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FOOD MICROBIOLOGY - RESEARCH PAPER

Development of a real-time nucleic acid sequence–based amplification assay for the rapid detection of *Salmonella* spp. from food

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Abstract

Salmonella spp. is one of the most common foodborne infectious pathogen. This study aimed to develop a real-time nucleic acid sequence–based amplification (NASBA) assay for detecting *Salmonella* in foods. Primers and a molecular beacon targeting the *Salmonella*-specific *xcd* gene were designed for mRNA transcription, and 48 *Salmonella* and 18 non-*Salmonella* strains were examined. The assay showed a high specificity and low detection limit for *Salmonella* (7×10^{-1} CFU/mL) after 12 h of pre-enrichment. Importantly, it could detect viable cells. Additionally, the efficacy of the NASBA assay was examined in the presence of pork background microbiota; it could detect *Salmonella* cells at 9.5×10^{3} CFU/mL. Lastly, it was successfully used to detect *Salmonella* in pork, beef, and milk, and its detection limit was as low as 10 CFU/25 g (mL). The real-time NASBA assay developed in this study may be useful for rapid, specific, and sensitive detection of *Salmonella* in food of animal origin.

Keywords *Salmonella* spp. · Real-time NASBA · Food · Detection

Introduction

Salmonella spp. is the leading cause of foodborne illnesses worldwide. Infection with these bacteria causes typhoid fever, gastroenteritis, and septicemia and may even be fatal [1]. To date, more than 93 million humans have been infected by *Salmonella* spp., and 155,000 deaths have been reported [2, 3]. A study found that 11% of all foodborne illnesses in the USA were caused by *Salmonella*, making it the most prevalent foodborne pathogen [4]. It has long been recognized that food animals in particular play an important role in the dissemination of *Salmonella*. Therefore, there is a zero tolerance for *Salmonella* in processing quality assurance for food animals [5, 6].

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The genus *Salmonella* currently includes two broad species: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* is further subdivided into six subspecies, namely, *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica*, and *houtenae*. Further, over 2600 serovars have been characterized for *Salmonella*, with almost 60% classified as *S. enterica*, which is also more commonly associated with disease than *S. bongori* [7, 8]. *Salmonella* strains are usually characterized by analyzing surface antigens: O antigens are part of the variable long–chain lipopolysaccharide on the outer membrane, and the organism also has two flagellar antigens [8].

Classically, *Salmonella* isolates are identified and detected using conventional culture methods and phenotyping, including pre-enrichment, selective enrichment, isolation, biochemical testing, and serotyping. These methods are often used as standard techniques to demonstrate the efficacy of *Salmonella* detection [9]; further, microbiological techniques require 4 days even to show negative results and 6–7 days to confirm the identity of positive isolates. Therefore, improved techniques have been developed for rapid isolation and detection of *Salmonella* in food. Molecular methods can circumvent the above problems and have shown high sensitivity and specificity for detecting *Salmonella* in different types of foods [10]. Many polymerase chain reaction (PCR) and real-time PCR techniques have been developed and applied for detection of foodborne pathogens [11]. One disadvantage of PCR,

however, is that since it is based on target DNA detection, it could amplify DNA from viable and dead cells [12].

Nucleic acid-based sequence amplification (NASBA), which was introduced by Compton (1991), is commonly used for selective amplification of RNA fragments [13, 14]. This assay relies on the activity of three enzymes, namely, T7 RNA polymerase, RNase H, and AMV reverse transcriptase, and requires the presence of a T7 promoter sequence at the 5′ end of the forward primer [15]. The technique is isothermal (41 °C), and the RNA is amplified to a billionfold in around 2 h [16]. In contrast to other detection techniques, such as PCR or real-time PCR, NASBA amplification obviates the need for a thermal cycler and might facilitate potential clinical in resource-poor settings. Although NASBA is more commonly used for detection of RNA viruses, it can also detect pathogenic bacteria in food and environmental samples, for example, *Campylobacter* spp., *Listeria monocytogenes*, *Vibrio cholerae*, and *Escherichia coli* [15]. As messenger (m)RNA molecules generally possess shorter half-lives, they have been considered more suitable than DNA for viability assays. The NASBA is able to amplify a RNA fragment, so NASBA would be a valuable method for detection of viable cell. The difference between real-time NASBA and traditional endpoint NASBA is that the former incorporates target-specific molecular beacon probes in the reaction mix, enabling simultaneous amplification and detection of the target [17].

