A novel real-time polymerase chain reaction-based method for the detection and quantification of lactose-fermenting *Enterobacteriaceae* in the dairy and other food industries

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ABSTRACT

The presence of lactose-fermenting *Enterobacteriaceae* and coliforms is routinely assessed to determine the hygienic quality of water and foods, particularly dairy products. This paper reports the use of *lacZ*-specific primers in an SYBR green I-based real-time PCR method for the easy and rapid detection of coliforms in dairy products. A large number of bacterial species were assayed to establish the specificity of the method. The sensitivity of the method was assessed using artificially contaminated cheeses. The limit of detection was 1 coliform cell in cheese samples enriched for 8 h in a culture medium. The entire procedure, including sample processing, enrichment, DNA extraction, and real-time PCR amplification, can be completed within 10 to 12 h, making it a single-day assay.

Key words: *Enterobacteriaceae*, coliform, real-time polymerase chain reaction detection, cheese

INTRODUCTION

Coliforms are a broad class of bacteria defined as rod-shaped, gram-negative, non-spore-forming microorganisms that ferment lactose with the production of acid and gas when incubated at 35 to 37°C. The presence of coliforms can be used to assess the hygienic quality of foods and water (Feng and Hartman, 1982; Bredie and de Boer, 1992). These microorganisms are abundant in the feces of warm-blooded animals, but are also found in aquatic environments, in soil, and on vegetation (Winfield and Groisman, 2003). Although coliforms do not usually cause sickness, their presence in food and the ease with which they can be cultured make them useful indicators of the possible presence of other, more pathogenic organisms of fecal origin (Leclerc et al., 2001).

Microbiologically, cheeses are considered to be among the safest of foods. Like other milk products, however, they can transmit pathogenic bacteria. The organisms responsible for infections associated with cheese consumption include *Salmonella*, *Listeria monocytogenes*, and verocytotoxin-producing *Escherichia coli* (Zottola and Smith, 1991; De Buyser et al., 2001). Careful investigations have shown the sources of contamination to be raw milk, inadequately pasteurized milk, or post-pasteurization contamination with organisms originally derived from raw milk or the manufacturing environment (Murphy and Boor, 2000).

Coliform bacteria are an important indicator of the hygienic standards maintained in dairy product manufacture, processing, and storage (including the storage of the milk used in their production; APHA, 2004). In the dairy farm setting, coliform counts provide a useful indicator of the extent of fecal contamination of milk, a recognized index of the hygienic standards maintained. Because milk pasteurization easily kills coliform bacteria, their detection in pasteurized products indicates inadequate practices during manufacture or packaging. Although the presence of these organisms certainly constitutes a food safety problem, they can also produce off flavors and reduce the shelf life of dairy products.

The traditional methods for detecting coliforms rely upon culturing in a medium that selectively permits the growth of gram-negative bacteria and differentially detects lactose-utilizing organisms (APHA, 2005). However, this is laborious, expensive, and time consuming (Bredie and de Boer, 1992); a more practical, rapid method is therefore needed. In recent years, methods based on PCR have been successfully used (Bej et al., 1990, 1991; Tantawiwat et al., 2005), and real-time quantitative PCR (**RT-qPCR**), which offers the advantages of speed and sensitivity, is now routinely used to detect pathogen bacteria in different food matrices, including pathogen coliform strains (Holicka et al., 2006; Bohaychuk et al., 2007; Elizaguível and Aznar, 2008; Cheng et al., 2009; Omiccioli et al., 2009). However, to our knowledge, the present study reports the first RT-qPCR method for the simultaneous detection of all coliform species in a single assay, which is based

Received June 12, 2009.

Accepted November 25, 2009.

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Table 1. Strains used in this work

