC in semisoft cheese (Brie) in 1983 (MacDonald *et al.* 1985). The outbreak in 1971 involved an EIEC O124 and was reported to be the first adequately documented occurrence of enteropathogenic *E. coli* food-borne disease in USA. The French authorities later identified a malfunctioning filtration system used to filter river water used in cleaning at the site of the cheesemaker and the organism was also isolated from a curdling tank and from ripening cheese.

Infections caused by VTEC, especially strains belonging to serotype O157: H7, can be potentially life threatening, especially if the disease progresses to HUS. In France a cluster of four cases of HUS in children, caused by *E. coli* O157 VT2, was traced to consumption of cheese made with unpasteurized mixed cows' and goats' milk (Deschênes *et al.* 1996). In Somerset England, a 12-year-old boy developed HUS following infection by *E. coli* O157 PT 2, VT2 after eating cheese made with unpasteurized milk (CDSC 1998). This case involved consumption of Wedmore, a Caerphilly-type cheese containing chives produced by a small company with limited national distribution (Anon 1998). In 1999, a cheese made with unpasteurized milk (Cotherstone cheese) was responsible for two cases of infection by *E. coli* O157 PT 21/28, VT2 in North Yorkshire, UK (CDSC 1999a, 1999b).

Despite the retail sale of raw cows' milk being prohibited in Scotland since 1983, there have been several documented outbreaks of *E. coli* O157 infection associated with cheese. Three outbreaks of *E. coli* O157 infection in Scotland, between 1994 and 1999, involved consumption of cheese made from unpasteurized milk (Reid 2001). In all three outbreaks the hygiene standards of the premises involved appeared to be satisfactory. However, the raw milk at two premises was being stored at temperatures that facilitated growth of bacteria. Furthermore, at one premises no starter

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culture was being used and in another the maturation step was found to be too short to enable sufficient reduction in pH and subsequent decrease in the bacterial population in the product. Poor traceability and a lack of adequate labelling of contaminated products were also highlighted in two of the outbreak investigations.

In 1998, an outbreak involving 55 laboratory confirmed cases of *E. coli* O157: H7 infection in Wisconsin, USA was linked to consumption of fresh cheese curds which had inadvertently been produced using vats previously used to produce cheese made from unpasteurized (raw) milk (Centers for Disease Control and Prevention 2000). In Alberta, Canada, unpasteurized gouda cheese was responsible for an outbreak of 13 cases of VTEC O157: H7 infection in 2002 (Honish *et al.* 2005) and in 2003 another outbreak involving contaminated cheese was responsible for at least 10 cases, including HUS in one child patient (Anon 2003). In 2004, two cases of HUS caused by VTEC O157 in France were traced to consumption of unpasteurized goats cheese made by an independent producer (Espié *et al.* 2006).

PREVALENCE OF VTEC IN RAW MILK AND DAIRY PRODUCTS

Although contaminated raw milk and cheese made from unpasteurized milk have been responsible for outbreaks of VTEC O157 infection, published studies indicate a low prevalence or a failure to detect this pathogen in these products (Table 1). However, as with prevalence studies for cattle and meats, the methodology used and the ability to recover these pathogens will influence the results. Although standard methods exist for detecting *E. coli* O157 and methods have been developed to detect non-O157 VTEC, many published studies have employed different approaches for the detection of these bacteria in milk and cheese samples. Despite this and irrespective of the methods used, past surveys and published studies report the incidence of VTEC O157 in these types of samples as consistently low. The findings of one study suggest that most raw milk intended for cheese making is of high microbiological quality with a low incidence of pathogens (D'Amico *et al.* 2008). Occasionally there are reports of higher isolation rates and there is evidence that the incidence can be higher on case farms linked to outbreaks (Wells *et al.* 1991).

