

Detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken meat samples by real-time nucleic acid sequence-based amplification with molecular beacons

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Abstract

A nucleic acid sequence-based amplification (NASBA) assay based on molecular beacons was used for real-time detection of *Campylobacter jejuni* and *Campylobacter coli* in samples of chicken meat. A set of specific primers and beacon probe were designed to target the 16S rRNA of both species. The real-time NASBA protocol including the RNA isolation was valid for both of the cell suspensions in buffered saline and the artificially contaminated chicken meat samples. The presence of rRNA could be correlated with cellular viability, following inactivation of the bacteria by heating, in inoculated chicken meat samples but not in RNase-free cell suspensions.

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1. Introduction

Thermophilic *Campylobacter* species, mainly *Campylobacter jejuni* and *Campylobacter coli*, are recognised worldwide as an important cause of food-borne illness and are a major concern to the poultry industry (World Health Organization, 2000; Newell and Fearnly, 2003). Conventional methods for *Campylobacter* spp. detection in food involve culturing in selective media at 42 °C under microaerobic conditions and identification of the isolates according to their biochemical and/or immunological characteristics. Although these methods are sensitive and are being continuously improved, they are relatively complex and time-consuming. The identification of a suspected campylobacter colony takes 4 to 6 days, and phenotypic identification schemes for *Campylobacter* spp. are often difficult to interpret (On, 2001). Furthermore, stressed and/or viable but non-culturable (VBNC) bacteria cannot grow in the selective culture media.

As an alternative, molecular amplification techniques can provide highly sensitive and specific methods for the detection, identification and characterization of food-borne organisms,

including *Campylobacter* spp. A number of PCR assays that involve DNA amplification have been developed (Giesendorf and Quint, 1995; Winters and Slavik, 2000; Lilja and Hanninen, 2001; Bolton et al., 2002; On and Jordan, 2003; Oliveira et al., 2005). However, due to the robustness of DNA, the signal is not necessarily related to the detection of viable infectious bacteria (Keer and Birch, 2003). Therefore, there has recently been interest the use of RNA as the molecular target for viability assessment. Both mRNA and rRNA have a shorter half-life than DNA, and so, should provide better indication of viable organisms than DNA. However, if mRNA is used there must be surety that the mRNA targeted for transcription is present under most assay conditions (Sheridan et al., 1998; Birch et al., 2001).

The most commonly used amplification techniques for detecting RNA are reverse transcriptase PCR (RT-PCR) and nucleic acid sequence based amplification (NASBA). RT-PCR is a two-stage process, in which a target RNA sequence is first transcribed into a complementary DNA that then serves as the template for PCR. In contrast, NASBA is a one-step process in which single-stranded RNA sequences are targeted and amplified. NASBA, first described by Kievits et al. in 1991, involves the simultaneous use of three enzymes, avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA

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polymerase, under isothermal conditions (41 °C). The final amplification product is a single-stranded RNA of opposite sense to the original target. NASBA has been applied for the detection of a number of pathogens, including *Campylobacter* spp. (Vandervliet et al., 1993; Uyttendaele et al., 1995; Simpkins et al., 2000).

More recently, real-time detection based on molecular beacon probes has been described (Leone et al., 1998). Molecular beacons are small, single-stranded nucleic acid, hairpin probes that brightly fluoresce when they are bound to their targets (Tyagi and Kramer, 1996). When NASBA is performed with use of molecular probes, the target amplicons are detected in a sealed reaction tube, which simplifies analysis and eliminates an important source of assay contamination. Molecular beacons have been used to detect viruses (Landry et al., 2005), bacteria (Rodríguez-Lázaro et al., 2004) and other microorganisms (Casper et al., 2004) under a variety of conditions, but to our knowledge they have not been applied for the detection of *Campylobacter* spp. in food samples.

The aim of this study was to combine NASBA with a molecular beacon probe for the detection of *C. jejuni* and *C. coli* based on their 16S rRNAs, and to use the method for detection of these organisms in chicken meat samples.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Ten strains of *C. jejuni*, eight of *C. coli*, and one each of *Campylobacter lari* and *Campylobacter fetus* were used (Table 1), and one strain each of *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Miami, and *Pseudomonas aeruginosa* were used.

