RESEARCH LETTER

tmRNA – a novel high-copy-number RNA diagnostic target – its application for *Staphylococcus aureus* detection using real-time NASBA

Justin O'Grady[1,2], Katrina Lacey[1,2], Barry Glynn[2], Terry J. Smith[1,2], Thomas Barry[1,2,3] & Majella Maher[1,2]

[1]The Biomedical Diagnostics Institute Program, National University of Ireland Galway, Galway, Ireland; [2]Molecular Diagnostics Research Group, National Centre for Biomedical Engineering Science, National University of Ireland Galway, Galway, Ireland; and [3]Microbiology, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

Correspondence: Justin O' Grady, Molecular Diagnostics Research Group, National Centre for Biomedical Engineering Science, National University of Ireland Galway, IDA Business Park, Dangan, Galway, Ireland. Tel.: +353 9149 2083; fax: +353 9149 5585; e-mail: justin.ogrady@nuigalway.ie

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Abstract

A real-time nucleic acid sequence-based amplification assay, targeting tmRNA, was designed for the rapid identification of *Staphylococcus aureus*. The selectivity of the assay was confirmed against a panel of 76 *Staphylococcus* strains and species and 22 other bacterial species. A detection limit of 1 cell equivalent was determined for the assay. A chimeric *in vitro* transcribed internal amplification control was developed and included in the assay. Application of the assay in natural and artificially contaminated unpasteurized (raw) milk enabled detection of 1–10 CFU *S. aureus* mL^{-1} in 3–4 h, without the need for culture enrichment. *Staphylococcus aureus* was detected in all artificially contaminated milk samples ($n = 20$) and none of the natural milk samples ($n = 20$). Microbiological analysis of the natural milk samples was performed in parallel according to ISO 6888-3 and confirmed the absence of *S. aureus*. The method developed in this study has the potential to enable the specific detection of *S. aureus* in raw milk in a significantly shorter time frame than current standard methods. The assay further demonstrates the usefulness of tmRNA/*ssrA* as a nucleic acid diagnostic target.

Introduction

The *ssrA* gene codes for tmRNA and has been identified in all bacterial phyla (Keiler *et al.*, 2000). The function of tmRNA in bacteria is to rescue stalled ribosomes and to clear the cell of incomplete polypeptides. tmRNA functions both as tRNA and mRNA, with the mRNA portion encoding a peptide tag that is incorporated at the end of the aberrant polypeptide, which targets it for proteolysis (Keiler *et al.*, 2000; O'Grady *et al.*, 2008). The application of the tmRNA/*ssrA* gene for use as a molecular diagnostic target for microbial species identification has been reported previously (Dimov *et al.*, 2008; O'Grady *et al.*, 2008, 2009; McGuinness *et al.*, 2009; Wernecke *et al.*, 2009).

Staphylococcus aureus is the primary cause of contagious mastitis in dairy herds, resulting in reduced milk production and economic loss, and is of major concern in the dairy industry (Hein *et al.*, 2005; Halasa *et al.*, 2007; Rysanek *et al.*, 2007). *Staphylococcus aureus* is a ubiquitous microorganism occurring on the skin and mucous membranes of most warm-blooded animals including humans. It is commonly detected in foods of animal origin such as raw meat and raw bulk milk; however, it is a poor competitor and rarely causes food poisoning in raw products, with an exception being milk from mastitic cows (Anonymous, 2007). Outbreaks of dairy product-related staphylococcal food poisoning, caused by ingestion of food containing staphylococcal enterotoxin, have been reported (Asao *et al.*, 2003; Jorgensen *et al.*, 2005).

Conventional methods of detection and enumeration of *S. aureus* in food, including milk and milk-based products, are labor-intensive and time-consuming, taking from 2 to 6 days to complete, depending on the method used (Anonymous, 2003a–c; Hein *et al.*, 2005). An alternative method for the detection of *S. aureus* with a similar performance, which reduces cost and turnaround time to results, would be of great value to the food and dairy industries.

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Previous studies have reported the detection of *S. aureus* in milk using real-time or conventional PCR (Chiang *et al.*, 2007; Riyaz-Ul-Hassan *et al.*, 2008; Trnčíková *et al.*, 2008). Real-time nucleic acid sequence-based amplification (NASBA) assays for the detection of foodborne pathogens have also been reported (Rodriguez-Lazaro *et al.*, 2004b; Nadal *et al.*, 2007). In this study, the development of a rapid method combining efficient harvesting of bacteria, RNA purification and real-time NASBA [including an internal amplification control (IAC)] for the detection of *S. aureus* in raw milk without enrichment is described. The NASBA assay targets tmRNA, a novel diagnostic target, and uses molecular beacon probe technology. Natural raw milk samples were tested using the rapid method and the ISO 6888-3 culture standard. Milk samples were then artificially contaminated with various levels of *S. aureus* cells and tested using the rapid method.