In Spain a study of VTEC in bovine and caprine milk and other dairy products showed the presence of VTEC in bulk tanks, fresh cheese curds and cheese, and one bulk tank was positive for VTEC O157 (Rey *et al.* 2006). In a survey of the prevalence of *E. coli* O157 in dairy industry in UK, this pathogen was not detected in 1146 samples comprising dairy products (raw milk, imported soft cheese), environmental samples and cattle faeces (Neaves *et al.* 1994). A study of 1097 samples of unpasteurized milk purchased from 242 retail outlets in England and Wales between May 1996 and July 1997 revealed the presence of *E. coli* O157 in three samples, and unacceptable aerobic plate counts or coliform counts in a fifth of samples not containing pathogens (de Louvois and Rampling 1998). This would tend to suggest that there is no correlation between high levels of indicator organisms such as coliforms and the presence of *E. coli* O157. Unfortunately, the numbers of *E. coli* O157 found in contaminated samples is often not reported, but where this information has been obtained it would appear to be very low. For example, Foschino *et al.* (2002) reported the level of *E. coli* O157 in the one positive sample of goats' milk was to be 1.5 cells/mL. In a study by D'Amico *et al.* (2008) the only positive sample was obtained after enrichment and it was reported that the level of *E. coli* O157 contamination in the sample was <1 cfu/mL. Interestingly, confocal scanning laser microscopy (Auty *et al.* 2005) has indicated that *E. coli* O157 can occur as single cells or in small clumps of about 10 cells, usually within the protein matrix of the cheese. Therefore some care should be taken in interpreting low cfu counts, as each cfu could comprise a greater number of individual cells.

DIVERSITY OF SEROTYPES AND VIRULENCE FACTORS

The presence of VTEC in cheese is undesirable because of their potential to cause disease. However, the problem is complicated because many VTEC serotypes have not been associated with human disease whereas others, notably O157: H7, O26: H11, O111: H^-, O145: H^-, O103: H2 and O121: H19, are known to cause disease in man, including HUS (EFSA 2007). The concept of seropathotype (Karmali *et al.* 2003) provides a useful method of assessing disease potential by dividing VTEC into categories (A to E) based on their relative prevalence and their association with outbreaks, severe disease, serotype and presence of OI-122 (a pathogenicity island linked to virulence, although its exact role has yet to be elucidated).

Studies to date indicate that VTEC serotypes carrying *eae* and toxin subtypes, commonly associated with those responsible for human disease, can be isolated from raw milk and raw milk cheese (Hussein and Sakuma 2005; Rey *et al.* 2006; Stephan *et al.* 2008). However, Pradel *et al.* (2008) showed that most VTEC isolated from dairy products in France were different from those isolated from patients and that the dairy VTEC strains predominantly carried *vtx1* with a minority carrying *eae*, *esp*A and/or *kat*P. In the study by

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Table 1 Prevalence of VTEC O157 in raw milk and unpasteurized dairy products

