Journal of Food Protection, Vol. 69, No. 4, 2006, Pages 920–924

Research Note

Detection, Occurrence, and Characterization of *Escherichia coli* O157:H7 from Raw Ewe's Milk in Spain

IRMA CARO,[1]* VICTOR M. FERNÁNDEZ-BARATA,[2] ANA ALONSO-LLAMAZARES,[2] AND M. ROSARIO GARCÍA-ARMESTO[2]

[1]*Research Centre for Food Science and Technology, Autonomous University of Hidalgo State, Tulancingo, Hidalgo 43600, México; and* [2]*Department of Food Hygiene and Food Technology, University of León, 24071 León, Spain*

MS 05-413: Received 11 August 2005/Accepted 1 November 2005

ABSTRACT

A study was carried out in the Castilla y León region of Spain to investigate the presence of *Escherichia coli* O157:H7 in raw ewe's milk samples collected from several cheese factories during 1 year. All specimens were examined for *E. coli* O157:H7 by selective enrichment at 41.5 ± 1.0°C, after both 6 and 22 h of incubation, and then immunomagnetically separated and plated on cefixime–potassium tellurite–sorbitol MacConkey agar. No growth was obtained in the enrichment broth after a 6-h incubation. Presumptive colonies obtained after 22 h of incubation were screened by a multiplex PCR assay for the presence of rfb_{O157} and $fliC_{H7}$ genes. Of all the ewe's milk samples studied, three were positive for *E. coli* O157:H7. The *E. coli* O157:H7 strains that were positive for the rfb_{O157} and $fliC_{H7}$ genes were then analyzed by multiplex PCR for the presence of virulence genes (stx_1, stx_2, *ehxA,* and *eaeA*). All *E. coli* O157:H7 isolates were Shiga toxigenic and harbored additional genes related to virulence (*ehxA* and *eaeA*). The predominant Stx toxin type was stx_2. These results demonstrate that raw ewe's milk used in cheesemaking may be sporadically contaminated with *E. coli* O157:H7 strains that are potentially pathogenic for humans.

Shiga toxin–producing *Escherichia coli* (STEC) O157:H7 has emerged as an important foodborne pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome *(24).* Because of its low infectious dose *(26)* and ability to survive under refrigeration conditions *(16),* this microorganism has been of concern for the dairy industry in recent years.

Cattle have been identified as a principal reservoir of *E. coli* O157:H7 *(5),* but recent studies have provided evidence that sheep may play a similar role *(1, 33).* Fecal contamination of milk during the milking process, along with poor hygienic practices, has been attributed special significance as a route of infection for milkborne *E. coli* O157:H7 diseases in humans *(3, 13).* Currently, there is little information about the presence of *E. coli* O157:H7 in raw cow's milk, even though this microorganism is found frequently in dairy cattle *(1).* Furthermore, few studies have examined the presence of *E. coli* O157:H7 in raw ewe's milk *(6, 9, 20, 29).*

Castilla y León is the prime production region in Spain for ewe's milk. This milk is mainly used for hard and semihard cheese production, with or without previous heat treatment. With regard to unpasteurized milk cheeses, *E. coli* O157:H7 seems to have the potential for survival, not only in high-moisture soft and semisoft cheeses but also in hard varieties *(21).* The objective of the present study was to investigate the overall and seasonal occurrence of *E. coli* O157:H7 in raw ewe's milk and to investigate the virulence characteristics of isolates from this origin.

MATERIALS AND METHODS

Milk samples. A total of 84 aseptically collected unpasteurized ewe's milk samples (1 liter each) were examined. Samples were taken from refrigerated holding tanks (from 10 to 150 tons in capacity) at five cheese factories in Castilla y León (at least once per month from January to December 2000). Milk samples were transported to the laboratory under refrigeration. On arrival, an immunochromatographic test from Z.E.U.-Inmunotec S.L. (Zaragoza, Spain) was used to determine whether the ewe's milk had been adulterated with cow's milk. Then, milk samples were tested for the presence of *E. coli* O157:H7 according to the analytical protocol summarized in Figure 1 and described below.