Strain ¹	RT-qPCR ²
<i>Klebsiella pneumoniae</i> ssp. <i>rhinoscleromatis</i> CECT 852	+
<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i> CECT 143	+
<i>Klebsiella pneumoniae</i> ssp. <i>ozaenae</i> CECT 851	+
<i>Klebsiella oxytoca</i> CECT 860	+
<i>Enterobacter cloacae</i> CECT 194	+
<i>Enterobacter aerogenes</i> CECT 684	+
<i>Enterobacter gergoviae</i> CECT 857	+
<i>Enterobacter sakazakii</i> CECT 858	+
<i>Citrobacter freundii</i> CECT 401	+
<i>Citrobacter amalonaticus</i> CECT 863	+
<i>Citrobacter koseri</i> CECT 856	+
<i>Escherichia coli</i> CECT 423	+
<i>Escherichia coli</i> CECT 428	+
<i>Escherichia coli</i> CECT 515	+
<i>Escherichia coli</i> CECT 434	+
<i>Escherichia coli</i> CECT 201	+
<i>Escherichia coli</i> CECT 470	+
<i>Escherichia coli</i> CECT 405	+
<i>Salmonella typhimurium</i> CECT 4594	–
<i>Salmonella virchow</i> CECT 4154	–
<i>Salmonella virchow</i> LSP 16/94	+
<i>Streptococcus thermophilus</i> CNRZ 1066	–
<i>Streptococcus thermophilus</i> LMD9	–
<i>Lactobacillus casei</i> ATCC 393	–
<i>Lactobacillus paracasei</i> CECT 4175	–
<i>Lactobacillus fermentum</i> CECT 4007	–
<i>Lactobacillus reuteri</i> CECT 925	–
<i>Lactobacillus plantarum</i> CECT 3801	–
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> CECT 4005	–
<i>Lactobacillus buchnerii</i> CECT 4683	–
<i>Lactobacillus curvatus</i> CECT 904	–
<i>Lactobacillus brevis</i> CECT 4121	–
<i>Lactococcus lactis</i> IL1403 (Chopin et al., 1984)	–
<i>Lactococcus lactis</i> CECT 697	–
<i>Lactococcus lactis</i> CECT 539	–
<i>Raoultella planticola</i> CECT 843	+
<i>Raoultella terrigena</i> CECT 4519	+
<i>Bacillus cereus</i> CECT 131	–
<i>Bacillus subtilis</i> CECT 356	–
<i>Bacillus coagulans</i>	–
<i>Clostridium argentinense</i> CECT 4615	–
<i>Clostridium sporogenes</i> CECT 892	–
<i>Clostridium perfringens</i> CECT 376	–
<i>Clostridium acetobutylicum</i> CECT 508	–
<i>Clostridium difficile</i> CECT 531	–
<i>Clostridium tyrobutyricum</i> CECT 4011	–
<i>Enterococcus casseliflavus</i> CECT 969	–
<i>Enterococcus faecalis</i> CECT 795	–
<i>Enterococcus faecalis</i> CECT 4176	–
<i>Enterococcus mundtii</i> CECT 972	–
<i>Enterococcus faecium</i> CECT 410	–
<i>Enterococcus durans</i> CECT 411	–
<i>Enterococcus hirae</i> CECT 279	–
<i>Listeria monocytogenes</i> CECT 940	–
<i>Listeria ivanovii</i> CECT 913	–
<i>Listeria innocua</i> CECT 910	–
<i>Micrococcus luteus</i> CECT 245	–
<i>Staphylococcus aureus</i> CECT 435	–
<i>Shigella flexneri</i> CECT 585	–
<i>Shigella sonnei</i> CECT 413	+
<i>Pseudomonas putida</i> CECT 324	–
<i>Pseudomonas fluorescens</i> CECT 378	–
<i>Serratia marcescens</i> CECT 846	–
<i>Yersinia enterocolitica</i> CECT 4315	–
<i>Hafnia alvei</i> CECT 158	–
<i>Edwardsiella ictaluri</i> CECT 885	–

Continued

Table 1 (Continued). Strains used in this work

Strain ¹	RT-qPCR ²
<i>Providencia stuartii</i> CECT 866	–
<i>Erwinia carotovora</i> CECT 314	–
<i>Rhodococcus equi</i> CECT 555	–
<i>Moraxella catarrhalis</i> ATCC 25238	–

¹CECT = Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain; LSP = Laboratorio de Salud Pública, Principado de Asturias, Spain; CNRZ = Centre National de Recherche Zootechniques, Jouy-en-Josas, France; LMD = Laboratory of Microbiology, Technical University, Delft, the Netherlands; ATCC = American Type Culture Collection, Rockville, Maryland.

²RT-qPCR = real-time quantitative PCR; + indicates a positive RT-PCR result, – indicates a negative result.

on oligonucleotide primers targeting a segment of the β -galactosidase gene (*lacZ*) and the use of SYBR green I as a fluorescent dye.

MATERIALS AND METHODS

Bacterial Strains

A total of 70 strains, including positive and negative controls, were used in this work (Table 1). All *Enterobacteriaceae* strains were grown at 37°C in brain heart infusion (BHI; Oxoid, Basingstoke, UK) with aeration. Viable numbers were determined on 2% violet red bile agar (with 4-methyl-umbelliferyl β -D-glucuronide; Oxoid) and brilliant green lactose bile (APHA, 2005). *Streptococcus thermophilus* was grown at 42°C in M17 broth (Oxoid) supplemented with 0.5% glucose and 0.5% lactose (GLM17) or 2% M17 agar. *Lactobacillus casei* was grown at 37°C in de Man, Rogosa, Sharpe (MRS) broth (Oxoid) or on 2% MRS agar. *Lactococcus lactis* was grown at 30°C in GLM17 broth or on 2% GLM17 agar.