To our knowledge, NASBA has not been used for the detection of *Salmonella* in food. Thus, the present study aimed to develop a sensitive and rapid real–time NASBA assay to detect viable *Salmonella* in food samples. Primers and a molecular beacon were targeted to mRNA sequences of the *Salmonella xcd* gene, putative protein (location of the gene is 3251654...3252577 in NC_006905.1) [18]. The specificity and sensitivity of this novel method were examined, and it was used for food analysis. The results showed that this protocol has considerable potential for detecting viable *Salmonella* cells.

Materials and methods

Bacterial strains and cultures

A total of 48 *Salmonella* strains representing 34 different serovars and an additional 18 non-*Salmonella* foodborne pathogens (Table 1) were acquired from the China Center of Industrial Culture Collection (CICC), the National Center for Medical Culture Collections (CMCC), Guangdong Culture Collections, and the American Type Culture Collection. Both *Salmonella* and non-*Salmonella* strains were used for specificity testing. *Salmonella* strains were grown on Luria-Bertani (LB) medium at 37 °C, and a final concentration of 10^7 CFU/mL was used for nucleic acid extraction.

Nucleic acid extraction

Bacterial RNA was isolated from enrichment cultures using a Total RNA Extractor kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The extracted RNA was stored at − 80 °C before use in NASBA reactions. The concentration of RNA was measured using a NanoDrop 2000 UV spectrophotometer (Thermo Scientific, US). The negative control was 1 mL of sterilized LB medium in every experiment.

Primer and molecular beacon design

The primers and molecular beacon used in this study were targeted to the *xcd* gene specific to *Salmonella* spp. (Table 2) [18], which encodes the important protein xylanase deacetylase. The set of primers and molecular beacon was created using Beacon Designer 7.0 (Premier Biosoft, Palo Alto, CA). The downstream primers included the bacteriophage T7 RNA polymerase promoter sequence at the 5′ end. The secondary structures of the molecular beacon and target sequence were analyzed using Mfold (http://mfold.rna.albany.edu/?q=mfold/). The beacon was labeled with FAM at its 5′ end and quencher DABCYL at its 3′ end. The specificity of the primers and molecular beacon was verified using online BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers and molecular beacon were obtained from Sangon Biotech and purified using high-performance liquid chromatography.

Real-time NASBA

The real-time NASBA reaction was carried out using the NucliSens basic NASBA kit (bioMerieux Ltd., Boxtel, the Netherlands) according to the manufacturer's instructions. The optimal final concentrations of the primers and molecular beacon used were 600 nM and 400 nM, respectively. Briefly, NASBA assays were carried out in a final volume of 10 μL, with a reaction mixture containing reagent mix (5.5 μL), KCl (80 nM), primers (0.2 μL), molecular beacon (0.1 μL), and target RNA (2.5 μL). The mixture was annealed at 65 °C for 5 min and then cooled at 41 °C for 2 min. The samples were incubated at 41 °C for 90 min in a PCR StepOnePlus™ system (Applied Biosystems, Foster City, USA) before the enzymes (1.5 μL) were added. A deionized H_2O blank was included as an amplification negative control with every assay. When the target amplification curves reached the threshold level, the result of detection was considered positive.

