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Quantitative RT-PCR ELISA to determine the amount and ratio of positive- and negative strand viral RNA synthesis and the effect of guanidine in poliovirus infected cells

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Abstract

Quantitative reverse transcription-polymerase chain reaction enzyme linked immunosorbent assay (RT-PCR ELISA) is the method of choice to study positive- and negative strand viral RNA synthesis during poliovirus replication. In comparison with other methods used for this purpose, it fulfils all necessary requirements to accurately determine RNA of different polarity. It combines high specificity, high sensitivity, safety, speed, and the ability to perform quantitative analysis. The enterovirus specific RT-PCR ELISA method described in this work, was used to determine quantitatively the amount of de novo poliovirus positive- and negative strand RNA synthesis at different time-points in the viral replication cycle, both in presence and absence of the viral RNA synthesis inhibitor guanidine hydrochloride.
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1. Introduction

In a poliovirus infected cell, the viral replication starts with translation of the infecting virion RNA [1]. The product of the translation process is a single polyprotein, that is post-synthetically cleaved in a number of virus-encoded proteins-including the viral protein $3D^{pol}$—of which many are necessary to replicate the viral genome [2,3]. The first step of the viral RNA replication involves the synthesis of negative strand RNA [1]. For this, a RNA dependent RNA polymerase (i.e. the viral protein $3D^{pol}$) is required [3], transcribing the parental positive stranded genomic RNA into a complementary negative RNA strand [2,4]. Finally, in the second step of the viral transcription process, the newly synthesised negative strand RNA is used as a template for the asymmetric synthesis of de novo positive stranded RNA copies [1].

Several methods have already been proposed for the (quantitative) detection of positive and negative strand RNA synthesis during the replication cycle of RNA viruses. Globally, a

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distinction can be made between hybridisation assays without prior amplification (i.e. RNase protection assays [4,5], Northern blotting [6], and dot or slot blotting [7]), and amplification assays (mostly reverse transcription-polymerase chain reaction (RT-PCR) based techniques) whether or not in combination with hybridisation detection [7–9]. Thus, the application of RT-PCR for the determination of positive- and negative viral RNA is not new. However, detection of the RT-PCR products has generally been performed by either ethidium bromide agarose gel electrophoresis or Southern blotting procedures in combination with densitometric scanning. Both these methods have the disadvantage of being semi-quantitative at best, and are less sensitive than newer technologies [10]. Moreover, ethidium bromide and radiolabels (commonly used in blotting procedures) are mutagenic substances. In addition, all higher mentioned blotting techniques are labour intensive, time consuming, and require complicated handling because of the health hazards and contaminated waste, when working with radioisotopes. Concerning sensitivity, Liu et al. [7] have compared several methods for the detection of the different RNA types formed during dengue virus type 2 replication (i.e. positive- and negative strand RNA, replicative intermediate (RI), and replicative form (RF)). They showed that RT-PCR, followed by Southern blot analysis or slot blot hybridisation, were the most sensitive methods for the detection of viral RNA and the determination of the single stranded forms [7]. Taken all the above mentioned facts into account, the method of choice to determine the amount of positive- and negative stranded viral RNA is RT-PCR enzyme linked immunosorbent assay (ELISA). This technique combines a double specificity (because of the target specific primer pair and probe), a high sensitivity (due to the exponential nature of PCR), and the ability to perform quantitative measurements. Moreover, in comparison with radiolabelled blotting procedures, it is a safe and high-speed technique.

2. Materials and methods

2.1. Virus

Purified poliovirus (serotype 1; strain Mahoney) was used for all experiments, because this enterovirus is one of the most extensively studied RNA viruses. The virus was grown, collected and purified as previously described [11], and the concentration was determined spectrophotometrically by assuming $A^{1\%}_{260}$ for virus being 81.6 [12].

2.2. Guanidine hydrochloride

Guanidine hydrochloride was purchased from the Sigma Chemical Co. (St. Louis, USA). Stock solutions (20 mg/ml) of the product were made in dimethyl sulfoxide (DMSO) and were subsequently diluted in the appropriate culture medium (Earle's Salts medium).

