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Research Note

Detection of *tet*(M) Gene from Raw Milk by Rapid DNA Extraction Followed by a Two-Step PCR with Nested Primers

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ABSTRACT

The likelihood that milk and milk products may act as a vehicle for antibiotic-resistant bacterial genes has become a concern to the food industry and a public health issue, and the demand for rapid tests has increased. The purity of DNA extracted from food samples is a key issue in the sensitivity and usefulness of biological analyses, such as PCR for pathogens and nonpathogens. A rapid, phenol-chloroform free method based on a modification of a sodium iodide DNA extraction, followed by a two-step PCR was developed for direct detection of the *tet*(M) gene in milk samples within a single working day. This study compares the proposed method with a traditional phenol solvent extraction method and with a commercial kit (QIAamp DNA blood mini kit, Qiagen). The three DNA extraction methods were used to ensure access to the *tet*(M) gene from 1 ml of raw milk, inoculated with a strain of *Enterococcus faecalis,* which carries the *tet*(M) gene. The proposed method, followed by a two-step PCR with nested primers specific for the *tet*(M) gene, was able to reach a detection limit below 10 CFU/ml in less than 4 h, including the two amplification cycles, thus outperforming in sensitivity and rapidity both the traditional and the commercial method.

Commensal bacteria that are present in the intestine of farmed animals exposed to antibiotics act as reservoirs of antibiotic resistance, thereby spreading antibiotic-resistant bacteria along the food chain. For several decades, the detection of antibiotic resistance in bacteria has been confined to cultivable clinical isolates and has been investigated by phenotypic-based methods mostly by evaluating the susceptibilities of bacteria to antibiotics using disc diffusion and dilution methods. For these reasons, information concerning antibiotic resistance in commensal microflora is scarce *(2, 3, 4, 12).* To increase our understanding of antimicrobial resistance mechanisms, new methods have been developed, including those designed to detect resistance genes by molecular techniques. An interesting model that is used to understand the mechanisms underlying antibiotic resistance along the food chain and in the environment detects genes that encode resistance to tetracyclines in commensal indicator organisms in foods and the environment *(2, 3, 14, 20).* Because of their broad spectrum, relative safety, and low cost, tetracyclines have been widely used in human and veterinary therapy, as growth promoters, and for prophylaxis in aquaculture *(3, 19, 22).* Presently, resistance to tetracyclines has spread to almost every bacterial genera, most probably as a consequence of previous overuse *(20, 25).* Resistance to tetracycline is often a result of the acquisition of new genes, which are carried on conjugative plasmids or transposons. Tetracycline resistance in bacteria is commonly mediated by two mechanisms: mainly by ribosomal protection by large cytoplasmic proteins and energy dependent efflux pumps, and enzymatic inactivation of tetracycline, which is relatively uncommon *(22).* Ribosomal protection proteins are encoded by eight genes: *tet*(M), *tet*(O), *tet*B(P), *tet*(Q), *tet*(S), *tet*(W), *tet*(T), and *oxtr*(A) *(22).* The tetracycline genes associated with efflux pumps are *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(I), *tet*(M), and *tet*(K) *(14, 24).* An example of a tetracycline resistance gene causing the enzymatic alteration of tetracycline is *tet*(X) *(22).* Tetracycline resistance genes have spread to different microbial species and over long distances. The best example of this spread is the *tet*(M) gene that was originally described in streptococci and subsequently found in various gram-positive and gram-negative bacteria including staphylococci, enterococci, clostridia, *Listeria,* and various enteric bacteria *(22, 26).* Among the abovementioned bacteria species, which have been shown to harbor genes encoding resistance to tetracycline, enterococci have recently captured the research community's attention because they are virtually resistant to all available antibiotics except vancomycin *(11, 16).* In the last decade, enterococci have emerged as important nosocomial pathogens in surgical wounds, bacteremia, and urinary tract infections *(19).* Antibiotic-resistant enterococci have been found in

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meat products, dairy products, ready-to-eat foods, and even within enterococcal strains proposed as probiotics *(6, 10, 11, 14, 23).* In light of the above-mentioned considerations, rapid detection of resistance genes in bacterial strains of food origin is becoming an important diagnostic and epidemiological tool, followed, if needed, by detection of genus- or species-specific genes. This has prompted the development of more sensitive molecular methods that make it possible to detect resistance genes in foods of animal origin. The most common approach has been to amplify specific DNA sequences by PCR, which allows direct detection of a target gene or target gene sequences in bacteria. In this study, a modification of the NaI-based method developed by Ishizawa *(15)* for DNA extraction, followed by a two-step PCR, is proposed as a safer alternative to a traditional phenol solvent extraction method and to a commercial kit, for a faster and more sensitive detection of the *tet*(M) gene from raw milk.