Selective enrichment. For each sample, 100 ml of milk was added to 900 ml of modified tryptone soya broth (mTSB), which contained 30 g of TSB (Oxoid, Hampshire, UK), 1.5 g of bile salts no. 3 (Oxoid), 1.5 g of dipotassium phosphate, and 20 mg of novobiocin (Sigma Chemical Co., St. Louis, Mo.) per liter *(15).* The mixture was swirled and incubated at 41.5 ± 1°C *(32).*

Immunomagnetic separation and plating. After 6 and 22 h of incubation, the enrichment broth was swirled, and 1 ml of the broth was added to 20 μl of magnetic beads coated with antibodies to *E. coli* O157 (Dynal, Oslo, Norway) *(27).* After immunomagnetic separation, 50 μl of the dynabeads-bacteria complex was plated on CT-SMAC agar: sorbitol MacConkey agar (SMAC; Oxoid) supplemented with cefixime and potassium tel-

* Author for correspondence. Tel: Int-34-987-291120; Fax: Int-34-987-291284; E-mail: dhticc@unileon.es.

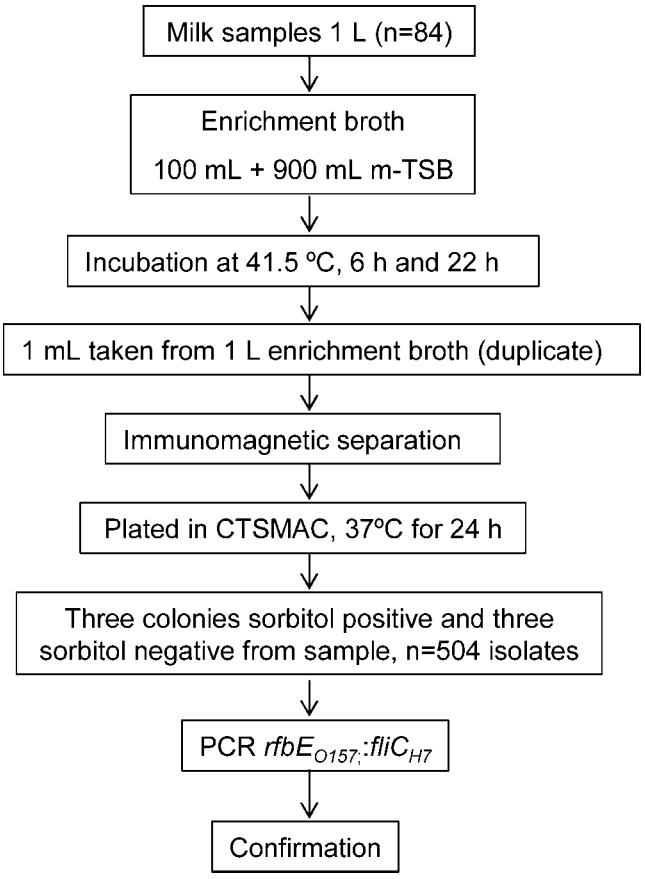

FIGURE 1. *Steps followed for detecting and characterizing* E. coli *O157:H7 from ewe's milk.*

lurite (0.05 and 2.5 mg/liter, respectively; CT Supplement, Oxoid) *(27).* After 18 to 20 h of incubation at 37 ± 1°C, nonsorbitol and sorbitol fermenting colonies were selected (three colonies per sample each) and isolated.