Extraction of DNA for the RT-qPCR Assay

Two methods were used to extract DNA from bacterial cells: lysis by boiling, or the use of the Genelute Bacterial Genomic DNA kit (Sigma, St. Louis, MO). Culture samples of 1 mL were used in both methods. In the first method, the bacterial suspension was centrifuged at $10,000 \times g$ for 5 min and the sediment was resuspended in 0.85% NaCl before being centrifuged again at $10,000 \times g$ for 5 min. The washed sediment was resuspended in 100 μ L of PCR buffer (10 mM Tris HCl, 25 mM KCl, 5 mM (NH₄)₂SO₄, 2 mM MgCl₂) and incubated at 95°C for 30 min (Oravcová et al., 2007). In the second method, the manufacturer's instructions were followed. The DNA was eluted in 100 μ L of molecular biology grade water (Sigma).

***lacZ* Sequencing and Nucleotide Sequence Analysis**

An internal fragment of the *lacZ* gene from different coliform species was amplified with the *lacZ*-1 (5'-ATGAAGCAGAACAACTTCAACGCCGT-3') and *lacZ*-4R (5'-CGCCGATGTCGTTGTCCAGCGG-3') oligonucleotides (Sigma Genosys, Haverhill, UK). For this, a loopful of bacteria from an agar culture was resuspended in a PCR mixture consisting of 1× PCR buffer, 0.2 mM deoxynucleoside triphosphates, 0.2 mM of each oligonucleotide primer, 2 U of DyNAzyme II DNA polymerase (Finnzymes Oy, Espoo, Finland), and nanopure water in a final volume of 50 µL. The PCR amplification was performed using a DNA thermocycler (iCycler, Bio-Rad, Hercules, CA). Denaturing was performed at 95°C for 5 min. This was followed by 30 cycles at 95°C for 60 s, 60°C for 60 s, and 72°C for 90 s, and a final incubation at 72°C for 10 min. The PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Buckinghamshire, UK). Nucleotide sequences were determined using an ABI Prism 373 Stretch automated sequencer (Secugen S.L., Madrid, Spain). Sequence data were assembled and analyzed using a sequence analysis software package available from the EMBL Spanish node (CNB, CSIC, Madrid, Spain). Alignment was performed using the CLUSTAL W algorithm (Thompson et al., 1994).

RT-qPCR Conditions

Real-time qPCR amplification of small regions of the *lacZ* genes was performed in a total volume of 20 µL containing 1 µL of template, 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), and 900 nM of each primer. The 7500 Fast Real-Time PCR System (Applied Biosystem) was used for thermocycling and to record changes in fluorescence. The PCR reaction was initiated with pre-incubation at 50°C for 2 min followed by denaturation at 95°C for 10 min, and then 40 cycles of denaturation at 95°C for 15 s plus annealing at 55°C for 60 s. Melt-curve analysis was performed immediately after the final PCR cycle to determine the specificity of the reaction. The reactants were incubated at 95°C for 15 s, annealing at 55°C for 1 min and then slowly increasing the temperature to 95°C. Fluorescence was monitored continuously. Negative controls were included, containing all the elements of the reaction mixture except the template. All samples were processed in duplicate.

Data Analysis

Data analyses were performed using Applied Biosystems 7500 Fast Real-Time PCR System software

(Sequence Detection System v. 1.3). In the 7500 Fast Real-Time PCR System, the fluorescence of SYBR green is calibrated dynamically against the fluorescence of Rox, a passive internal reference dye. The cycle threshold (C_t) is defined as the PCR cycle during which the increase in the SYBR green fluorescence crosses the chosen threshold (in this case, the default set by the manufacturer). The C_t value is inversely related to the copy number of the target gene. A sample was considered positive for the presence of coliforms if the C_t value was at least 2 U below that recorded for the negative control.