Country	*Type of products tested*	*No. positive/ No. tested (%)*	*Reference*
Australia	Raw milk[a]	0/147 (0)	Desmarchelier *et al.* (1998)
Belgium	Soft/semisoft (cow, ewe and goat milk) cheeses	0/153 (0)	Vivegnis *et al.* (1999)
Egypt	Raw cows' milk	3/50 (6.0)	Abdul-Raouf *et al.* (1996)
France	Raw milk[a], unpasteurized cheeses	0/205 (0) 0/180 (0)	Fach *et al.* (2001)
Germany	Raw milk[a]	1/273 (0.4)	Klie *et al.* (1997)
Italy	Raw cows' milk	0/227 (0)	Colombo *et al.* (1998)
Italy	Raw milk[a]	0/100 (0)	Massa *et al.* (1999)
Italy	Raw goats' milk for cheese making	1/60 (1.7)	Foschino *et al.* (2002)
Italy	Various dairy products (Italian cheeses and butter)	0/1313 (0)	Conedera *et al.* (2004)
The Netherlands	Raw cows' milk from bulk tanks	0/1011 (0)	Heuvelink *et al.* (1998)
Peru	Soft cheese	8/102 (7.8)	Mora *et al.* (2007)
Portugal	Raw milk cheeses	0/70 (0)	Almeida *et al.* (2007)
Spain	Raw Ewes' milk	3/84 (3.6)	Caro *et al.* (2006)
Spain	Raw milk soft cheese	1/221 (0.4)	Quinto and Cepeda (1997)
Switzerland	Raw (cows', sheep's, goat's milk) milk cheeses—soft, semi hard, hard cheese)	0/796 (0)	Stephan *et al.* (2008)
UK	Raw cows' milk	3/1097 (0.3)	de Louvois and Rampling (1998)
UK (Scotland)	Raw cows' milk	0/500 (0)	Coia *et al.* (2001)
UK (Scotland)	Raw milk cheeses (Scottish and imported)	0/739 (0)	Coia *et al.* (2001)
USA	Raw milk[a]	11/115 (9.6)	Padhye and Doyle (1991)
USA	Raw cows' milk	0/603 (0)	Hancock *et al.* (1994)
USA	Raw milk[a] Soft and semisoft cheeses	0/42 (0) 0/65 (0)	Ansay and Kaspar (1997)
USA	Raw milk (cows', goat and sheep) for cheese making)	1/133 (0.75) goat's milk	D'Amico *et al.* (2008)

[a]origin (animal species) not specified (assumed to be cows' milk).

Caro and Garcia-Armesto (2007), *vtx1* was the only toxin gene carried by the three strains of VTEC isolated from Spanish raw ewes' milk cheese and none of these strains possessed the *eaeA* or *ehxA* genes. The diversity of VTEC serogroups/serotypes isolated from raw milk and cheeses is shown in Tables 2 and 3, respectively. It is evident from the results of various studies that a diverse range of VTEC serotypes carrying several virulence-associated genes can be encountered in these products. The prevalence of VTEC O157 is generally very low (Table 1), whereas non-O157 VTEC can be encountered more frequently (Tables 2 and 3). Our understanding of VTEC in cheese is, however, currently restricted to a limited number of studies and only where it has been possible to obtain and further characterize isolates. Isolates from cheese samples that are polymerase chain reaction (PCR) positive for the presence of *vtx* genes can be difficult to obtain. Vivegnis *et al.* (1999) reported obtaining isolates from only 5 (29%) of 17 PCR (*vtx*)-positive samples and Vernozy-Rozand *et al*. (2005) recovered strains from 18 (13%) of 136 *vtx*-positive samples. Greater isolation success was reported by Stephan *et al.* (2008), who recovered VTEC from 16 (41%) of the 39 *vtx*-positive samples with the aid of a colony dot-blot hybridisation procedure.

SURVIVAL OF VTEC DURING CHEESE MANUFACTURE

Although the reported prevalence of VTEC in raw milk is low, it is known that raw milk intended for cheese making does occasionally contain pathogenic bacteria, including *E. coli* O157 (Table 1) and other VTEC serotypes (Tables 2 and 3). The manufacture of cheese involves a series of different stages, including curd formation, drainage, setting, ripening and storage. The exact process will vary depending on the type of cheese, and there are additional processes such as brining which are associated with the manufacture of some cheeses. Owing to the wide diversity of cheeses made throughout the world, it is therefore difficult to completely understand how pathogens such as VTEC will

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Table 2 Prevalence of VTEC in milk

