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Viral RNA extraction for in-the-field analysis

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Abstract

Retroviruses encode their genetic information with RNA molecules, and have a high genomic recombination rate which allows them to mutate more rapidly, thereby posting a higher risk to humans. One important way to help combat a pandemic of viral infectious diseases is early detection before large-scale outbreaks occur. The polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) have been used to identify precisely different strains of some very closely related pathogens. However, isolation and detection of viral RNA in the field are difficult due to the unstable nature of viral RNA molecules. Consequently, performing in-the-field nucleic acid analysis to monitor the spread of viruses is financially and technologically challenging in remote and underdeveloped regions that are high-risk areas for outbreaks. A simplified rapid viral RNA extraction method is reported to meet the requirements for in-the-field viral RNA extraction and detection. The ability of this device to perform viral RNA extraction with subsequent RT-PCR detection of retrovirus is demonstrated. This inexpensive device has the potential to be distributed on a large scale to underdeveloped regions for early detection of retrovirus, with the possibility of reducing viral pandemic events.
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Keywords: Viral RNA extraction; In-the-field analysis

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1. Introduction

Retroviruses encode their genetic information in RNA molecules, and have a high genomic recombination rate, which allows them to mutate more rapidly and thereby pose higher risk to humans. International travel and rising population densities render infectious retroviruses such as HIV, severe acute respiratory syndrome (SARS) virus, hepatitis virus (A, C, D and E) and influenza virus serious health threats to the public worldwide. A clear example is the recent virulent strain of avian flu (H5N1), which has the potential to transcend the species barrier and become a deadly infectious disease for humans. One important way to help combat these viral infectious diseases is early detection before large-scale outbreaks occur. In addition to combating infectious diseases, precise determination of the presence of pathogenic organisms (e.g. certain viruses, bacteria, fungi and protozoa) is an essential task in many aspects of public health, such as detecting bioterrorist agents, identifying food contaminations, and excluding infected donors from the blood bank system.

For the identification of microorganisms, nucleic acid analysis has advantages over other assays, including immunoassays, because it can precisely identify and distinguish different strains of closely related pathogens. The genetic information also provides information pertaining to virulence, antibiotic resistance and epidemiology of the analyzed pathogens. The key instruments for nucleic acid analysis are nucleic acid extraction devices and thermal cyclers for performance of PCR. PCR amplification allows the precise detection and identification of a specific DNA molecule by using specific primers to amplify a defined fragment of the target DNA molecule. In order to detect specific RNA molecules, hence specific RNA viruses, the RNA molecules must first be converted to DNA molecules for PCR detection. This process is referred to as reverse transcription-PCR (RT-PCR). The specificity, sensitivity and effectiveness of PCR and RT-PCR have been well demonstrated in the detection

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of viruses, providing the basis for a number of molecular diagnostic assays (Castro et al., 2004; Gibbs et al., 2005; Kaltenboeck and Wang, 2005; Nagasse-Sugahara et al., 2004). To date most of these assays depend upon access to quite sophisticated laboratories, having the necessary equipment and expertise. However, the risk of disease outbreak may be substantially higher in remote areas and underdeveloped regions, where the performance of nucleic acid analysis is financially and technologically challenging, particular for mobile 'in-the-field' testing. Some inexpensive thermal cyclers having low power usage for in-the-field nucleic acid analysis have been developed (Belgrader et al., 1999, 2001; Kopp et al., 1998; Krishnan et al., 2002; Wheeler et al., 2004). These devices perform temperature cycling by flowing reactions through different temperature zones. They can be powered by battery and have successfully detected bacterial DNA with PCR (Belgrader et al., 1999, 2001). However, extracting viral RNA for use with these cyclers is more challenging than obtaining bacterial DNA by boiling the samples (Belgrader et al., 1999). Also, when developing devices for large-scale distribution in underdeveloped areas, low cost, simple and durable devices are most desirable. Electricity is essential for most laboratory equipments in the developed world, but is rarely available and luxury in many of those areas most at risk of viral outbreaks. Therefore, simple portable free standing devices for easy in-the-field usage must be developed.