Table 1
Strains of *Campylobacter* spp. used in this study

Species	Strain	Origin	Country
<i>C. jejuni</i>	NCTC 11168	Collection	United Kingdom
<i>C. jejuni</i>	CNET 016	Flock	Netherlands
<i>C. jejuni</i>	CNET 017	Flock	Netherlands
<i>C. jejuni</i>	CNET 026	Birds	Finland
<i>C. jejuni</i>	CNET 056	Cattle	Denmark
<i>C. jejuni</i>	CNET 057	Cattle	Denmark
<i>C. jejuni</i>	CNET 111	Canine	Sweden
<i>C. jejuni</i>	CPM 012	Chicken	Spain
<i>C. jejuni</i>	CPM 032	Chicken	Spain
<i>C. jejuni</i>	CH 157	Human	Spain
<i>C. coli</i>	CCUG 11283	Collection	Belgium
<i>C. coli</i>	CNET 019	Flock	Netherlands
<i>C. coli</i>	CNET 021	Flock	Netherlands
<i>C. coli</i>	CNET 064	Poultry	Denmark
<i>C. coli</i>	CNET 066	Pig	Netherlands
<i>C. coli</i>	CNET 069	Pig	Northern Ireland
<i>C. coli</i>	CPM 057 ₁	Chicken	Spain
<i>C. coli</i>	CH 160	Human	Spain
<i>C. lari</i>	CCUG 23947	Collection	United Kingdom
<i>C. fetus</i>	CCUG 6823	Collection	United Kingdom

CCUG: Culture Collection University of Göteborg (Sweden). NCTC: National Collection of Type Cultures (UK). CNET: CAMPYNET collection.

Campylobacter strains were routinely grown at 37 °C on *Campylobacter* agar base (Oxoid, Basingstoke, UK) supplemented with 5% laked horse blood (Oxoid) for 24 h, under a microaerobic atmosphere in the GENbag microaer system (bioMérieux, Marcy l'Etoile, France).

For survival experiments, *C. jejuni* strains were grown in nutrient broth no.2 (Oxoid) for an additional 24-h period, harvested by centrifugation and suspended in 100 ml sterile phosphate-buffered saline (PBS), pH 7.3, at numbers of 10⁹ cells/ml as determined by direct counts (Lázaro et al., 1999). Cell suspensions were then incubated at 4 °C without shaking in the dark for up to 6 months. At the time of inoculation and at intervals of 15 days until no cfus were detected, the numbers of viable cells were determined by plate counts as before. Thereafter, the numbers of total and viable cells were determined by using a double-staining, 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) and 4, 6-diamidino-2-phenylindole (DAPI) technique (Cappelier et al., 1997).

The other enteric bacteria were grown at 37 °C, except for *A. hydrophila* which was grown at 30 °C, on Plate Count Agar (Oxoid) over night prior to all experiments.

2.2. Inactivation treatments

To determine if detected 16S rRNA could be exclusively attributed to viable bacteria, RNA extractions were also carried out on suspensions autoclaved at 121 °C for 30 min. Autoclaved suspensions were kept at 4 °C under the same conditions as were the other suspensions. Immediately after autoclaving and at 1, 2, 5, 10, 30 and 60 days post-treatment, samples were removed for RNA extraction and for further viability checks.

2.3. RNA isolation

Total RNA isolation was performed with NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany), according to the supplier's instructions. RNA quality of the extractions was tested by spectrophotometric determinations using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and absence of DNA was checked by PCR according to Mateo et al. (2005).

