

# Preparation of milk samples for PCR analysis using a rapid filtration technique

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## ABSTRACT

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**Aims:** To investigate the usefulness of a straightforward filtration method for the isolation of *Escherichia coli* O157:H7 contaminants from milk for PCR detection.

**Methods and Results:** *Escherichia coli* O157:H7 is grown in milk and enriched in Luria–Bertani (LB) medium. Samples are filtered through a 0.45- $\mu$ m pore membrane. The membrane is immersed in 200- $\mu$ l lysis buffer and incubated at 95°C for 10 min to release bacterial DNA for subsequent PCR detection. Under current conditions, the overall duration from filtration to PCR-ready DNA generation is <20 min, and the detection level for PCR was as low as 10 CFU of bacteria in 1 ml of milk.

**Conclusion:** Bacterial contaminants of milk can be concentrated and isolated by a simple, one-step filtration and their DNA can be released for subsequent PCR detection by heating the filter membrane at 95°C for 10 min.

**Significance and Impact of the Study:** The simplicity of this method allows inexpensive, high throughput automation that meets the demands of modern food hygiene monitoring.

**Keywords:** detection, *Escherichia coli*, filtration, food analysis, PCR.

## INTRODUCTION

The detection of microbial pathogens in foods is critical for consumer safety. Traditionally, detection of food-borne bacteria relies on biochemical and immunological methods that necessitate prolonged procedures for culturing and isolation of the organisms in question. However, advances in molecular biology techniques for nucleic acid manipulation have led to the suggestion that DNA-based tests will be faster, more sensitive and more specific for monitoring microbial contaminants (de Boer and Beumer 1999; Rudi *et al.* 2002; Alocilja and Radke 2003).

Polymerase chain reaction (PCR) is the most acknowledged and adaptable DNA-based assay technique partly due to the amplification of target DNA sequence by, as much as five orders of magnitude, reducing or eliminating the

lengthy enrichment and isolation of micro-organisms (Hill 1996; Barrett *et al.* 1997). However, the complexity of the food samples and the presence of PCR inhibitors have restricted such theoretical capacity and, consequently, the efficacy of PCR for detecting food pathogens is compromised. For example, the presence of high levels of protein and calcium in the milk can diminish amplification efficiency (Rossen *et al.* 1992; Bickley *et al.* 1996). Various methods, such as immunomagnetic separation (Gooding and Choudary 1997; Grant *et al.* 2000; Djonne *et al.* 2003), centrifugation (Lindqvist 1997; Hsu and Tsen 2001), proteinase digestion (Bickley *et al.* 1996; Hein *et al.* 2001), direct purification of bacterial DNA (Romero and Lopez-Goñi 1999), have been employed to concentrate bacteria and to remove PCR inhibitors. Despite their value in PCR-grade sample preparation, this array of methods still require a certain amount of time and labour. Moreover, the requirement of specific reagents and/or equipment reduces the cost-effectiveness and general usefulness.

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Apparently, more rapid, inexpensive and reliable methods for sample preparation are still needed to fully exploit the potential of PCR in food-borne pathogen detection. The aim of the present study, therefore, was to investigate the usefulness of a straightforward method of a quick enrichment and one-step filtration to prepare samples for PCR detection of bacterial pathogens. We evaluated this method by detecting as little as 10 CFU *Escherichia coli* O157:H7 in milk. Milk inoculated with *E. coli* O157:H7 was first diluted in Luria-Bertani (LB) medium for bacterial enrichment. This was followed by a one-step filtration. The filter membrane was subsequently immersed in lysis buffer and heated to release bacterial DNA. The lysate was then used directly for PCR detection. Fast, accurate and reproducible results demonstrate the value of this efficient method for detecting food contaminants.

## MATERIALS AND METHODS

### Bacterial strains and culture media

The enterohaemorrhagic *E. coli* O157:H7 *stx*<sup>+</sup> strain used in this study was a kind gift from Mrs F. McCleskey (Armstrong Laboratory, Brooks Air Force Base, San Antonio, TX, USA). Nonpathogenic *E. coli* strain TOP10 was obtained from Invitrogen (Carlsbad, CA, USA). Cells were grown at 37°C in LB broth (yeast extract, 5 g; tryptone, 10 g; NaCl, 5 g and H<sub>2</sub>O, 1 l) with agitation at the rate of 200 rev min<sup>-1</sup> or on LB plates. For liquid cultures, media were prefiltered through 0.45 µm membranes prior to autoclave to remove any insoluble particles that can interfere with subsequent experiments.