MATERIALS AND METHODS

Bacterial strain and artificial specimen preparation. The *E. faecalis tet*(M) isolate used in this study is a laboratory strain provided by the Department of Pathology and Microbiology, School of Medical Science, University of Bristol. The nucleotide sequence of *E. faecalis tet*(M) gene has been previously reported (GenBank accession number 1065723) *(21). E. faecalis tet*(M)-adulterated milk specimens were prepared as follows. An *E. faecalis tet*(M) culture was grown aerobically in nutrient broth (Oxoid CM0001, Basingstoke, UK) at 37°C for 16 h. Total viable cells count (on nutrient agar, Oxoid CM0003, incubated at 37°C on air for 24 h) at 16 h was 1×10^8 CFU/ml. A 10-ml aliquot of the culture was then added to 90 ml of raw milk to obtain a final concentration of 10^7 CFU/ml. Decimal dilutions were performed to obtain the following concentration in milk: 10^6, 10^5, 10^4, 10^3, 10^2, and 10 CFU/ml. Moreover, serial dilutions (1:1) were performed with the 10 CFU/ml concentration to obtain the following concentration in milk: 5, 2.5, and 1.25 CFU/ml. Total viable cells counts from all samples were recorded, as a control, on nutrient agar. Raw milk used in this experiment had been previously tested for the absence of contaminating bacteria resistant to tetracycline.

DNA extraction. Three methods were compared, (i) a NaI extraction, (ii) a traditional solvent extraction, and (iii) a commercial kit (QIAamp DNA blood mini kit, Qiagen, 52306, Milan, Italy). Milk samples (1 ml) containing *E. faecalis tet*(M) (0 to 10^6 CFU/ml) were each mixed with 1 ml of phosphate-buffered saline (80 g NaCl, 11.6 g Na_2HPO_4, 2 g KH_2PO_4, 2 g KCl, and distilled water to 10 liters, pH 7.2 to 7.4)–Tween-20 (0.05%, vol/vol), vortexed, and centrifuged at 10,000 $\times$ *g* for 5 min. This procedure was performed to pellet the bacterial cells and to remove proteins and lipids in milk samples, which may interfere with PCR amplification. After the removal of the supernatant fluid, DNA was extracted using the following procedures.

(i) Sodium iodide (NaI) method. The NaI extraction procedure was a modification of the method proposed by Ishizawa et al. *(15).* The pellet was resuspended in 1 ml of Tris-EDTA (TE) buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0), transferred to a 2-ml microcentrifuge tube, and added with 200 μl of ammonium hydroxide, 200 μl of ethanol absolute, 400 μl of petroleum ether, and 20 μl of sodium dodecyl sulfate (SDS) 10%. The mixture was vortexed and then centrifuged at 15,000 $\times$ *g* for 10 min. After supernatant removal, 1 ml of phosphate-buffered saline–Tween-20 was added and 100 μl was transferred to a 2-ml microcentrifuge tube. A 300-μl solution containing 6 M NaI, 13 mM EDTA, 0.5% sodium *N*-lauroylsarcosine, 10 μg of glycogen, and 26 mM Tris-HCl, pH 8.0 was added to the tube, mixed, and incubated at 60°C for 15 min in a heating block. After addition of an equal volume of isopropanol, the mixture was vigorously agitated and left to stand for 15 min. The sample was then centrifuged at 10,000 $\times$ *g* for 5 min to precipitate DNA, and the supernatant was discarded. One ml of isopropanol 40% was added, and the mixture was vortexed. After centrifugation at 10,000 $\times$ *g* for 5 min to recover DNA, the pellet was vacuum dried. All operations were conducted at room temperature. Extract DNA was stored at −20°C prior to PCR.