Materials and methods

Bacterial strains, culture media and growth conditions

Seventy-six *Staphylococcus* strains (63 *S. aureus*, one *Staphylococcus capitis*, one *Staphylococcus caseolyticus*, two *Staphylococcus chromogenes*, two *Staphylococcus cohnii*, four *Staphylococcus epidermidis*, one *Staphylococcus lactis*, one *Staphylococcus warneri* and one *Staphylococcus xylosus* strain) and 22 non-*Staphylococcus* strains were used in this study (Table 1). All *S. aureus* food isolates were kindly donated by Dr Ingeborg Hein, Institute of Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine (Vienna, Austria). All other bacterial species and strains were laboratory stocks previously obtained from culture collections. *Staphylococcus* strains were grown on trypticase soy broth/agar plates (TSB) at 37 °C overnight. All non-*Staphylococcus* were grown in TSB or Luria–Bertani broth/agar plates at either 30 or 37 °C overnight. All media were purchased from Oxoid (Hampshire, UK).

Table 1. Species and strains included in the study

Species name	Source	Real-time NASBA tmRNA	Real-time NASBA IAC
Staphylococcus aureus (*n* = 7)	DSM 346, DSM 6236, NCTC 6571, DSM 6732, ATCC 9144, DSM 12463, DSM 15676	+	+
Staphylococcus aureus (*n* = 46)	Raw milk isolates	+	+
Staphylococcus aureus	Cream cheese isolate	+	+
Staphylococcus aureus (*n* = 8)	Cheese-making milk isolates	+	+
Staphylococcus aureus	Cheese isolate	+	+
Staphylococcus capitis	DSM 20326	–	+
Macrococcus caseolyticus (*Staphylococcus caseolyticus*)	DSM 20597	–	+
Staphylococcus chromogenes (*n* = 2)	DSM 20454, ATCC 43764	–	+
Staphylococcus cohnii (*n* = 2)	DSM 20260, 20261	–	+
Staphylococcus epidermidis (*n* = 4)	DSM 1798, 20044, 20042, NCTC 11047	–	+
Kocuria varians (*Staphylococcus lactis*)	DSM 20033	–	+
Staphylococcus warneri	CCM 2730	–	+
Staphylococcus xylosus	DSM 6179	–	+
Lactococcus lactis	NCTC 662	–	+
Bacillus cereus	NCTC 7464	–	+
Enterococcus faecalis	DSM 20371	–	+
Enterococcus faecium	DSM 20477	–	+
Enterobacter cloacae	NCTC 11933	–	+
Listeria monocytogenes	NCTC 11994	–	+
Streptococcus agalactiae (*n* = 3)	BCCM 15081, 15086, 15094	–	+
Streptococcus bovis	DSM 20480	–	+
Klebsiella aerogenes	NCTC 9528	–	+
Klebsiella oxytoca	ATCC 43086	–	+
Escherichia aninodolica	DSM 30042	–	+
E. coli (*n* = 2)	DSM 301, 30083	–	+
Klebsiella pneumoniae	DSM 30104	–	+
Proteus mirabilis	DSM 4479	–	+
Citrobacter freundii	NCTC 8090	–	+
Aeromonas hydrophila	ATCC 35654	–	+
Salmonella Dublin	NCTC 9676	–	+
Salmonella Enteritidis	ATCC 13076	–	+
Salmonella Typhimurium	NCTC 12416	–	+

Raw milk samples

Raw milk samples from individual udder quarters of cows from a farm in Ireland were sourced by Enfer Scientific (Kildare, Ireland) and stored at −20 °C until testing.

RNA extraction from culture

RNA was extracted using the RiboPure™-Yeast Kit (Ambion, Applied Biosystems, CA) from all organisms in Table 1, from 1 mL overnight culture according to the manufacturer's instructions.

