ELSEVIER

FEMS Microbiology Letters 210 (2002) 143–147

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Rapid detection of human rotavirus using colorimetric nucleic acid sequence-based amplification (NASBA)–enzyme-linked immunosorbent assay in sewage treatment effluent

Julie Jean [a], Burton Blais [c], André Darveau [b], Ismaïl Fliss [a,*]

[a] *Centre de Recherche STELA, Département de Sciences des Aliments et de Nutrition, Université Laval, Laval, QC, Canada G1K 7P4*
[b] *Département de Biochimie et Microbiologie, Université Laval, Laval, QC, Canada G1K 7P4*
[c] *Canadian Food Inspection Agency, Laboratory Services Division, Bldg 22, C.E.F., Ottawa, ON, Canada K1A 0C6*

Received 7 January 2002; received in revised form 28 February 2002; accepted 5 March 2002

First published online 11 April 2002

Abstract

A colorimetric nucleic acid sequence-based amplification–enzyme-linked immunosorbent assay (NASBA-ELISA) was developed for rapid detection and identification of human rotavirus. Oligonucleotide primers targeting gene 9 encoding a serotype-specific antigen VP7 were selected and used for the amplification of viral RNA by the isothermal NASBA process, resulting in the accumulation of biotinylated RNA amplicons. Amplicons were hybridized with a specific amino-linked oligonucleotide probe covalently immobilized on microtiter plates. The DNA–RNA hybrids were colorimetrically detected by the addition of streptavidin–peroxidase conjugate and tetramethylbenzidine substrate. Using the NASBA-ELISA system, as little as 0.2 PFU (4×10^1 PFU ml^{-1}) and 15 PFU (3×10^3 PFU ml^{-1}) of rotavirus were detected within 6 h in spiked MQ water and sewage treatment effluent respectively. No interference was encountered in the amplification and detection of rotavirus in the presence of non-target RNA or DNA. Moreover, the presence of non-target bacteria and virus does not generate any non-specific signal, confirming the specificity of the developed NASBA-ELISA system and its effectiveness in specifically detecting rotavirus. The NASBA-ELISA system offers several advantages in terms of sensitivity, rapidity and simplicity. This technique should be readily adaptable for detection of other RNA viruses in both foods and clinical samples.

Keywords: Rotavirus; Nucleic acid sequence-based amplification; Microplate hybridization

1. Introduction

In recent years, viral gastroenteritis has become extremely common in humans worldwide. Since 1982, 40% of the confirmed food-borne outbreaks reported throughout the world have been of viral etiology. Over 110 known enteric viruses are excreted in human feces. However, only a relatively small number have been epidemiologically linked to viral disease, namely Norwalk, rotavirus and hepatitis A virus. In the USA, rotaviruses cause about 3.5 million infections every year, resulting in 70 000 hospitalizations, 75–125 deaths of children and annual losses of nearly $250 millions [1]. Epidemiological evidence suggests that rotaviruses may be transmitted to humans by various foods, such as shellfish and crops. A major source of contamination is treated or raw waste water into which rotaviruses are excreted in large numbers from infected persons and where it can persist for a long period [2]. As for most enteric viruses, rotaviruses implicated in food-borne illness are often found in very low concentrations in contaminated water samples since they are not able to multiply in vitro. The ability to detect traces of rotavirus contamination in water and related samples is essential in developing tools for the investigation and possible prevention of viral disease outbreaks. Traditional methods for the detection of rotaviruses in water include tissue culture techniques in which viruses are concentrated from water samples and allowed to multiply in sensitive cell cultures in which characteristic cytopathic effects can be visualized. Although sensitive, these techniques remain cumbersome,

* Corresponding author. Tel.: +1 (418) 656-2131 ext. 6825; Fax: +1 (418) 656-3353.
E-mail address: ismail.fliss@aln.ulaval.ca (I. Fliss).

