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An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*

S.A. Simpkins, A.B. Chan[1], J. Hays, B. Pöpping and N. Cook

Central Science Laboratory, Sand Hutton, York, and [1]Organon Teknika, Cambridge, UK

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S.A. SIMPKINS, A.B. CHAN, J. HAYS, B. PÖPPING AND N. COOK. 2000. Possession of mRNA is indicative of cell viability. RTPCR is not appropriate for mRNA detection as it cannot unambiguously detect mRNA in a DNA background. The alternative amplification technique, NASBA, avoids the disadvantages of RTPCR. We have devised a method for detection of viable *Salmonella enterica*. This involves NASBA amplification of mRNA transcribed from the *dnaK* gene. Amplification of mRNA extracted from viable and heat-killed cells from the same population produced consistent and highly significant ($P > 0{\cdot}01$) differences between the respective signals. The signal obtained from viable cells was completely eradicated by RNase treatment, while PCR amplification of treated and untreated samples was unaffected, indicating that NASBA was unaffected by background DNA.

INTRODUCTION

Routine detection of viable bacteria can be slow, commonly relying on growth of cells, which may take several days. Furthermore, additional tests are often necessary to confirm the identity of the cultured micro-organisms.

Rapid molecular-based systems for microbial detection have been devised, the best-known of which is the polymerase chain reaction (PCR) (Saiki *et al.* 1985). PCR offers high sensitivity and specificity, and can give a result in a few hours. A positive PCR result, however, gives no indication of microbial viability, as DNA is present whether the cell is alive or not. A variant technique, reverse transcription (RT)–PCR (Byrne *et al.* 1988) amplifies RNA. Several types of RNA are produced in cells and one, messenger RNA (mRNA), functions as an intermediary in protein synthesis, and is thus found only in viable cells. The use of mRNA as a diagnostic of cell viability has been proposed (Bej *et al.* 1991), but approaches to its detection have not produced unambiguous results. Generally, RT–PCR has been employed (Sheridan *et al.* 1998; Vaitilingom *et al.* 1998). In RT–PCR, reverse transcription produces a DNA copy of the mRNA target sequence which is subsequently amplified by PCR to produce a signal. However, within the cell mRNAs are generated from their template genetic sequences, and if cellular DNA is present in the reaction these sequences will become amplifiable during the denaturation steps of the PCR. PCR is exquisitely sensitive, and theoretically can detect a single target molecule per reaction; therefore, unless all traces of genomic DNA are removed, they can produce a false positive signal in a viability assay (Sails *et al.* 1998). Although DNase treatment can be employed to remove genomic DNA, it is not completely effective (Simon and Cook, unpublished results). The use of intron-flanking primers to distinguish between mRNA- and DNA-derived amplicons (Tong *et al.* 1994) is not suitable for prokaryotes. Besides, additional steps such as these add to the complexity of an already variant PCR.

PCR relies upon denaturation of double-stranded DNA, to allow oligonucleotide primers to bind to matching sequences. Denaturation is performed at temperatures which are normally in excess of 80 °C. In an alternative technique using transcription-based amplification, NASBA (nucleic acid sequence-based amplification) (Compton 1991; Kievits *et al.* 1991), RNA is amplified below the melting temperature of DNA. NASBA can be used to detect a range of pathogens (Chan and Fox 1999). It has also been proposed for the detection of viable cells (van der Vliet *et al.* 1994) but, so far, the target employed for this has been ribosomal RNA (rRNA), which can persist for lengthy periods in dead cells (Uyttendaele *et al.* 1997). Messenger RNA, in contrast, has a short half-life within viable cells, and is rapidly degraded by enzymes (RNases) which are very stable even in environments

Correspondence to: N. Cook, Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK.

outside the cell itself (Sela *et al.* 1957). Messenger RNA would thus be a better indicator of cell viability.

We have devised a method for detection of viable *Salmonella enterica* using NASBA amplification of mRNA. The target was mRNA transcribed from the bacterial *Hsp*70 homologue *dnaK* (Bardwell and Craig 1984). The results indicate that this approach has great potential as a means of assessing cell viability.

MATERIALS AND METHODS

Bacterial strains

- *Citrobacter freundii* ATCC 10787;
- *Enterobacter faecalis* ATCC 29212;
- *Escherichia coli* ATCC 23724;
- *Klebsiella pneumoniae* NCIB 714;
- *Salmonella enterica* var. *enteritidis* PT4 FMG1 (an environmental isolate);
- *Salmonella enterica* var. *typhimurium* FMG2 (an environmental isolate).

All strains were routinely grown on TSYE agar and/or broths at 37 °C as appropriate. In broth culture, cells were grown to exponential phase, enumerated using a Helber cell counting chamber, and the concentration adjusted to 10^7 cells ml^{-1} before use.

The NASBA-based protocol (including RNA release, extraction, NASBA amplification and detection) was carried out using the NucliSens® Basic Kit (Organon Teknika, Cambridge, UK).