RNA extraction from raw milk

Five hundred microlitres of milk clearing solution [50 mL milk clearing solution contains 49.7 mL 0.25 M EDTA, 250 μL IGEPAL detergent (Sigma), and 50 μL latex beads (Sigma)] was added to 1 mL of either natural raw milk or artificially contaminated raw milk with various concentrations of *S. aureus* cells (10^8–1 CFU) added. Samples were inverted 10 times to mix and centrifuged at top speed in a bench-top microcentrifuge for 10 min. The top layer of fat was removed and the supernatant was discarded. RNA was then extracted from the pellet using the RiboPure™-Yeast Kit.

Table 2. Real-time NASBA primers and probes

Name	Sequence
NASBA-F	*5′-aattgtaatacgactcactatagggagTTCGTCATCAACACACA-3′
NASBA-R	5′-CAGAGGTCCTGATACACA-3′
NASBA-Beacon	5′FAM-**CCAGC**AAGTTTCTTCTAAACAGACT**GCTGG**-3′BHQ1†
Composite F	5′-TTCGTCATCAACACACAATACCCAACTTGGAATG-3′
Composite R	5′-CAGAGGTCCTGATACACATCTTCACCAGAATAAAATTG-3′
IAC-Beacon	5′ROX-**CCGAG**TGAATGTATCCCCTGGA**CTCGG**-3′BHQ2†

*Lower case sequence is T7 RNA polymerase-binding site.
†Molecular beacon stem sequence is given in bold.

Traditional culture method

Microbiological analysis of the naturally contaminated milk samples was performed according to ISO 6888-3 (Anonymous, 2003a) by Enfer Scientific.

Real-time NASBA primers and molecular beacon probes

Oligonucleotide primers and molecular beacon probes for the *S. aureus* real-time NASBA assay were designed according to recommended guidelines (Deiman *et al.*, 2002), following alignment of tmRNA sequences of species and strains (Table 1) generated for this study or available on the tmRNA website (Williams, 2000). Primers and molecular beacon probes were supplied by Eurofins MWG Operon (Ebersberg, Germany) (Table 2).

Real-time NASBA

Real-time NASBA was performed using the Nuclisens Basic Kit (bioMérieux, Marcy l'Etoile, France) and the LIGHTCYCLER 1.2 (Roche Diagnostics, Mannheim, Germany), in accordance with the manufacturer's instructions. Briefly, 4 μL of the target and 1 μL IAC RNA were added to each 10-μL aliquot of reagent/KCl (70 mM final concentration)/primers and molecular beacon probes (0.2 μM final concentration). The reaction was incubated at 65 °C for 5 min to denature the RNA secondary structure and was then immediately cooled to 41 °C for 5 min to allow primer and probe annealing. Next, 5 μL of an enzyme mixture containing avian myeloblastosis virus reverse transcriptase, RNAse H and T7 RNA polymerase was added to the reaction. Each reaction mixture was then transferred to a LIGHTCYCLER capillary and incubated at 41 °C for 90 min with a fluorescence measurement recorded every minute. Fluorescence was plotted against time to yield real-time NASBA curves.

IAC development

An IAC was developed for the real-time NASBA assay according to Rodríguez-Lázaro *et al.* (2004a) with some modifications. Composite primers containing both *Candida albicans als1* gene and *S. aureus* tmRNA primer sequence (Table 2) were used to amplify *C. albicans ALS1* gene producing a 213-bp chimeric PCR product (PCR conditions: 30 cycles of 95 °C for 30 s; 55 °C for 30 s; 72 °C for 30 s followed by 7 min final extension at 72 °C) (O'Grady *et al.*, 2008). *Staphylococcus aureus* real-time NASBA primers were then used to reamplify the PCR product to introduce a T7 RNA polymerase-binding site (239 bp). This PCR product was *in vitro* transcribed using T7 RNA polymerase to produce a 217-nt IAC cRNA. The IAC cRNA was amplified using the *S. aureus* real-time NASBA primers and detected by the ROX-labelled IAC molecular beacon in channel 2 on the LIGHTCYCLER 1.2.

Results and discussion

Design and optimization of the *S. aureus* real-time NASBA assay

NASBA primers and molecular beacon probes were designed to target regions of *S. aureus* tmRNA that did not contain a high degree of secondary structure. Positioning NASBA primers in areas on a low secondary structure has been demonstrated to improve assay sensitivity (Nadal *et al.*, 2007). The M-FOLD RNA and DNA programs were used to predict the secondary structure of *S. aureus* tmRNA and the molecular beacon probes, respectively (Zuker, 2003). Molecular beacons with multiple secondary structures were avoided where possible and were designed to have a free energy of approximately $-3\,\text{kcal}\,\text{mol}^{-1}$, as recommended by Deiman *et al.* (2002). A number of NASBA primers and probes were designed and tested for specificity and sensitivity before the optimum assay was chosen (data not shown).