Artificial Contamination and Enrichment of Cheese

Cheeses were artificially contaminated based on a procedure previously described (Hein et al., 2001). *Escherichia coli* CECT 515, *Enterobacter cloacae* CECT 194, *Citrobacter freundii* CECT 401, or *Klebsiella pneumoniae* ssp. *pneumoniae* CECT 143 were cultured in BHI broth overnight at 37°C and 10-fold serially diluted in BHI. The number of bacteria per milliliter was determined by the viable count on 2% violet red bile agar (with 4-methyl-umbelliferyl β-D-glucuronide). For the enrichment step, 25 g of Emmental cheese samples were homogenized in 225 mL of BHI (Oxoid) using a Lab-Blender 400 Stomacher (Seward Ltd., London, UK). Equal numbers of the 4 bacterial species were added to the stomacher bags to final concentrations of 1, 10, and 100 cfu/mL. These samples were enriched at 37°C for 0, 2, 3, 4, 5, 6, 7, 8, or 24 h. All cheese samples used were previously confirmed to be negative for coliforms.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the *lacZ* genes were deposited in the European Molecular Biology Laboratory database ([http://www.ebi.ac.uk/Clustal W/](http://www.ebi.ac.uk/Clustal_W/)) under accession numbers FN297863 (*E. coli* CECT 515), FN297864 (*E. coli* CECT 428), FN297865 (*E. coli* CECT 423), FN297866 (*Enterobacter sakazakii* CECT 858), FN297867 (*K. pneumoniae* ssp. *rhinoscleromatis* CECT 852), FN297868 (*K. pneumoniae* ssp. *ozaenae* CECT 851), FN297869 (*K. pneumoniae* ssp. *pneumoniae* CECT 143), FN297870 (*C. freundii* CECT 401), FN297871 (*Citrobacter koseri* CECT 856), FN297872 (*Citrobacter amalonaticus* CECT 863), and FN297873 (*Raoultella planticola* CECT 853).

The accession numbers of the sequences obtained from databases are *C. freundii* MF466 (AY746954), *C. freundii* OS60 (AY746953), *E. coli* K12 W3110 (AC_000091), *E. coli* K12 MG1655 (U00096), *E. coli* O157:H7 EDL933 (NC_002655), *E. coli* O157:H7 Sakai (NC_002695), *Ent. cloacae* B5 (DQ266449), *Ent. cloacae* (D42077),

Figure 1. Alignment of a 63-bp region of the β -galactosidase (*lacZ*) gene from 22 representative coliform strains (*Citrobacter freundii*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Enterobacter sakazakii*, *Citrobacter koseri*, *Citrobacter amalonaticus*, and *Raoultella planticola*) using Clustal W software (Altschul et al., 1997). The locations of the primers are shown by arrows, an asterisk denotes identical sequences, and a dash indicates a mismatch with the consensus sequence.

Ent. cloacae 10.2-45 (AY746948), *Ent. cloacae* E482 (AY746947), and *K. pneumonia* (M11441).

RESULTS

Sequencing of the *lacZ* Genes and Primer Design

Near-complete DNA sequences of the *lacZ* gene were obtained by sequencing a 1.2-kb internal fragment obtained by PCR amplification (primers lacZ-1 and lacZ-4R) from 11 different coliforms strains. The obtained sequences, together with 11 sequences from databases, were compared and aligned using BLAST (Altschul et al., 1997) and Clustal W software (Chenna et al., 2003). The alignment of the 22 *lacZ* nucleotide sequences allowed the design of a degenerate oligonucleotide primer pair for RT-qPCR: forward primer lacZ-7 5'-CGCTACGGYCTGTAYGTS GT-3' and reverse primer lacZ-6R 5'-TCATCGGCACCATSCCGTG-3' (Figure 1).

Optimization of RT-qPCR

Real-time qPCR reaction mixes were produced to provide at least 3 replicates of each of the following

forward:reverse primer concentration (nM) combinations: 100:100, 100:300, 100:600, 100:900, 300:100, 300:300, 300:600, 300:900, 600:100, 600:300, 600:600, 600:900, 900:100, 900:300, 900:600, and 900:900. The lowest and highest C_t values were obtained with the 900:900 and 100:100 combinations, respectively (data not shown). The 900:900 combination was chosen as the optimal combination for use in subsequent experiments. Thermocycling was optimized in terms of annealing temperature (tests were performed between 50 and 60°C with a 60 s elongation step; a temperature of 55°C produced the earliest C_t responses) and extension step time.

Specificity and Sensitivity

The specificity of the assay was tested using a panel of different bacterial strains from culture collections (Table 1). The specificity of the method was assessed by comparing the amplification products and melting curves. Only *lacZ*-positive *Enterobacteriaceae* were detected. As expected, *Shigella flexneri* and *Salmonella virchow* CECT 4154, which are lactose-negative *Enterobacteriaceae*, were undetected. However, it is important to note that *Shigella sonnei* and

Figure 2. Standard curves for the log number of coliform cells per reaction versus the cycle threshold (C_t) value for the fluorescent signal for A) *Escherichia coli* CECT 515, B) *Enterobacter cloacae* CECT 194, C) *Citrobacter freundii* CECT 401, and D) *Klebsiella pneumoniae* ssp. *pneumoniae* CECT 143. The error bars indicate standard deviations for 3 independent experiments. CECT = Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain.