2.4. Primer design and molecular beacon

The designs of the primers and beacon probe were based on target sequences of the three 16S rRNA genes of *C. jejuni* subsp. *jejuni* NCTC 11168 using the PrimerExpress software package (Applied Biosystems, Foster City, CA, USA). All the oligonucleotides were synthesized by Invitrogen-Life Technologies (Glasgow, UK). The primers OTCj559 and OTCj619 were designed to target an internal region of the 16S rRNA sequence with amplification of an 80-bp fragment. The probe OTCj585 matches a central region of the NASBA amplicons produced by the primers. The sequence of forward primer OTCj559 was 5'-AGGCGGATTATCAAGT-3', and the sequence of the reverse primer OTCj619 was 5'-aattctaatacactcactataggagCCCTCACTCTAGACTATCAG-3', where the T7

promoter sequence is in lower case. The sequence of the molecular beacon OTCj585 was 5'-FAM-GCGCAGATCT-AATGGCTTAACCATTAAACCTGCGC-DABCYL-3', where additional sequences of the stem structure are underlined. FAM is 6-carboxyfluorescein (fluorescent label), and DABCYL is 4-(4-dimethyl-aminophenylazo)-benzoic acid (universal quencher). The secondary structure of the molecular beacon probe was obtained using the Mfold 3.1 web server (Zuker, 2003), and is shown in Fig. 1.

2.5. Real-time NASBA

The NASBA reactions were performed with the NucliSens Basic Kit[®] amplification reagents (bioMérieux). Reactions were carried out in 20 µl final volumes. The reaction mixture was prepared by sequential addition of 10 µl of NASBA reagent-primer mix containing 0.2 µM of each primer and 0.1 µM of the molecular probe, 5 µl of RNA template and 5 µl of enzyme-mix containing RNase H, T7 polymerase and AMV-reverse transcriptase. Before addition of the enzyme-mix, the mixture was incubated at 65 °C for 5 min to uncoil secondary and tertiary structures, and then held at 41 °C for 5 min. The final mixture was incubated at 41 °C for 90 min to complete the amplification and real-time detection in an ABIPRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). As negative control for each run, RNase-free water and rodent RNA as foreign RNA were added to the NASBA reaction instead of the *Campylobacter* RNA template controls. RNA from *C. jejuni* strain NCTC 11168 was used as positive control. Fluorescence was measured every 75 s. The results were considered positive when a typical logarithmic curve above the threshold line shown by the negative control was obtained.

2.6. Artificial contamination of retail chicken meat

Portions of chicken breast were purchased from local supermarkets. To avoid interferences from naturally present *Cam-*

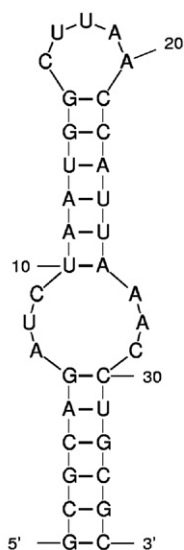


Fig. 1. Secondary structure of the oligonucleotide chosen as molecular probe.

pylobacter spp., only portions that gave samples which were *Campylobacter*-free as determined by PCR directed to the 16S rRNA gene (Mateo et al., 2005) were used to prepare artificially contaminated samples.

Samples were contaminated using *C. jejuni* suspensions of three types which contained culturable cells from a 24-h culture, viable but non-culturable cells from suspensions exposed to cold temperatures, or autoclaved cells. *Campylobacter*-free breasts were aseptically divided into 5 g samples, which were arranged in groups of four samples. Three samples from each group were submerged in 20 ml of the same *C. jejuni* suspension for 10 min, and then the samples were drained on sterile filter pads. One inoculated sample from each group was used for isolation of RNA immediately after inoculation. The other two inoculated and that uninoculated samples were stored at 4 °C. RNA was isolated from one inoculated portion after 24 h and from the remaining portions after 48 h.

For total RNA isolation, each portion was rinsed in 20 ml of PBS in an orbital shaker for 10 min at 200 rpm. Then, 1 ml of the rinse fluid was transferred to a tube for RNA isolation as described above.

3. Results and discussion

3.1. Development of the real-time NASBA with molecular beacons protocol

The real-time NASBA protocol was first assayed with *C. jejuni* strain NCTC 11168. Then, the method was applied to other *C. jejuni*, *C. coli*, *C. lari* and *C. fetus* strains. Finally, eight enteric bacteria other than *Campylobacter* spp. were tested.