A simplified viral RNA extraction procedure that can be performed in the field with a syringe and a stable denature buffer is described. With this rapid viral RNA extraction procedure, as little as one functional lentivirion carrying a green fluorescent protein (GFP) gene was detected using a one-step fluorogenic RT-PCR assay. This inexpensive and simple RNA extraction procedure can be used with existing portable thermal cyclers for rapid viral detection, providing a possible method that can be distributed worldwide to monitor the spread of retroviral agents, such as bird flu, where early detection of outbreaks may play a role in combating a potential pandemic.

2. Materials and methods

2.1. Retroviral vector

To quantify the ability of the procedure incorporating the viral RNA extraction device, a retroviral SIN vector (pRRL-Sin.HCMV-EGFP-pre) containing the GFP gene in a HIV back bone (Follenzi et al., 2000) was used to generate virions. Packaging of the virions was performed by transient transfections with three plasmids on 293T cells as described in other reports (Naldini et al., 1996; Zufferey et al., 1997). Briefly, confluent 10 cm plates of 293T cells were split 1:5 on the day prior to transfection (approximately 5×10^5 cell/plate). Calcium phosphate precipitation of 2 μg VSVG envelope expression plasmid, 10 μg of pCMV delta 8.91 (the gag, pol plasmid) and 10 μg of the lentiviral expression plasmid (pRRL-Sin.HCMV-EGFP-pre) was performed on 293T cells with a CalPhos kit (Clontech, USA) as instructed (Aguilar et al., 2003; Chen and Okayama, 1987; Christodoulopoulos and Cannon, 2001; Soneoka et al., 1995; Zufferey et al., 1997). The medium was changed the following morning and the virions were collected 24 h later by filtration through a 0.45 μM filter. The resulting virions were used to infect 293T cells for titer calculation. One millilitre of serial three-fold dilutions of the viral supernatant with 8 μg/ml polybrene (Millipore, USA) was added to each well of a 6-well dish. Each well contained approximately 2×10^4 293T cells. The cells were incubated with the vector for 2–4 h after which the diluent was replaced with 2 ml growth medium. FACS analysis was carried out 48 h later to identify GFP positive cells, which are infected by the retrovirus, at the FITC channel of the EPICS XL flow cytometer (Beckman Coulter, USA). The titer was calculated by a linear regression of percentages of infected cells and concentrations of viral supernatant used to infect the cells. The titer was determined to be 6.5×10^5 virions/ml.

2.2. Design of viral RNA extraction device

A number of commercial RNA extraction kits based on RNA-binding glass membrane are available. These kits usually require centrifugation or vacuum force to perform RNA extraction, and are designed for laboratory usage. With both methods, residues of liquid samples on the membrane or in its container (usually a plastic column) are not a concern. The strong centrifugation or vacuum force can remove most liquid from the membrane into the collection tubes. However, centrifuge or vacuum pumps are not conveniently available in remote areas where in-the-field testing is desirable. Designed for portable rapid RNA extraction, the procedure reported minimizes the residual liquid in the device. As shown in Fig. 1, the device consists of two plastic parts, and a gasket. The structure seals a RNA-binding membrane (Qiagen, USA) in the middle with very small dead volume. The gasket is tightly fitted to the RNA-binding membrane (Fig. 1B), and the two plastic caps are in contact with the membrane. The gasket also makes the device air tight, and allows the syringe to expel residual liquids out with the air. Various samples such as body fluids can be mixed with guanidine

