

Detection of *Vibrio cholerae* by Real-Time Nucleic Acid Sequence-Based Amplification[∇]

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A multitarget molecular beacon-based real-time nucleic acid sequence-based amplification (NASBA) assay for the specific detection of *Vibrio cholerae* has been developed. The genes encoding the cholera toxin (*ctxA*), the toxin-coregulated pilus (*tcpA*; colonization factor), the *ctxA* toxin regulator (*toxR*), hemolysin (*hlyA*), and the 60-kDa chaperonin product (*groEL*) were selected as target sequences for detection. The beacons for the five different genetic targets were evaluated by serial dilution of RNA from *V. cholerae* cells. RNase treatment of the nucleic acids eliminated all NASBA, whereas DNase treatment had no effect, showing that RNA and not DNA was amplified. The specificity of the assay was investigated by testing several isolates of *V. cholerae*, other *Vibrio* species, and *Bacillus cereus*, *Salmonella enterica*, and *Escherichia coli* strains. The *toxR*, *groEL*, and *hlyA* beacons identified all *V. cholerae* isolates, whereas the *ctxA* and *tcpA* beacons identified the O1 toxigenic clinical isolates. The NASBA assay detected *V. cholerae* at 50 CFU/ml by using the general marker *groEL* and *tcpA* that specifically indicates toxigenic strains. A correlation between cell viability and NASBA was demonstrated for the *ctxA*, *toxR*, and *hlyA* targets. RNA isolated from different environmental water samples spiked with *V. cholerae* was specifically detected by NASBA. These results indicate that NASBA can be used in the rapid detection of *V. cholerae* from various environmental water samples. This method has a strong potential for detecting toxigenic strains by using the *tcpA* and *ctxA* markers. The entire assay including RNA extraction and NASBA was completed within 3 h.

Vibrio cholerae is the etiological agent of epidemic cholera, which causes watery diarrhea that can result in the rapid dehydration and death of infected persons. Coastal waters are an important reservoir of *V. cholerae*, and cholera is generally transmitted to humans via contaminated water or seafood (12, 13). Of more than 200 known serogroups of *V. cholerae*, the two well-known serogroups O1 and O139 have been associated with epidemic cholera (11). The serogroup O1 can be divided into three serotypes, Inaba, Ogawa, and Hikojima, and each serotype can be divided into two biotypes, classical and El Tor (30). The other serogroups of *V. cholerae*, collectively referred to as non-O1 and non-O139 serogroups, have not been associated with epidemics but have been associated with occasional outbreaks of cholera-like disease. These other serogroups are usually isolated from patients with mild diarrhea or from the environment (27, 39). *Vibrio* species such as *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* are found in blue mussels harvested along the coastline of Norway (5).

The pathogenesis of cholera is a complex process, and the major virulence factors of *V. cholerae* are the cholera toxin (CT) encoded by the *ctxAB* genes and the toxin-coregulated pilus (TCP) encoded by the *tcpA* gene (30). CT leads to increased intestinal secretion of electrolytes and water into the lumen. TCP is a type IV pilus that is essential for intestinal colonization in humans and in animal models of cholera (26, 50). The *ctxAB* genes are located in the genome of the CTX Φ filamentous prophage, and *tcpA* is located in the large *Vibrio*

pathogenicity island, which functions as a receptor for the CTX Φ prophage (31, 33, 53). The *toxR* gene encodes a transcription factor that directly regulates the expression of CT, among others (37). Most toxigenic strains and non-O1 and non-O139 strains of *V. cholerae* possess the *toxR* gene (48). Another genetic target is the virulence factor hemolysin, which is proposed to cause diarrhea (29). The hemolysin produced by *V. cholerae* non-O1 and non-O139 strains is identical to the hemolysin product of the *V. cholerae* El Tor strains (54). However, some El Tor strains do not express the *hly* gene. Still, the *hlyA* gene is detected in several nonhemolytic strains by PCR (48).

PCR has now become a frequently used detection method, and several PCR protocols have been developed for *V. cholerae* (24, 25, 35, 36, 48, 49). PCR detection confirms the presence of specific genetic regions in a target organism. However, RNA-based methods such as the nucleic acid sequence-based amplification (NASBA) method and reverse transcription-PCR may reveal the expression of various genes. Viable but nonculturable (VBNC) forms of bacterial cells are now recognized as a common phenomenon in many bacterial species, and these organisms may escape detection if only culture methods are used (28). It has been demonstrated that the viability of VBNC cells may be monitored by reverse transcription-PCR analysis (17).

The NASBA assay, initially introduced by Compton (14), is a sensitive, transcription-based amplification system specifically designed for detecting RNA. The technology relies on the simultaneous activity of three different enzymes: RNaseH, avian myeloblastosis virus reverse transcriptase, and T7 RNA polymerase. The presence of a T7 promoter sequence at the 5' end of the forward primer is essential and is used by the T7

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RNA polymerase during the synthesis of new RNA amplicons. In contrast to PCR and reverse transcription-PCR, the NASBA method is isothermal (41°C). At 41°C, genomic DNA remains double-stranded, and it is therefore not a substrate in NASBA. Although NASBA is specifically designed for the detection of RNA targets, in some NASBA systems DNA may be amplified (19, 42). However, the DNA amplification is ineffective and occurs only in the absence of RNA targets or in the case of a 1,000-fold excess of target DNA. Successful reverse transcription-PCR requires residual DNA to be eliminated in order to provide accurate detection of specific bacterial RNA sequences. Such extensive extraction of the nucleic acids may be time-consuming and may reduce the recovery of target RNA.

In NASBA, single-stranded RNA amplicons are produced, and these can easily be detected by hybridization with a sequence-specific probe such as a molecular beacon. Molecular beacons are single-stranded oligonucleotide hybridization probes designed such that they can form a stem-loop structure (51). The loop sequence is complementary to the target sequence to be detected, and the stem sequences are complementary to each other. A fluorescent label is linked to the end of one arm and a quencher to the other. Therefore, the molecular beacon is not fluorescent when it is free in a solution. A molecular beacon is designed so that its probe sequence is just long enough for a perfect complementary probe-target hybrid to be more stable than the stem hybrid. Consequently, the molecular beacon spontaneously forms probe-target hybrids in which the effect of the quencher is eliminated (19). Molecular beacon probes are designed according to specific rules (19), and the design is critical for the specificity and sensitivity of real-time NASBA (34).