0378-1097/02/$22.00
PII: S0378-1097(02)00614-6

costly and time-consuming (up to 2 weeks), with the cytopathic effect often requiring more than 1 week to observe. Moreover, several rotavirus strains are unable to induce any cytopathic effect. Faster and more sensitive molecular biology techniques have been proposed for the specific detection of some enteric viruses including rotaviruses in food and water samples. Nucleic acid amplification techniques such as the reverse transcription-polymerase chain reaction (RT-PCR) have been shown to offer the possibility of direct detection of rotaviruses in food and environmental samples [3,4]. An alternative to RT-PCR potentially applicable to the detection of rotaviruses is the nucleic acid sequence-based amplification (NASBA) technique. NASBA is a homogeneous, isothermal RNA amplification process involving the action of three enzymes, reverse transcriptase, T7 RNA polymerase and RNase H, as well as two target sequence-specific oligonucleotide primers (one of which bears a bacteriophage T7 promoter sequence appended to its 5′ end), acting in concert to amplify target sequences more than 10^8-fold [5]. Its advantage over the traditional RT-PCR technique is its suitability for the direct amplification of RNA targets, obviating the need for thermal cycling equipment. NASBA was successfully used for the detection of *Listeria monocytogenes* [6], *Campylobacter* [7] and human immunodeficiency virus [8]. In recent work, we have demonstrated the effectiveness of NASBA for the detection of hepatitis A virus in wastewater, fruits and vegetables [9]. In this paper, a NASBA technique combined with a simple microplate hybridization system was developed for the specific and sensitive detection of human rotavirus.

2. Materials and methods

2.1. Viral and bacterial strains

The human rotavirus strain Wa, kindly provided by Dr. P. Payment (Institut Armand-Frappier, Montreal, QC, Canada), was propagated in MA-104 cells using previously published method [1] and stored in 1-ml fractions at −80°C until use. Hepatitis A virus HM-175 (HAV) and *Escherichia coli* (ATCC 11775) were used to evaluate the specificity of the NASBA reaction. HAV was propagated in FRhK-4 cells as previously described [10]. *E. coli* was grown in tryptic soy broth and bacterial counts were determined by plating on tryptic soy agar.

2.2. Nucleic acids

Purified yeast tRNA (Roche Diagnostic, Laval, QC, Canada) and *E. coli* total RNA were used to evaluate the impact of non-homologous nucleic acids on the specificity of the NASBA reaction. Total RNA was purified from *E. coli* using Trizol reagent (Gibco-BRL, Burlington, ON, Canada).

2.3. Titration of rotavirus by immunofluorescence microscopy

The viral titer was determined in 96-well microtiter plates containing a 24-h MA-104 cell culture (150 000 cells ml^{-1}) in maintenance medium containing 5% fetal calf serum. The viral suspension was treated with 20 U trypsin ml^{-1} for 30 min at room temperature and diluted in maintenance medium. 25 μl of each dilution and 200 μl of maintenance medium were added to microtiter plate wells. The microplates were incubated at 37°C for 24 h in 5% CO_2. The medium was discarded and 100 μl of 80% (v/v) acetone was added, followed by 30 min of incubation at 4°C. In emptied wells, 50 μl per well of anti-rotavirus antibody (1:300, Accurate Chemical, Westbury, NY, USA) was added and incubated for 30 min at 37°C. The microplates were washed five times with 200 μl of phosphate-buffered saline (PBS) and 50 μl of fluorescein isothiocyanate-labeled anti-sheep IgG (H+L) antibody (1:3000, Sigma, Oakville, ON, Canada) was added, followed by incubation for 30 min at 37°C. The microplate was then washed four times with 200 μl of PBS, 50 μl of glycerol/PBS (3:1) was added to each well and the microplate was sealed until observation by epifluorescence microscopy. Each viral titer was done in quadruplicate and calculated using the Reed–Muench method [11].

2.4. Primers and oligonucleotide probes

A primer pair was selected from published rotavirus gene 9 nucleic acid sequences encoding the serotype-specific antigen VP7 (GenBank accession number K02033), synthesized and gel-purified before use (Table 1). The reverse primer (Rota-2) bears the bacteriophage T7 RNA polymerase promoter binding region and preferred transcriptional initiation sequence at its 5′ end. Digoxigenin (DIG)-labeled and amino-linked oligonucleotide probes corresponding to an internal region defined by the different primer pairs were synthesized and used for the detection of amplified target RNA.