Nucleic acid extraction

This was performed using a silica–guanidine thiocyanate protocol (Boom *et al.* 1990), provided as proprietary lysis buffer and isolation reagent modules in the NucliSens® kit. Cell lysis was improved by sonication of cell suspensions for 60 s at 14% amplitude using a Branson Sonifier. Recovery of mRNA was aided by the incorporation of 8 M urea (final concentration) into the elution buffer.

Primers and probes

NASBA primers and capture probe were designed with reference to the published sequence for *S. enterica* var. *typhimurium dnaK* (EMBL ref. no. Q60004) and according to the specifications set out by Compton (1991) and the NucliSens® kit.

Primer SDnaK1 (5′-aattctaatacgactcactatagggAGAGGCAGTCGGTTCGTTGATGATA-3′) carried the T7 promoter sequence at its 5′ end (lower case), which was followed by a 3-bp GA-rich sequence. Primer SDnaK2 (5′-gatgcaaggtcgcatatgagCTTGATGTGAAAGGTCA-3′) contained at its 5′ end a 20-bp sequence (lower case) that is specific for the NucliSens® ruthenium-linked oligonucleotide detection probe (5′-ruthenium-GATGCAAGGTCGCATATGAG-3′). In addition a 22-mer capture probe, SBIOT4 (5′-biotin-TGAAGATTATCTGGGCGAACCG-3′) was synthesized with a biotin moiety attached at its 5′ end, enabling it to be linked to streptavidin-coated magnetic beads (Dynal).

NASBA amplification of mRNA

This was carried out using reagents and protocols included in the NucliSens® kit. Reactions were carried out in a final volume of 20 μl containing 40 mM Tris-HCl pH 8·5, 12 mM $MgCl_2$, 70 mM KCl, 5 mM dithiothreitol, 15% DMSO, 1 mM of each dNTP, 2 mM of each NTP, 0·5 μM of each primer, 5 μl of nucleic acid extract, 0·08 U RNase H, 32 U T7 RNA polymerase and 6·4 U AMV-RT. Before addition of the enzyme mix, the SDnaK1 and SDnaK2 primers were annealed to the target at 65 °C for 5 min, and the mix was then cooled to 41 °C for a further 5 min. After addition of the enzyme mix, an incubation was carried out at 41 °C for 90 min to allow exponential amplification of the mRNA.

A water blank was included as an amplification negative control with every assay.

Detection of amplicons

A 5-μl aliquot of the NASBA reaction was added to 100 μl of NucliSens® detection diluent and 5 μl of this was then added to a 20-μl hybridization reaction containing 16·5 nM SBIOT4, and 27·5 nM detection probe. Hybridization was carried out for 30 min at 41 °C, with intermittent vortexing.

Following hybridization, each reaction was diluted with 300 μl of assay buffer containing tripropylamine (TPA), and then read on a NucliSens® ECL Reader (Organon Teknika).

The NucliSens® ECL Reader detects light produced when a low voltage is applied to an electrode (magnetic), which subsequently triggers the cyclical oxidation–reduction reaction of a ruthenium metal ion (Blackburn *et al.* 1991; Gudibande *et al.* 1992; Kenten *et al.* 1992). The metal ion is attached to a detection probe which hybridizes to the RNA target sequence which, in turn, is anchored to the electrode via the magnetic bead on the capture probe. TPA, which is present in excess, is consumed in the oxidation–reduction reaction and the ruthenium ion is recycled. The ECL signal is amplified during the cycling of these two components and is proportional to the amount of RNA target anchored to the electrode surface. Light is emitted from the reaction and detected at 620 nm using a photomultiplier tube. The information generated is digitally stored.

A proprietary instrument reference solution-positive control was included in every assay, along with a hybridization

assay negative control, consisting of the two probes with H_2O in place of the amplification reaction components.

Sample treatment for comparison of NASBA signals from viable and non-viable cells

Salmonella enterica cells were grown at 30 °C in TSYE broth into mid-exponential phase (approximately 10^7 ml^{-1}). Two 5-ml aliquots were taken from the culture. One aliquot was placed on ice and the other incubated at the pasteurization temperature of 65 °C for 5 min to kill the cells; this was then also placed on ice. RNA was extracted from both aliquots, and NASBA was performed.

RNAse treatment and PCR amplification conditions

Aliquots of extracted nucleic acid were incubated with 50 U DNase-free RNase (Promega, UK) at 37 °C for 1 h. Following a phenol : chloroform extraction step to remove the RNase, the aliquots were used in both NASBA and PCR amplifications. PCR was performed in 50 μl reaction volumes containing 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 8·4), 50 mM KCl, 0·2 mM dNTP mix, 1 U Taq DNA polymerase (Perkin-Elmer, UK), 0·325 μM each of primers SDnaK1 and SDnaK2 and 5 μl of nucleic acid extract. Cycle conditions were 94 °C for 2 min, 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 74 °C for 1 min, and a final extension step of 72 °C for 5 min. The product was visualized by electrophoresis of 10 μl of the reaction mix on a 2% agarose gel containing 5 ng/ml ethidium bromide and UV transillumination.

