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Development of a real-time NASBA assay for the detection of *Campylobacter jejuni* cells

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Abstract

The objectives of this study were the development of a real-time NASBA assay for the detection of *Campylobacter jejuni* mRNA and the evaluation of its potential to determine the viability of the detected *C. jejuni* cells. A set of specific primers and probes was chosen to amplify the mRNA of the *tuf*-gene and the *GTPase*-gene. Only the *tuf*-assay was able to detect as low as 10^2 cells per NASBA reaction and was specific for *Campylobacter*. However, as the assay was able to detect dead cells, it cannot be used to demonstrate the viability of *C. jejuni* cells. The *tuf*-gene mRNA is no good viability indicator due to its stability.

Keywords: NASBA; mRNA; Lethal treatment; *tuf*-gene; *GTPase*-gene

1. Introduction

Campylobacter jejuni, a Gram-negative spiral shaped rod was first observed in 1886 but it was not until 1972 that certain campylobacters were recognised as causes of foodborne illness (Park, 2002). In 2002, *Campylobacter* was responsible for 31% of all foodborne infections in the USA. (http://www.cdc.gov/foodnet/annual/2002/2002executive_summary.pdf).With an infective dose as low as 800 cells (Black et al., 1988), the significance of detecting low numbers of this organism is evident. *C. jejuni* is currently identified and quantified by several techniques including the conventional culture method (ISO 10272, 1995), PCR (Lubeck et al., 2003) and reverse transcriptase PCR (RT-PCR) (Sails et al., 1998). All of these techniques have their respective advantages and disadvantages. The classical culture method is the reference method for the detection of *C. jejuni* and is continuously optimized to obtain good performance in conjunction with foods. However, it is laborious and time-consuming and due to the use of selective media, it may fail to resuscitate stressed micro-organisms, e.g. in water and on surfaces (Cools et al., 2003, 2005). PCR is a rapid method with increased specificity (dependent on the choice of the primer set) and without the need for prior culturing. However, due to the robustness of DNA, the signal is not necessarily related to the detection of viable infectious bacteria. Therefore, our attention turned to the use of RNA as a marker of viability. As 16S rRNA is also known not to be related with viability (Uyttendaele et al., 1995a; Keer and Birch, 2003)

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mRNA was considered. mRNA is a highly labile molecule with a very short half-life and hence, it should prove a more suitable indicator of viability status. The most commonly used amplification techniques for detecting mRNA are RT-PCR and nucleic acid sequence based amplification (NASBA). When performing RT-PCR, prior DNase steps are necessary to yield pure RNA to be subjected to reverse transcription and PCR. Hence, RT-PCR is susceptible to contamination with DNA products. On the contrary, NASBA, first described by Kievits et al. in 1991, is an isothermal method of RNA amplification that utilizes the simultaneous activities of reverse transcriptase, RNase H and T7 RNA polymerase. The end product is single-stranded RNA anti-sense to the original RNA template. In previous studies on foodborne pathogens, NASBA has been used for the detection of *C. jejuni* (Uyttendaele et al., 1995a), *Listeria monocytogenes* (Uyttendaele et al., 1995b), *Salmonella* (Simpkins et al., 2000) and mycobacteria (Vandervliet et al., 1993). NASBA has several potential advantages over RT-PCR. It generates the same number of copies in a shorter time than RT-PCR because every cycle results in an exponential increase, whereas PCR progresses in a binary fashion (Chan and Fox, 1999). Incubation times for NASBA are shorter i.e. 90 to 150 min versus 3–5 h for RT-PCR. Since there is no DNA denaturation step in NASBA, contaminating genomic DNA is not amplified (Edwards et al., 2004). Finally, as NASBA is an isothermal reaction, it obviates the need for a thermal cycler. NASBA, however, also suffers from a number of drawbacks in comparison to RT-PCR: the impossibility to control the extent of the reaction by adjusting the number of cycles because NASBA is isothermal, an increased likelihood of non-specific reactions because the amplification temperature cannot exceed 41 °C without the risk of enzymatic denaturation (Tai et al., 2003) and the need for a subsequent hybridisation step as identification cannot be supported by the presence or absence of amplification products on an ethidium bromide-stained gel (Uyttendaele et al., 1995a). More recently, however, real-time procedures incorporating amplification and detection in a single step have been reported and applied to a wide range of targets, e.g. viruses, bacteria, parasites and yeasts (Edwards et al., 2004). For NASBA, the real-time procedure involves the use of a molecular beacon. The beacon generates a fluorescent signal during amplification when hybridising with its target. The fluorescence is measured continuously during amplification as the reaction proceeds.

In a previous study, NASBA with end-point detection was developed for the detection of *C. jejuni* in foods (Uyttendaele et al., 1995a). However, this assay was based on the detection of rRNA and therefore not related to viability. The objective of the present study was to develop a real-time NASBA assay for the detection of *C. jejuni* mRNA and to evaluate whether this would be related to viability.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Campylobacter* spp. were grown for 24 h at 37 °C under micro-aerobic atmosphere in Bolton broth (Oxoid, Basingstoke, UK) supplemented with the Bolton selective supplement (Oxoid). Other Gram negative bacteria were grown overnight at 37 °C in Brain heart infusion broth (BHI: Oxoid). Gram positive bacteria were grown overnight at 30 °C in BHI (Oxoid).

2.2. mRNA isolation

One ml of a 24 h *C. jejuni* culture was used for mRNA isolation by the Qiagen RNeasy minikit (Qiagen, Venlo, The Netherlands). The eluate was subjected to a DNase (Promega, Leiden, The Netherlands) treatment (15U DNase per 50 µl eluate). After 30 min incubation at 37 °C, stop solution (Promega) was added and the residual DNase was inactivated by heat treatment (10 min 65 °C).

Table 1
Bacterial species tested by the NASBA procedure

Species	Strain	Origin
Campylobacter jejuni	KC 592	Poultry
Campylobacter jejuni	KC 25	Poultry
Campylobacter jejuni	RIZA 488	Water
Campylobacter jejuni	RIZA 622	Water
Campylobacter jejuni	CN 31	Human
Campylobacter jejuni	CN 40	Human
Campylobacter jejuni	LMG[a] 6629	Human
Campylobacter jejuni	79	Human
Campylobacter jejuni	Bof	Human
Campylobacter coli	KC 7	Poultry
Campylobacter coli	KC 8	Poultry
Escherichia coli	EE 5	Pork
Enterobacter cloaca	LMG 2783	Cerebrospinal fluid
Citrobacter freundii	LMG 3246	Collection strain[c]
Salmonella Enteritidis	LMG 10395	Collection strain
Listeria monocytogenes	ATCC[b] 5105	Human
Listeria innocua	LMG 11387	Bovine
Bacillus subtilis	LMG 8197	Collection strain
Staphylococcus aureus	ATCC 6538	Human Lesion

[a] LMG = Bacteria Collection of the Laboratory of Microbiology Ghent University (BCCM™, Ghent , Belgium).
[b] ATCC = American Type Culture Collection.
[c] Collection strain: origin could not be determined.