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PCR-ELISA detection of *Escherichia coli* in milk

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Aims: The purpose of this study was to develop a reliable molecular procedure for the detection of *Escherichia coli* in milk.

Methods and Results: Robust and expeditious DNA extraction and PCR techniques were evaluated using Enzyme-Linked Immunosorbent Assay (ELISA) detection of biotin-labelled amplicons to facilitate optimal detection of *E. coli* DNA.

Conclusions: It was found that 5 *E. coli* colony-forming units (cfu) could be detected per PCR reaction using the PCR-ELISA system, equating to a sensitivity of detection of 100 *E. coli* cfu ml^{-1} pasteurized milk.

Significance and Impact of the Study: This approach should facilitate evaluation of milk contamination and enable rapid detection of *E. coli* mastitis, leading to correct deployment of relevant antibiotic therapy and improved animal welfare.

INTRODUCTION

In recent years *Escherichia coli* has become recognized as a serious food-borne pathogen and has been associated with numerous outbreaks of disease in the UK, Japan and USA (Uyttendaele *et al*. 1999; Scotter *et al*. 2000). Raw milk and dairy products, such as pasteurized milk and yoghurt contaminated with *E. coli*, have been the main cause of several outbreaks of milk-borne disease since the 1980s and thus remain a serious health risk (Allmann *et al*. 1995; Seo *et al*. 1998). Detection and enumeration of *E. coli* in food products can be assessed by various methods including plate counting, immuno-magnetic separation, flow cytometry and chromogenic-fluorogenic substrate technology (Venkateswaran *et al*. 1996; de Boer 1998; Seo *et al*. 1998; Yu 1998).

The standard detection method for *E. coli* involves the use of substrates that detect *E. coli* and can be time-consuming. Up to 97% of *E. coli* produce β-D-glucuronidase which hydrolyses 4-methylumbelliferyl-β-D-glucuronide (MUG) to form a blue fluorescent product (Venkateswaran *et al*. 1996; Manafi 2000). However, a mutation in the *uid*A gene in *E. coli* leads to false-negative identification when using β-D-glucuronidase as a method of detection, while certain strains of *E. coli*, such as O157, fail to produce this enzyme (Yokoigawa *et al*. 1999). In addition, β-D-glucuronidase is also produced by members of the *Salmonella*, *Shigella* and some *Yersinia* spp., while *Staphylococcus* spp. can also hydrolyse MUG. Thus, more accurate methods for detecting *E. coli* are necessary (de Boer 1998).

Polymerase chain reaction (PCR) has led to rapid and sensitive detection of *E. coli* from clinical samples and various food types (Fratamico *et al*. 2000; McKillip and Drake 2000; Hsu and Tsen 2001). However, many food types contain PCR inhibitors which co-purify with the target DNA (González *et al*. 1999), thereby requiring extensive sample preparation to remove, dilute or inactivate inhibitors prior to PCR amplification (Fratamico *et al*. 2000). In many cases, the low level of *E. coli* in food types requires a pre-enrichment step to overcome poor sensitivity, which increases the overall assay time by up to 24 h (Seo *et al*. 1998; Scotter *et al*. 2000). Thus, a more sensitive, specific and rapid method for detecting *E. coli* is required.

Alanine racemase is produced by all *E. coli* strains, including O157, and is essential for cell-wall biosynthesis by providing D-alanine to peptidoglycan. Thus, detection of the alanine racemase (*alr*) gene may be useful for the detection of *E. coli* in foods (Yokoigawa *et al*. 1999). In the present study, the successful design and application of a robust PCR-ELISA for the detection of low levels *E. coli* in milk is described.

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MATERIALS AND METHODS

Bacterial strain and artificial specimen preparation

Escherichia coli strain JM109 was obtained from Promega (Madison, WI, USA). *Escherichia coli*-adulterated milk specimens were prepared as follows. An *E. coli* culture was grown in Luria–Bertani (LB) Broth at 37°C for 16 h with shaking. A 25 ml aliquot of the culture was centrifuged at 3200 *g* for 15 min. After removal of the supernatant fluid, the pellet was resuspended in 1 ml fresh pasteurized milk, raw milk or phosphate-buffered saline (0·05% (v/v) Tween-20; PBST) and each sample serially diluted in unadulterated matrix to determine the sensitivity of PCR amplification. A 100 μl aliquot of each dilution was also plated, in triplicate, on LB Agar and colony counting was performed using an Eagle-Eye II gel documentation system (Stratagene, La Jolla, CA, USA). Fresh pasteurized milk and raw milk were individually plated to determine the presence of naturally-contaminating microflora.