Salmonella virchow LSP 16/94, both of which are lactose-positive strains (Martín et al., 2001), were detected.

In the first step of the validation procedure, the dynamic range of quantification and the sensitivity of the assay were investigated. For this purpose, a 10-fold dilution series of *E. coli* CECT 515 was used (Figure 2a). The limit of detection was 10 cells per reaction, when 2 out of 3 measurements were positive. The linear range of quantification was 5 logarithmic decades, with a correlation coefficient of 0.9802 for triplicate measurements. The slope of the curve for *lacZ* gene was -3.463 . Another important variable, the PCR reaction efficiency ($E = 10^{1/-S} - 1$, where S = slope), can be obtained from the standard curve if the correlation coefficient is high (Klein et al., 1999); in this case the calculated efficiency was 0.9443.

The limit of detection for *C. freundii* CECT 401, *Ent. cloacae* CECT 194, and *K. pneumoniae* ssp. *pneumoniae* CECT 143 were 10, 100, and 100 cells per reaction, respectively (Figure 2b, 2c, and 2d). The linear range of quantification was 5 logarithmic decades: the r^2 values were 0.9968, 0.9897, and 0.9906, respectively. The slopes of the curves were -3.4138 , -3.2729 , and -3.5912 , respectively; the efficiency of the reaction was 0.9630, 1.02, and 0.8987, respectively.

Melting Curve Analysis of the Amplified DNA

Real-time qPCR data were further analyzed by determining the melting temperature of each amplification product. Melting temperature is dependent upon the length of the amplified DNA and the G/C content of the sequence. The values obtained were 76.3°C for *E. coli* CECT 515, 78°C for *C. freundii* CECT 401, 77.2°C for *Ent. cloacae* CECT 194, and 76.4°C for *K. pneumoniae* ssp. *pneumoniae* CECT 143.

RT-qPCR Detection Limits

To develop a universal protocol for food monitoring, 25 g of cheese was artificially contaminated in 2 independent experiments with decreasing amounts of an overnight culture of *E. coli* CECT 515, *C. freundii* CECT 401, *Ent. cloacae* CECT 194, or *K. pneumoniae* ssp. *pneumoniae* CECT 143 (100 μL of 10-fold dilutions in BHI to provide 10^6 down to 10 cfu/g). The contaminated cheeses were subjected to enrichment in BHI for 2, 3, 4, 5, 6, 7, 8, or 24 h. The DNA extraction was then performed; this was best done using the Genelute Bacterial Genomic DNA kit (Sigma). Real-time qPCR amplification was performed as described in Materials and Methods. The results are shown in Table 2.

Table 2. Detection of coliforms in cheese by culture enrichment and real-time quantitative PCR¹

Enrichment (h)	<i>Escherichia coli</i> CECT 515			<i>Enterobacter cloacae</i> CECT 194			<i>Klebsiella pneumoniae</i> CECT 143			<i>Citrobacter freundii</i> CECT 401		
	1	10	100	1	10	100	1	10	100	1	10	100
0	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	+	–	–	+	–	–	+	–	–	+
6	–	+	+	–	+	+	–	+	+	–	+	+
7	+	+	+	+	+	+	+	+	+	–	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+

¹CECT = Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain; 1, 10, and 100 indicate the contamination level of coliforms (cfu/mL) before enrichment; + indicates a positive RT-PCR result, – indicates a negative result.

When cheeses were contaminated with 1 cfu/mL, *E. coli*, *Ent. cloacae*, and *K. pneumoniae* were detectable after 7 h of enrichment (Table 2). At this time, the average number of cfu/mL reached 1×10^5 for *E. coli* and *Ent. cloacae* and 2×10^5 for *K. pneumoniae*. Eight hours of enrichment were necessary for the detection of *C. freundii*, at which time the culture contained a mean 1×10^5 cfu/mL. When the initial contamination was 10 cfu/mL and 100 cfu/mL, detection was possible after 6 and 5 h of enrichment, respectively.