Specificity and sensitivity of real-time NASBA

The specificity of real-time NASBA was verified using 48 strains of *Salmonella* spp. belonging to different

Table 1 *Salmonella* and non-*Salmonella* strains used for the real-time nucleic acid sequence–based amplification

Salmonella	Source	Number	*xcd*	*Salmonella*	Source	Number	*xcd*	Non-*Salmonella*	Source	*xcd*
S. Paratyphi A	CMCC50001	1	+	*S.* Kentucky	CICC21488	1	+	*Escherichia coli*	ATCC35150	−
S. Paratyphi A	CICC21501	1	+	*S.* Bazenheid	CICC21587	1	+	*Escherichia coli*	ATCC43889	−
S. Saint Paul	CICC21486	1	+	*S.* Typhi	CMCC50071	1	+	*Enterococcus faecalis*	ATCC12953	−
S. Paratyphi B	CICC21495	1	+	*S.* Enteritidis	CICC21527	1	+	*Enterococcus faecalis*	ATCC29212	−
S. Agona	CICC21586	1	+	*S.* Enteritidis	CICC21482	1	+	*Enterococcus avium*	ATCC14025	−
S. Heidelberg	CICC21487	1	+	*S.* Enteritidis	CVCC3374	1	+	*Klebsiella pneumoniae*	ATCC13884	−
S. Typhimurium	CMCC51005	1	+	*S.* Enteritidis	CMCC50041	1	+	*Staphylococcus aureus*	ATCC29213	−
S. Typhimurium	CICC21483	1	+	*S.* Enteritidis	CMCC50071	1	+	*Staphylococcus aureus*	ATCC25923	−
S. Typhimurium	CVCC3384	1	+	*S.* Enteritidis*	Pork	2	+	*Serratia marcescens*	CICC10187	−
S. Typhimurium*	Pork	3	+	*S.* Dublin	CICC21497	1	+	*Bacillus pumilus*	CMCC63202	−
S. Bredeney*	Pork	1	+	*S.* Dublin	CMCC50761	1	+	*Bacillus cereus**		−
S. Derby*	Beef	1	+	*S.* Miami	CICC21509	1	+	*Pseudomonas fluorescens**		−
S. Paratyphi C	CICC21512	1	+	*S.* Eastbourne	CICC21508	1	+	*Listeria grayi*	CICC21670	−
S. Montevideo	CICC21588	1	+	*S.* Anatum	CICC21498	1	+	*Listeria seeligeri*	CICC21671	−
S. Jerusalem	CICC21651	1	+	*S.* Meleagridis	CICC21511	1	+	*Listeria welshimeri*	CICC21672	−
S. Bonn	CICC21677	1	+	*S.* London*	Pork	1	+	*Listeria monocytogenes*	CICC21662	−
S. Choleraesuis	CICC21493	1	+	*S.* Senftenberg	CICC21502	1	+	*Listeria ivanovii*	CICC21663	−
S. Choleraesuis	ATCC13312	1	+	*S.* Aberdeen	CICC21492	1	+	*Listeria innocua*	CICC10417	−
S. Thompson	CICC21480	1	+	*S.* Blockley	CICC21489	1	+			
S. Potsdam	CICC21500	1	+	*S.* Adelaide	CICC21505	1	+			
S. Braenderup	ATCC19812	1	+	*S.* Wandsworth	CICC21504	1	+			
S. Bonariensis	CICC21496	1	+	*S.* Dakar	CICC21507	1	+			
S. Bovismorbificans	CICC21499	1	+	*S.* Arizonae	CICC21506	1	+			

+, positive result; −, negative result; *laboratory-isolation strain

serogroups and 18 strains of non-*Salmonella* foodborne pathogens (Table 1). Nucleic acid extraction and real-time NASBA were conducted for each strain.

Salmonella *ser*. Choleraesuis (CICC21493) was used to evaluate the detection limit of the assay. This strain was cultured overnight after tenfold serial dilution with buffered peptone water. Each dilution, which corresponded to a cell concentration determined using the plate-count method, was subjected to nucleic acid extraction in triplicate. Each dilution and space was detected by this real-time NASBA assay.

Viability testing

Samples of Salmonella *ser*. Enteritidis (approximately 10^6 CFU/mL) were autoclaved (121 °C) for 20 min and then incubated at room temperature for 0, 12, 24, or 48 h, after which DNA and RNA were extracted from the samples and analyzed by real-time NASBA, PCR (139–141 primers) [19] and culturing (Table 2). Samples of non-autoclaved cells were used as a control to examine the effects of this treatment.