Curves representative of the results obtained with *C. jejuni* NCTC 11168 are shown in Fig. 2. The results obtained by NASBA amplification and molecular beacon probe were validated by gel electrophoresis followed by Northern blotting detection (data not shown). The protocol, including the RNA isolation, was valid for both of the cell suspensions in PBS and the artificially contaminated chicken samples as with both clear signals above the negative controls were obtained.

Similar target-specific fluorescent signals were obtained with RNA extracts from the other *C. jejuni* strains and *C. coli*, but did not with extracts from *C. lari*, *C. fetus* and any of the other enteric bacteria tested. These results indicated that the method was specific for the detection of *C. jejuni* and *C. coli* in chicken meat.

Post-NASBA monitoring steps were not necessary because RNA amplification and target-specific fluorescent signalling were accomplished simultaneously in a one-tube system. The risk of contamination was minimized by performance of the entire reaction in a closed tube. The assay seemed to be robust as its satisfactory use with chicken breast samples indicated that it could be used for complex samples. Together, these results made the real-time NASBA with molecular beacons a more convenient assay than conventional NASBA with Northern blotting.

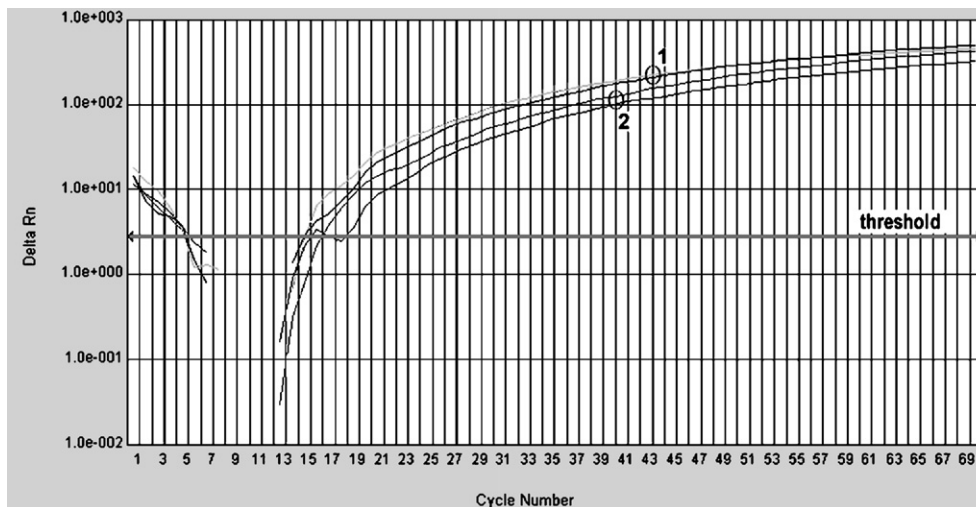


Fig. 2. Typical amplification plot of *C. jejuni* rRNA using the real-time NASBA assay with molecular beacon probe. Representative amplification plots correspond to culturable cells in PBS (1), and artificially contaminated chicken meat (2). Samples were run in duplicate. The horizontal line on the figure indicates the threshold fluorescence at which the signal reaches exponential growth. Rn, relative fluorescence.

3.2. 16S rRNA stability as evaluated by real-time NASBA assay

In stressful environments, *C. jejuni* may be able to enter a viable but non-culturable (VBNC) state (Colwell, 2000). Such cells cannot be detected by a routine culture method; however they retain a spiral morphology and cell viability (Lázaro et al., 1999; Tholozan et al., 1999). The significance of this state in transmission of disease is uncertain, as the recovery of VBNC-forms of *Campylobacter* cells is still controversial. While several authors have reported *C. jejuni* resuscitation from the VBNC state after passage in embryonated eggs or experimental animals (Baffone et al., 2006) there are others who referred the inability to resuscitate of the VBNC forms (Ziprin and Harvey, 2004). The potential threat from VBNC *Campylobacter* spp. may be a critical issue for the food industry if VBNC forms are present in foods or drinking water.