2.3. RT-PCR sample preparation

HeLa cell monolayer cultures were infected with purified poliovirus at a multiplicity of infection of 25, and incubated at 36.5 °C in drug-free (0.1% DMSO) or drug-containing (200 μg/ml guanidine hydrochloride) culture medium. At 1 h intervals (i.e. at 0, 1, 2, 3, 4, 5, and 6 h post-infection [p.i.]), a drug-free and a drug-containing culture were rinsed three times with sterile cold PBS buffer (150 mM NaCl, 13 mM KCl, 0.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM sodium phosphate, pH 7.4), and subsequently treated with a cell lysis buffer (0.1% Nonidet P40, 0.05% deoxycholic acid sodium salt and 0.005% bovine serum albumin [fraction V] in PBS buffer [pH 7.4]). Cell lysates were collected, cellular debris was removed by a low speed centrifugation and viral RNA was extracted using a RNA isolation kit (PURESCRIPT® RNA isolation kit, GENTRA systems, Minneapolis, USA) [13]. Mock infected drug-free and guanidine hydrochloride-containing HeLa cell monolayer cultures were also included in the experiment and used as negative controls. The extracted and precipitated RNA was dissolved and diluted (100 and 1000 ×) in sterile RNase- and DNase free

water (Sigma Chemical Co.) and stored at −80 °C before RT-PCR analysis was performed.

The quantitative aspect of the RT-PCR ELISA method was achieved by analysing a standard curve simultaneously with the samples. Therefore, a $\log_{10}$ dilution series of known concentrations of poliovirus RNA (10^0–10^8 genomes) was prepared. Briefly, purified poliovirus (500 μg/500 μl) was submitted to a RNA extraction (PURESCRIPT® RNA isolation kit) [13], and the obtained viral RNA pellet was dissolved in sterile RNase- and DNase free water. The RNA concentration and the A_{260}/A_{280} ratio were spectrophotometrically determined, assuming $A^{1\%}_{260}$ for RNA being 250 [14]. The ratio was found to be approximately 1.9, as expected for pure RNA [14]. The genome number was then calculated considering the molecular weight of poliovirus [15]. Prior to RT-PCR analysis, the viral RNA suspension was diluted in sterile RNase- and DNase free water in order to obtain the appropriate genome concentrations (10^0–10^8 genomes) for RT-PCR analysis of the standard curve.

2.4. Primers and probe

The sequences of the primers and probe are all situated in a 148 basepairs (bp) segment with absolute sequence conservation among all sequenced enteroviruses in the 5′ untranslated region of the viral genome. The primers and probe are, therefore, able to detect all available enterovirus prototypes, including the three serotypes of poliovirus [16]. The downstream primer (5′-TTGTCACCATAAGCAGCCA-3′) and the 3′ digoxigenin (DIG)-labelled capture probe (5′-CCAAAGTAGTCGGTTCCGC-3′) are antisense to genomic viral RNA. The 5′ biotin-labelled upstream primer (5′-CGGCCCCTGAATGCGGCTAAT-3′) is sense to genomic viral RNA. Both primers and probe were purchased at Pharmacia Biotech (Uppsala, Sweden).

2.5. RT-PCR

Before reverse transcription, 5 μl of the diluted RNA samples (100 and 1000 ×) as well as 5 μl of the different RNA concentrations of the standard curve (10^0–10^8 genomes) were incubated at 65 °C for 5 min, followed by 5 min at 4 °C (= pre-RT step). The following components (all values correspond with final concentrations in the reaction mixture) were then added to the incubated pre-RT samples: RNasin (25 U; Promega, Madison, USA), RT-buffer (50 mM Tris–HCl pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol; Roche Molecular Biochemicals, Manheim, Germany), dNTP mix (0.4 mM of each dATP, dCTP, dGTP, and dTTP [deoxy-adenosine, -cytidine, -guanosine, -thymidine triphosphate]; Roche Molecular Biochemicals), and avian myeloblastosis virus (AMV) reverse transcriptase (2 U; Roche Molecular Biochemicals). To obtain specificity of the assay for positive or negative strand detection, downstream primer (1 μM) was added in the case of positive strand detection and upstream primer (1 μM) was added in the case of negative strand detection. The mixture was adjusted to a volume of 10 μl with sterile RNase- and DNase-free water and incubated for 60 min at 42 °C, 5 min at 95 °C, and 5 min at 4 °C.