Detection of *Salmonella* spp. in the presence of background microbiota

This detection was not subjected to selective enrichment; we decided to test our detection method in the presence of non-*Salmonella* microorganisms. For this, the detection limits of real-time NASBA were investigated in the presence of pork background microbiota. Salmonella *ser*. Typhimurium (CMCC51005) was cultured overnight after tenfold serial dilution using buffered peptone water. The pork sample was confirmed to be *Salmonella* free by standard microbiological methods, and 10 g of this sample was inoculated in 90 mL of LB at 37 °C for 12 h for enrichment culture. A 500-μL sample of each dilution of Salmonella *ser*. Typhimurium was added to 500 μL of the pork background microbiota suspension. This mixture was subjected to nucleic acid extraction and tested using real-time NASBA.

Artificial contamination of food samples

In order to validate the method for detection of *Salmonella* spp., it was conducted using artificially contaminated food samples. Portions of beef, pork, and milk were purchased

Table 2 Primers and molecular beacon for the *Salmonella* spp. real-time nucleic acid sequence–based amplification assay

Target organism	Gene	Primer beacon	Sequence(5′-3′)	Reference
Salmonella spp.	*xcd*	Pxcd-f	5′-GTTAGCTGGTATCTGGATGA-3′	This study
		Pxcd-f	5′-AATTCTAATACGACTCACTATAGGG$_1$ AAACTGATGGTTATAAG CATAGGT-3′	This study
		Bxcd	FAM-CGATCG$_2$CGTATACCGGT AACCAGGAGGGGACGATCG$_2$-DABCYL	This study
	*inv*A	139	GTGAAATTATCGCCACGTTCGGGCAA	[16]
		141	TCATCGCACCGTCAAAGGAACC	[16]

1: Recognition sequence of T7 RNA polymerase

2: Reverse repetitive sequence

from local supermarkets. They were confirmed to be free of *Salmonella* spp. by standard methods (GB4789.4-2010). In replicates, 25-g food samples were artificially contaminated with dilutions of Salmonella *ser*. Enteritidis at the following approximate concentrations: 10 [2], 10 [1], 10^0, 10^{-1}, and 0 CFU/25 g. These dilutions were inoculated in LB culture medium at 37 °C for 12 h. Then, 1 mL of each pre-enriched sample was processed for nucleic acid extraction and real-time NASBA.

Results

Specificity and sensitivity of real-time NASBA

The specificity of real-time NASBA was detected using RNA isolated from 48 *Salmonella* spp. strains and 18 non-*Salmonella* strains. The *xcd* gene was successfully amplified from all the *Salmonella* spp. strains using the primers and molecular beacon designed in this study, but not from all the non-*Salmonella* strains. As show in Table 1, the results showed that our protocol was specific to the target strains, and non-specific reactions did not occur with non-*Salmonella* strains.

The real-time NASBA assay enabled successful amplification of different bacterial concentrations (700, 70, 7, and 7×10^{-1} CFU/mL) after 10 h of enrichment (Table 3). When concentration was less than 7×10^{-1} CFU/mL, the results of detection were negative, as they were for the negative control. The detection limit of the real-time NASBA assay for *Salmonella* spp. was found to be 7×10^{-1} CFU/mL.

Viability detection

In order to confirm that NASBA is able to detect viable cells because it uses mRNA as the amplification target, Salmonella *ser*. Enteritidis was heat treated at 121 °C and incubated at 0, 12, 24, and 48 h at room temperature. The samples were examined using standard culture, PCR (*inv*A), and real-time NASBA (*xcd*). PCR (*inv*A) showed positive results for detection of Salmonella *ser*. Enteritidis after treatment at 121 °C and further incubation for 0, 12, 24, and 48 h. In contrast, the results of the standard culture method were negative (Table 4). The *xcd* gene was detected by real-time NASBA in Salmonella *ser*. Enteritidis subjected to heat treatment at 121 °C and further incubated for 0 and 12 h. However, real-time NASBA was not able to detect organism after heat treatment and 24 h of incubation. These results indicated that dead cells did not interfere with the detection of viable *Salmonella* spp. using the real-time NASBA method.