NASBA has been extensively applied in clinical microbiology in detecting RNA viruses (19). However, NASBA is also used successfully for detection of microbial pathogens in food and environmental samples (16). Descriptions of such methods for the detection of *Campylobacter* spp. (52), *Listeria monocytogenes* (7), *Salmonella enterica* in various foods (15), *Cryptosporidium parvum* (3), and *Escherichia coli* in water (38) have been published. It has been shown that as few as 10 viable spores of *Bacillus anthracis* can be detected by NASBA (4).

In the present study, a real-time multitarget NASBA assay for the specific detection of *V. cholerae* cells has been developed. Primers and molecular beacons were targeted to five different genomic regions. These were the constitutively expressed housekeeping gene *groEL*, the hemolysin A virulence gene *hlyA*, the CT gene *ctxA*, the TCP gene *tcpA*, and the regulator for the expression of CT, the *toxR* gene. Serial dilutions of *V. cholerae* RNA were used to determine the detection limits of the single assays (50 CFU/ml), and RNA extraction and NASBA were performed within 3 h. The specificity of the assay was tested against several isolates of *V. cholerae*, *Vibrio* spp., and strains of *Bacillus cereus*, *S. enterica*, and *E. coli*. Furthermore, *V. cholerae* was detected in spiked environmental water samples (5×10^3 CFU/ml). This is to our knowledge the first report describing the use of NASBA for the specific detection of *V. cholerae*. Our results indicate that the *ctxA* and *tcpA* markers may be used to monitor the environmental population of *V. cholerae* strains expressing the virulence factors.

MATERIALS AND METHODS

Bacterial strains, media, and cultures. The *Vibrio* spp. strains used in this study are shown in Table 1. The isolates, collected from clinical and environmental samples, were kindly provided by the Norwegian School of Veterinary Science, the Norwegian Institute of Public Health, and the Istituto Superiore di Sanità, Italy. The strains of *B. cereus* and the *V. cholerae* strain Cip 106855 O1 Inaba El Tor were obtained from the American Type Culture Collection, and the *V. cholerae* strain NCTC 8457 was from the National Collection of Type Cultures, United Kingdom. The strains of *S. enterica* and the *E. coli* strain CCUG 29188 were obtained from the Culture Collection, University of Göteborg, Sweden. The *E. coli* strain DSM 4230 was from the German Collection of Microorganisms and Cell Cultures. The *Vibrio* spp. strains were grown in tryptic soy broth (TSB; Merck, Germany). The *E. coli* and *S. enterica* strains were grown in Luria-Bertani broth (Merck, Germany), and the *B. cereus* strains were grown in brain heart infusion broth (Acumedia Manufacturers Inc., Baltimore, MD). All cultures were grown aerobically at 37°C and harvested by centrifugation at $2,000 \times g$ for 10 min in the logarithmic phase of growth. The cells were washed once in sterile phosphate-buffered saline (PBS). The numbers of structurally intact cells were determined with a phase-contrast microscope (Zeiss, Germany). Cultures of *V. cholerae* were serially diluted, and CFU were enumerated by plating 100 μ l of each dilution onto TSB agar and incubating at 37°C for 24 h. RNAlater (QIAGEN) was added to aliquots of the cell pellets to be used in RNA isolation prior to storage at -80°C.

Primers and molecular beacon probes. The primers and probes used in this study were targeted to the constitutively expressed housekeeping gene *groEL*, the virulence gene *hlyA*, the CT gene *ctxA*, the TCP gene *tcpA*, and the *toxR* gene. *V. cholerae*-specific sequences of the mentioned genes were identified by performing BLASTP and BLASTN database searches. The results were shown as multiple alignments, and regions unique to *V. cholerae* could be identified and used in primer and probe design using the Primer3 program (43). Primers and molecular beacon probes were synthesized by Invitrogen and Eurogentec (Belgium) (Table 2). The structures of the probes were tested using mfold, a DNA folding program (Zucker program; <http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi>). The sequences of the target genes were selected from gi9657252:c3783-3667 (*groEL*), gi40548577:c268-403 (non-O1, non-O139 El Tor hemolysin gene, *hlyA*), gi497317:c83-185 (*tcpA*), gi18448890:c180-331 (*ctxA*), and gi155248:c397-536 (*toxR*). The general guidelines described previously (19) and advice from personnel at NorChip AS (personal communication) were followed in primer and probe construction.

RNA isolation. Unless otherwise stated, the nucleic acids (DNA and RNA) used in all experiments were extracted from the toxigenic *V. cholerae* strain Cip 106855 O1 Inaba El Tor. RNA was isolated from cell pellets (as described above) according to the method for the NucliSens basic kit (bioMérieux Ltd., Boxtel, The Netherlands) (8). Briefly, 900 μ l of lysis buffer was added to a cell pellet from either 100 or 500 μ l of bacterial cells grown as described above. Fifty microliters of silica suspension was added to the lysis buffer to bind the nucleic acids. The silica was washed with washing buffer two times and air dried. Nucleic acids were eluted with 50 or 100 μ l of elution buffer, and the eluates were aliquoted and stored at -80°C. In some experiments, pure RNA was isolated using the RNeasy kit according to the procedure of the manufacturer (QIAGEN).

NASBA. The NASBA reaction was carried out by using the NucliSens basic NASBA kit according to the guidelines of the manufacturer (BioMérieux Ltd., Boxtel, The Netherlands) and the personnel at NorChip AS (personal communication). The NASBA assays were carried out with a total of 25 μ l containing the reagent mix, primers (0.2 μ M [each]), the molecular beacon (0.25 μ M), KCl (70 mM), and the template (5 μ l). Samples were incubated at 65°C for 4 min before the enzymes were added. The samples were then incubated at 41°C for 90 min. Each NASBA assay included a deionized H₂O blank as a negative control. The RNA amplicons were identified in real-time and online by using specific fluorescent molecular beacon probes (Table 2). Real-time measurements with these probes were made with a SmartCycler thermocycler (Cepheid). The amplification of a specific NASBA product is indicated by the detection time in minutes (the *t* value). The *t* value represents the time point at which the fluorescence value crosses a fixed threshold that is 10 times the standard deviation of the baseline intensity. In some experiments, the RNA amplicons were analyzed by electrophoresis on a Bioanalyzer (Agilent Technology, Germany). The predicted sizes of the amplicons were between 102 and 151 nucleotides (Table 2). The expression of the selected target genes was tested at different time points during the logarithmic growth phase and in the late stationary growth phase. These results provided a basis for further experiments in which cells were grown to mid-logarithmic phase (about 4×10^7 CFU/ml) before harvesting. A variety