PCR-based detection of rfb_{O157} and $fliC_{H7}$ genes. All isolates ($n = 504$) were subjected to multiplex PCR analysis for the detection of rfb_{O157} and $fliC_{H7}$ genes *(12, 25).* PCR primers, their locations, and the length of the amplified products are listed in Table 1. Two sets of primers, O157F and O157R, FLICH7F and FLICH7R, were used to amplify the 259- and 625-bp fragments of the rfb_{O157} and $fliC_{H7}$ genes, respectively. The multiplex PCR assay was performed under the conditions described by Paton and Paton *(25).* A positive control (*E. coli* O157:H7 ATCC 700728) and two negative controls (no DNA template and no *E. coli* C600 laboratory strain) were used for the experiment. Amplified PCR products were analyzed by 2% agarose gel electrophoresis containing ethidium bromide.

Serological and biochemical confirmation. To further verify multiplex PCR specificity, positive isolates to rfb_{O157} and $fliC_{H7}$ genes were confirmed as serogroup O157 and as serotype H7 by agglutination to titer with antiserum to *E. coli* O157 and H7 at the *E. coli* Reference Laboratory, University of Santiago de Compostela, Lugo, Spain. In addition, the positive isolates were confirmed as *E. coli* by an API 20E biochemical test strip (bioMérieux, Lyon, France) and two additional biochemical tests: (i) cellobiose utilization plus growth in the presence of potassium cyanide to differentiate *E. coli* O157:H7 from *Escherichia hermanii (10, 22)* and (ii) β-glucuronidase activity on ID medium (Merck, Darmstadt, Germany) to differentiate *E. coli* O157 from most non-O157:H7 *E. coli* strains *(22).*

Virulence traits of isolates. All isolates confirmed as *E. coli* O157:H7 were analyzed for the presence of stx_1, stx_2, *ehxA,* and *eaeA* genes by multiplex PCR according to the method of Paton and Paton *(25).* A positive control (an *E. coli* O157:H7 reference strain harboring stx_1, stx_2, *ehxA,* and *eaeA* genes; kindly provided by Dr. Blanco, *E. coli* Reference Laboratory, University of Santiago de Compostela) and a negative control (*E. coli* C600) were used in the assay. More details on the location of the primers are listed in Table 1.

RESULTS

Detection and occurrence of *E. coli* O157:H7 in raw ewe's milk. Initial tests for adulteration with cow's milk were negative in all raw ewe's milk samples. All presumptive isolates were obtained from the plates of CT-SMAC after a 22-h incubation in mTSB plus novobiocin at 41.5°C; no growth was observed in plates after 6 h of incubation. The multiplex PCR assay—targeting the *rfb* and *fliC* genes—for the *E. coli* O157:H7 reference strain yielded two clear PCR products of the predicted size (259 bp for

TABLE 1. *Primers used in the study*

Primer	Specificity	Location within gene	PCR product (bp)	Reference
(A) O157F O157R	O157 *rfbE*	393–651	259	*25*
FLICH7F FLICH7R	*fliC* gene encoding the H7 flagellar antigen	69–694	625	*12*
(B) stx1F stx1R	Shiga toxin 1 (stx_1)	454–633 of subunit A	180	*25*
stx2F stx2R	Shiga toxin 2 (stx_2)	603–857 of subunit A	255	*25*
eaeAF eaeAR	*Escherichia coli* attaching and effacing gene (*eaeA*)	27–410[a]	384	*25*
ehxAF ehxAR	Enterohemolysin (*ehxA*)		534	*25*

[a] This region is conserved between enteropathogenic *E. coli* and STEC.