### PCR primers and amplification conditions

The gene encoding for *E. coli* O157:H7 Shiga-like toxin (genebank accession no. AB048837) was targeted for PCR detection using primers 5'-CGGTTTCCATGACAACGGACAG-3' and 5'-CACAGGAGCAGTTTCAGACAGTG-3'. The size of the expected PCR amplicon is 251 bp.

The specificity of the primers were verified by isolating total DNA from agar grown, single colonies of strains O157:H7 and TOP10. Each colony was suspended in 200 µl of lysing solution composed of 0.5% Triton X-100, 20 mM Tris (pH 8.0), and 2 mM EDTA (Fratamico *et al.* 1995). The suspension was boiled for 10 min; 5 µl of the colony lysate and 50 pmol of each primer were added to the reaction mixture in a total volume of 100 µl.

DNA polymerase used in this study was AmpliTaq Gold (Applied Biosystem, Foster City, CA, USA) and the reaction mixture was according to manufacturer's suggestion except the MgCl<sub>2</sub> concentration was adjusted to 2 mM. Amplification was carried out in a Perkin-Elmer Gene Amp 2400 thermal cycler (Perkin-Elmer Corp., Norwalk, CT, USA). An initial

denaturation of 94°C for 10 min was followed by 40 cycles of denaturation at 94°C for 30 s and annealing/polymerization at 68°C for 90 s, and a final extension at 72°C for 5 m. Following amplification, 10 µl of the PCR reaction was analysed by agarose (2%) gel electrophoresis and subsequent visualization with ethidium bromide.

### Milk inoculation

A 100-µl overnight culture of *E. coli* O157:H7 was mixed in 100 ml LB broth and allowed to grow at 37°C for 3 h to dilute nondividing cells. Serial dilutions were made thereafter and the quantity of bacteria in each dilution was determined by plating on LB plates.

Ten-millilitre samples of pasteurized whole milk (purchased from the local supermarket) were inoculated with 100 µl of each dilution. A 100-µl of the inoculated milk was then transferred to a flask containing 100 ml fresh LB broth and allowed to grow at 37°C for 4 h.

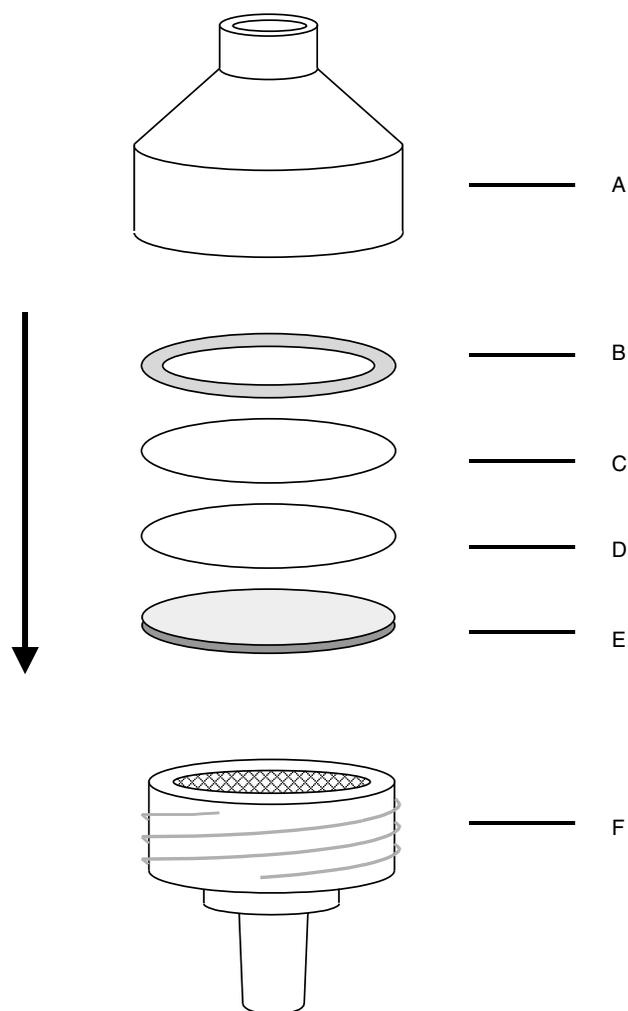
### Preparation of DNA samples from milk