In previous studies (Lázaro et al., 1999) we determined that starvation of *C. jejuni* cells after long-term exposure to low temperatures caused the loss of culturability in about 2 months of incubation and then survival continued for up to 7 months based on respiratory activity determined by direct CTC-DAPI counts. Non-culturable cells also maintained spiral morphology as well as DNA integrity. These features were assumed to indicate the presence of VBNC forms of *C. jejuni* in our starved cultures long after no cfus were detected.

A major concern of this research was to identify a molecular target for assessment of the viability of non-culturable cells. To evaluate the ability of the rRNA in non-culturable cells to support nucleic acid amplification, both starved non-culturable and heat-killed cells of *C. jejuni* were analyzed by the real-time NASBA assay.

In starved cultures, target 16S rRNA was detected at all times during 6 months of incubation. The rRNA signals, although lower than for non-starved cultures were clearly above the negative controls, in the NASBA-beacon assays. At first in the absence of culturable cells, the 16S rRNA signals were pre-

sumed to indicate the presence of viable but non-culturable cells. However, with autoclaved cultures, 16S rRNA signals were detected immediately after autoclaving and, surprisingly, during subsequent incubation at 4 °C for 2 months. Obviously, an extreme heat treatment may denature RNases as well as other bacterial enzymes. Although McKillip et al. (1998) detected no rRNA in autoclaved cultures of *E. coli* and *Staphylococcus aureus*, Sheridan et al. (1998) detected amplification of *E. coli* 16S rRNA by RT-PCR for 16 h after thermal inactivation of the organism and Uyttendaele et al. (1997), using NASBA followed by an enzyme-linked gel assay (ELGA) found that rRNA was stable for 5 h following treatment at 100 °C. In our study, 16S rRNA from heat-killed *C. jejuni* did not disappear during 2 months incubation at 4 °C. This long period of rRNA resistance was probably due to the chiller rather than room storage temperature used in this study. Thus, our unexpected results were more likely related to the maintenance of the intact rRNA templates in an RNase-free medium than an indication of cell viability under starvation conditions.

3.3. Detection of rRNA from *C. Jejuni* in artificially contaminated chicken samples

C. jejuni 16S rRNA was detected in the samples inoculated with cells from either fresh or starved cultures at 0, 24 and 48-h (Fig. 3). However, in samples inoculated with heat-killed cells no rRNA signals were detected. Negative results obtained in heat-killed cells could not be due to dilution of RNA in the heat-killed suspensions as all meat samples, positive and negative, were processed in the same way. Probably, RNA in those suspensions was degraded by enzymes in the chicken meat. These findings suggest that in starved cell suspensions rRNA is protected inside VBNC cells.

The presence of *C. jejuni* 16S rRNA, in artificially contaminated chicken meat samples, could correspond to the presence of viable cells and a lack of signal coincided with heat-killed