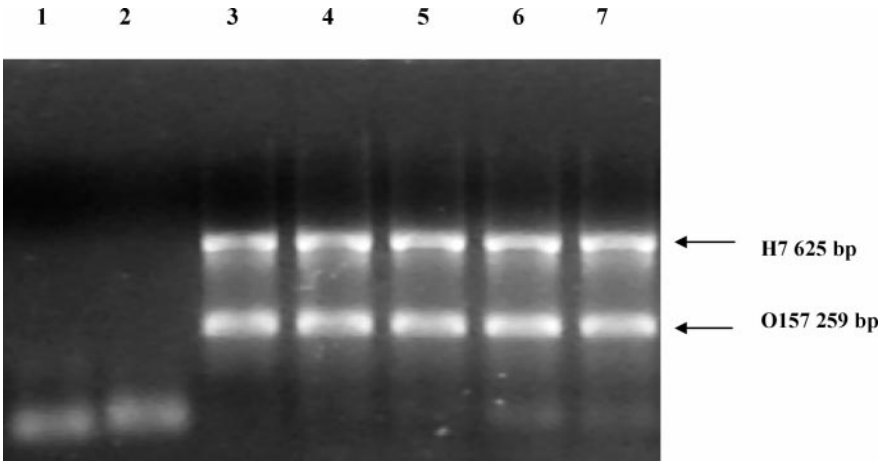

FIGURE 2. *Agarose gel electrophoresis of DNA fragments amplified by multiplex PCR of* rfb_{O157} *and* $fliC_{H7}$ *genes. Lane 1, non-DNA control; lane 2,* E. coli *C600 negative control; lane 3,* E. coli *O157:H7 (ATCC 700728), positive control; lanes 4, 5, 6, and 7, positive isolates.*

the O157 genes and 625 bp for the H7 genes), and no gene products were observed from the DNA template or the *E. coli* C600 laboratory strain (Fig. 2).

By this multiplex PCR assay, serotype O157:H7 was detected in 7 of the 504 colonies tested. No O157 isolates were detected that were not also of serotype H7. These seven colonies originated from three raw ewe's milk samples (3.5% of the samples), which were collected in the spring (one sample) and summer (two samples) (Table 2).

Positive strains by PCR were all confirmed as *E. coli* O157:H7 by conventional and microbiochemical methods. These strains neither used cellobiose nor grew in the presence of potassium cyanide. They were β-glucuronidase negative. Finally, these seven strains also tested positive for O157:H7 by serology.

Virulence genes. *E. coli* O157:H7 strains isolated from raw ewe's milk were all STECs and exhibited two distinct ''virulence patterns'' (Table 3). PCR testing showed that, among the seven STEC isolates, six were positive for stx_2, and the remaining isolate harbored both stx_1 and stx_2. Moreover, all isolates were characterized by the presence of *eaeA* and *ehxA* virulence factors.

DISCUSSION

Detection and occurrence of *E. coli* O157:H7 in raw ewe's milk. A 22-h incubation period of the enrichment broth proved to be significantly more sensitive for the detection of *E. coli* O157:H7 in refrigerated raw ewe's milk than a 6-h enrichment. This is in good agreement with the studies of Voitoux et al. *(32),* who used a method for the detection of *E. coli* O157:H7 similar to that used in the present study, as did Uyttendaele et al. *(31).* In both studies, an 18- to 24-h enrichment was recommended so that the presence of *E. coli* O157:H7 in refrigerated foods would not be underestimated. The latter authors observed that cold stress may increase the lag-phase time of different enterohemorrhagic *E. coli* strains. On the contrary, Fratamico et al. *(11)* found that the ability to detect *E. coli* O157:H7 in ground beef after 8 h of enrichment was not affected by cold stress. Not only differences in cold storage or strain characteristics, as was suggested by Fratamico et al. *(11)* when referring to the study of Uyttendaele et al. *(31),* but also differences in food substrate and isolation procedures could have accounted for the differences in results between the study of Fratamico et al. *(11)* and the other studies.