(ii) Phenol method. The preliminary steps were the same as described above. Briefly, the pellet was resuspended in 1 ml of TE buffer, transferred to a 2-ml microcentrifuge tube, and added with 200 μl of ammonium hydroxide, 200 μl of ethanol absolute, 400 μl of petroleum ether, and 20 μl of SDS 10%. The mixture was vortexed and then centrifuged at 15,000 $\times$ *g* for 10 min. Two hundred microliters of 6 M urea, 200 μl of ethanol absolute, 400 μl of petroleum ether, 80 μl of SDS 10%, and 13 μl of 3 M sodium acetate were added to the pellet. The mixture was vortexed and centrifuged at 15,000 $\times$ *g* for 10 min. The pellet was resuspended in 0.6 ml of TE buffer pH 8.0 and 100 μl of lysozyme (50 mg/ml) and incubated at 37°C for 1 h in a heating block. After the addition of 35 μl of SDS 10% and 10 μl of RNase DNase free (10 mg/ml) the sample was incubated at 37°C for 30 min. Ten microliters of proteinase K (20 mg/ml) was added and the mixture incubated at 37°C for 30 min. Then 130 μl of 6 M sodium perchlorate and 0.9 ml of phenol-chloroform-isoamyl alcohol (25:24:1) were added and the mixture centrifuged at 15,000 $\times$ *g* for 5 min. The upper phase was transferred to a 2-ml microcentrifuge tube, and the phenol-chloroform-isoamyl alcohol step was repeated. Next 0.6 volumes of isopropanol were added, mixed gently, and centrifuged at 15,000 $\times$ *g* for 15 min. The pellet was washed with 700 μl of ethanol 70%, centrifuged at 15,000 $\times$ *g* for 5 min, and then dried. The pellet was resuspended in 100 μl of TE pH 8.0. All operations were conducted at room temperature. Extract DNA was stored at −20°C prior to PCR.

(iii) QIAamp DNA blood mini kit (Qiagen). Extraction was performed according to the manufacturer's instructions.

Design of oligonucleotide primers and DNA amplification. All samples were tested to detect the presence of the *tet*(M) gene by means of PCR. To obtain very sensitive detection, a two-step PCR amplification procedure was developed with two nested sets of primers. In the first PCR, the primers tetM-f (5′ ACC CGT ATA CTA TTT CAT GCA CT 3′) and tetM-r (5′ CCT TCC ATA ACC GCA TTT TG 3′), derived from *tet*(M) sequence (GenBank accession number 1065723) *(21),* were used (MWG Biotech, Ebersberg, Germany). The primers tetM-f and tetM-r are located at positions 538 to 560 and 1633 to 1652, respectively, of the coding sequence. By means of these primers, a fragment of 1115 bp was amplified. As nested primers, tetMi2-f (5′ CTT AGG AAA ATG GGG ATT CC 3′) and tetMi2-r (5′ GCG GTG ATA CAG ATA AAC C 3′) were applied (MWG Biotech). They are located at positions 559 to 578 and 1549 to 1567, respectively, of the above-mentioned *tet*(M) sequence. The nested PCR amplified a DNA fragment of 1009 bp. Five microliters of each extracted sample were used for PCR, which also contained 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 2.5 U of *Taq*

TABLE 1. *PCR amplification of* E. faecalis tet*(M) following extraction by sodium iodide, phenol, and QIAmp DNA blood mini kit methods from raw milk*

*E. faecalis tet*M (CFU/ml)	Extraction methods:					
	Sodium iodide (1 h extraction + 1 h PCR + 1 h nested = 3)		Phenol (8 h extraction + 1 h PCR + 1 h nested = 10 h)		QIAamp DNA (2 h extraction + 1 h PCR + 1 h nested = 4 h)	
	First PCR	Nested	First PCR	Nested	First PCR	Nested
0	−	−	−	−	−	−
1.25	−	+	−	+	−	+
2.5	−	+	−	+	−	+
5	−	+	−	+	−	+
10	−	+	−	+	−	+
10^2	−	+	−	+	−	+
10^3	−	+	+	+	−	+
10^4	−	+	+	+	−	+
10^5	−	+	+	+	−	+
10^6	+	+	+	+	−	+

DNA polymerase (*Taq* DNA polymerase in storage buffer A, M1865, Promega Corporation, Madison, Wis.), 0.1 mM of each appropriate primer, in a total volume of 25 μl. For the first PCR, 5 μl of sample DNA was added; for the second PCR, 5 μl of the first PCR product was used as template. DNA amplification reactions were carried out using a PCR Mastercycler (Eppendorf AG, Hamburg, Germany) with the following program: denaturation at 95°C for 3 min, 30 cycles each consisting of 1 min denaturation at 95°C, 1 min annealing at 48°C, 1 min extension at 72°C, and a final extension for 10 min at 72°C. In each PCR assay, a positive control with 100 ng of *E. faecalis tet*(M) DNA and a negative control without any bacterial DNA were included. A 10-μl aliquot of each PCR product was subjected to 1% (wt/vol) agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide (Promega Corporation, M5041) for 30 min at 100 V. PCR products (1115 bp and 1009 bp, respectively, for the first PCR and for the nested PCR) were visualized under UV illumination. Their size was estimated using a standard DNA molecular weight marker (Novagen 69278-3, Madison, Wis.).