DISCUSSION

The enumeration of coliforms, and more specifically of *E. coli*, in food and water is of major importance because it provides a means of assessing the hygienic quality of the latter (Feng and Hartman, 1982; Bredie and de Boer, 1992). The traditional methods for detecting bacteria in food are labor intensive and time consuming; the past 10 yr has therefore seen the development of several faster and less tedious procedures. Those based on RT-qPCR have demonstrated great potential, not only because of the ability to quantify the target but also because of their specificity, sensitivity, and rapidity. Primers directed toward the *E. coli* enterotoxin gene, used as part of a suite of real-time SYBR green assays for the detection of food- and waterborne pathogenic strains in feces (Fukushima et al., 2003), and RT-qPCR methods that use fluorescent probes for monitoring microbial levels in water and different foods (Foulds et al., 2002; Cheng et al., 2009; Omiccioli et al., 2009), have been developed. To our knowledge, however, a single RT-qPCR assay for the detection of all coliform species has not been previously proposed. In the present study, a set of *lacZ*-targeted, species- and group-specific primer pairs was designed and optimized for the accurate detection of coliforms in cheese samples. These were analyzed by RT-qPCR involving SYBR green I, a nonspecific, double-stranded, DNA-binding dye that

does not require the design of specific probes and the binding of which is not affected by potential mutations of the target gene. The method is reliable and sensitive with a wide dynamic detection range (at least 5 orders of magnitude), shows good linearity (r^2 between 0.9802 and 0.9968) and PCR efficiency (E between 0.8987 and 1.02), and has a very low detection limit (as few as 10 coliforms per reaction). The ranges in these values could be caused by mismatches between the oligonucleotide primers and the target sequences of the different strains, which would affect the annealing temperature.

Because the method is based on the detection of *lacZ*, it is able to detect coliform bacteria, including members of the genera *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*, but also lactose-positive *Shigella* and *Salmonella* strains. Lactose fermentation is a biochemical property used to distinguish *Shigella* ssp. from *E. coli*. However, there are some *Shigella* strains that can ferment lactose (although relatively slowly); after cultivation for 2 d or more, their colonies show a lactose-fermenting phenotype (Powe and Gross, 1984). Southern hybridization analysis has revealed that a region homologous to *E. coli lacZ* is present in lactose-positive *Shigella* strains *Shigella dysenteriae* serovar 1 and *Shigella sonnei* (Ito et al., 1991). Indeed, the analysis of the complete genome sequences of *Sh. dysenteriae* and *Sh. sonnei* has revealed the presence of the *lacZ* gene (Yang et al., 2005). It has not been found, however, in *Shigella flexneri* (Wei et al., 2003).

A substantial number of genes can be inserted into, deleted from, or rearranged within genomes during evolution. The idea that the *lac* operon could be acquired via horizontal transfer, allowing it to invade a new niche and form new species, has become a paradigmatic example of bacterial adaptation and specialization (Ochman et al., 2000).

Traditionally, *Salmonella* are considered non-lactose-fermenting bacteria. However, a small but important number of strains in this highly diverse group are able

to ferment this sugar. Lactose-positive *Salmonella* ssp. strains have been detected sporadically in the past (Usera et al., 1998; Martín et al., 2001). The RT-qPCR method proposed in this work was able to detect an atypical lactose-fermenting *Salmonella virchow* isolated during an outbreak of food poisoning associated with the consumption of infant formula.

The genus *Klebsiella* is a heterogeneous group that has been divided into 3 clusters (Drancourt et al., 2001). Cluster I includes *K. pneumoniae* ssp. *pneumoniae*, *K. pneumoniae* ssp. *rhinoscleromatis*, and *K. pneumoniae* ssp. *ozaenae*; cluster II comprises *Klebsiella ornithinolytica*, *Klebsiella planticola*, *Klebsiella trevisanii*, and *Klebsiella terrigena*; and cluster III includes *Klebsiella oxytoca*. Recently, the analysis of 16S ribosomal DNA and *rpoB* sequences led to the subdivision of the genus *Klebsiella* into 2 genera—*Klebsiella* and *Raoultella*—the latter of the 2 containing the species of cluster II (Drancourt et al., 2001). As expected, the method proposed in this work detected species of the 3 clusters, including those from the new genus *Raoultella*. Current molecular microbiology methods allow a more precise identification of bacteria and highlight the imprecision of certain traditional methods. Nowadays it would seem more appropriate to speak of lactose-fermenting *Enterobacteriaceae* than coliforms.