DNA extraction

Milk samples (100 μl) containing *E. coli* (0–10^4 cfu ml^{-1}) were each mixed with 1 ml PBST, vortexed, and centrifuged at 10 000 *g* for 5 min. This procedure was performed to pellet the bacterial cells and to remove interfering amounts of protein and lipid in milk samples, which may interfere with PCR amplification. Following removal of the supernatant fluid, DNA was extracted using the following procedures.

Alkaline extraction. The alkaline extraction procedure was a modification of the method proposed by Millar *et al.* (2000). The bacterial pellet was resuspended in 1 ml alkaline extraction solution (0·5 mol l^{-1} sodium hydroxide, 0·05 mol l^{-1} sodium citrate). This mixture was shaken for 10 min and centrifuged at 13 000 *g* for 5 min. The supernatant fluid was again removed and the pellet resuspended in 500 μl 0·5 mol l^{-1} Tris-HCl pH 8·0, followed by centrifugation at 13 000 *g* for 5 min. This step was repeated. The final pellet was resuspended in 100 μl 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA pH 8·0, and placed in a heating block for 1 h at 100°C. The sample was then freeze–thawed twice, centrifuged at 13 000 *g* for 15 min and the supernatant fluid removed for PCR analysis.

Detergent extraction. The extraction method of Yokoigawa *et al.* (1999) to detect *E. coli* strains in foods, including raw milk, was employed. Here, following the initial centrifugation of milk samples in PBST, the supernatant fluid was removed and 100 μl lysis buffer (10% (w/v) NaCl, 2% (v/v) Tween-20 and 2 mmol l^{-1} EDTA) was added. Samples were then treated at 100°C on a heating block for 10 min and centrifuged at 5000 *g* for 5 min. Supernatant fluids were then used directly as PCR templates.

Sodium iodide (NaI) extraction. The NaI extraction procedure was that proposed by Ishizawa *et al.* (1991) for isolation of DNA from serum.

Oligonucleotide primers and DNA amplification

Primers specific for a conserved region situated within the *E. coli* alanine racemase gene were selected (Yokoigawa *et al.* 1999) using a modified amplification procedure: forward: 5′ Biotin-CTGGAAGAGGCTAGCCTGGACGAG 3′ and reverse: 5′-AAAATCGGCACCGGTGGAGCGATC-3′ (Sigma-Genosys, Cambridge, UK). A 5 μl volume of each extracted sample was used for PCR (PCR Express Thermal Cycler; Hybaid, Ashford, Middlesex, UK), which also contained 10 mmol l^{-1} Tris-HCl pH 9·0, 50 mmol l^{-1} KCl, 0·1% (v/v) Triton X-100, 2·0 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP (Promega), 1 mol l^{-1} betaine (Sigma) and 1·0 μmol l^{-1} of the above primers in a total volume of 49·5 μl. Hot-start PCR was performed on each sample by adding 1·25 U *Taq* polymerase (Promega) after the initial 6 min denaturation step at 95°C. The PCR reaction was then continued with 35 cycles consisting of 20 s denaturation at 95°C, primer annealing/extension at 72°C for 90 s, and a final extension for 5 min at 72°C. A 10 μl aliquot of each PCR product was subjected to 1% (w/v) agarose gel electrophoresis containing 0·5 μg ml^{-1} ethidium bromide (Sigma) for 30 min at 100 V. Amplicon visualization (366 bp) was performed using an Eagle-Eye II gel documentation system (Stratagene).