TABLE 1. Bacterial strains used for NASBA

Bacterium	Strain and serogroup	Source, ^e date of collection, and type of isolate	Detection of genetic targets ^f :				
			<i>tcpA</i>	<i>ctxA</i>	<i>groEL</i>	<i>hlyA</i>	<i>toxR</i>
<i>V. cholerae</i> O1	O1 Inaba El Tor Cip106855	ATTC 393	+	+	+	+	+
	O1 Inaba El Tor ATTC 14033	NCTC 8457	+	+	+	+	+
	VC242, O1 Ogawa El Tor	Feces ^a	+	+	+	+	+
	VC243, O1 Ogawa El Tor	Feces ^a	+	+	+	+	+
	VC 5/77, O1	Peru, 1991 ^b	+	+	+	+	+
	VC 2/60, O1	Albania, 1994 ^b	+	+	+	+	+
	VC 2/70, O1	Albania, 1994 ^b	+	+	+	+	+
	VC 2/67, O1	Albania, 1994 ^b	+	+	+	+	+
	VC 2/57, O1	Albania, 1994 ^b	+	+	+	+	+
	VC 2/21, O1	Italy, 1994 ^b	+	+	+	+	+
	VC 2/23, O1	Italy, 1994 ^b	+	+	+	+	+
	VC 2/26, O1	Italy, 1994 ^b	+	+	+	+	+
	VC 2/32, O1	Italy, 1994 ^b	+	+	+	+	+
	VC 6/31, O1	Italy, 2005 ^{b,c}	+	+	+	+	+
	VC 4/53, O1	Italy, 1973 ^b	+	+	+	+	+
	VC 4/57, O1	Italy ^b ; environmental, <i>ctx</i> positive	+/-	+/-	+	+	+
VC 6/23, O1	Italy ^b ; environmental, <i>ctx</i> negative	-	-	+	+	+	
<i>V. cholerae</i> non-O1	VC 5/47, O2	Italy ^b ; septicemic	+/-	+/-	+	+	+
	VC 5/42, O158	Italy ^b ; septicemic	-	-	+	+	+
	VC 4/76, O111	Italy ^b ; septicemic	-	-	+	+	+
	VC084, non-O1	Scampi ^d	-	-	+	+	+
	VC216, non-O1	India ^d	-	-	+	+	+
	VC229, non-O1	Wound ^a	-	-	+	+	+
	VC230, non-O1	Feces ^a	-	-	+	+	+
	VC246, non-O1	Norway ^a ; mussel	-	-	+	+	+
	VC344, non-O1	Norway ^a ; mussel	-	-	+	+	+
	VC503, non-O1	Norway ^d ; water	-	-	+	+	+
Other <i>Vibrio</i> species ^d							
<i>V. fluvialis</i>	VF 062		-	-	-	-	-
<i>V. mimicus</i>	VM 034		-	+/-	-	-	-
	VM 052		-	-	-	-	-
	VM 345		-	-	-	-	-
<i>V. metschnikovii</i>	VM 116		-	-	-	-	
<i>V. parahaemolyticus</i>	VP 160		-	-	-	-	-
	VP 363		-	-	-	-	-
	VP 438		-	-	-	-	-
<i>V. alginolyticus</i>	VA 054		-	-	-	-	-
	VA 647		-	-	-	-	-
Other bacterial species							
<i>B. cereus</i>	ATCC 14579	ATCC	-	-	-	-	-
	ATCC 10987	ATCC	-	-	-	-	-
<i>E. coli</i>	DSM 4230	DSMZ	-	-	-	-	-
	ATCC 43888, O157:H7	CCUG 29188	-	-	-	-	-
<i>S. enterica</i>	ATCC 13076 Cip 82.97	CCUG 34136 T	-	-	+/-	-	-
	ATCC 43971 Cip 60.62	CCUG 43971	-	-	-	-	-

^a Strain received from J. Lassen, The Norwegian Institute of Public Health, Norway.

^b Strain received from A. Carotolli, Istituto Superiore di Sanita, Italy.

^c Imported case of *V. cholerae*.

^d Strain(s) received from L. M. Rørvik, The Norwegian School of Veterinary Science, Norway.

^e ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures); NCTC, National Collection of Type Cultures (United Kingdom); CCUG, Culture Collection of the University of Göteborg (Sweden).

^f +, specific amplified product detected; -, no amplified product detected; +/-, product was amplified at a late stage of the NASBA assay with a high *t* value and a weak fluorescence signal.

of primers were tested, but only the optimal primer pair is shown in this report. Several isolates of *Vibrio* spp. and non-*Vibrio* spp. were used to test the specificities of the primers and the molecular beacons in NASBA (Tables 1 and 2).

Isolated nucleic acids were subjected to DNase or RNase treatment. Aliquots (100 µl) of nucleic acids were incubated with 10 U of RNase (EC 3.1.27.5; Sigma chemical Co.) in Tris (10 mM)-EDTA (1 mM) buffer, pH 7.5, at 37°C for 2 h or 120 U of DNase (EC 232-667-0; Sigma Chemical Co.) in sodium acetate (100 mM) buffer, pH 5.25, containing MgCl₂ (5 mM) for 2 h at 25°C. These treatments were followed by phenol-chloroform extraction and NASBA.

Sensitivity. The detection limit and the reproducibility were determined by amplifying samples from 10-fold serial dilutions of *V. cholerae* RNA. Each dilution, which corresponded to cell concentrations ranging from 5 to 5 × 10⁵ CFU/ml, was tested in triplicate, and two separate experiments were performed. RNA was also isolated from a 10-fold-dilution series of *V. cholerae* cells ranging from 5 to 5 × 10⁵ CFU/ml, and isolated nucleic acids were amplified by NASBA.

Analysis of spiked environmental water samples. Environmental samples were collected from the Oslo fjord (marine) and from two different freshwater lakes. These samples were spiked with *V. cholerae* cells (Cip 106855 O1 Inaba El Tor),

TABLE 2. Primers and molecular beacon probes for the *V. cholerae* real-time NASBA assay