Statistics

Where appropriate, results were analysed using Student's *t*-test.

RESULTS

Specificity of NASBA *S. enterica* primers

Messenger RNA was extracted from *S. enterica* strains and four other closely related enteric bacteria. NASBA amplification of *dnaK* sequences was performed using the SDnaK1 and SDnaK2 primer set. Only *S. enterica* strains produced a signal significantly above background (Table 1).

NASBA amplification of mRNA distinguished viable from non-viable *S. enterica*

Table 2 shows the results obtained after NASBA analysis of pasteurized and untreated *S. enterica* cells. The pasteurized aliquots gave low ECL readings; but the untreated aliquots gave significantly ($P > 0{\cdot}01$) higher readings.

Table 1 ECL signals after NASBA amplification of mRNA from a range of enteric bacteria

Organism	ECL reading*
Citrobacter freundii	496 (± 700)
Enterobacter faecalis	17 (± 23)
Escherichia coli	1199 (± 585)
Klebsiella pneumoniae	210 (± 295)
Salmonella enterica var. *typhimurium*	398793 (± 71325)
Salmonella enterica var. *enteritidis*PT4	327471 (± 37183)
Assay negative control	339 (± 232)

* Mean of triple-replicated experiments. Standard deviations are give in parentheses.

Table 2 ECL signals after NASBA amplification of mRNA from viable and non-viable *S. enterica*

Treatment	ECL reading*
Viable	283908 (± 56818)
Pasteurized	39582 (± 31849)

* Mean of triple-replicated experiments. Standard deviations are give in parentheses.

Confirmation of cell death in the pasteurized samples was made by spreading an aliquot of each onto TSYE agar and incubating at 37 °C for up to 3 d. No colonies were obtained in any instance.

NASBA did not detect genomic DNA present in the nucleic acid extract

NASBA was performed on RNAse-treated extracts obtained from cultures of *S. enterica* (approximately 10^7 cells ml^{-1}). The signals obtained were significantly lower ($P > 0{\cdot}01$) than for untreated extracts, and indeed were not different to the assay negative control signals (Table 3). These extracts were

Table 3 ECL signals after NASBA amplification of mRNA from viable *S. enterica* before and after RNase treatment

Treatment	ECL reading*
None	456956 (± 123922)
RNase-digested	86·7 (± 121)

* Mean of triple-replicated experiments. Standard deviations are give in parentheses.

subjected to PCR using primers SDnaK1 and SDnaK2, and strong signals were obtained (Fig. 1, showing bands of 236 bp, the expected size of the region of *dnaK* targeted by the primer pair).

DISCUSSION

We have demonstrated unambiguous detection of viable *S. enterica* by NASBA amplification of mRNA. This approach is more effective than any previously published amplification-based viability detection method. DNA can not interfere with NASBA. Thus, significantly different results were obtained from live and pasteurized samples containing equal amounts of DNA, and DNase was not necessary to achieve this. NASBA amplification of RNase-treated extracts gave no signal; however, these extracts contained high concentrations of DNA, as shown by the intensity of the PCR signal obtained. It was noted that pasteurized cells gave higher ECL readings than RNase-treated extracts: this was due most probably to the relatively reduced exposure to (cellular) RNases when RNA was extracted from the cells immediately after killing.

The *Hsp*70 gene, the protein product of which functions as a molecular chaperone (Hendrick and Hartl 1993), is found among all bacteria, and indeed within most organisms (Craig *et al.* 1993). By appropriate design of primers and probes it would be useful for a wide range of specific and general microbial detection assays, including environmental and clinical analyses. We are currently investigating the use of NASBA mRNA detection for validation of liquid egg pasteurization, and it should be similarly applicable to the evaluation of other food decontamination procedures.

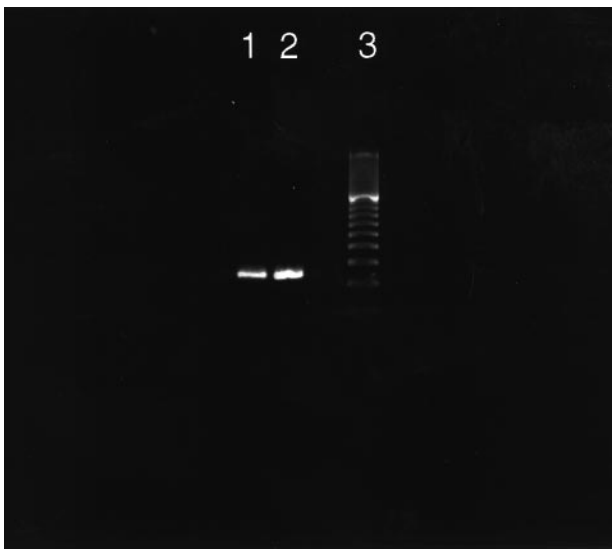

Fig. 1 PCR amplification of *dnaK* sequences from untreated and RNase-treated extracts of *S. enteritidis* cells. Lane 1, RNase-treated extract; lane 2, untreated extract; lane 3, molecular size markers.

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