Five millilitres of the enrichment broth was filtered through a 0.45-µm membrane filter (HA type, 13 mm diameter, Cat. No. HAWP 013 00; Millipore, Bedford, MA, USA) with a Swinnex syringe filter holder (Millipore) and a vacuum unit. The detailed assembly of the system is illustrated in Fig. 1. After filtration, the filter membrane was removed from the filter holder and placed in a 0.5 ml sterilized microfuge tube containing 200 µl of cell lysis buffer [0.25% Triton X-100, 10 mM Tris (pH 8.0) and 1 mM EDTA]. The tube was then heated at 95°C for 10 min in order to lyse the cells. Ten microlitres of the lysates was then subjected directly to PCR amplification as described above.

## RESULTS

### Selectivity of the primers for PCR-based analysis of spiked milk

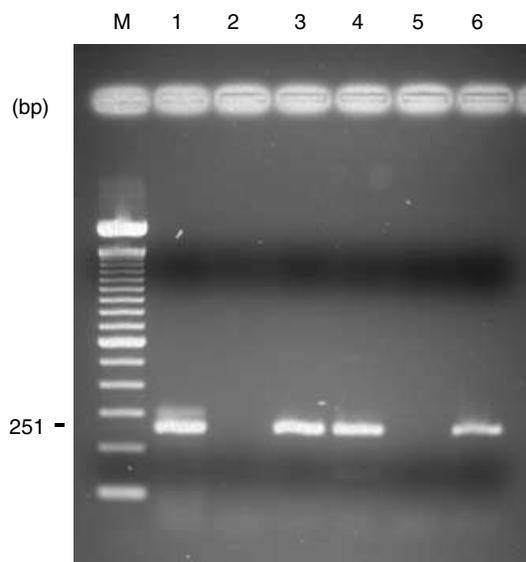
PCR using the Shiga-like toxin gene-specific primers and DNA template prepared from *E. coli* O157:H7 strain generated a single DNA fragment, as visualized by ethidium bromide staining of an agarose electrophorogram. The length of the DNA fragment was estimated to be *ca* 250 bp, which is the expected size of the amplicon. Sequencing result confirmed that the amplicon was amplified from *E. coli* O157:H7 Shiga-like toxin gene (data not shown). No DNA bands were found in an avirulent *E. coli* strain TOP10 that was analysed in an identical manner (data not shown). The high reproducibility of the PCR indicates that the high-melting point primers and the two-step amplification cycles are sufficient for the PCR detection experiment.



**Fig. 1** Schematic drawing of the filtration unit assembly. (a) Filter holder cover; (b) Teflon O-ring; (c) first filter membrane; (d) second filter membrane; (e) 10 mm thick prefilter membrane; and (f) filter holder base. The first filter membrane is used to collect the bacterial cells that will be lysed for DNA amplification. The second filter membrane cushions the first, allowing easy removal. The thicker prefilter membrane is required to ensure the dryness of the first filter membrane after vacuuming. Arrow indicates flow direction

### Efficacy and sensitivity of filtration

Before testing the milk samples, pure LB broth was used to analyse the efficacy of the cell isolation and lysis procedures. Ten-millilitre LB broth samples containing varying amounts of *E. coli* O157:H7 cells were passed through the filtration unit. No obstruction of the filter membrane was observed when the bacterial titre was below  $10^4$  CFU ml<sup>-1</sup> (the maximum titre tested in this study). The filter was transferred to a 0.5-ml microfuge tube and the cells on the filter were lysed at 95°C, 10 min in lysis buffer as described