RESULTS

E. faecalis tet(M) was cultured in nutrient broth and added to raw milk. Following plating of each dilution on nutrient agar, the numbers of CFU/ml were in accordance with expected values based on the decimal dilution factor. No *E. faecalis tet*(M) DNA was detectable in unadulterated milk. Bacterial DNA was successfully extracted from adulterated raw milk by NaI and phenol methods. Results are summarized in Table 1. After the first PCR, the NaI method provided a detection limit of 10^6 CFU/ml, the phenol method provided a detection limit of 10^3, while the QIAamp DNA blood mini kit did not show any visible amplicon (Fig. 1). The second PCR with nested primers allowed a 10^5-fold improvement in the detection limit, when applied to milk extracted with NaI method, with visible amplicons obtained at concentration levels of 10 CFU/ml. Nested PCR applied to samples extracted with the phenol method allowed a 10^2-fold improvement in detection limit, with visible amplicons, again, obtained at concentration levels of 10 CFU/ml. Nested PCR applied to samples extracted with the QIAamp allowed a 10^6-fold improvement, with visible amplicons, again, obtained at concentration levels of 10 CFU/ml (Fig. 2). Nested PCR applied to serial dilutions of 10 CFU/ml, namely 5, 2.5, and 1.25 CFU/ml, were always positive for all extraction methods (Fig. 3 shows results for NaI and phenol method extractions). No visible amplicons were obtained from control samples where no *E. faecalis tet*(M) had been added.

DISCUSSION

The detection limits of PCR-based bacterial screening methods for foods are directly dependent on the efficiency of the nucleic acid extraction method employed. Direct DNA extraction from a variety of foods has been applied, with varying degrees of detection sensitivity *(1, 8, 9)*. One of the main problems encountered using PCR in food analysis is the presence of inhibitors, proteases, and nucleases that interfere with amplification. In this study, a modification of the NaI-based DNA method developed by Ishizawa *(15)* was compared with a traditional solvent extraction method, using raw milk. The present findings indicate that the NaI method coupled with nested PCR outperformed the phenol method at a fraction of the time. The extraction time for NaI method was less than 1 h, while the phenol method required 8 h (Table 1), and most of the times the operator had to split the procedure into 2 days. Both methods provided better results than the commercial kit, which can be performed in approximately 2 h. The proposed method is, therefore, able to give results within a single working day (including the nested PCR), does not requires phenol-chloroform steps, and is cheaper than the commercial kit. The advantages and disadvantages of these methods vary depending on time constraints, PCR detection limits desired, level of contamination, specific project objectives, and decision to use or to avoid the carcinogen mixture phenol-chloroform-isoamyl alcohol. The NaI method, in fact, involves fewer steps than the phenol method and does not require the extensive use of organic solvents. Since the phenol method, although more sensitive than the NaI one after the first PCR, was not able to go beyond the 1,000 CFU/