Product	*Country*	*No. positive/ No. tested (%)*	*Serotypes/serogroup*	*Reference*
Raw milk	Canada	15/1720 (0.9)	No VTEC O157 isolated. Serotypes isolated: O?: H-, O121: H7, O163: H19, O136: H16, O26: H11, O136: H12, O91: H21, O?: H8, O1: H20, O113: H21, O142: H38, O?: H21	Steele *et al.* (1997)
Raw milk	France	44 (21.5)[b] 14/205 (7.0)[c]	No VTEC O157 isolated. Serogroups isolated: O3 O91, O110, (*vtx1* only). O6, O117, O77 (*vtx2* only). O76/O22 (both *vtx1* and *vtx2*)	Fach *et al.* (2001)
Raw milk cheeses	France	136/1039 (13.1)	No VTEC O157 isolated. Serotypes isolated: O6: H10, O76: H19, O109: H25, O174: H8 (*vtx1* only).O22: H8, O109: H25 (*vtx2* only).O?: H8, O174: H8*, O76: H19 O6: H1, O175: H16, O6: H10, O162: H10 (both *vtx1* and *vtx2*). *Only *eae*-positive strain	Vernozy-Rozand *et al.* (2005)
Raw bulk tank milk (goat's and ewe's milk)	Switzerland	55/344 (16.3) Goat's milk 8/63 (12.7) ewe's milk	No VTEC O157 isolated. Serotypes isolated O5:H$^-$, O76:H19 (all *vtx2c*), O91: H?, O113: H4, O113: H4, O174 (OX3): H8 (all *vtx2d*) ONT: H19 (*vtx2*) Only ONT: H19 was *eae*-positive. All EHEC-*hly* positive except O87: H16 and O174 (OX3): H8	Muehlherr *et al.* (2003)

VTEC, Verocytotoxin (Shiga toxin)-producing *Escherichia coli*; EHEC, enterohaemorrhagic *Escherichia coli*; O?, O antigen cannot be determined; H?, H antigen not determined/unidentified.
[b]*vtx (stx)* positive by PCR ELISA. [c]positive by Vero cell assay.

behave during the cheesemaking process and their survival during storage and maturation. Furthermore, some strains of *E. coli* O157: H7 appear to be more acid-resistant, being able to withstand pH values as low as 3.0 (Jordan *et al.* 1999), and there have been outbreaks associated with low pH foods including fermented meats (Getty *et al.* 2000; MacDonald *et al.* 2004) and yoghurt (Morgan *et al.* 1993). Consequently, there is a potential risk that some VTEC strains, and especially strains of *E. coli* O157: H7, could survive the low pH associated with the cheese manufacturing process.

In raw milk cheese operations there are different factors and complex interactions that occur during production. During the ripening process the indigenous microflora of the raw milk can outcompete the pathogens. These bacteria, together with the presence of starter culture and low pH, are important factors for controlling pathogens in cheese. Whilst the effects of intrinsic factors such as pH on the growth and survival of micro-organisms are well known, less is understood about the interactions that can occur between the specific pathogens such as *E. coli* O157 and the starter culture organisms or natural microflora found in cheese. Commercial cheese manufacturers generally use defined starter cultures for cheese production. In contrast, producers of artisan cheeses or small-scale cheesemakers may rely upon the natural microflora from the milk or starter cultures that have not been clearly defined or even properly identified. Rash and Kosikowski (1981) studied the influence of lactic acid starter bacteria on EPEC survival during the manufacture of Camembert cheese. They showed that successful elimination of the pathogen could be achieved and that using a combination of *Streptococcus cremoris* and *Lactobacillus helveticus* or *Lactobacillus bulgaricus* as starter cultures was more effective than using a single species or *S. cremoris* and *Streptococcus diacetylactis* in combination. During the

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Table 3 Prevalence of VTEC in cheese