Primer or MB ^a	Nucleotide sequence, 5' → 3' ^b	Predicted size of amplicon (bp)
Pvc55-1 <i>hlyA</i>	aattctaatacagactactataggg ^c AATCTCTTCCGTCCGATCAA	135
Pvc56-2 <i>hlyA</i>	TGATGCTGAAGGTCAAGCAG	
MBvc10- <i>hlyA</i>	ccgac ^d TCAGAAAGGCTTATGGGGTGgatcgg	102
Pvc62-1 <i>tcpA</i>	aattctaatacagactactatagggCGCTGAGACCACACCCATA	
Pvc60-2 <i>tcpA</i>	GAAGAAGTTTGTAAAAGAAGAACACG	151
MBvc11- <i>tcpA</i>	ccgacAGAAAACCGGTCAAGAGGGTgatcgg	
Pvc64-1 <i>ctxA</i>	aattctaatacagactactatagggAGAAGGTGGGTGCAGTGGCTATAACA	116
Pvc61-2 <i>ctxA</i>	TGATCATGCAAGAGGAACTCA	
MBvc-12 <i>ctxA</i>	ccgacTTGTTAGGCACGATGATGGAgatcgg	139
Pvc65-1 <i>groEL</i>	aattctaatacagactactatagggATGATGTTGCCACGCTAGA	
Pvc66-2 <i>groEL</i>	GGTTATCGCTGCGGTAGAAAG	139
MBvc13- <i>groEL</i>	ccgacCTGTCTGTACCTTGTGCCGAgatcgg	
Pvc69-1 <i>toxR</i>	aattctaatacagactactatagggCGGAACCGTTTTGACGTATT	139
Pvc72-2 <i>toxR</i>	CTCGCAATGATTTGCATGAC	
MBvc14- <i>toxR</i>	ccgacTTAACCCAAGCCATTCGACgatcgg	

^a MB, molecular beacon. 5' ends of molecular beacons were labeled with 6-carboxyfluorescein, and 3' ends were labeled with dabsyl.

^b Nucleotide sequences of primers and molecular beacons are in capital letters; lowercase letters represent surrounding sequences.

^c T7 RNA polymerase promoter sequence.

^d Stem sequence.

and RNA was isolated using the NucliSens basic kit. Briefly, 100 μ l of *V. cholerae* cells was added to 0.9 ml of the environmental samples to final concentrations of 5×10^3 or 5×10^5 CFU/ml. The cell suspensions were centrifuged at $2,000 \times g$ for 10 min, the pellets were suspended in 900 μ l of lysis buffer, and RNA was isolated as described above. RNA isolated from one of the freshwater lake samples spiked with 5×10^5 CFU/ml was amplified using all five primer sets and molecular beacons. RNA from the other freshwater lake sample and from the seawater sample (spiked with 5×10^5 and 5×10^3 CFU/ml) was amplified using primers and molecular beacons targeting the *groEL* and *tcpA* genes. Spiked samples of PBS were used as positive controls for RNA isolation and amplification. Environmental water samples not spiked with *V. cholerae* cells were used as negative controls, and neither of the targets was amplified.

Viability testing. Viable *V. cholerae* cells were investigated in comparison with nonviable cells. Samples of *V. cholerae* cells (5×10^5 CFU/ml) were subjected to the following treatments: autoclaving (121°C) for 20 min and further incubation at room temperature for 4 or 24 h and heating at 98°C for 20 min and further incubation at room temperature for 4 or 24 h. As controls, cells were kept on ice but otherwise treated similarly. The treatments were followed by the isolation of nucleic acids and subsequent analysis with all NASBA primers and beacons immediately after the treatment and after 4 h and 24 h. The viability was tested by plating samples onto TSB agar and incubating at 37°C for 24 h. Nucleic acids isolated from cells heated at 98°C were subjected to DNase and RNase treatment (as described above) to confirm that RNA and not DNA was amplified.

Real-time PCR. Real-time PCR was performed using a LightCycler (Roche, Germany) real-time PCR instrument. The NASBA primers and molecular beacons were used for PCR. The real-time PCR assays were carried out as described previously (22) using a Lithos qPCR kit (Eurogentec, Belgium). Briefly, the reaction mixture contained deoxynucleoside triphosphates, *Taq* DNA polymerase and reaction buffer, MgCl₂ (4.5 mM), primers (1 μ M [each]), and the probe (0.75 μ M) in a final volume of 20 μ l. The PCR program was as follows: initial denaturation at 95°C for 3 min and then 40 cycles at 95°C for 5 s, 58°C for 10 s, and 72°C for 15 s.

RESULTS

NASBA. Five different genetic markers (*groEL*, *toxR*, *ctxA*, *tcpA*, and *hlyA*) were used for the specific detection of *V. cholerae*. The selection of target genes was based on previous work (41, 48). The DNA sequences of the *groEL*, *ctxA*, *tcpA*, *toxR*, and *hlyA* markers showed no significant similarity, with one exception, to sequences of other genetic regions as determined using BLASTN. The *toxR* genetic target was 98% similar to the *Serratia marcescens toxR* gene.

The five different molecular beacons were evaluated using serial dilutions of nucleic acids isolated from *V. cholerae* cells. All target sequences were amplified by the corresponding primers, and the amplicons were detected by molecular beacons as a significant increase in fluorescence (Fig. 1). The results were confirmed by the amplification of an RNA sample, containing no DNA, that had been isolated by using the RNeasy kit (results not shown). The sensitivity of the single-target NASBA assays was evaluated using 10-fold dilutions of nucleic acids extracted from *V. cholerae* cells. Two separate assays were performed, and each assay was run in triplicate. Consistent detection (Table 3) was obtained at 50 CFU/ml for *groEL* and *tcpA*, 500 CFU/ml for *toxR* and *ctxA*, and 5×10^5 CFU/ml for *hlyA*. As shown in Fig. 1 and Table 3, lower concentrations were occasionally detected, but these levels of detection could not be reproduced in all NASBA experiments (Table 3).

The sensitivity of NASBA detection is also dependent on the yield of RNA extracted from cells. The *groEL* and *tcpA* targets were easily detected even when RNA was isolated from *V. cholerae* cultures containing only 50 CFU/ml. This result indicates that the NucliSens basic kit was able to effectively isolate RNA even from a low cell concentration. Furthermore, a prerequisite for NASBA detection is the expression of the selected target gene. The *groEL*, *toxR*, *ctxA*, *tcpA*, and *hlyA* targets were detected in all samples collected from early logarithmic to late stationary phase, indicating that these targets are all expressed during these growth phases.