To the complete RT mixture, the following components were added to perform a Hot-Start PCR: PCR-buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$; Applied Biosystems, Foster City, USA), AmpliTaq Gold® DNA polymerase (2.5 U; Applied Biosystems), and 0.2 μM upstream primer (in the case of positive strand detection), or 0.2 μM downstream primer (in the case of negative strand detection). The mixture was adjusted to a volume of 40 μl with sterile RNase- and DNase-free water, and submitted to the following thermal profile: 15 min at 95 °C (pre-PCR step), 25 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, finally followed by an elongation period at 72 °C for 7 min. All thermal profiles were performed using an Amplitron II® thermocycler (Barnstead/Thermolyne Corporation, Dubuque, USA). In each analysis, a no template control was taken along to detect any form of contamination.

2.6. ELISA

The ELISA detection of the biotin-labelled RT-PCR products was essentially done as previously

described elsewhere [17,18]. A global overview of the subsequent ELISA steps is given in Table 1.

3. Results and discussion

The time-course of poliovirus RNA replication was investigated in infected HeLa cells by means of a quantitative RT-PCR ELISA method. In particular, the amount of newly synthesised positive- and negative strand viral genomes was quantified. A standard curve of known concentrations of poliovirus RNA was analysed simultaneously with the samples, in order to accurately quantify the measured absorbances. At different time-points p.i., the ratio of positive- to negative strands was determined. The effect of guanidine hydrochloride on positive- and negative RNA synthesis was also determined. Guanidine hydrochloride is known as a model inhibitor of poliovirus RNA synthesis [3], primarily inhibiting the synthesis of viral RNA, probably at the initiation step [2,19].

In order to study the quantitative time-course of poliovirus RNA replication in HeLa cells, viral RNA was isolated from infected and control (mock-infected) cell lysates, prepared at different time-points, i.e. 0, 1, 2, 3, 4, 5, and 6 h post infection. These samples were diluted (i.e. 100 and 1000 ×) prior to RT-PCR ELISA, as to obtain accurate quantitative information. The latter can only be achieved if a standard curve is analysed simultaneously with the samples.

Therefore, a $\log_{10}$ dilution series of known concentrations of poliovirus RNA (10^0–10^8 genomes) was prepared and subjected to RT-PCR ELISA, using the downstream primer in the RT step (see Section 2) along with the diluted samples. In this way, the linear range of the assay for positive strand RNA molecules could be determined, in order to perform linear regression analysis. Linearity is seen over an approximately 4-log interval (10^4–10^7 genomes), from which the equation of the linear response curve was calculated (Fig. 1). This linear response curve for positive strand RNA molecules was comparable to previously published ones [17,18]. A nearly identical linear response curve was found for negative strand RNA molecules in a comparable assay using the upstream primer in the RT step (see Section 2).

Since quantification is only possible in the linear range of the assay, for each sample prepared during poliovirus RNA replication, two dilutions (100 and 1000 ×) were prepared. The appropriate dilution, from which the absorbance values of the control samples were subtracted, was then chosen as a function of the measured absorbance, which should be more or less centrally situated in the linear range of the standard curve to assure

Table 1
Global overview of the subsequent steps of the ELISA detection

Biotin-labelled PCR product
↓
Binding biotin-streptavidin
↓
Denaturation double stranded PCR product
↓
Hybridisation DIG-labelled capture probe
↓
Binding anti-DIG-POD conjugate
↓
Addition ABTS® substrate
↓
Green-coloured end product ($A_{405/490}$)

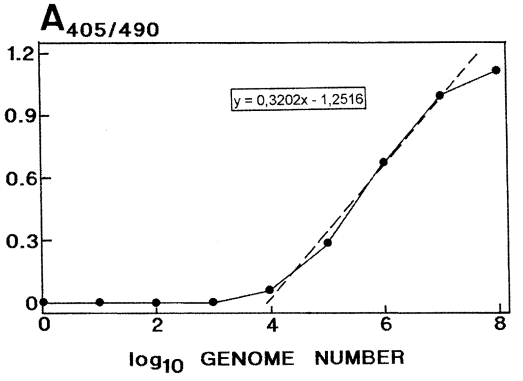

Fig. 1. Standard curve for the enterovirus specific RT-PCR ELISA analysis (absorbances ($A_{405/490}$) of the ELISA detection as a function of the $\log_{10}$ dilution series of poliovirus RNA: 10^0–10^8 genomes). The experimental conditions of the RT-PCR and the ELISA detection are, respectively, described in Section 2.