Microplate preparation and ELISA format

Streptavidin-coated microplates were prepared by coating microwells (Nunc Maxisorp, Roskilde, Denmark) with streptavidin (2·5 μg ml^{-1}) in 50 mmol l^{-1} sodium carbonate pH 9·4, and stabilized by the addition of 1% (w/v) albumin in the same diluent. Biotinylated PCR products were diluted 1/20 in 6× SSC to give a final volume of 200 μl; they were then added to streptavidin-coated microwells and incubated at 37°C for 30 min. After two washes with PBST, 100 μl of 125 mmol l^{-1} NaOH, 100 mmol l^{-1} NaCl were added to the microwells, incubated at room temperature for 3 min and washed four times with PBST. A 100 μl volume of dinitrophenol (DNP)-labelled oligonucleotide (100 ng ml^{-1}; 5′ $(DNP)_3$-ATATTCACCGGCTGACGAAC-3′) in 6× SSC/0·1% (w/v) sodium dodecyl sulphate was added to each microwell and incubated for 1 h at 60°C, followed by washing four times with PBST. Microwells were then blocked with 2·5% (w/v) milk powder in PBST at 20°C for 1 h. Following blocking solution removal, IgG [anti-DNP]

horseradish peroxidase conjugate was added, incubated at 20°C for 30 min and washed four times with PBST. Substrate (100 μl tetramethylbenzidine) was then added and incubated at 20°C for 15 min. The reaction was terminated by the addition of 0·5 mol l^{-1} sulphuric acid and measured spectrophotometrically at 450/630 nm (Dynatech MRX Dynex Technologies, Ashford, Middlesex, UK).

RESULTS

Extraction of *E. coli* and detection of *alr* gene amplicons by agarose gel electrophoresis

Escherichia coli was cultured in LB broth and added to raw milk, fresh pasteurized milk and PBST. Following plating of each dilution on LB agar, the numbers of colony-forming units per millilitre (cfu ml^{-1}) were determined. *Escherichia coli* DNA was extracted from adulterated samples ranging from 10^0 to 10^4 cfu ml^{-1} using the alkaline, detergent and NaI extraction procedures.

No *E. coli* DNA was detectable in either unadulterated pasteurized or raw milk, the latter having been obtained under sterile conditions. Bacterial DNA was successfully extracted from adulterated raw and pasteurized milk samples, and PBST, by the alkaline extraction and NaI methods. A minimum of 50 *E. coli* cfu were reproducibly detectable by PCR using the *alr* gene oligonucleotide primers (Table 1 and Fig. 1). Since 5 μl extracted sample (initial volume 100 μl) were used in each PCR reaction, the PCR detection limit was determined to be 10^3 cfu ml^{-1}. Significantly, the detergent extraction method proposed by Yokoigawa *et al.* (1999) failed to result in the generation of any PCR product (Table 1). In fact, amplification of target DNA failed even after high levels of *E. coli* (10^8 cfu ml^{-1}) were extracted by the detergent method (data not shown). It was also found

Table 1 PCR amplification of *Escherichia coli* DNA following extraction by sodium iodide, detergent and heat from raw milk (milk (R), pasteurized milk (milk (P) and PBST (control diluent). The intensity of ethidium bromide amplicon binding is given by the following abbreviations: high (3 +), moderate (2 +), low (+) and negative (−)

	Extraction methods								
	Sodium iodide			Detergent			Heat		
E. coli (cfu 5 μl^{-1} PCR template)	Milk (R)	Milk (P)	PBST	Milk (R)	Milk (P)	PBST	Milk (R)	Milk (P)	PBST
0	−	−	−	−	−	−	−	−	−
0·5	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	+
50	+	2 +	2 +	−	−	−	−	−	2 +
500	3 +	3 +	3 +	−	−	−	−	−	3 +