NASBA is specifically designed to amplify RNA. However, it has been shown that DNA can also be amplified (19, 42). To confirm that the origin of the NASBA signal was RNA, the nucleic acids were subjected to RNase and DNase treatment as described in Materials and Methods. In the presence of RNase, no targets were amplified, whereas DNase treatment did not affect NASBA. This result was further confirmed with the electrophoresis of all amplicons (Bioanalyzer). These re-

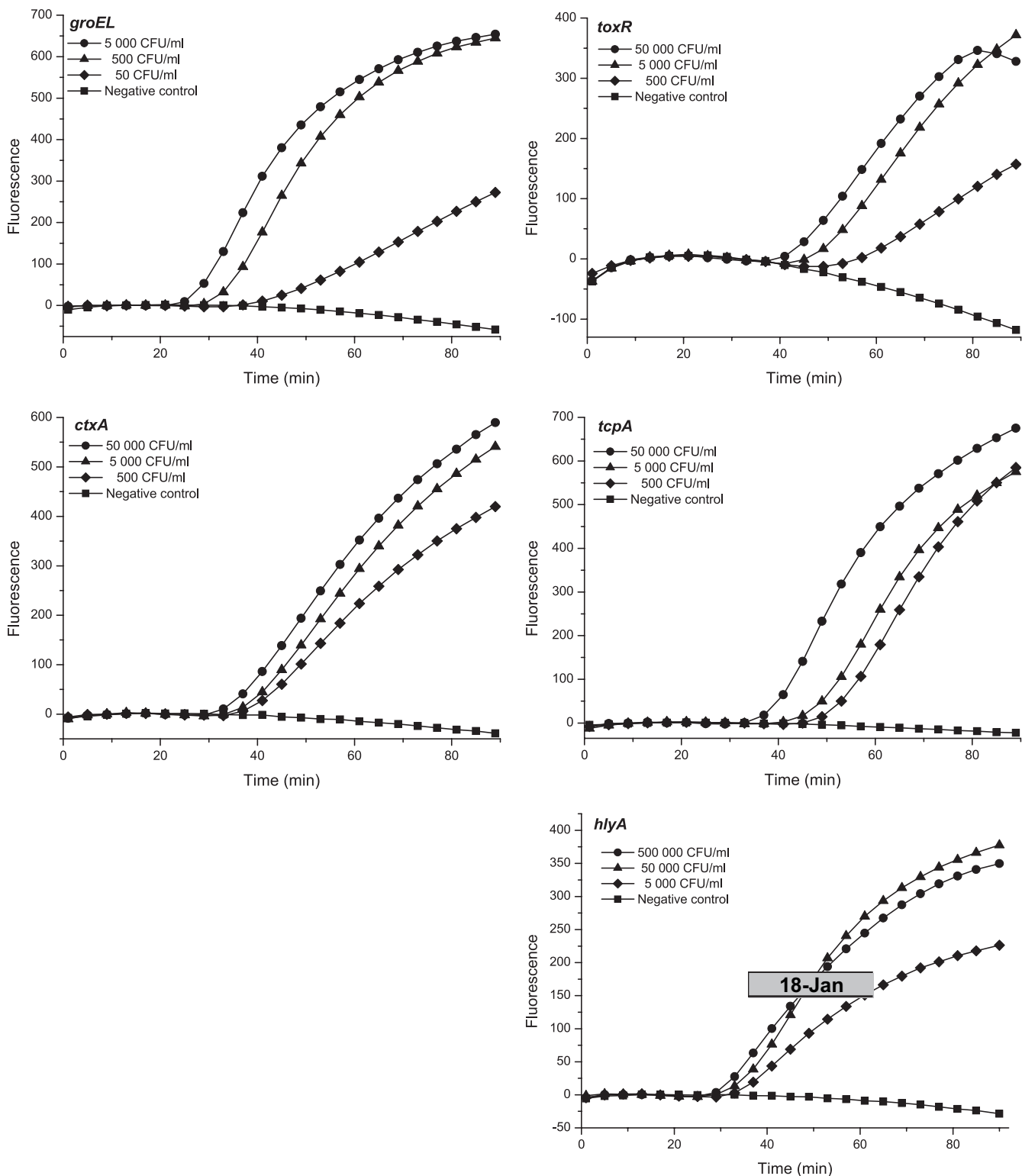


FIG. 1. Real-time NASBA of *V. cholerae* RNA. The five different molecular beacons were evaluated by using a 10-fold dilution series of RNA isolated from *V. cholerae* cells (Cip 106855 O1 Inaba El Tor). Data shown are representative of results from multiple experiments. Fluorescence is measured in relative units.

results are illustrated for the *hlyA* and *tcpA* targets in Fig. 2. Similar results were obtained for all other targets. However, for *toxR*, a slight decrease in the level of RNA amplicons was observed after DNase treatment. The decrease in RNA did not

affect the *t* value, but a small decrease in the fluorescence intensity was observed for *toxR* after DNase treatment. These results show that *V. cholerae* RNA was detected by NASBA in a DNA-contaminated background.

TABLE 3. Sensitivity and reproducibility of the single-target NASBA assays^a

Target	No. of specific NASBA products detected/no. of total reactions for concn (CFU/ml) of:						
	5	50	250	500	5×10^3	5×10^4	5×10^5
<i>groEL</i>	4/6	6/6	NT	6/6	3/3	3/3	NT
<i>tcpA</i>	2/6	6/6	NT	6/6	6/6	NT	NT
<i>ctxA</i>	No signal	3/6	3/3	6/6	6/6	6/6	NT
<i>toxR</i>	No signal	5/6	3/3	6/6	6/6	6/6	NT
<i>hlyA</i>	No signal	No signal	No signal	No signal	1/6	3/6	6/6

^a Two separate NASBA experiments, each run in triplicate, were performed. NT, not tested.

Specificity. The specificity of the NASBA assay was determined by amplifying specific RNA targets from several *Vibrio* spp. strains, those of *V. cholerae* (27 strains), *V. mimicus* (3 strains), *V. parahaemolyticus* (3 strains), *V. alginolyticus* (2 strains), *V. fluvialis* (1 strain), and *V. metschnikovii* (1 strain), and from strains of other bacterial species, *B. cereus* (2 strains), *S. enterica* (2 strains), and *E. coli* (2 strains). The results are summarized in Table 1. In general, the *ctxA* and *tcpA* RNA was detected in all O1 (clinical) isolates of *V. cholerae* but not in the environmental non-O1 isolates. Although the *ctxA* and *tcpA* targets were detected in the VC 4/57 O1 environmental isolate and in the VC 5/47 strain of the O2 serogroup (Table 1), these potential target sequences were amplified at a late stage of the NASBA assay with high *t* values and low fluorescence intensities. The *toxR*, *hlyA*, and *groEL* gene targets were detected in all *V. cholerae* strains examined, and no fluorescence signal was detected for the other *Vibrio* spp. However, to our surprise, in

two of three separate experiments the *groEL* target was detected in RNA from *S. enterica* (ATCC 13076). The *t* value was 49 min for *S. enterica* compared to 20 min for the *V. cholerae groEL* target. The maximum fluorescence levels (*F*) were 100 and 600 relative units, respectively. The *ctxA* target in *V. mimicus* (VM034) was also amplified. In this case, the sequence was amplified at *t* of 59 min and *F* of 100 relative units compared to *t* of 34 min and *F* of 600 relative units for *V. cholerae* (Cip 106855). Electrophoresis of the nonspecific RNA amplicons from *S. enterica* and *V. mimicus* showed that these products were larger than the specific products from *V. cholerae* (not shown). Furthermore, real-time PCR using the same molecular beacon probes was also used to verify the specificity of the *groEL* and *ctxA* targets. These DNA targets were not amplified in the *S. enterica* (ATCC 13076) and *V. mimicus* (VM034) strains. In conclusion, specific NASBA detection of *V. cholerae* was obtained using a combination of *groEL* and *toxR* as general primers and beacons and the *ctxA* and *tcpA* primers and beacons for detecting toxigenic strains.