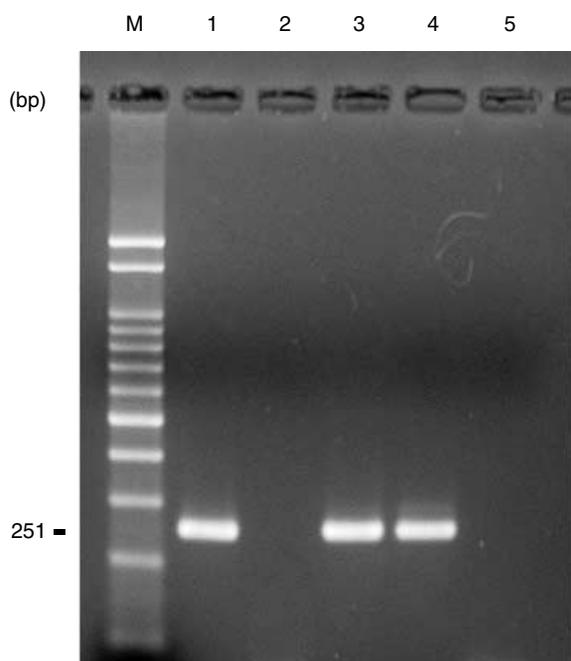


**Fig. 2** Agarose (2%) gel electrophoretogram of PCR-amplified products from bacteria cells lysed on the filter. Various cell concentrations of *Escherichia coli* O157:H7 in 10 ml LB broth were collected on the filter of the filtration unit illustrated in Fig. 1. Lane 1, positive control using DNA sample derived from a single colony of *E. coli* O157:H7 as PCR template. Lane 2, LB broth negative control. Lanes 3–6, 10 ml of LB broth containing  $10^3$ ,  $10^2$ ,  $10^1$  and  $10^0$  *E. coli* O157:H7 cell(s), respectively. Lane M, a 100-bp DNA ladder

in 'Material and methods'. When subjected to PCR amplification using our specially designed primers, the targeted Shiga-like toxin gene was amplified at cell concentrations as low as 100 cells per 10 ml of broth, and occasionally even below 10 cells per 10 ml of broth. A representative test is shown in Fig. 2. As only a 5- $\mu$ l sample is withdrawn from the 200  $\mu$ l of cell lysate for PCR amplification, the probability of obtaining a sample containing template DNA is small when cell numbers are below 100. No amplicon was detected in LB broth alone or when *E. coli* TOP10 was prepared at the sample dilution levels as that of strain O157:H7 (data not shown).

### Detection of *E. coli* O157:H7 in milk

*Escherichia coli* O157:H7-inoculated milk was diluted in LB broth to decrease the concentration of potential PCR inhibitors present in milk and incubated for 4 h at 37°C to enrich the cells. The number of *E. coli* cells in the enrichment broth was determined prior to, and after, incubation by plating on LB agar plates. Five millilitres of the enriched broth was passed through the filtration unit. The flow rate of the LB/milk sample was slightly, although not significantly, slower than that of milk-free LB broth. Following filtration, the filter was transferred to a microfuge tube and the cells were lysed in 200  $\mu$ l of lysis buffer at



**Fig. 3** Agarose (2%) gel electrophoretogram of PCR-amplified products from DNA extracted from enrichment broths of milk samples seeded with varying amounts of *Escherichia coli* O157:H7. Samples of 100  $\mu$ l each were transferred into 100 ml LB broth for enrichment, and a 5 ml aliquot of the mixture was filtered through the filtration unit described in Fig. 1. Lane 1, purified DNA from *E. coli* O157:H7. Lane 2, milk sample without added bacteria. Lanes 3–5, filtered 5 ml LB broth from 100 ml of LB broth inoculated with 100  $\mu$ l milk samples spiked with  $10^3$ ,  $10^2$ , and  $10^1$  CFU ml $^{-1}$  *E. coli* O157:H7, respectively. Lane M contains a 100 bp DNA ladder

95°C. This was followed by PCR amplification of a 5- $\mu$ l sample containing the DNA template for amplification under the direction of Shiga-like toxin gene primers. As shown in Fig. 3, a specific band was consistently observed in the agarose gel electrophoretograms when cell numbers were 100 CFU ml $^{-1}$  or more in 10 ml of milk. Occasionally, an identical amplicon was observed when cell numbers of 10 CFU ml $^{-1}$  in 10 ml of milk were used. Again, as only 100  $\mu$ l of the milk sample was transferred for enrichment, the probability of acquiring an *E. coli* cell became very small when 10 CFU ml $^{-1}$  or less in 10 ml of milk was used. No amplicon was detected when *E. coli* TOP10 was substituted for strain O157:H7 (data not shown).