Product	*Country*	*No. positive/ No. tested (%)*	*Serotypes/serogroups and virulence factors identified*	*Reference*
Cheeses (NS)	France	60/603 (9.9)	No VTEC O157 isolated. Serotypes and serogroups isolated: OX3: H2, O91: H21 (both *vtx1* and *vtx2*), O113, O8, O15, O103 (toxin type not stated). All strains *eae*-negative	Pradel *et al.* (2000)
Unpasteurized cheeses	France	55/180 (30.5)[b] 16/180 (9.0)[c]	No VTEC O157 isolated. Serogroups isolated: O5, O76, O110, O117, O136 (*vtx1* only). O6, O77, O91, O113 (*vtx2* only). O79, (both *vtx1* and *vtx2*). Only O5 strains *eae*-positive	Fach *et al.* (2001)
Pasteurized cheeses	France	4/45 (8.9)[b] 0/45 (0)[c]	N/A, no isolates obtained	Fach *et al.* (2001)
Unpasteurized bulk tank milk (sheep and goat's milk)	Spain	39/360 (10.8)	O157: H7 serotype (*vtx2* and *ehxA*) positive was isolated from one (0.3%) bulk tank sample. Other serotypes identified: O27: H18*, O76: H19*, O91: H28* (*vtx1*). ONT: H7, ONT: H9, O157: H7[a], O45: H38 (*vtx2*). ONT: H21 (both *vtx1* and *vtx2*). Only O157: H7 serotype showed *eae* virulence gene. **ehxA*-positive	Rey *et al.* (2006)
Fresh (sheep and goat's milk) cheese curd	Spain	4/103 (3.9)	No VTEC O157 isolated. No isolates obtained for characterization	Rey *et al.* (2006)
Cheeses (sheep and goat's milk)	Spain	2/39 (5.1)	No VTEC O157 isolated. No isolates obtained for characterization	Rey *et al.* (2006)
Raw ewe's milk cheese	Spain	3/83 (2.4)	ONT, O14 (2 isolates). All *vtx1* (*eaeA* and *ehxA* negative)	Caro and Garcia-Armesto (2007)
Raw milk cheeses (goats, sheep and cows milk)	Switzerland	5/52 (9.6) Soft cheese 34/744 (4.6) Semihard and hard cheese	No VTEC O157 isolated. Serotypes isolated: O2:H27, O15:H16, O22:H8, O91:H21, O109:H16, O113:H4, O174:H21 1 strain of Or: H45 was *vtx1*-positive. All other strains carried *vtx2* or *vtx2* variants 2 strains of O91:H21 and 1 strain Or: HNT were EHEC *hly*-positive	Stephan *et al.* (2008)

NS, not specified; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; VTEC, Verocytotoxin-producing *Escherichia coli*; EHEC *hly/ehxA*: enterohaemolysin gene; HNT, H antigen not typeable; ONT, O antigen not typeable.
[a]origin not specified (assumed to be cows' milk); [b]*vtx* (*stx*) positive by PCR ELISA; [c]positive by Vero cell assay.

production of a typical Brazilian fresh cheese (Minas cheese), the addition of a type O lactic culture to the milk has been shown to improve reduction of *E. coli* O157 (Saad *et al.* 2001).

Concern over the safety of traditional raw milk cheeses eaten in some countries has prompted research into the survival of *E. coli* O157 in these products. Soft Hispanic cheeses have become popular in USA. These typically contain no starter culture, have a pH value of 6.6, a moisture content of 60% and low brine (1.61%) and have been shown to support the growth of *E. coli* O157 if the storage temperature is ≥10°C (Kasrazadeh and Genigeorgis 1995). The manufacturing process of Paneer, which is a popular cheese in India, has been shown to allow the growth and survival of *E. coli* O157: H7 (Wahi *et al.* 2006) and this pathogen has also been shown to survive during the manufacture and storage of other cheeses (Tsegaye and Ashenafi 2005; Lekkas *et al.* 2006). In Camembert and feta cheeses, *E. coli* O157 has been shown to survive the manufacturing process and persist for 65 and 75 days' storage, respectively (Ramsaran *et al.* 1998). One of the most popular cheeses consumed is Cheddar, which can be made with unpasteurized or pasteurized milk, although the latter is more common. In a study by Reitsma and Henning (1996), *E. coli* O157: H7 was reported to survive the Cheddar cheese manufacturing process and could be recovered after 60 days' ripening. When the cheese was made using milk containing 10^3 cfu/mL *E. coli* O157, a 2-log reduction was demonstrated after 60 days' ripening, but the pathogen could still be detected after 158 days. When the milk contained 1 cfu/mL of this pathogen, the 60 days' ripening period yielded a reduction to 1 or <1 cfu/g in the cheese, but it could not be detected at 158 days. A later study by Schlesser *et al.* (2006) confirmed that the 60-day ageing period was inadequate to eliminate *E. coli* O157: H7 during ripening of Cheddar cheese. However, it is worthy to note that two of the strains used by Schlesser *et al*. (2006) were chosen for their tolerance of acidic conditions, being strains associated with outbreaks linked to cider and fresh apple juice.