Spiked environmental water samples. Highly sensitive detection of *V. cholerae* from environmental samples is dependent on efficient RNA extraction and the removal of potential NASBA inhibitors, as well as other factors. RNA was extracted

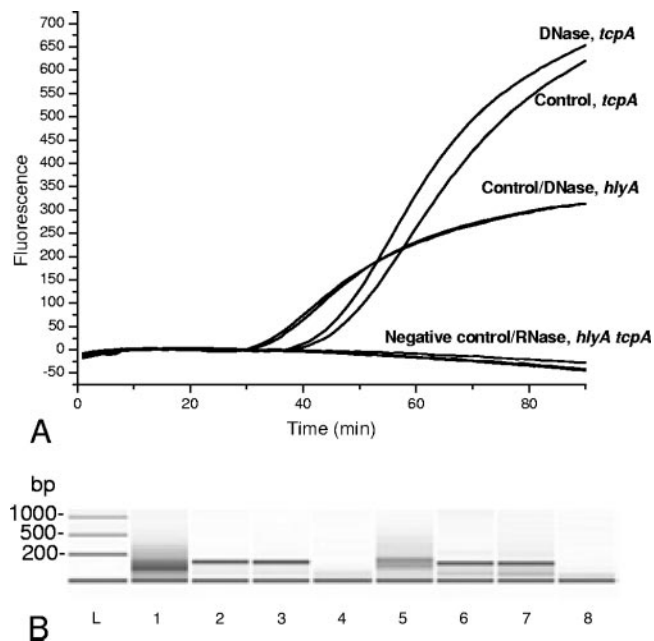


FIG. 2. Detection of RNA isolated from *V. cholerae* cells (Cip 106855 O1 Inaba El Tor) after RNase and DNase treatment of the nucleic acids isolated with the NucliSens basic kit. Fluorescence is measured in relative units. (A) Real-time NASBA of the *hlyA* and *tcpA* gene targets. (B) Electrophoresis (Bioanalyzer) of the *hlyA* and *tcpA* real-time NASBA products. Lanes: L, molecular weight standard; 1, *hlyA* negative control; 2, *hlyA* control; 3, *hlyA* DNase; 4, *hlyA* RNase; 5, *tcpA* negative control; 6, *tcpA* control; 7, *tcpA* DNase; 8, *tcpA* RNase.

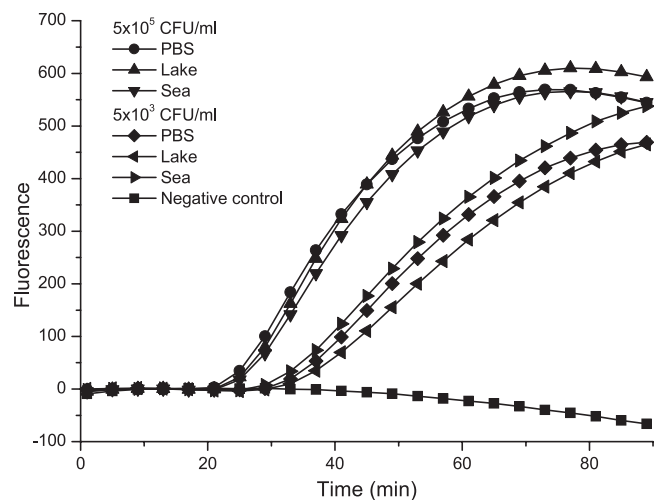


FIG. 3. Real-time NASBA detection (*groEL*) of *V. cholerae* RNA isolated from environmental water samples spiked with *V. cholerae* cells (Cip 106855 O1 Inaba El Tor). Seawater, freshwater, and a positive control (PBS) were spiked with 5×10^3 and 5×10^5 CFU/ml, and RNA was isolated using the NucliSens basic kit. Fluorescence is measured in relative units.

TABLE 4. Detection of *V. cholerae* cells in spiked water samples^a

Sample	Amplification of genetic target from <i>V. cholerae</i> cells at:									
	5 × 10 ³ CFU/ml					5 × 10 ⁵ CFU/ml				
	<i>groEL</i>	<i>tcpA</i>	<i>ctxA</i>	<i>hlyA</i>	<i>toxR</i>	<i>groEL</i>	<i>tcpA</i>	<i>ctxA</i>	<i>hlyA</i>	<i>toxR</i>
Seawater	+	+	NT	NT	NT	+	+	NT	NT	NT
Freshwater lake 1	+	+	NT	NT	NT	+	+	NT	NT	NT
Freshwater lake 2	NT	NT	NT	NT	NT	+	+	+	+	+
PBS	+	+	NT	NT	NT	+	+	+	+	+

^a +, specific amplified product detected; NT, not tested. Environmental water samples without spiking had no effect on amplification.

and amplified from three different water samples, one from seawater and two from different freshwater lakes, spiked with *V. cholerae* cells. The genetic targets were specifically detected in *V. cholerae*, and no inhibition was observed. The results are illustrated by the amplification of the *groEL* target from seawater, freshwater, and a positive control sample (PBS) (Fig. 3). Similar results were obtained with all environmental samples and for all genetic targets (Table 4).

Viability tests. Previous studies have shown that NASBA may be used to detect viable cells by using mRNA as the amplification target. In order to verify this possibility, *V. cholerae* cells were subjected to heat treatments at 98°C and 121°C, followed by further incubation for 4 or 24 h at room temperature. Cell death after these treatments was confirmed by CFU enumeration. The *ctxA*, *hlyA*, and *toxR* targets were not detected after treatment at 121°C and further incubation at 24 h. After 4 h, no *hlyA* and *ctxA* amplicons were detected, but *toxR* was still detectable. In contrast, the *groEL* and *tcpA* targets were detected in the heat-killed (121°C) cells. These results are illustrated for the *toxR* and *groEL* targets in Fig. 4A. Heating of the cells at 98°C was less effective, resulting in the detection of all targets, and treatment with RNase and DNase confirmed that RNA and not DNA was amplified. These results are illustrated for *groEL* in Fig. 4B. These results indicate that care must be taken when genetic targets are selected if NASBA is to be used for the detection of viable cells.