Detection of *Salmonella* spp. in the presence of background microbiota

The total aerobic plate count of the pork sample enriched for 12 h was 1.9×10^7 CFU/mL. The detection limit of the real-time NASBA assay was evaluated in the presence of background microbiota. Additionally, the sensitivity of the assay for detection of *Salmonella* spp. was tested by combining various dilutions of *Salmonella* spp. with pork background microbiota. The detection limit of the assay for *Salmonella* spp. was approximately 9.5×10^3 CFU/mL (Table 5). Real-time NASBA did not show an amplification curve in the

Table 3 Sensitivity of the real-time nucleic acid sequence–based amplification assays

Salmonella spp. (CFU/mL)	*xcd* (Ct ± SD) $n = 3$	Result
7×10^2	8.7 ± 1.69	+
7×10^1	9.26 ± 1.14	+
7×10^0	10.6 ± 3.6	+
7×10^{-1}	11 ± 0.66	+
7×10^{-2}	ND	−
0	ND	−

ND, not determined; +, positive result; −, negative result

Table 4 Real-time nucleic acid sequence–based amplification, PCR, and standard culture with heat-treated non-viable *Salmonella* samples

Detection of methods	Duration of incubation of non-viable cells at room temperature (h)			
	0	12	24	48
Real-time NASBA (*xcd*)	5.75 ± 0.87	11.03 ± 2.61	ND	ND
PCR(*inv*A)	+	+	+	+
GB/T4789.4-2008	−	−	−	−

ND, not determined; +, positive result; −, negative result

presence of pork background microbiota at 9.5×10^2 CFU/mL (Table 5).

Artificial contamination of food sample

Food samples of pork, beef, and milk spiked with four concentrations of *Salmonella* from $n \times 10^{-1}$ to $n \times 10^2$ CFU/25 g (mL) ($1 < n < 10$) were subjected to the real-time NASBA assay after 10 h of enrichment. Positive signals were obtained from all food samples artificially contaminated with n CFU/25 g (mL) of *Salmonella* spp. (Table 6). Control samples containing with 0 CFU/25 g (mL) of *Salmonella* spp. showed no amplification curve for any sample. These results indicated that the detection limit of the assay for *Salmonella*-contaminated food samples was n CFU/25 g (mL).

Discussion

Nowadays, the demand for highly reliable and specific methods for detection of foodborne pathogens has peaked mainly because of these infections are common and cause substantial economic loss worldwide. The present study aimed to develop a real-time NASBA assay for the detection of *Salmonella* [20]. The *xcd* gene specific to *Salmonella* was selected as target sequence and used for primer and molecular beacon development [18]. Use of the molecular beacon to monitor amplicon generation during NASBA ensures result reliability in a one-tube system and minimizes the risk of contamination. Real-time NASBA approaches certainly cannot completely replace the traditional culture method, but they do yield results within a short time and have minimum processing errors. The real-time NASBA assay can differentiate between viable and non-viable cells and also avoids other disadvantages of real-time PCR, such as interference of dead cells.

Table 5 Detection of *Salmonella* cells in the presence of pork background microbiota

Salmonella (CFU/mL)	*xcd* (Ct ± SD) $n = 3$	Result
9.5×10^6	4.807 ± 0.453	+
9.5×10^5	7.908 ± 3.89	+
9.5×10^4	4.491 ± 0.556	+
9.5×10^3	4.399 ± 0.722	+
9.5×10^2	ND	−
0	ND	−