Understanding the behaviour of pathogens during cheese manufacture is difficult to determine and it is inevitable that much of our information has been derived from artificially contaminated cheese and cheese produced in the laboratory using

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artificially contaminated milk. Using a laboratory-scale smear-ripened cheese produced from raw milk, Maher *et al.* (2001) showed that *E. coli* O157: H7, at an initial level of 1.52 log cfu/mL in the milk, was able to grow by 1.3 log cycles during the cheese manufacture stage. A dramatic increase was observed following the heating of the curd from 32 to 37°C, and the pathogen subsequently survived 70 days during the ripening process and could be detected by enrichment after 90 days. A laboratory procedure reported by Leuschner and Boughtflower (2002) for producing soft cheese artificially contaminated with low levels of pathogens also showed that *E. coli* O157 can survive the cheese manufacture process, including curd formation, drainage, setting and ripening. Other studies have shown that *E. coli* O157 can survive for up to 21 days in pasteurized and unpasteurized whey (Marek *et al.* 2004) and in cheese brine for several weeks under typical brining conditions (Ingham *et al.* 2000).

METHODS FOR THE DETECTION AND ISOLATION OF VTEC IN CHEESE

In the past there has been less information available on the prevalence of non-O157 VTEC in raw milk and cheese, although with the more widespread adoption of molecular methods and the availability of immunomagnetic beads for O26, O111, O103 and O145, this information has started to emerge. Methods for the detection and confirmation of non-O157 VTEC are still developing and improving. However, most tests for these bacteria are currently being performed by government agencies and specialist laboratories in hospitals or those involved with public health protection and research. In contrast, there are a number of methods available for the detection and confirmation of VTEC O157, including commercial media and test kits (Baylis and Mitchell 2008). Some methods have also become published standards which have been widely adopted and are used for regulatory testing of foods. Examples include the International Organisation for Standardization (ISO) method for the detection of *E. coli* O157 (Anon 2001), which has been validated for use with milk (Scotter *et al.* 2000), and the US Food and Drug Administration Bacteriological Analytical Manual method (Feng and Weagant 2002).

One of the difficulties of comparing results from different studies is the diversity of methods that have been used and the potential impact that this could have on the recovery and isolation of VTEC from milk and cheese products. Although there are several media that have been developed and used successfully for the isolation of *E. coli* O157 in foods, some of these media may not necessarily be suitable for use with dairy products. Compared with other foods, dairy products, especially cheese, can present several practical difficulties to the microbiologist trying to isolate VTEC. Interference by competitor organisms can be particularly problematic and selective agars such as Sorbitol MacConkey (SMAC) agar plates can be difficult to read because of heavy growth by competing bacteria (Hammack *et al.* 1997; Vernozy-Rozand *et al.* 2005). Cheese can contain particularly high numbers of competing bacteria so it is important that these are suppressed or inhibited by the enrichment and plating media to enable successful growth and isolation of the target organism. The population of background microflora in cheese has been reported to be 10^6 cfu/g or above (Drysdale *et al.* 2004). Increasing the incubation temperature of the enrichment medium to *ca* 42°C for samples containing high numbers of competing bacteria has been shown to improve isolation of *E. coli* O157 from meats and dairy samples (Baylis *et al.* 2001; Drysdale *et al.* 2004).