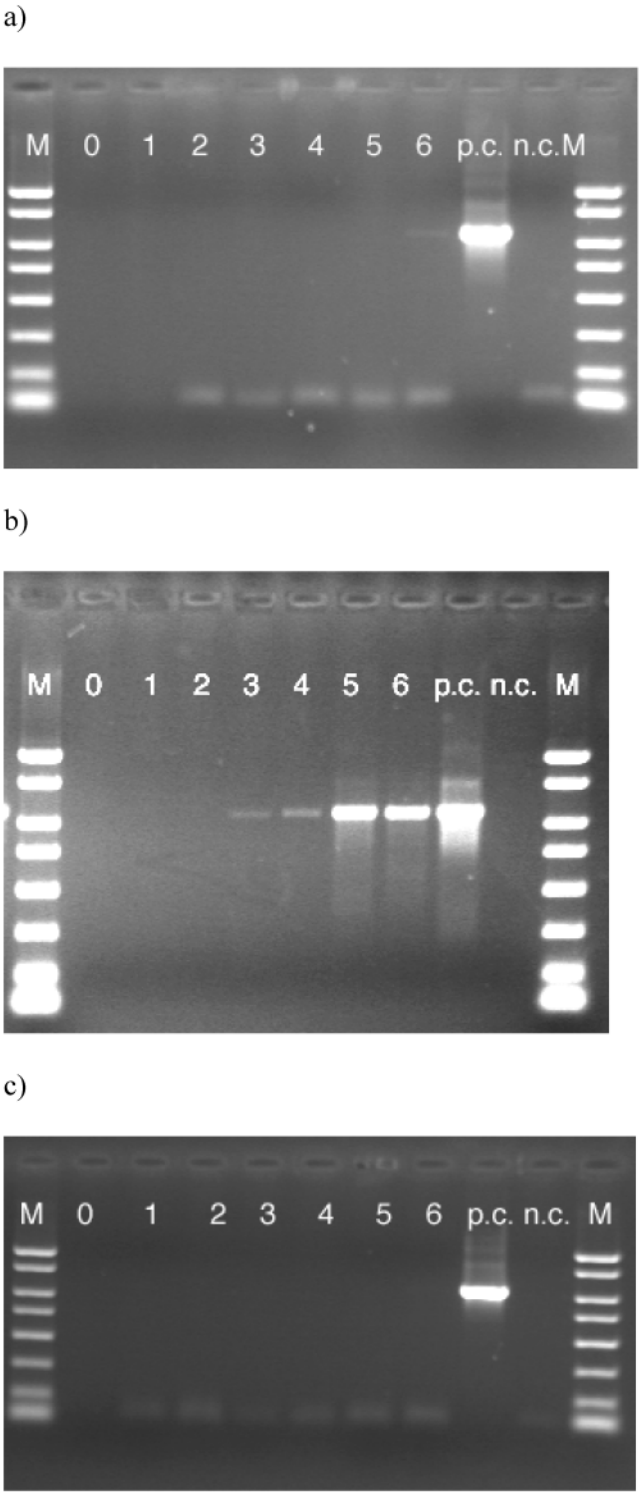

FIGURE 1. *PCR amplification of* E. faecalis tet*(M) DNA isolated from raw milk using the NaI method (a), the phenol method (b), or the QIAamp DNA blood mini kit (c). M, molecular weight marker (2,000, 1,500, 1,000, 750, 500, 300, 150, 50 bps); n.c., negative (no template) control; p.c., positive control (100 ng of* E. faecalis tet*(M) DNA); 0 to 6 raw milk inoculated with 0 to* 10^6 *CFU/ml of* E. faecalis tet*(M).*