2.5. NASBA procedure

The NASBA procedure used in this study has been previously described by Jean et al. [9] except that in this case, biotinylated 16-uracil triphosphate (biotin-16-UTP, Roche Diagnostics) was incorporated at a concentration of 0.4 mM into the NASBA reaction mixture. The amplification products were analyzed by denaturing agarose gel electrophoresis, by Northern blot [9] or by the microplate hybridization system described below.

2.6. Microtiter plate hybridization and detection of NASBA-amplified RNA

Amino-linked rota-probe oligoprobe (0.2 μM, 100 μl)

Table 1
Nucleotide sequences of oligonucleotide primers and probes used in this study

Primers and probe	Sequence	Location
Rota-1	5′-GTAAGAAATTAGGTCCAAGAG-3′	794–814
Rota-2+T7[a]	5′-AATTCTAATACGACTCACTATAGGGAGAGGTCACATCGAACAATTC-3′	1045–1062
Rota-probe-DIG	5′-CAAACTGAGAGAATGATGAGAGTGAATTGG-3′	886–915

[a]The T7 promoter sequence is underlined.

diluted in coating buffer (500 mM NaH_2PO_4 plus 1 mM EDTA pH 8.5) was incubated in a 96-well DNA-bind microtiter plate (Corning, Acton, MA, USA) at 37°C for 30 min. The microtiter plate was washed three times with 250 μl of 1×TBS (50 mM Tris–HCl plus 150 mM NaCl, pH 7.6) and blocked for 30 min at 37°C with 200 μl of blocking solution [1× TBS containing 0.05% Tween 20 (TBS-T) plus 1% blocking reagent (Roche Diagnostics)]. 10 μl of NASBA product was heated 2 min at 95°C to inactivate RNase H enzyme and added to 490 μl hybridization solution [5× SSC (75 mM sodium citrate plus 750 mM NaCl) and 0.1% SDS]. 100 μl of this NASBA product were added to the amino-linked oligoprobe-coated DNA-bind microtiter plates and hybridized for 1 h at 55°C. Plates were washed three times with TBS-T and blocked again for 30 min at room temperature. 100 μl of streptavidin–peroxidase conjugate diluted 1:4000 in blocking solution was added to each well and microtiter plates were incubated for 30 min at room temperature. After several washes, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) substrate were added to each well and the color was allowed to develop before reading absorbance at 650 nm in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Validation of NASBA-ELISA by analysis of human rotavirus spiked sewage treatment effluent

Ten-fold serial dilutions of human rotavirus starting at 3×10^4 PFU ml^{-1} in sewage treatment effluent (treatment plant, Saint-Nicolas, QC, Canada) were prepared. 5 μl of each sample was heated to 100°C for 5 min to lyse the virus particles and subjected to the NASBA and microtiter plate hybridization procedure described above. Unspiked sewage treatment effluent samples were also analyzed as negative controls. The detection limit achieved in sewage treatment effluent was compared to that obtained in MQ water (ultrafiltration grade deionized water, Millipore). For the evaluation of the specificity, non-target microorganisms including *E. coli* (2×10^4 CFU ml^{-1}) and hepatitis A virus (10^5 PFU ml^{-1}) as well as non-target nucleic acids, notably yeast tRNA and total *E. coli* RNA (50 μg ml^{-1}) were added, alone or in the presence of rotavirus and analyzed by the NASBA-ELISA.