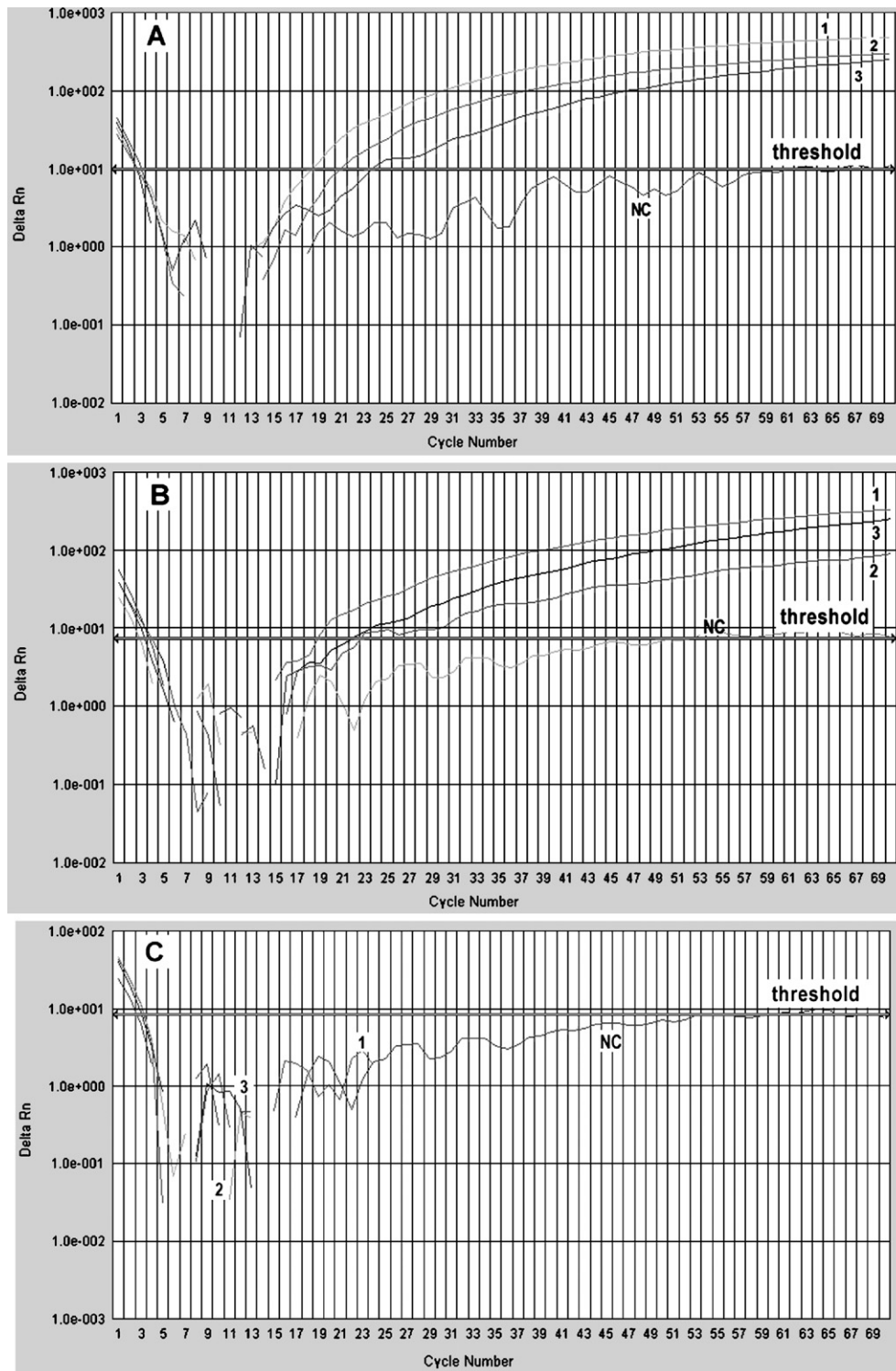


Fig. 3. Real-time NASBA analysis of chicken samples artificially contaminated with *C. jejuni* in three physiological states: A, culturable cells; B, viable but non-culturable cells; C, heat-killed cells by autoclaving. The samples were kept under survival conditions at 4 °C without shaking in the dark and analysed immediately after inoculation (1), at 24 h after inoculation (2) and at 48 h after inoculation (3). (NC) Negative control.

cells. Therefore, a correlation between viability and cellular rRNA content was observed. Several authors concluded that the presence of rRNA can be correlated with cellular viability following inactivation of the bacteria with extreme temperatures i.e., autoclaving (McKillip et al., 1998; Sheridan et al., 1998;

Birch et al., 2001). Our data were in part consistent with this conclusion, but they depended on the environmental conditions in which the experiments were carried out. The rRNA could be related with cell viability following extreme temperature treatment in model food systems but it could not in RNase-free media.

The protocol applied to artificially contaminated chicken samples could be used in conjunction with conventional culturing procedures, as real-time NASBA results can be obtained in less than 4 h after sample processing began. This is important because the method could then be used as a method for detecting *C. jejuni* and *C. coli* positive samples, with only rinses from positive samples be subjected to enrichment procedures for strain isolation and typing.

The Real-time NASBA would seem to be a convenient and rapid method for the detection of *C. jejuni* and *C. coli* in food samples. The specificity of the method was apparent in the lack of amplification of non-target sequences from other enteric bacteria. The method is also compatible with conventional culturing procedures allowing, in positive samples, the strain isolation and typing which is of great interest in epidemiological studies.

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