Method specificity is also improved by using the immunomagnetic separation (IMS) technique. This has become an integral part of many *E. coli* O157 detection methods, including EN ISO 16654. The technique uses paramagnetic beads coated with antibodies to specifically capture the target organism, which can then be separated from other organisms and food debris in the enriched sample using a magnetic field. After gently washing the beads, the target organism can be concentrated into a smaller volume and the beads are typically spread over the surface of a suitable selective medium where cells can grow and develop into typical colonies. Although beads were originally available only for serogroup O157, others are now commercially available for the major VTEC serogroups (O157, O26, O111, O103 and O145) previously identified as being commonly associated with human diseases by the World Health Organization Scientific Working Group (1999). However, the fat content of dairy products can adversely affect the IMS technique by preventing the capture of immunomagnetic beads by the magnetic field (Baylis *et al.* 2001). In a study by Vernozy-Rozand *et al.* (1997), detection of *E. coli* O157 in replicate raw milk cheese samples artificially contaminated at levels of 8 and 20 cfu/25 g yielded fewer positives by traditional enrichment and IMS procedure compared to detection by immunoassay (VIDAS *E. coli* O157; bioMérieux s.a., Marcy l'Etoile, France). The authors attributed the fewer positive results by IMS to be due to the fatty matrix interfering with the settling of bead particles.

The ability to grow low numbers of cells to detectable levels is a critical step in the isolation of VTEC from foods. The choice of enrichment medium and the type and concentration of selective

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agents must be chosen with care, especially if the method is intended to detect and isolate a wide range of VTEC. Growth of some VTEC, including strains of *E. coli* O157, can be poor in some enrichment media, including those developed for the detection of serogroup O157 (Baylis 2008). Antibiotics and other selective agents are often used to reduce interference from background microflora, although some VTEC can also be sensitive to these compounds (Hussein and Bollinger 2008). The enrichment medium stipulated in EN ISO 16654 is modified Tryptone soya broth supplemented with 20 mg/L novobiocin. For the detection of *E. coli* O157 in cheese and other dairy products using the VIDAS system, the addition of 2.25 g/L acriflavin to the enrichment medium mTSB has been used (Cohen and Kerdahi 1996; Vernozy-Rozand *et al.* 1997). Ogden *et al.* (2001) found that buffered peptone water (BPW) supplemented with 8 mg/L vancomycin (V) incubated at 42°C, followed by IMS and plating beads onto SMAC and a chromogenic medium (Rainbow O157 agar, Biolog Inc. Hayward, USA; CHROMagar O157, CHROMagar, Paris, France), gave optimum recovery of *E. coli* O157 from spiked samples including cheese. Further work by this group confirmed that enrichment in BPW or BPW + V at 42°C gave the best recovery of VTEC O157, O26 and O111 from foods including cheese (Drysdale *et al.* 2004).

Alternative methods have become popular because of their potential to reduce the overall test time and their greater specificity compared with conventional culture methods. This is highlighted by two published studies where *E. coli* O157 in cheese (Vernozy-Rozand *et al.* 1997) and milk (Foschino *et al.* 2002) was not detected following enrichment and plating on CT SMAC agar (SMAC containing cefixime and tellurite). In both studies the same sample tested by an immunological method (VIDAS; bioMérieux) yielded a positive result and the bacteria in the sample were subsequently isolated and confirmed. Detecting all VTEC in a food normally involves confirming the presence of *vtx* genes or VT in an enriched sample. Immunological methods, e.g. enzyme-linked immunosorbent assay, are now available to detect VT, and the PCR technique is most commonly used to detect *vtx* gene sequences. Inhibitors or competing microflora in the food sample can, however, sometimes prevent the growth of the target organisms, which then fail to reach detectable levels after enrichment. Furthermore, components in the food can inhibit certain techniques such as the PCR. In cheese, fats and proteinases have been reported to adversely affect PCR reactions (Wernars *et al.* 1991; Rossen *et al.* 1992; Powell *et al.*1994; McKillip *et al.* 2000). Some success in removing inhibitors found in cheese has been achieved by utilizing proprietary DNA extraction kits (Fratamico *et al.* 2000). Centrifugation is a step commonly incorporated into many DNA extraction protocols, including commercial kits to aid removal of inhibitory compounds. Buoyancy centrifugation is another approach which has been used to separate bacteria from food and which can help to remove potential PCR inhibitors. This technique has been used to enable successful PCR detection of *E. coli* O157 in a range of foods including milk, blue cheese and Camembert cheese (Lindqvist 1997).