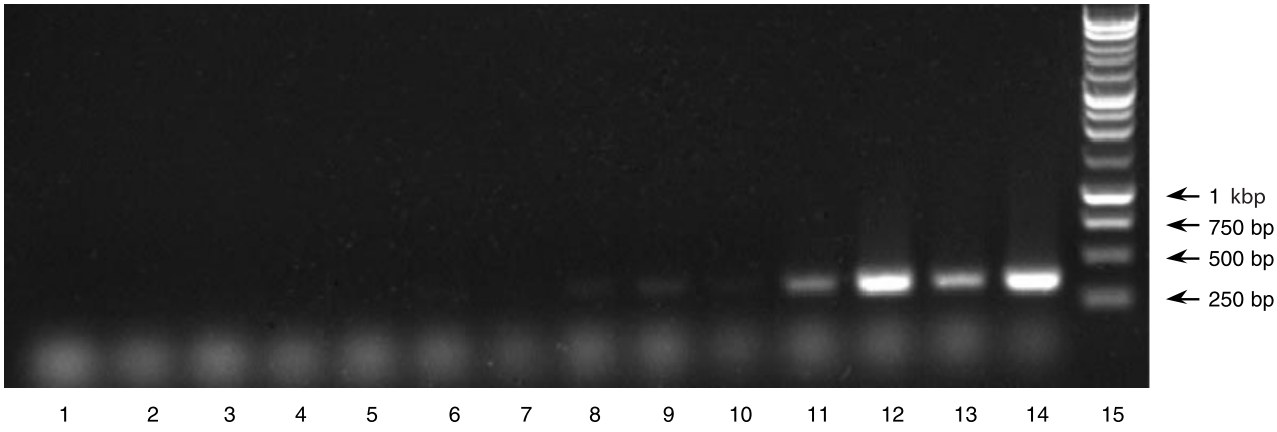

Fig. 1 Agarose gel electrophoresis of 366 bp PCR products from alkaline extraction of raw milk, pasteurized milk and PBST containing 0–500 cfu *Escherichia coli*. Lane 1: negative control; lanes 2–4: raw milk, pasteurized milk and PBST, following alkaline extraction, containing no *E. coli*; lanes 5–7: raw milk, pasteurized milk and PBST, following alkaline extraction, containing 5 cfu *E. coli*; lanes 8–10: raw milk, pasteurized milk and PBST, following alkaline extraction, containing 50 cfu *E. coli*; lanes 11–13: raw milk, pasteurized milk and PBST, following alkaline extraction, containing 500 cfu *E. coli*; lane 14: purified *E. coli* DNA (strain JM109); lane 15: 1 kbp molecular size marker

that heat treatment alone of bacterial cells (100°C for 15 min) obtained from either raw or pasteurized milk was insufficient to facilitate successful PCR amplification of *E. coli* DNA, yet DNA was detectable after heat treatment only, at identical *E. coli* cfu ml^{-1}, in PBST (Table 1).

Agarose gel electrophoresis of PCR products shows the detection of 50 and 500 cfu using the alkaline extraction method for DNA extraction from PBST and raw milk, respectively (Fig. 1). Occasionally, as few as 5 *E. coli* cfu (equivalent to 100 cfu ml^{-1}) were detectable after alkaline extraction from pasteurized milk. However, ethidium bromide staining could not reproducibly detect *E. coli* at this low level.

PCR-ELISA of *alr* gene amplicons

Following amplicon detection by gel electrophoresis, PCR products were then further analysed by ELISA in an attempt to improve the sensitivity of detection. The results of the ELISA of PCR products from alkaline extraction are presented in Fig. 2. Results show specific detection of 50–500 cfu by the DNP-labelled oligonucleotide probe when *E. coli* was extracted from raw milk and PBST. Pasteurized milk samples showed enhanced extraction of *E. coli* DNA relative to raw milk and PBST, whereby as few as 5 cfu could be reliably detected using PCR-ELISA after DNA purification by the alkaline extraction method; this confirmed the increased sensitivity of the ELISA detection method over conventional agarose gel electrophoresis and ethidium bromide detection of DNA (i.e. 10^2 *vs* 10^3 cfu ml^{-1}, respectively).

DISCUSSION

A rapid and sensitive PCR-ELISA system for detecting *E. coli* in milk has been developed. Using serial dilutions of *E. coli* in raw and pasteurized milk and PBST, the specific detection of low levels of *E. coli* and the removal of potential inhibitory substances by alkaline extraction or NaI extraction have been demonstrated. The PCR-ELISA used in this study detected between 5 and 50 *E. coli* cfu and did not require a pre-enrichment step.

Hsu and Tsen (2001) found levels of 10^6 cfu ml^{-1} (10^3 *E. coli* cfu ml^{-1} using plate count agar). Beerens *et al.* (2000) observed a mean level of 119 *E. coli* cfu ml^{-1} in 58 samples of raw milk. The level of endogenous microflora discovered in raw milk in the present study was 2×10^2 cfu ml^{-1}, with observed levels of *E. coli* between 1 and 10 cfu ml^{-1}. This lower level of bacteria in raw milk could be attributed to obtaining the milk from cows under aseptic conditions and from healthy cows with no evidence of mammary infection. Uyttendaele *et al.* (1999) reported that the presence of 10^6 cfu ml^{-1} of endogenous microflora did not influence the detection limit of their PCR assay, which was in agreement with our observations. It should be noted that the *alr* primers used in this study detect a wide range of *E. coli* strains but also lead to the detection of *Shigella* spp. (Yokoigawa *et al.* 1999). However, the incidence levels of *Shigella* in foods is ordinarily very low (González *et al.* 1999) and therefore would not be expected to complicate the detection limits of the PCR-ELISA assay presented here.