3. Results and discussion

3.1. Specificity of the rotavirus NASBA products

As shown in Fig. 1, the analysis of the rotavirus NASBA product by denaturing agarose gel electrophoresis shows a unique band corresponding to the expected size of 286 nucleotides (Fig. 1A). No band was observed with the negative control. The specificity of the NASBA was confirmed by Northern blotting using a DIG-labeled oligoprobe. The amplification product generated a strong hybridization signal of a molecular size of 286 nucleotides (Fig. 1B). These results confirm the effectiveness of the NASBA system developed and the specificity of the selected primer pair for the amplification of the target region in gene 9. Several primer sets have been reported in the literature for the amplification of rotavirus by RT-PCR. However, considering the results obtained in our previous study [9] on the amplification of hepatitis A virus, which showed that primers designed for RT-PCR were not necessarily the best candidates for amplification by NASBA, a new primer set was designed from a highly conserved region near the end of gene 9 for the specific amplification of human rotavirus.

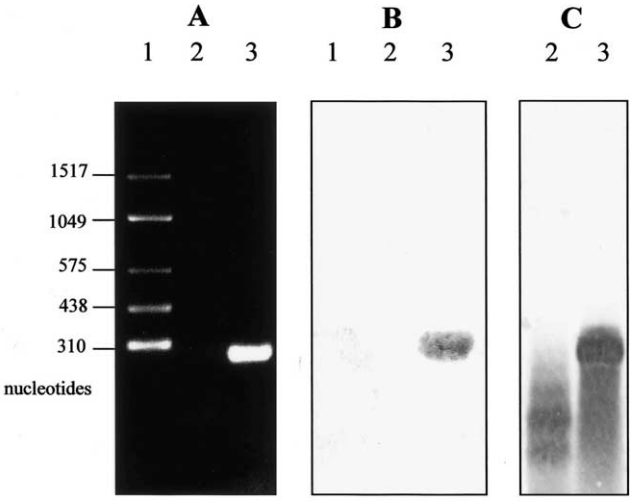

Fig. 1. Analysis of primer specificity on target human rotavirus in NASBA. A: Analysis of the NASBA-amplified rotavirus RNA by denaturing agarose gel electrophoresis. B: Northern blot analysis of NASBA product using the DIG-labeled rota-probe oligoprobe. C: Detection of the biotinylated NASBA product using streptavidin–peroxidase conjugate and TMB substrate. Lane 1: RNA molecular marker; lane 2: NASBA product negative control; lane 3: NASBA product in the presence of human rotavirus.

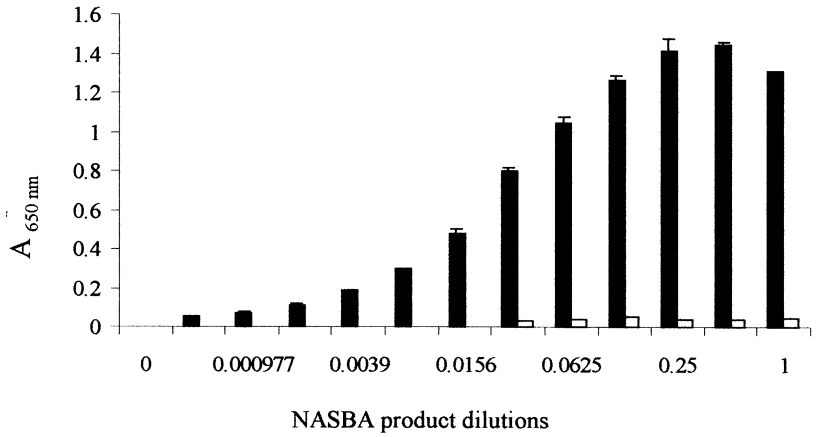

Fig. 2. Detection of biotinylated NASBA products by the microplate hybridization system. NASBA amplification was performed in the presence of 10^6 PFU ml^{-1} and then the biotinylated amplified viral RNA was two-fold serially diluted before detection by the microplate hybridization system (■) described above. Negative control (□) consists of NASBA reaction performed in the absence of human rotavirus. Results are means of triplicate analyses.