To our knowledge, there are, at present, only four studies related to *E. coli* O157:H7 in raw ewe's milk: two in the United Kingdom *(6, 20),* one in Italy *(29),* and one in Greece *(9).* The contamination frequency found in the present study (3.5%) was higher than that found in those countries (0, 0.6, and 1%, respectively). It was also higher than the frequency detected in cow's milk *(8, 30).* There are several reasons for these variations, such as differences in hygienic practices during milking, differences in geographic location *(5),* differences in sampling, e.g., pooled raw milk versus milk from farm bulk tanks *(30),* differences in seasonal trends, and differences in sensitivity of isolation methodology for *E. coli* O157:H7 *(2, 4).* With regard to methodology, more severe enrichment conditions were used in the present study, i.e., direct exposure to both novobiocin and bile salts and 41.5°C incubation, than those used or recommended in several recent studies for the recovery of cold-stressed *E. coli* cells *(4, 14, 31),* in which, to improve recovery performance, selective agents were omitted, lower

TABLE 2. *Seasonal incidence of* Escherichia coli *O157:H7 in ewe's raw milk samples*[a]

	Winter	Spring	Summer	Autumn	Total
Milk samples	0 (21)	1 (21)	2 (21)	0 (21)	3 (84)

[a] Numbers in parentheses refer to the number of tested samples.

TABLE 3. *Genetic virulence factors of* Escherichia coli *O157:H7 isolates from ewe's milk*

Strain no.	Source (dairy/ewe milk sample no.)	Virulence profile			
		stx_1	stx_2	*eaeA*	*ehxA*
642	C/41	+	+	+	+
1189	D/60	−	+	+	+
1193	D/60	−	+	+	+
1197	D/60	−	+	+	+
360	E/25	−	+	+	+
361	E/25	−	+	+	+
362	E/25	−	+	+	+

incubation temperatures were used, or a preenrichment or resuscitation step was implemented.

In Spain, there are no previously published data on the occurrence of *E. coli* O157:H7 in ewe's milk. However, the presence of *E. coli* O157:H7 in sheep feces has been studied *(23).* Percentages between 0.4 and 1% were found in fecal swab samples taken from healthy lambs. Several other studies in different countries have found occurrence rates in sheep feces between <0.8 and 7.4% *(5, 12, 13, 17).*

Virulence genes. In agreement with our results, stx_2 and stx_1-stx_2 toxin genotypes have been detected for *E. coli* O157:H7 from ovine sources *(7, 9, 18, 29).* In most of these studies as well as in the present study, the predominant toxin genotype was stx_2; this toxin is considered the most important virulence factor associated with hemolytic uremic syndrome disease *(28).*

Our results also agree with previous findings that indicate that the *eaeA* gene is expressed by most, if not all, STEC O157 isolates *(19).* The *eaeA* gene is a potential *E. coli* O157:H7 adherence factor, which may be responsible for the intimate attachment of STECs to epithelial cells, causing attaching and effacing lesions in the intestinal mucosa *(19, 22).* In addition, all isolates harbored the *ehxA* gene that encodes for the production of hemolysin. It has been reported that hemolysin and Shiga toxin production are closely associated in most of the STECs isolated from cattle, sheep, and goats *(3).*

The major finding of the present investigation is that a considerable percentage of ewe's milk samples are contaminated with *E. coli* O157:H7.

ACKNOWLEDGMENT

This study was supported by grant 1FD97-1921 from the Ministry of Education and Culture of Spain (FEDER-CICYT).