Yokoigawa *et al.* (1999) quote a sensitivity of detection of 10^3 *E. coli* cells ml^{-1} using detergent extraction and an identical primer pair to that employed in the present study. However, it proved impossible to replicate this finding, possibly due to the inhibitory nature of EDTA in the PCR reaction. In order to obtain an equivalent level of sensitivity to that of Yokoigawa *et al.*, the following modifications were required. First, an increase in PCR cycle number from 25 to 35 cycles had a significant effect, resulting in at least a 10^6 increase in sensitivity of detection. Secondly, the

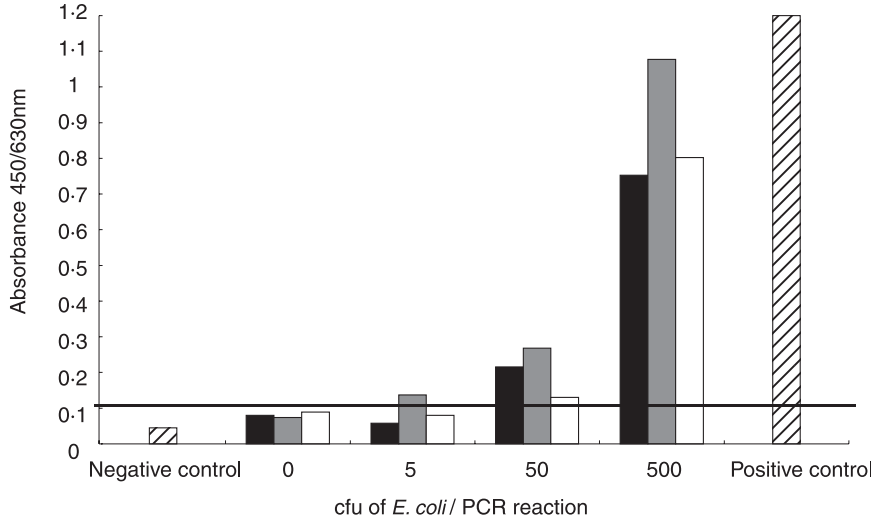

Fig. 2 PCR-ELISA analysis of raw milk (■), pasteurized milk (▨) and PBST (□) containing 0–500 cfu *Escherichia coli* obtained following alkaline extraction procedure. Negative and positive controls are also indicated. Assay cut-off (horizontal line) was calculated by replicate analysis of eight negative specimens and was determined to be 0·105 O.D. units ($mean_{negatives}$ + 2 standard deviations, i.e. 0·065 + 2 (0·02))

wash/centrifugation step prior to extraction was included to enhance the recovery of bacteria and also to help remove the high levels of fat and protein. Interestingly, the elimination of potential PCR inhibitors by the inclusion of a washing step while recovering the bacterial pellet prior to DNA extraction was also achieved by Uyttendaele *et al*. (1999). Thirdly, hot-start PCR was used to prevent reduced activity of *Taq* polymerase during the initial denaturation step. Finally, betaine (1 mol l^{-1}) was included as a PCR additive to enhance amplification. Investigation into the failure of amplification using the method of Yokoigawa *et al*. (1999) illustrated that even when 10^6 cfu *E. coli* were added to the PCR master mix containing 5 μl of detergent extraction buffer, inhibition of amplification resulted.

The higher sensitivity of *E. coli* detection in pasteurized milk samples using alkaline extraction can be explained by enhanced removal of inhibitors, thereby facilitating increased amplification efficiency. Furthermore, failure of sample heating alone to release detectable *E. coli* DNA from milk further highlights the importance of the removal of inhibitory compounds prior to amplification.