DISCUSSION

There is a need for rapid, accurate, and sensitive detection of target organisms responsible for food and water poisoning. To our knowledge, this paper describes for the first time a sensitive multitarget real-time NASBA application for the specific detection of *V. cholerae* cells in water.

In general, NASBA is a sensitive method, and more than 10⁹ copies of RNA can be amplified in the assay in 90 min (14). In the present study, as little as 50 CFU/ml was detected using the *groEL* or *tcpA* primers and molecular beacons. The *groEL* marker is a general marker for detecting all *V. cholerae* strains. The *tcpA* marker is specific for the detection of toxigenic strains. The sensitivity of the presented method was in agreement with previous NASBA results for other bacterial species. Min and Baeumner (38) were able to detect 40 *E. coli* cells/ml of water, and as few as 10 spores of *B. anthracis* could be detected (4). In the latter case, the spores were inoculated into a culture medium for 30 min in order to germinate the spores and express target RNA. In a recent study, the levels of toxigenic *V. cholerae* cells in water in Mathbaria, Bangladesh, were

monitored. Seasonal variation was observed, and the concentration of *V. cholerae* cells varied from <10 to 3.4 × 10⁷ cells/liter (1). In the NASBA assay in the present study, 5 × 10⁴ CFU/liter was detected. Preliminary studies in our laboratory indicate that 5 × 10⁴ CFU/ml corresponds to 1 × 10⁶ cells/liter. The sensitivity might be further increased by the filtration of large volumes of water, which has not been done in this study. The infectious dose of *V. cholerae* from water is assumed to be 10³ to 10⁶ bacteria (44). Thus, such doses may be detected using the present NASBA method either directly or after filtration of the water.

A report of the use of molecular beacons for the multiplex real-time PCR detection of *V. cholerae* cells has recently been published, stating a detection limit of 5 × 10³ CFU/ml (25). The sensitivity of our assay proved to be 100-fold higher (50 CFU/ml). Previous reports revealed that real-time PCR using TaqMan probes or SYBR green can detect 10 to 10⁶ CFU of *V. cholerae*/ml (24, 36). It is well known that the nucleotide sequences and the binding efficiencies of primers and probes for the target strand are of importance in obtaining high sensitivity and specificity for real-time PCR and NASBA assays. The kinetics of the NASBA reaction are mainly determined by the efficiency of primer binding and the extent of nonspecific product synthesis due to mispriming events (20). In this study, the sensitivity of the amplification of *hlyA* was lower than that of the other targets. This might be due to primer or probe construction or a lower level of expression of the *hlyA* marker compared to the other targets.

Theoretically, the possibility that the *groEL* primers and molecular beacon can amplify and detect nontarget strains is low, confirmed by BLAST analysis. Surprisingly, the amplification of RNA isolated from the *S. enterica* ATCC 13076 strain occurred in two out of three separate experiments. Sequence alignments of the *groEL* target gene of *V. cholerae* (>gi15640032:2832629-2834254) and the *groEL* gene of *S. enterica* (gi7527386) showed approximately 45% similarity at the DNA level, but the sequence of the *groEL* molecular beacon probe appeared to be unique (BLASTN analysis). Therefore, the detection of *groEL* in *S. enterica* was not expected. Furthermore, the amplification of *S. enterica* was not reproducible. Also, in a few experiments a background signal in the negative control was achieved with the *groEL* molecular beacon probe. These two observations might be due to the binding of the molecular beacon to primers (19). However, *S. enterica* ATCC 13076 was not detected at all using real-time PCR and molecular beacon probe detection.

Pathogenicity is dependent on the expression of virulence

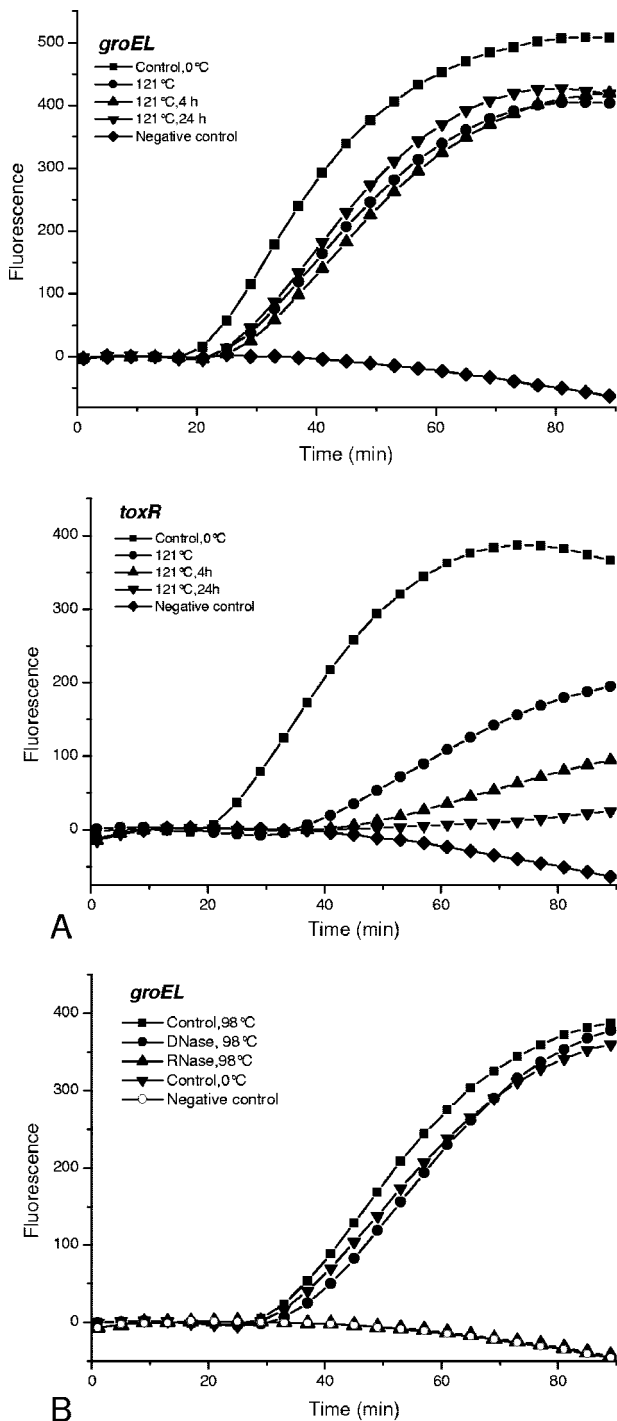


FIG. 4. Real-time NASBA of RNA isolated from heat-treated nonviable *V. cholerae* cells (Cip 106855 O1 Inaba El Tor). (A) NASBA of the *toxR* and *groEL* genes in cells treated at 121°C and further incubated for 4 and 24 h at room temperature. (B) NASBA of the *groEL* gene target in cells treated at 98°C and further incubated for 24 h at room temperature. To confirm that RNA and not DNA was amplified, the nucleic acids were treated with RNase and DNase as described in Materials and Methods. No amplification was detected after RNase treatment. Fluorescence is measured in relative units.