## DISCUSSION

The dearth of rapid and user-friendly sample preparation methods is one of the major obstacles that needs to be overcome to fully exploit the benefits of PCR-based food-borne pathogen detection. The use of a filtration technique

to accomplish this goal has been applied to some food types (Starbuck *et al.* 1992; Tsuchiya *et al.* 1992; Venkateswaran *et al.* 1997; Lantz *et al.* 1999). Nevertheless, they either involved multiple filtration steps or required complicated postfiltration treatments that substantially diminished its efficiency. Therefore, a simplified yet effective protocol was developed in the study presented here. Using *E. coli* O157:H7-spiked milk sample, we have demonstrated that a one-step filtration followed by heating the filter membrane is sufficient for preparing DNA samples for subsequent PCR detection. This procedure greatly simplifies the equipment and reagents employed, and reduces the duration of the assay (from filtration to PCR ready DNA) to <20 min.

In addition to the fat and suspension particles that can clog filtration membranes, milk contains high concentrations of several PCR inhibitors such as proteinase and calcium ions (for review, see Wilson 1997 and references therein). These PCR inhibitors make the direct filtration of milk samples impractical, and necessitate an enrichment procedure to dilute these components. LB broth was employed for this purpose, and showed no inhibitory effect on subsequent PCR. Moreover, a 1 : 1000 dilution of milk during enrichment significantly reduced the adverse effect of those components on both filtration and PCR efficiencies. According to the report from Bickley *et al.* (1996), our milk : diluent ratio is lower than it needs to be for successful PCR amplification, provided the magnesium concentration in the reaction is optimized. This suggests that, decreasing the milk dilution factor to shorten the enrichment time and/or increase the detection sensitivity is plausible, assuming the filtration efficiency is not compromised.

In current experimental design, the minimal bacterial titre of milk to obtain a reproducible PCR-positive result was 100 CFU ml $^{-1}$ . Considering that 4 h of enrichment can increase the titre up to 4000-fold as *E. coli* grows under optimum conditions can have a doubling time as low as 20 min, and that bacteria with titres below 10 CFU ml $^{-1}$  can occasionally be detected, the limiting factor for detection is most likely the probability of selecting viable cells from milk for enrichment. If so, the existing sensitivity could potentially be increased. For example, the previously described milk dilution factor in enrichment broth can be reduced to enhance the probability of selecting one of the sparse bacterial cells. Otherwise, if the efficiency of filtration and PCR is a cause for concern, the volume for enrichment and filtration can be scaled up to keep the milk dilution factor constant. Accordingly, a 10-fold increase in the enrichment volume should reproducibly yield a detection sensitivity down to 10 CFU ml $^{-1}$  milk sample. A stepwise enrichment procedure can also be developed to increase the initial quantity of milk, thereby increasing the probability of selecting a dilute micro-organism.

Due to inherent differences of foodstuffs and the contaminating micro-organisms, more studies are required to examine the applicability of this method for the detection of various microbial pathogens in numerous foodstuffs. Nevertheless, the method presented here is simple and rapid, and yields consistent and reliable results. The time required from collecting milk sample for enrichment to viewing the electropherogram is <8 h. Moreover, several existing techniques can be incorporated to further strengthen its specificity and efficiency. For example, using a multiplex PCR that can simultaneously amplify DNA fragments corresponding to the serotypic antigens and virulent factors of *E. coli* O157:H7 would allow unambiguous detection and identification (Hu *et al.* 1999). Real-time PCR techniques that amplify and detect amplicon concurrently can eliminate the need for gel electrophoresis and staining (Bellin *et al.* 2001; Hein *et al.* 2001). Finally, the simplicity of this method will allow inexpensive, high throughput automation that meets the demands of modern food monitoring.