An IAC was developed for the assay, amplified by the same primers as *S. aureus* tmRNA, but producing a 217-nt *C. albicans* NASBA product. The optimum number of IAC cRNA copies for inclusion in the NASBA reaction was determined by performing titrations of serial dilutions of the IAC cRNA (2×10^5–20 copies), with known concentrations of *S. aureus* RNA (10^5–1 cell equivalents). An IAC concentration of 2×10^4 copies per reaction enabled detection of the IAC without affecting the detection of the primary *S. aureus* tmRNA target.

Analytical sensitivity and selectivity

The analytical sensitivity of the *S. aureus* real-time NASBA assay was determined using serial dilutions of *S. aureus* RNA in triplicate. A detection limit equivalent to 1 cell was determined. RNA content per cell was estimated by extracting RNA from 1 mL of an overnight *S. aureus* culture and determining the $\text{CFU}\,\text{mL}^{-1}$ by plate counts. tmRNA is present at approximately 1000 copies per cell in *S. aureus* (Glynn *et al.*, 2007). This natural amplification of the target in the cell has the potential to improve the limit of detection of the assay. Detection of 0.1 cell equivalents of *S. aureus* RNA was observed on occasion, but detection at this level was not consistent possibly due to dilution inaccuracies.

Molecular beacon probes were used for the real-time NASBA assay because of the specificity they display at low temperatures. NASBA is an isothermal reaction performed at 41 °C and hydrolysis or hybridization probes do not display sufficient specificity for the target sequence at this temperature. It is crucial for the molecular beacon to be designed such that it unfolds at 41 °C when the target sequence is present and forms a stem–loop structure in the absence of the exact target sequence (Niesters, 2001). The annealing of the primers must also occur at this temperature, which may lead to nonspecific primer annealing and amplification of nonspecific NASBA products.

The selectivity of the assay was confirmed against a panel of RNA from the species listed in Table 1. All 63 *S. aureus* strains were detected in the assay, while the 35 other strains (27 species) including closely related staphylococci species were not detected. The *S. aureus* samples were sourced from culture collections and isolated from raw milk and cheese. The real-time NASBA assay was sensitive and specific for the detection of *S. aureus* when testing pure RNA samples.

Detection of *S. aureus* in natural and artificially contaminated raw milk

Current conventional methods for the detection of *S. aureus* in milk take between 2 and 6 days, depending on the method chosen. Recently, real-time PCR assays for the detection of *S. aureus* in milk have been reported (Chiang *et al.*, 2007; Riyaz-Ul-Hassan *et al.*, 2008; Trnčíková *et al.*, 2008). To the best of our knowledge, this is the first description of real-time NASBA-based detection of *S. aureus* RNA.

NASBA assays can be used for the direct detection of pathogens in sample matrices without the need for culture enrichment, therefore reducing the time to result from days to hours. RNA-based amplification methods may have an advantage over DNA-based methods in terms of detection of viable organisms (Cook, 2003; Rodríguez-Lázaro *et al.*, 2006, 2007). Real-time NASBA has, however, been demonstrated to amplify DNA (Rodríguez-Làzaro *et al.*, 2004c), and as the half-life of *S. aureus* tmRNA has not been demonstrated, further studies would have to be performed to assess the potential of the assay developed for the detection of viable *S. aureus*.

In this study, a real-time NASBA-based method was developed for the detection of *S. aureus* in natural and