After enrichment, one of the most common media used for the isolation of *E. coli* O157 is CT SMAC, a medium that was originally developed for clinical specimens. In a study by Hammack *et al.* (1997), HC agar incubated at 43°C was reported to recover significantly more unstressed *E. coli* O157 from Brie cheese than a variety of SMAC formulations. To estimate the number of *E. coli* O157 in samples generally involves direct plating onto a selective diagnostic medium. Whilst CT SMAC remains a common medium for the isolation of *E. coli* O157 from food enrichments, this medium is often too inhibitory and therefore unsuitable for direct plating of samples containing stressed cells (McCarthy *et al.* 1998). In cheese VTEC cells may be sublethally injured by the presence of lactic acid and the low pH of the product. Under these circumstances, using a chromogenic medium (ID O157:H7 ID medium now chrom ID O157:H7; bioMérieux) supplemented with 0.005 mg/L cefixime and 2.5 mg/L potassium tellurite and a pour plate method was shown to give better recovery and higher counts of *E. coli* O157 in cheese during ripening compared with CT SMAC (Jordan and Maher 2006). For the isolation of non-O157 VTEC there are few truly diagnostic media available. In the case of O26 strains, Hiramatsu *et al.* (2002) demonstrated that rhamnose MacConkey agar with and without cefixime (50 μg/mL) and potassium tellurite (2.5 mg/L) provided good diagnostic and selective properties. For other VTEC serotypes, detection often involves primary isolation on a suitable *E. coli*-specific chromogenic medium such as Tryptone Bile X-Glucuronide (TBX) medium, followed by confirmation of *vtx* genes in a selection of typical colonies. However, some chromogenic media have been developed to assist with the isolation of non-O157 VTEC (Bettelheim 1998) or specific serogroups, e.g. O111, O26, O103 and O145 (Hara-Kudo *et al.* 2002; Possé *et al.* 2008).

CONCLUSIONS

Direct consumption of raw milk remains a high risk and there is a large amount of documented evidence highlighting the different types of pathogens that can be transmitted by this product, and outbreaks that have been traced to this source. Compared with other bacterial pathogens, *E. coli* O157: H7 and

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other VTEC can cause severe disease and even death. The manufacture of cheese made with raw milk is commonplace in some countries and has increased in popularity in others. Studies show that despite the low incidence of *E. coli* O157 in these products, occasionally this pathogen can be isolated, although other VTEC serotypes are generally present at a higher frequency. The serotypes/serogroups isolated from these products can be quite diverse and often include VTEC that are less frequently associated with human disease or which do not carry virulence-associated genes such as *eae*, which is often found in VTEC isolated from patients. Therefore, the clinical significance of these VTEC is not fully understood. These results show the complexity of the current situation, especially when it comes to risk assessment of these products. Studies with artificially contaminated cheese confirm that *E. coli* O157 is capable of surviving the cheese manufacture process and subsequent ripening and storage periods. More research is needed to fully appreciate the survival and prevalence of VTEC in cheese made with raw milk. Strict hygiene is essential during the production of these products. In the UK there have been few if any reported outbreaks associated with a milk product since 2003. Although the hazards still exist, this would suggest that the dairy industry now has better control over the manufacturing process and hygiene practices have been maintained or improved.

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