REFERENCES

1. Bastian, S. N., and S. K. Sivelä. 2000. *Escherichia coli* O157:H7: aspects of concern to the dairy industry. *Bull. IDF* 357:58–62.
2. Bennett, A. R., S. MacPhee, and R. P. Betts. 1996. The isolation and detection of *Escherichia coli* O157 by use of immunomagnetic separation and immunoassay procedures. *Lett. Appl. Microbiol.* 22:237–243.
3. Bielaszewska, M., J. Janda, K. Bláhová, H. Minaríková, E. Jíková, M. A. Karmali, J. Laubová, J. Sikulová, M. A. Preston, R. Khakhria, H. Karch, H. Klazarová, and O. Nyc. 1997. Human *Escherichia coli* O157:H7 infection with the consumption of unpasteurized goat's milk. *Epidemiol. Infect.* 119:299–305.
4. Blackburn, C. W., and J. D. McCarthy. 2000. Modifications to methods for the enumeration and detection of injured *Escherichia coli* O157:H7 in foods. *Int. J. Food Microbiol.* 55:285–290.
5. Chapman, P. A. 2000. Sources of *Escherichia coli* O157:H7 and experiences over the past 15 years in Sheffield, UK. *J. Appl. Microbiol.* 88:51S–60S.
6. Chapman, P. A., A. T. Cerdán Malo, M. Ellin, R. Ashton, and M. A. Harkin. 2001. *Escherichia coli* O157:H7 in cattle and sheep at slaughter, on beef and lamb carcasses and in raw beef and lamb products in South Yorkshire, UK. *Int. J. Food Microbiol.* 64:139–150.
7. Chapman, P. A., C. A. Siddons, A. T. Cerdan Malo, and M. A. Harkin. 2000. A one year study of *Escherichia coli* O157 in raw beef and lamb products. *Epidemiol. Infect.* 124:207–213.
8. De Louvois, J., and A. Rampling. 1998. One fifth of samples of unpasteurised milk are contaminated with bacteria. *Br. Med. J.* 316: 625.
9. Dontorou, C., C. Papadopoulou, G. Filioussis, V. Economou, I. Apostolou, G. Zakkas, A. Salamoura, A. Kansouzidou, and S. Levidiotou. 2003. Isolation of *Escherichia coli* O157:H7 from foods in Greece. *Int. J. Food Microbiol.* 82:273–279.
10. Ewing, W. H. 1986. Edwards and Ewing's identification of Enterobacteriaceae, 4th ed. Elsevier, New York.
11. Fratamico, P. M., L. K. Bagi, and T. Pepe. 2000. A multiplex polymerase Caín reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. *J. Food Prot.* 63:1032–1037.
12. Gannon, V. P. J., S. D'Souza, T. Graham, R. K. King, K. Rahn, and S. Read. 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *J. Clin. Microbiol.* 31:1268–1274.
13. Griffin, P. M., and R. V. Tauxe. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other entero-hemorrhagic *E. coli,* and associated hemolytic uremic syndrome. *Epidemiol. Rev.* 13: 60–98.
14. Hara-Kudo, Y., M. Ikedo, H. Kodaka, H. Nakagawa, K. Goto, T. Masuda, H. Konuma, T. Kojima, and S. Kumagai. 2000. Selective enrichment with a resuscitation step for isolation of freeze-injured *Escherichia coli* O157:H7 from foods. *Appl. Environ. Microbiol.* 66: 2866–2872.
15. Heuvelink, A., T. M. J. Zwartkruis-Nahuis, and E. De Boer. 1997. Evaluation of media and test kits for detection and isolation of *Escherichia coli* O157 from minced beef. *J. Food Prot.* 7:817–824.
16. International Commission on Microbiological Specifications for Foods. 1996. Intestinally pathogenic *Escherichia coli,* p. 126–140. *In* T. A. Roberts, A. C. Baird-Parker, and R. B. Tompkin (ed.), Microorganisms in foods. 5. Microbiological specifications of food pathogens. Blackie Academic & Professional, London.
17. Johnsen, G., O. I. Berget, Y. Wasteson, and H. Heriksa. 2000. *E. coli* O157:H7 in faeces from cattle, sheep and pigs in the south west part of Norway during 1998, p. 186. *In* G. Duffy, P. Garvey, J. Coia, Y. Wasteson, and D. A. McDowell (ed.), Verocytotoxigenic *E. coli* in Europe. 3. Pathogenicity and virulence of verocytotoxigenic *E. coli.* Concerted Action CT98-3935.). Teagasc, The National Food Centre, Dublin.
18. Kudva, I. T., P. G. Hatfield, and C. J. Hovde. 1996. *Escherichia coli* O157:H7 in microbial flora of sheep. *J. Clin. Microbiol.* 34:431–433.
19. Law, D. Virulence factors of *Escherichia coli* O157 and other Shiga toxin–producing *E. coli.* A review. *J. Appl. Microbiol.* 88:729–745.
20. Little, C. L., and J. de Louvois. 1999. Health risks associated with unpasteurized goats' and ewes' milk on retail sale in England and Wales. A PHLS dairy products working group study. *Epidemiol. Infect.* 122:403–408.
21. McClure, P. J., and S. Hall. 2000. Survival of *Escherichia coli* in foods. *J. Appl. Microbiol.* 88:61S–70S.
22. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli. Clin. Microbiol. Rev.* 11:142–201.
23. Orden, J. A., J. A. Ruiz-Santa-Quiteria, M. Blanco, J. E. Blanco, A. Mora, D. Cid, E. A. González, J. Blanco, and R. De la Fuente. 2003. Prevalence and characterization of Vero cytotoxin–producing *Escherichia coli* isolated from diarrhoeic and healthy sheep and goats. *Epidemiol. Infect.* 130:313–321.
24. Parry, S. M., and S. R. Palmer. 2000. The public health significance of VTEC O157. *J. Appl. Microbiol.* 88:1S–9S.
25. Paton, A. W., and J. C. Paton. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA,* enterohemorrhagic *E. coli hlyA,* rfb_{O111}, and rfb_{O157}. *J. Clin. Microbiol.* 36:598–602.
26. Paton, J. C., and A. W. Paton. 1998. Pathogenesis and diagnostics of Shiga-toxin-producing *Escherichia coli* infection. *J. Clin. Microbiol.* 28:803–805.
27. Public Health Laboratory Service. 1998. PHLS standard operating procedures. Food method manual. Standard method F17.