genes in a microorganism. The *ctxA* and *tcpA* genes are known to play a crucial role in maintaining the virulence of *V. cholerae*. These genes are usually associated with clinical strains of the O1 and O139 serogroups. However, outbreaks of cholera caused by non-O1 and non-O139 have also been reported (18, 45), and some of these strains produce CT or CT-like toxins (10). Interestingly, the *ctxA* and *tcpA* genes were detected by NASBA in a strain belonging to the O2 serogroup (VC 5/47). However, the *t* value was high and the fluorescence low. This result was most likely due to mispriming or a low yield of RNA. PCR studies have also revealed the presence of the *ctxA* and *tcpA* genes in various non-O1 and non-O139 serogroups of environmental *V. cholerae* strains (10). An environmental O1 isolate (VC 4/57) was also detected in this study. As said, the *t* value was high and the increase in the fluorescence signal was low and most likely due to mispriming or the level of expression. Furthermore, isolates of *V. mimicus* that express the two main virulence factors (CT and TCP) of *V. cholerae* have been identified (9), and this finding might explain why the *V. mimicus* (VM034) strain was detected using the *ctxA* primers and a molecular beacon. Thus, CT or CT-like proteins are associated with non-O1 and non-O139 serogroups of *V. cholerae* and strains of *V. mimicus* as well. Amplification of the *ctxA* gene target in environmental samples by NASBA indicates the detection of a potential pathogen. The potential nonspecific detection in environmental samples of, e.g., *ctxA* in *V. mimicus* or *toxR* in *S. marcescens*, found in water, soil, insects, plants, and vegetables (23), is not a problem since the specificity of the presented NASBA assay is based on the use of several genetic targets. Using a combination of *groEL* and *tcpA*, the presented multitarget NASBA assay is specific for the detection of *V. cholerae* cells. For the monitoring of strains expressing virulence factors, the *ctxA* and *tcpA* targets can be used. A direct comparison of the specificity of NASBA and real-time PCR using molecular beacons is difficult since different targets and different strains of bacteria were used. A real-time PCR assay using molecular beacons used four different genetic targets in a multiplex assay (*rtxA*, *epsM*, *ompW*, and *tcpA*) in order to detect *V. cholerae* cells; in this assay, *V. mimicus* was detected nonspecifically through *ompW* (25). The *tcpA* marker used in PCR was designed to detect the toxigenic El Tor strains. However, in one of these strains no amplification was detected (25). To our knowledge, these two studies are the only studies describing the use of molecular beacon probes for the detection of *V. cholerae*.

Using NASBA for analyzing environmental samples requires efficient methods for sample preparation since the samples generally contain substances that inhibit NASBA (16). *V. cholerae* was detected by NASBA in spiked environmental seawater and freshwater samples. Potential inhibitors were effectively removed, and the whole analysis including RNA extraction and NASBA was completed within 3 h. If the samples contain large amounts of inhibitory substances, the extraction methods might require modifications. In contrast, conventional culture methods and subsequent bacterial identification are time-consuming and require several days to complete (21). *Vibrio* spp. in the environment are known to be in a VBNC state, a state that is described as a survival mechanism of bacteria facing environmental stress conditions (28). Prelimi-

nary results show that *V. cholerae* cells in the VBNC state could be detected by NASBA.

In principle, the presence of RNA in bacterial cells may serve as an indicator for viable cells (32), and mRNA species are supposed to have an average half-life of only minutes in metabolizing cells (2). However, in several studies it has been shown that mRNA species may persist in a detectable form for many hours after cell death (6, 46, 47). The persistence of mRNA species may vary depending on the method of cell death (46, 47) and the physiological state of the cell population before killing. In another study, only viable cells of *E. coli* were detected using NASBA (38). Other studies have also demonstrated that the selection of the genetic target is important for providing good correlations with cell viability (40, 55). This was also the case in this study. A correlation between cell viability and NASBA was demonstrated for the *ctxA*, *hlyA*, and *toxR* targets, whereas such correlation was not demonstrated for the *groEL* and *tcpA* targets. Our results therefore indicate that care must be taken in using NASBA for viability studies. This finding also highlights the need for increased knowledge of the persistence and decay characteristics of the potential mRNA targets. False-positive results due to the amplification of RNA from dead cells can be eliminated by preenrichment in a selective medium, and the analysis time might be shortened from days (culture methods) to hours (52). This strategy also solves the problems with NASBA inhibitors contained in environmental samples.

In conclusion, the described multitarget NASBA assay for the detection of *V. cholerae* is a novel, sensitive, specific, and rapid method in which nucleic acid extraction and NASBA can be completed in less than 3 h. The NASBA assay is run at a single temperature, and a thermocycler is not required. The advantages, including the use of a crude RNA extract, have a potential for being further improved for field applications. Environmental water samples spiked with *V. cholerae* cells were used to demonstrate that the combination of a rapid method for the extraction of nucleic acids (DNA and RNA) and NASBA could be a promising strategy for the rapid and sensitive detection of *V. cholerae*. The monitoring of particular strains expressing virulence factors known to cause human disease is of great value for hazard evaluation of water. Preliminary studies also show that the NASBA method has potential as a detection tool for VBNC cells isolated from water samples.

The *groEL* and *toxR* targets are general markers and detected all *V. cholerae* strains. The sensitivity of the *groEL* assay was 10-fold higher than that of the *toxR* assay. In order to detect toxigenic strains, we suggest that the *tcpA* and *ctxA* markers be used. However, the sensitivity of the *tcpA* assay was 10-fold higher than that of the *ctxA* assay. A combination of the *groEL*, *toxR*, *tcpA*, and *ctxA* markers provides maximum specificity for the detection of all *V. cholerae* strains.

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