In comparison with previous RT-qPCR assays, which are focused on the detection of pathogen species such as *Salmonella* ssp. (Omiccioli et al., 2009) or even just pathogen strains of *E. coli* O157, the proposed method allowed the detection of all the lactose-fermenting *Enterobacteriaceae* species, including those belonging to *Klebsiella*, *Citrobacter*, and *Enterobacter* genera. Although most of the coliform strains are not pathogens, their quantification allows an investigator to determine the hygienic quality of foods and indicates the possible presence of pathogen organisms of fecal origin. In this regard, it is noteworthy that this method was faster (detection in less than 12 h) and more sensitive (1 cfu/mL) than previous methods, including culture and PCR methods.

The reliability of PCR detection methods depends, in part, on the presence of a sufficient number of target cells (Feng, 1997). Because foods comprise complex matrices, most commercially available detection systems require selective enrichment steps to overcome problems of low pathogen numbers (Hill, 1996). A major challenge in developing a rapid RT-qPCR-based method for the detection of pathogenic bacteria in food is the optimization of the enrichment procedure to make it as short as possible, but providing sufficient target cells for downstream analysis. In the most difficult case met in the present work in this respect—that of *C. freundii*

CECT 401—a minimum 8 h of enrichment culture was required for detection to be possible. Therefore, the recommended time of enrichment to ensure the hygienic quality of the cheeses would be at least 8 h. The processing of the samples, the extraction of bacterial DNA, and RT-qPCR amplification takes between 2 and 4 h.

In conclusion, the proposed RT-qPCR was shown to be an effective, sensitive, and rapid single-day method for the detection of lactose-fermenting *Enterobacteriaceae* in cheese. It is important to note that small modifications to the procedure should allow the detection of these bacteria in drinking water (which should be easier because of the more favorable characteristics of the matrix) and in other types of food, including other dairy products.

ACKNOWLEDGMENTS

We are grateful to Corporación Alimentaria Peñasanta S.A. (CAPSA, Granda, Asturias, Spain) for its support of this work. N. Martínez, B. del Río, and V. Ladero were beneficiaries of I3P CSIC contracts financed by the European Social Fund (Brussels, Belgium). This research was also supported by project IE05-125 from the Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología (FICYT; Asturias, Spain).

REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–3402.
- APHA. 2004. Standard Methods for the Examination of Dairy Products. 17th ed. American Public Health Association, Washington, DC.
- APHA. 2005. Standard Methods for the Examination of Water and Wastewater. 21st ed. American Public Health Association, Washington, DC.
- Bej, A. K., S. C. McCarty, and R. M. Atlas. 1991. Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: Comparison with defined substrate and plating methods for water quality monitoring. *Appl. Environ. Microbiol.* 57:2429–2432.
- Bej, A. K., R. J. Steffan, J. DiCesare, L. Haff, and R. M. Atlas. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Appl. Environ. Microbiol.* 56:307–314.
- Bohaychuk, V. M., G. E. Gensler, M. E. McFall, R. K. King, and D. G. Render. 2007. A realtime PCR assay for the detection of *Salmonella* in a wide variety of food-animal matrices. *J. Food Prot.* 70:1080–1087.
- Bredie, W. L. P., and E. de Boer. 1992. Evaluation of the MPN, Anderson-Baird-Parker, Petrifilm *E. coli* and Fluorocult ECD method for enumeration of *Escherichia coli* in foods of animal origin. *Food Microbiol.* 16:197–208.
- Cheng, C.-M., K. T. Van, W. Lin, and R. M. Ruby. 2009. Interlaboratory validation of a real-time PCR 24-hour rapid method for detection of *Salmonella* in foods. *J. Food Prot.* 72:945–951.
- Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. J. Higgins, and J. D. Thompson. 2003. Multiple sequence alignment