ND, not determined; +, positive result; −, negative result

In the present study, the real-time NASBA assay had 100% specificity because of the use of the *xcd* gene. The specificity of the *xcd* gene was evaluated from its complete sequence in the NCBI database; in PCR-based verification, the primer sets showed good specificity and did not yield false negative or false positive results for *Salmonella* and non-*Salmonella* organisms, respectively [18]. This molecular beacon–based real-time NASBA also showed high specificity by combined use of specific primers [21]. The method for *Salmonella* detection included a short enrichment step, RNA extraction, and real-time NASBA. The purpose of the short enrichment step was to increase the possibility of detecting even low amounts of the pathogen and to overcome the effects of any NASBA inhibitors [22]. The detection limit was found to be 7×10^{-1} CFU/mL within 1 working day. In a previous study, a novel PCR instrument for detection of *Salmonella* yielded positive results when the concentration of the target strains was 2.5 CFU/mL. [11] The presence-absence assay with real-time NASBA described here could detect 5 CFU/mL of *Salmonella* [23]. The detection limit in our study was better than those in previous studies. The NASBA reaction involves three enzymes, because of which it may be more sensitive to inhibitors than PCR, which only involves a single enzyme [13]. However, culture enrichment could effectively reduce the influence of inhibitors.

In principle, the presence of RNA should indicate cell viability [24]. mRNA has an average half-life of only a few minutes in metabolizing strains [25]. However, previous studies have shown that mRNA may persist for several days after cell death [26]. The ability of NASBA to detect viable cells was evaluated in the present study, and positive results were obtained in the assay for heat-treated samples incubated at room temperature for less than 24 h. However, for heat-treated samples incubated for more than 24 h, real-time NASBA was not able to discriminate between dead and viable cells: it yielded the same results as PCR. A reason for this may be that viable cells contained sufficient *xcd*-mRNA (in the 24 h after heat treatment) to produce a positive real–time

Table 6 Detection of *Salmonella* cells in spiked food samples

Strain	Number of cell	Food sample		
	(CFU/25 g (mL))	Pork	Beef	Milk
S. Enteritidis	$N \times 10^2$	24.186 ± 1.887	12.65 ± 4.826	13.89 ± 1.892
CICC21527	$N \times 10^1$	15.85 ± 7.426	10.596 ± 0.262	15.618 ± 4.577
	$N \times 10^0$	14.211 ± 6.82	27.498 ± 4.569	23.07 ± 2.77
	$N \times 10^{-1}$	ND	ND	ND
	0	ND	ND	ND

ND, not determined

NASBA reaction. However, the *xcd*-mRNA of non-viable cells was degraded by RNase in the environment 24 h after heat treatment and thereafter. Another reason could be changes in specific features related to mRNA stability and secondary structures due to prolonged incubation after heat treatment.

The detection limits for *Salmonella* strains in different foods may depend on the complexity of food components and background microbiota [27]. The introduction of an enrichment step before NASBA could reduce this influence of food material on the sensitivity of detection. In the present study, we investigated the detection limits of real-time NASBA for *Salmonella* in the presence of natural background microbiota. The minimum concentration of *Salmonella* for detection was 9.5×10^3 CFU/mL in the presence of pork background microbiota. This result is similar to that of Chen et al. (2010), who found that the sensitivity of *Salmonella* detection by real-time PCR was 1.3×10^3 CFU/mL in the presence of nature background microbiota. Our protocol applied to three different artificially contaminated in food products of animal origin yielded positive results in less than 1 working day with initial inoculum levels as low as 10 CFU/25 g (mL). Our method was more sensitive than a previously conducted NASBA assay followed by electrochemiluminescence detection (10^2 CFU per 25 g) [28]. Thus, we showed that this real-time NASBA assay is applicable to the detection of *Salmonella* in pork, beef, and dairy products. Food samples of animal origin spiked with *Salmonella* cells were used to verify that the combination of a rapid procedure for the extraction of RNA and the cell density was very necessary.

In conclusion, the present study described a novel, rapid, and sensitive real–time NASBA assay for the detection of *Salmonella* in food products. This method targeted the *xcd* gene and could discriminate viable and non-viable cells. The protocol included a pre-enrichment step, nucleic acid extraction, and real-time NASBA and could be completed in less than 1 working day. The real-time NASBA assay was specific and sensitive for *Salmonella* detection from food samples of animal origin. Because of its efficiency, we believe this assay can be used in efforts to prevent outbreaks of foodborne illnesses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interest.

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