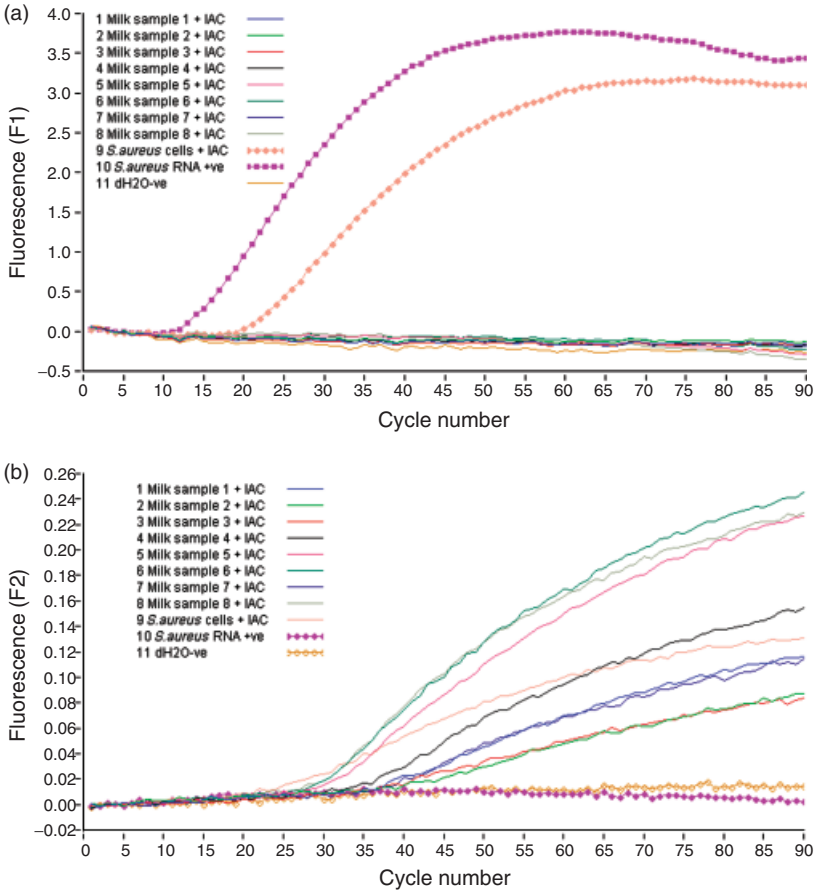

Fig. 1. Detection of *Staphylococcus aureus* in natural and artificially contaminated milk samples using the *S. aureus* NASBA assay. This example demonstrates detection of eight natural (milk sample 1–8+IAC) and one artificially contaminated milk (*S. aureus* cells+IAC) samples and positive (*S. aureus* RNA +ve) and negative (dH_2O − ve) controls in (a) channel 1 on the LIGHTCYCLER 1.2 for *S. aureus* detection and (b) channel 2 for IAC detection.

artificially contaminated raw milk that could be performed in 3–4 h. Twenty unpasteurized milk samples were collected from a farm in Ireland and tested for the presence of *S. aureus* using the ISO 6888-3 (Anonymous, 2003a) culture-based method. No *S. aureus* were found in any of the samples tested; therefore, both natural and artificially contaminated raw milk samples were tested using the real-time NASBA assay (Fig. 1). No *S. aureus* were detected in the natural raw milk and all milk samples artificially contaminated with various levels of *S. aureus* were detected. The rapid method had a limit of detection of 1–10 CFU *S. aureus* mL^{-1} of artificially contaminated milk sample.

The IAC was detected in channel 2 of the LIGHTCYCLER for all milk samples tested, controlling for PCR inhibition and validating the results obtained (Fig. 1b). The IAC was not detected in the *S. aureus* RNA-positive control sample. This is because the IAC and *S. aureus* RNA compete for the NASBA primers. In the presence of a high concentration of *S. aureus* RNA, the IAC was not detected. A valid assay result must consist of one of the following three outcomes: positive in both channels; positive in channel 1 and negative in channel 2; positive in channel 2 and negative in channel 1. A negative result in both channels signifies an invalid result, indicating a problem with the NASBA reaction.

The quantitative potential of the real-time NASBA assay was investigated as food legislation concerning the presence of *S. aureus* in dairy products requires quantitative rather than qualitative detection. Although there was a correlation between the input template and the time to positive using RNA extracted from pure culture, no linear relationship was observed using RNA extracted from milk samples (negative for *S. aureus*) spiked with dilutions of *S. aureus* culture (data not shown).

The *S. aureus* real-time NASBA assay developed in this study demonstrates the potential of tmRNA as a nucleic acid diagnostic (NAD) target. The *ssrA* gene, which codes for tmRNA, has previously been established as a suitable diagnostic target in food and clinical environments (Glynn *et al.*, 2006; O'Grady *et al.*, 2008, 2009; McGuinness *et al.*, 2009; Wernecke *et al.*, 2009). Targeting the high-copy-number tmRNA transcript may improve the limit of detection of the NAD assay, making it possible to directly detect the pathogen of interest in the sample environment without the need for culture enrichment. NAD assays based on tmRNA/*ssrA* gene, including the *S. aureus* NASBA assay, have the potential to be used in the human clinical, food, dairy, veterinaryand environmental sectors.

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