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## REFERENCES

- Alocilja, E.C. and Radke, S.M. (2003) Market analysis of biosensors for food safety. *Biosensors & Bioelectronics* **18**, 841–846.
- Barrett, T., Feng, P. and Swaminathan, B. (1997) Amplification methods for detection of foodborne pathogens. In *Nucleic Acid Amplification Techniques: Application to Disease Diagnosis* ed. Lee, H.H., Morse, S.A. and Olsvik, O. pp. 171–181. Boston, MA: Eaton Publishing.
- Bellin, T., Pulz, M., Matussek, A., Hempen, H.G. and Gunzer, F. (2001) Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. *Journal of Clinical Microbiology* (January) **39**, 370–374.
- Bickley, J., Short, J.K., McDowell, D.G. and Parkes, H.C. (1996) Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in milk and reversal of PCR inhibition caused by calcium ions. *Letters in Applied Microbiology* **22**, 153–158.
- de Boer, E. and Beumer, R.R. (1999) Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology* **12**, 119–130.
- Djonne, B., Jensen, M.R., Grant, I.R. and Holstad, G. (2003) Detection by immunomagnetic PCR of *Mycobacterium avium* subsp. *paratuberculosis* in milk from dairy goats in Norway. *Veterinary Microbiology* **92**, 135–143.
- Fratamico, P., Sakitey, S.K., Wiedmann, M. and Deng, M.Y. (1995) Detection of *Escherichia coli* O157:H7 by multiplex PCR. *Journal of Clinical Microbiology* **33**, 2188–2191.
- Gooding, C.M. and Choudary, P.V. (1997) Rapid and sensitive immunomagnetic separation-polymerase chain reaction method for the detection of *Escherichia coli* O157:H7 in raw milk and ice-cream. *Journal of Dairy Research* **64**, 87–93.
- Grant, I.R., Pope, C.M., O’Riordan, L.M., Ball, H.J. and Rowe, M.T. (2000) Improved detection of *Mycobacterium avium* subsp. *Paratuberculosis* in milk by immunomagnetic PCR. *Veterinary Microbiology* **77**, 369–378.
- Hein, I., Klein, D., Lehner, A., Bubert, A., Brandl, E. and Wagner M. (2001) Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. *Research in Microbiology* **152**, 37–46.
- Hill, W.E. (1996) The polymerase chain reaction: application for the detection of foodborne pathogens. *Critical Review in Food Science and Nutrition* **36**, 123–173.
- 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.
- Hu, Y., Zhang, Q. and Meitzler, J.C. (1999) Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. *Journal of Applied Microbiology* **87**, 867–876.
- Lantz, P.G., Stålhandske, F.I.T., Lundahl, K. and Råetrom, P. (1999) Detection of yeast by PCR in sucrose solution using a sample preparation method based on filtration. *World Journal of Microbiology & Biotechnology* **15**, 345–348.
- Lindqvist R. (1997) Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* O157:H7. *International Journal of Food Microbiology* **37**, 73–82.
- Romero, C. and Lopez-Goñi, I. (1999) Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella spp.* by PCR. *Applied and Environmental Microbiology* **65**, 3735–3737.
- Rossen, L., Norskov, P., Holmstrom K. and Rasmussen O.F. (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International Journal of Food Microbiology* **17**, 37–45.
- Rudi, K., Nogva, H.K., Moen, B., Nissen, H., Bredhold, S., Møretro, T., Naterstad, K. and Holck, A. (2002) Development and application of new nucleic acid-based technologies for microbial community analyses in foods. *International Journal of Food Microbiology* **78**, 171–180.
- Starbuck, M.A.B., Hill, P.J. and Stewart, G.S.A.B. (1992) Ultra sensitive detection of *Listeria monocytogenes* in milk by the polymerase chain reaction (PCR). *Letters in Applied Microbiology* **15**, 248–252.
- Tsuchiya, Y., Kaneda, H., Kano, Y. and Koshino, S. (1992) Detection of beer spoilage organisms by polymerase chain reaction technology. *Journal of the American Society of Brewing Chemists* **51**, 63–66.
- Venkateswaran, K., Kamijoh, Y., Ohashi, E. and Nakanishi H. (1997) A simple filtration technique to detect enterohemorrhagic *Escherichia coli* O157:H7 and its toxins in beef by multiplex PCR. *Applied and Environmental Microbiology* **63**, 4127–4131.
- Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63**, 3741–3751.