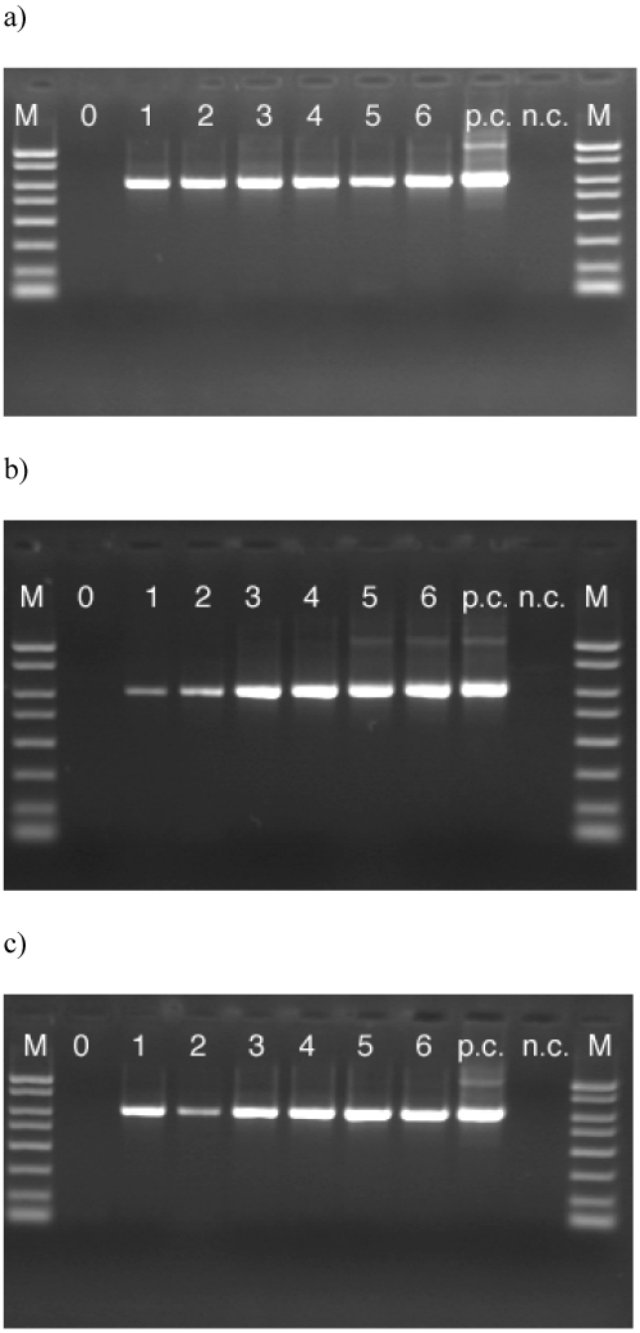

FIGURE 2. *Nested-PCR amplification of amplicons obtained from the first PCRs: NaI method (a), the phenol method (b), or the QIAamp DNA blood mini kit (c). M, molecular weight marker (2,000, 1,500, 1,000, 750, 500, 300, 150, 50 bps); n.c., negative (no template) control; p.c., positive control of the nested PCR; 0 to 6 raw milk inoculated with 0 to* 10^6 *CFU/ml of* E. faecalis tet*(M).*

ml limit, which is common in food samples, a two-step PCR should as well be coupled with it in order to increase its sensitivity. These results show that for all extraction methods the nested PCR allows the detection at less than 10 CFU/ml. For this reason we propose to streamline the whole procedure using a modification of the NaI method described by Ishizawa *(15)* followed by a two-step PCR. Nested PCR is also recommended to overcome the presence of PCR inhibitors in milk *(7, 13, 17, 23).* It should be noted here that any phenol extraction, followed by ethanol precipitation, is able to remove ionic detergents, such as SDS, which inhibit PCR even at very low concentrations (<0.01%) *(18).* Differences observed after the first PCR

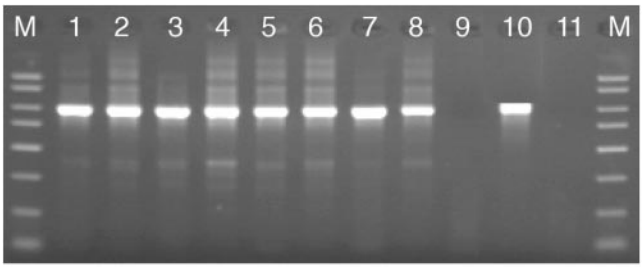

FIGURE 3. *Nested-PCR amplification of amplicons obtained from the first PCRs for serial dilutions of 10 CFU/ml. M, molecular weight marker (2,000, 1,500, 1,000, 750, 500, 300, 150, 50 bps); 1 to 4, raw milk inoculated with 10, 5, 2.5, 1.25 CFU/ml of* E. faecalis tet*(M), NaI method; 5 to 8, raw milk inoculated with 10, 5, 2.5, 1.25 CFU/ml of* E. faecalis tet*(M), phenol method; 9, 0 CFU/ml; 10, positive control of the nested PCR; 11, negative (no template) control.*

are, therefore, related to the phenol-chloroform extraction step, absent in the proposed NaI method. With the method described in this paper a detection limit of less than 10 bacteria per ml of raw milk was achieved. This detection limit is at least 100 times lower than that of a single PCR. In conclusion, the results from this study suggest that a two-step PCR approach can be used to detect the presence of *tet*(M) genes in milk after a rapid, solvent free, DNA extraction. The detection could be performed in a matter of a few hours. While some minor procedural modifications may be needed when adapting this method to different dairy commodities, it is expected that it could readily be adapted for a variety of food products and other genes encoding antibiotic resistance in foodstuffs. Future improvements may result in a further reduction in detection time and the association with sets of primers for genes specific for *Enterococcus* spp. (even at the species level) in a multiplex PCR *(5).* Because conventional phenotypic identification methods are cumbersome, require up to 72 h for results, are sometimes inaccurate, and provide only data related to selected microorganisms, the method described here has the potential for providing rapid detection of *tet*(M) genes in milk. The assay may prove useful for rapid assessment of the food matrix, increasing our knowledge regarding the diffusion of genes that encode resistance to tetracyclines in commensal indicator organisms in foods.

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