The development of this novel PCR-ELISA method offers several advantages over traditional microbiological culture techniques. The PCR step takes just over 1 h to complete due to the simultaneous annealing and polymerization at 72°C. Specific amplicon detection via the DNP oligonucleotide probe used in the ELISA confers increased assay specificity with optimal sensitivity. The method is straightforward, does not require expensive equipment and should be capable of detecting the suggested *E. coli* O157 infective dose (4–24 organisms) as proposed by Strachan *et al*. (2001). Automation and multiple sample analysis can also be more easily performed on streptavidin-coated microtitre plates in comparison with agarose gel electrophoresis. In addition, the subjectivity of band identification and the use of carcinogenic DNA binding agents are also eliminated. This novel PCR-ELISA should help in the assessment of the hygienic quality of milk collected from farms and facilitate rapid diagnosis of *E. coli* mastitis, which would allow for early antibiotic therapy helping to reduce loss in milk production and damage to mammary tissue.

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REFERENCES

Allmann, M., Höfelein, C., Köppel, E. et al. (1995) Polymerase chain reaction (PCR) for detection of pathogenic microorganisms in bacteriological monitoring of dairy products. *Research in Microbiology* **146**, 85–97.

Beerens, H., Hass Brac de la Perriere, B. and Gavini, F. (2000) Evaluation of the hygienic quality of raw milk based on the presence of bifidobacteria: the cow as a source of faecal contamination. *International Journal of Food Microbiology* **54**, 163–169.

de Boer, E. (1998) Update on media for isolation of Enterobacteriaceae from foods. *International Journal of Food Microbiology* **45**, 43–53.

Fratamico, P.M., Bagi, L.K. and Pepe, T. (2000) A multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. *Journal of Food Protection* **63**, 1032–1037.

González, I., Garcia, T., Fernandez, A., Sanz, B., Hernandez, P.E. and Martin, R. (1999) Rapid enumeration of *Escherichia coli* in oysters by a quantitative PCR-ELISA. *Journal of Applied Microbiology* **86**, 231–236.

Hsu, S.-C. and Tsen, H.-Y. (2001) PCR primers designed from malic acid dehydrogenase gene and their use for detection of *Escherichia coli* in water and milk samples. *International Journal of Food Microbiology* **64**, 1–11.

Ishizawa, M., Kobayashi, Y., Miyamura, T. and Matsuura, S. (1991) Simple procedure of DNA isolation from human serum. *Nucleic Acids Research* **19**, 5792.

Manafi, M. (2000) New developments in chromogenic and fluorogenic culture media. *International Journal of Food Microbiology* **60**, 205–218.

McKillip, J.L. and Drake, M. (2000) Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. *Journal of Food Protection* **63**, 855–859.

Millar, B.C., Jiru, X., Moore, J.E. and Earle, J.A.P. (2000) A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. *Journal of Microbiological Methods* **42**, 139–147.

Scotter, S., Aldridge, M. and Capps, K. (2000) Validation of a method for the detection of *E. coli* O157:H7 in foods. *Food Control* **11**, 85–95.

Seo, K.H., Brackett, R.E. and Frank, J.F. (1998) Rapid detection of *Escherichia coli* O157:H7 using immuno-magnetic flow cytometry in ground beef, apple juice, and milk. *International Journal of Food Microbiology* **44**, 115–123.

Strachan, N.J.C., Fenlon, D.R. and Ogden, I.D. (2001) Modelling the vector pathway and infection of humans in an environmental outbreak of *Escherichia coli* O157. *FEMS Microbiology Letters* **203**, 69–73.

Uyttendaele, M., van Boxstael, S. and Debevere, J. (1999) PCR assay for detection of the *E. coli* O157:H7 eae-gene and effect of the sample preparation method on PCR detection of heat-killed *E. coli* O157:H7 in ground beef. *International Journal of Food Microbiology* **52**, 85–95.

Venkateswaran, K., Murakoshi, A. and Satake, M. (1996) Comparison of commercially available kits with standard methods for detection of coliforms and *Escherichia coli* in foods. *Applied and Environmental Microbiology* **62**, 2236–2243.

Yokoigawa, K., Inoue, K., Okubo, Y. and Kawai, H. (1999) Primers for amplifying an alanine racemase gene fragment to detect *E. coli* strains in foods. *Journal of Food Science* **64**, 571–575.

Yu, H. (1998) Comparative studies of magnetic particle-based solid phase fluorogenic and electrochemiluminescent immunoassay. *Journal of Immunological Methods* **218**, 1–8.