accurate quantification. Taking into account all dilution factors of the entire procedure, the amount of de novo positive- and negative strand viral RNA synthesis (by subtracting the value at 0 h p.i. from each sample) could then be calculated from the equation of the linear response curve. The obtained results are shown in Fig. 2. The time-course of positive- and negative strand synthesis is very similar. In both cases, RNA synthesis is already observed at 1 h p.i., but the greater part starts at 2 h p.i., increasing linearly until 5 h p.i., where both positive- and negative RNA synthesis are at their peaks. At 6 h p.i., a slight decrease is observed. At 5 h p.i., the total yield is 4.4 $\log_{10}$ positive strands and 3.2 $\log_{10}$ negative strands. From these results, the ratio of the amount of positive- to negative strands could also be determined at each time-point (Fig. 2). This ratio increases from approximately 3:1 (1 and 2 h p.i.) to 21:1 (6 h p.i.). Although other cell-lines were used here, the obtained results, but also the findings of Andino et al. [6], are in agreement with the results of Lopez-Guerrero et al. [20], Novak and Kirkegaard [5], and Bolten et al. [4].

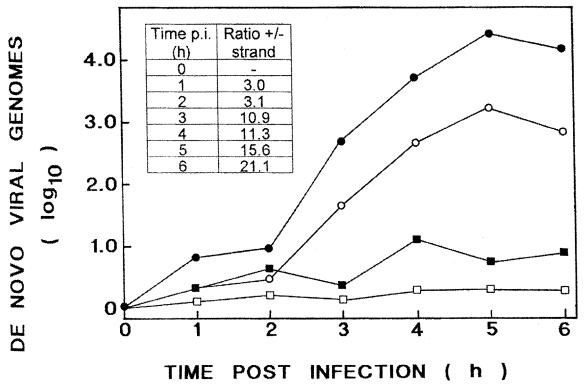

Fig. 2. Time-course of de novo viral RNA synthesis and effect of guanidine hydrochloride in poliovirus infected HeLa cells as determined by RT-PCR ELISA: time p.i. vs. amounts ($\log_{10}$) of RNA synthesised in the absence (positive strand (—●—), negative strand (—○—)), and in the presence of guanidine hydrochloride (positive strand (—■—), negative strand (—□—)). The ratio of positive- to negative strands at each time point p.i. in the absence of guanidine hydrochloride is included in the figure as a table.

However, when comparing results, it also has to be taken into account that all of the earlier findings were based on semi-quantitative methods at best.

The effect of guanidine on positive- and negative strand RNA synthesis at different time-points in the viral replication cycle was also investigated (Fig. 2). This was done in the same way (i.e. RT-PCR ELISA analysis and calculations) as for the samples in absence of guanidine. At all time points p.i., guanidine causes a complete inhibition of both positive- and negative stranded viral RNA synthesis. This is in accordance with the fact that guanidine inhibits the initiation of viral RNA synthesis [3–19]. Particularly, it blocks the initiation of negative strand RNA synthesis by inhibiting a specific function of protein 2C, required for RNA replication [1].

In conclusion, the applied RT-PCR ELISA method enabled us to quantitatively study the time-course of de novo poliovirus positive- and negative strand RNA synthesis, both in presence and absence of the inhibitor guanidine.

Moreover, the proposed method can also be applied to determine the amounts of positive- and negative stranded RNA of other enteroviruses, since the sequences of the primers and probe are situated in a segment with absolute sequence conservation among all sequenced enteroviruses in the 5′ untranslated region of the viral genome [16]. Moreover, using a human rhinovirus specific primer pair and probe, this technique has already been used to define the target of a new antiviral compound [B. Verheyden, K.Andries and B. Rombaut, submitted for publication].

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