3.2. Detection of amplified RNA by NASBA-ELISA

To verify if the incorporation of biotin-16-UTP could affect the NASBA specificity, amplification reactions were performed in the presence of biotin-16-UTP. The amplified product was separated by denaturing gel electrophoresis, transferred to a nylon membrane and detected by streptavidin–peroxidase conjugate. As shown in Fig. 1C, the incorporation of biotin-16-UTP did not interfere with the amplification reaction, since a characteristic amplification band was obtained. For the NASBA-ELISA, the biotinylated NASBA product was hybridized with the amino-linked probe immobilized on microtiter plates. The hybrid formed was detected using streptavidin–peroxidase conjugate. The efficiency of the NASBA-ELISA was confirmed by analyzing two-fold serial dilutions of biotinylated amplified rotavirus RNA. Specific signals proportional to the amount of added RNA were obtained (Fig. 2).

3.3. Specificity of the NASBA-ELISA

The specificity of the NASBA-ELISA was confirmed by the analysis of non-target microorganisms and nucleic acids. The presence of non-target microorganisms such as *E. coli* and hepatitis A virus, as well as non-target nucleic acids such as yeast tRNA and *E. coli* RNA, did not generate any non-specific signal (Fig. 3). Moreover, the addition of such non-target microorganisms and nucleic acids to rotavirus samples did not interfere with the specificity or the sensitivity of the detection system, since the hybridization signals obtained were comparable in terms of intensity to that of rotavirus alone.

3.4. Sensitivity of the NASBA-ELISA

As shown in Fig. 4, the detection signal generated by the NASBA-ELISA was proportional to the viral concen-

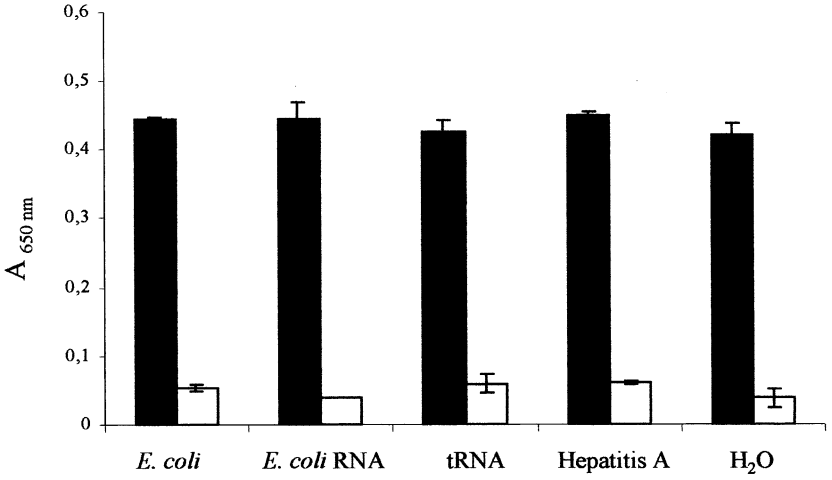

Fig. 3. Specificity of the rotavirus NASBA-ELISA. NASBA reactions were performed with non-homologous RNA (tRNA and total *E. coli* RNA) at a concentration of 50 μg ml^{-1} and with non-target microorganisms (*E. coli* and hepatitis A virus) at a concentration of 10^5 CFU or PFU ml^{-1} in the absence (□) or presence (■) of rotavirus. Results are means of triplicate analyses.