28. Ramachandran, V., M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic. 2001. The common ovine Shiga toxin 2-containing *Escherichia coli* serotypes and human isolates of the same serotypes possess a Stx2d toxin type. *J. Clin. Microbiol.* 39:1932–1937.
29. Rubini, S., G. Cardeti, S. Amiti, G. Manna, R. Onorati, A. Caprioli, and S. Morabito. 1999. Verocytotoxin-producing *Escherichia coli* O157 in sheep milk. *Vet. Rec.*144:56.
30. Steele, M. L., W. B. McNab, C. Poppe, M. W. Griffiths, S. Chen, S. A. Degrandis, L. C. Fruhner, C. A. Larkin, J. A. Lynch, and J. A. Odumeru. 1997. Survey of Ontario bulk tank raw milk for foodborne pathogens. *J. Food Prot.* 60:1341–1346.
31. Uyttendaele, M., C. Grangette, F. Rogerie, S. Pasteau, J. Debevere, and M. Lange. 1998. Influence of cold stress on the preliminary enrichment time needed for detection of enterohemorrhagic *Escherichia coli* in ground beef by PCR. *Appl. Environ. Microbiol.* 64: 1640–1643.
32. Voitoux, E., V. Lafarge, C. Collette, and B. Lombard. 2002. Applicability of the draft standard method for the detection of *Escherichia coli* O157 in dairy products. *Int. J. Food Microbiol.* 77:213–221.
33. Wales, A. D., F. A. Clifton-Hadley, A. L. Cookson, M. P. Dibb-Fuller, C. M. Hayes, R. M. La Regione, J. M. Roe, K. A. Springings, G. R. Pearson, and M. J. Woodward. 2001. Preliminary observations on *E. coli* O157:H7 in sheep. *In* G. Duffy, P. Garvey, J. Coia, Y. Wasteson, and D. A. McDowell (ed.), Verocytotoxigenic *E. coli* in Europe. 5. Epidemiology of verocytotoxigenic *E. coli*. Concerted Action CT98-3935. Teagasc, The National Food Centre, Dublin.