- with the Clustal series of programs. *Nucleic Acids Res.* 31:3497–3500.
- Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella. 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* 11:260–263.
- De Buyser, M. L., B. Dufour, M. Maire, and V. Lafarge. 2001. Implication of milk and milk products in food-borne diseases in France and in different industrialized countries. *Int. J. Food Microbiol.* 67:1–17.
- Drancourt, M., C. Bollet, A. Carta, and P. Rousselier. 2001. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int. J. Syst. Evol. Microbiol.* 51:925–932.
- Elizagüivel, P., and R. Aznar. 2008. A multiplex RTi-PCR reaction for simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimally processed vegetables. *Food Microbiol.* 25:705–713.
- Feng, P. 1997. Impact of molecular biology on the detection of foodborne pathogens. *Mol. Biotechnol.* 7:267–278.
- Feng, P. C. S., and P. A. Hartman. 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli*. *Appl. Environ. Microbiol.* 43:1320–1329.
- Foulds, I., R. A. Guy, A. Kapoor, C. Xiao, U. J. Krull, and P. A. Horgen. 2002. Application of quantitative real-time PCR with dual-labeled hydrolysis probes to microbial water quality monitoring. *J. Biomol. Tech.* 13:272–276.
- Fukushima, H., Y. Tsunomori, and R. Seki. 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. *J. Clin. Microbiol.* 41:5134–5146.
- Hein, I., A. Lehner, P. Rieck, K. Klein, E. Brandl, and M. Wagner. 2001. Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. *Appl. Environ. Microbiol.* 67:3122–3126.
- Hill, W. E. 1996. The polymerase chain reaction: Applications for the detection of foodborne pathogens. *Crit. Rev. Food Sci. Nutr.* 36:123–173.
- Holicka, J., R. A. Guy, A. Kapoor, D. Shepherd, and P. A. Horgen. 2006. A rapid (one day), sensitive real-time polymerase chain reaction assay for detecting *Escherichia coli* O157:H7 in ground beef. *Can. J. Microbiol.* 52:992–998.
- Ito, H., N. Kido, Y. Arakawa, M. Ohta, T. Sugiyama, and N. Kato. 1991. Possible mechanisms underlying the slow lactose fermentation phenotype in *Shigella* spp. *Appl. Environ. Microbiol.* 57:2912–2917.
- Klein, D., P. Janda, R. Steinborn, M. Müller, B. Salmons, and W. H. Günzburg. 1999. Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: Influence of mismatches on quantification. *Electrophoresis* 20:291–299.
- Leclerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk. 2001. Advances in the bacteriology of the coliform group: Their suitability as markers of microbial water safety. *Annu. Rev. Microbiol.* 55:201–234.
- Martín, M. C., M. A. González-Hevia, J. A. Alvarez-Riesgo, and M. C. Mendoza. 2001. *Salmonella* serotype Virchow causing salmonellosis in a Spanish region. Characterization and survey of clones by DNA fingerprinting, phage typing and antimicrobial resistance. *Eur. J. Epidemiol.* 17:31–40.
- Murphy, S. C., and K. J. Boor. 2000. Trouble-shooting sources and causes of high bacteria counts in raw milk. *Dairy Food Environ. Sanit.* 20:606–611.
- Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405:299–304.
- Omiccioli, E., G. Amagliani, G. Brandi, and M. Magnani. 2009. A new platform for real-time detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiol.* 26:615–622.
- Oravcová, K., T. Kuchta, and E. Kacliková. 2007. A novel real-time PCR-based method for the detection of *Listeria monocytogenes* in food. *Lett. Appl. Microbiol.* 45:568–573.
- Powe, B., and R. J. Gross. 1984. *Shigella* Castellani and Chalmers, 1919, 936. Pages 423–427 in *Bergey's Manual of Systematic Bacteriology*. Vol. 1. N. R. Krieg and J. G. Holt, ed. Williams & Wilkins Co., Baltimore, MD.
- Tantawiwat, S., U. Tansuphasiri, W. Wongwit, V. Wonghtigul, and D. Kitayaporn. 2005. Development of multiplex PCR for detection of total coliform bacteria for *Escherichia coli* and *Clostridium perfringens* in drinking water. *Southeast Asian J. Trop. Med. Public Health* 36:162–169.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673–4680.
- Usera, M. A., A. Rodriguez, A. Echeita, and R. Cano. 1998. Multiple analysis of a foodborne outbreak caused by infant formula contaminated by an atypical *Salmonella virchow* strain. *Eur. J. Clin. Microbiol. Infect. Dis.* 17:551–555.
- Wei, J., M. B. Goldberg, V. Burland, M. M. Venkatesan, W. Deng, G. Fournier, G. F. Mayhew, G. I. I. Plunkett, D. J. Rose, A. Darling, B. Mau, N. T. Perna, S. M. Payne, L. J. Runyen-Janecky, S. Zhou, D. C. Schwartz, and F. R. Blattner. 2003. Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. *Infect. Immun.* 71:2775–2786.
- Winfield, M. D., and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol.* 69:3687–3694.
- Yang, F., J. Yang, X. Zhang, L. Chen, Y. Jiang, Y. Yan, X. Tang, J. Wang, Z. Xiong, J. Dong, Y. Xue, Y. Zhu, X. Xu, L. Sun, S. Chen, H. Nie, J. Peng, J. Xu, Y. Wang, Z. Yuan, Y. Wen, Z. Yao, Y. Shen, B. Qiang, Y. Hou, J. Yu, and Q. Jin. 2005. Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. *Nucleic Acids Res.* 33:6445–6458.
- Zottola, E. A., and L. B. Smith. 1991. Pathogens in cheese. *Food Microbiol.* 8:171–182.