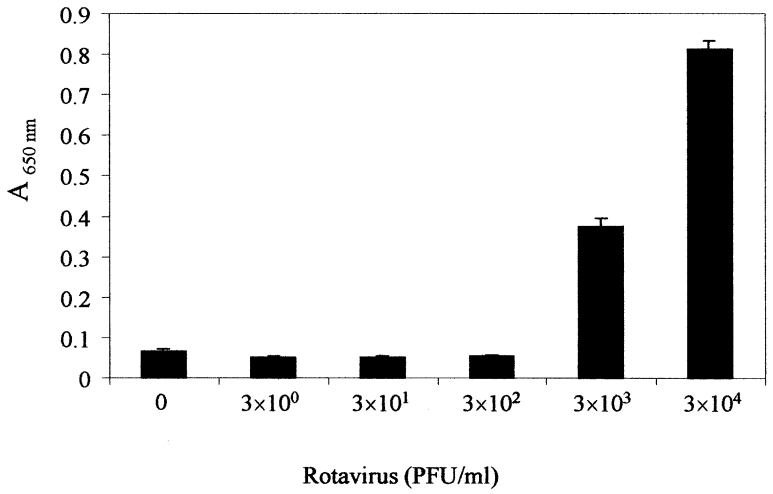

Fig. 4. Sensitivity of the rotavirus NASBA-ELISA in sewage treatment effluent. Ten-fold serial dilutions of rotavirus were prepared in sewage treatment effluent and 5 μl of each dilution was subjected to NASBA amplification and detection by the microplate hybridization system. Each dilution was analyzed in triplicate and the detection limit is defined as the highest dilution giving an absorbance significantly higher than $\bar{A}+3\sigma$; where $\bar{A}$ is the mean absorbance generated by the negative control and σ is the standard deviation.

tration with a detection limit of 4×10^1 PFU ml^{-1} in MQ water (data not shown) and 3×10^3 PFU ml^{-1} in sewage treatment effluent. No signal was obtained with negative controls. This minimum viral concentration corresponds to as little as 0.2 PFU and 15 PFU in MQ water and sewage treatment effluent respectively if we consider that only 5 μl from each viral dilution was added to the reaction mixture and amplified by NASBA. This detection limit is 10 times more sensitive than that obtained by NASBA for HAV [9]. In conclusion, this work has strongly confirmed the effectiveness of the NASBA approach for the direct and specific amplification of viral RNA. The combination of NASBA with the simple and quantitative microplate hybridization system offers several advantages over other molecular techniques, including high sensitivity and specificity. It is simple to perform, quantitative and allows the analysis of several samples simultaneously.

Acknowledgements

This work was supported by a grant from the Conseil de recherches en pêche et en agro-alimentaire du Québec (CORPAQ).

References

[1] Sattar, S.A., Jacobsen, H., Rahman, H., Cusack, T.M. and Rubino, J.R. (1994) Interruption of rotavirus spread through chemical disinfection. Infect. Control Hosp. Epidemiol. 15, 51–756.

[2] Smith, E.M. and Gerba, C.P. (1982) Development of a method for detection of human rotavirus in water and sewage. Appl. Environ. Microbiol. 43, 40–1450.

[3] Barardi, C., Yip, H., Emsile, K.R., Vesey, G., Shanker, S.R. and Williams, K.L. (1999) Flow cytometry and RT-PCR for rotavirus detection in artificially seeded oyster meat. Int. J. Food Microbiol. 49, 9–18.

[4] Tsai, Y.-L., Tran, B., Sangermano, L.R. and Palmer, C.J. (1994) Detection of poliovirus, hepatitis A virus, and rotavirus from sewage and ocean water by triplex reverse transcriptase-PCR. Appl. Environ. Microbiol. 60, 2400–2407.

[5] Compton, J. (1991) Nucleic acid sequence-based amplification. Nature 350, 91–92.

[6] Blais, B.W., Turner, G., Sooknanan, R. and Malek, L.T. (1997) A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes hlyA* sequences. Appl. Environ. Microbiol. 63, 310–313.

[7] Uyttendaele, M., Schukkink, R., Van Gemen, B. and Debevere, J. (1994) Identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBA. J. Appl. Bacteriol. 77, 694–701.

[8] Kievits, T., Van Gemen, B., Van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R. and Lens, P. (1991) NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J. Virol. Methods 35, 273–286.

[9] Jean, J., Blais, B., Darveau, A. and Fliss, I. (2001) Detection of hepatitis A virus by the nucleic acid sequence-based amplification technique and comparison with reverse transcription-PCR. Appl. Environ. Microbiol. 67, 5593–5600.

[10] Ansari, S.A., Sattar, S.A., Springthorpe, S., Wells, G.A. and Tostowaryk, W. (1988) Rotavirus survival on human hands and transfer of infectious virus to animate and nonporous inanimate surfaces. J. Clin. Microbiol. 26, 513–1518.

[11] Payment, P. and Trudel, M. (1989) Manuel de techniques virologiques. Presses de l'Université du Québec/AUPELF, Sillery, QC.