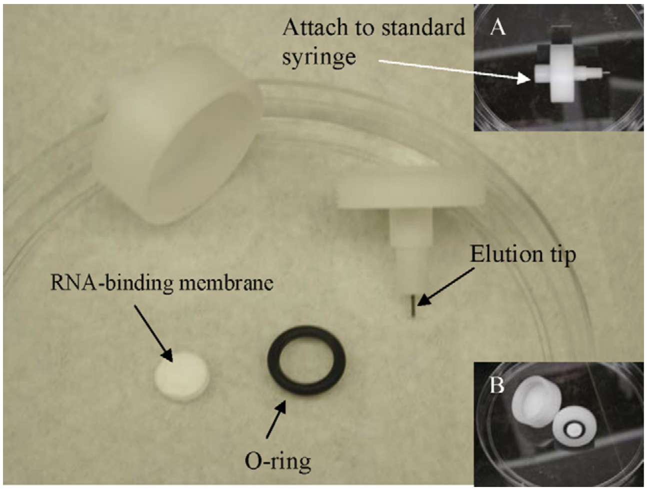

Fig. 1. The viral RNA extraction device. The device consists of a plastic cap which can attached to standard syringe, an elution tip, a gasket O-ring and a RNA-binding membrane. Inset A shows the assembled device. Inset B shows the RNA-binding membrane and the air tight position of the O-ring.

isothiocyanate (GITC)-containing denature buffer (Qiagen) with a syringe. The resulting lysates are introduced to the device through a 0.2 mm diameter channel in the plastic cap using a syringe. Passing through the RNA-binding membrane, the lysate is discharged through a 35 gauge needle on the other side of the device (Fig. 1A). Therefore, while an almost unlimited volume of samples can be passed through the device with a syringe, the resulting RNA can be eluted in just a small volume of water (as little as 50 μl when using a 1 ml syringe). This feature leads to the rapid enrichment of viral RNA from samples.

2.3. Viral RNA extraction and real-time one-step RT-PCR

Viral RNA was extracted from the supernatant (titer 6.5×10^5) or 293T cells infected with the replication deficient HIV vector. One millilitre of virion containing supernatant or cell suspension (∼1×10^5 cells) was denatured by adding 3 ml of guanidine isothiocyanate (GITC)-containing buffer (Qiagen). One millilitre of lysate was passed through a customized filter (Fig. 1) containing a RNA-binding membrane. After washing with 95% ethanol, viral RNA was eluted with 50 μl of RNase-free water. To rule out the possibility of plasmid DNA contamination, eluted RNA samples were processed with and without DNase before RT-PCR analysis. From the 50 μl elution, 2 μl was used in a one-step RT-PCR reaction (QuantiTect, SYBR Green RT-PCR Kit, Qiagen, USA) with a lightcycler (Roche, USA). The thermal cycle profile was: 95 °C 1 min and 40 °C 20 min for RT; 50 cycles of 95 °C 20 s, 60 °C 20 s and 72 °C 50 s for PCR. The GFP specific primers were: gacgtaaacggccacaagtt (forward primer) and gaactccagcaggaccatgt (reverse primer). A 609 bp product was detected with SYBR Green.

3. Results

3.1. Infecting 293T cells with HIV retroviral vector

For testing this viral RNA extraction device, replication deficient lentiviral virions carrying GFP gene in two single-strand RNA genomes were constructed. The constructed retroviral virions are capable of infecting human cells (Fig. 2), and serve as a surrogate for pathogenic retroviruses. By infecting human 293T cells with serial dilutions of supernatant containing virions, the titer of the supernatant was determined to be 6.5×10^5 virions/ml. Using known amounts of plasmids containing the viral sequence, a standard curve was generated to estimate the amount of total viral particles including non-infectious particles. The ratio of infectious to total viral particles in this viral supernatant is approximately 13/20 (data not shown).

One millilitre of retroviral supernatant containing 6.5×10^5 functional virions was added into a 10 cm plate of 293T cells. As shown in Fig. 2, not all cells are infected. Approximately, 2% cells were successfully transduced. The infected cells expressed GFP proteins 48 h after transduction, and showed strong green fluorescence. The GFP expression indicated the existence of retroviral virions in the supernatant, and most importantly allowed the estimation of functional virion numbers by titer. With these surrogate virions, it is possible to test the sensitivity and efficiency of the device quantitatively with minimal risk of viral infection.

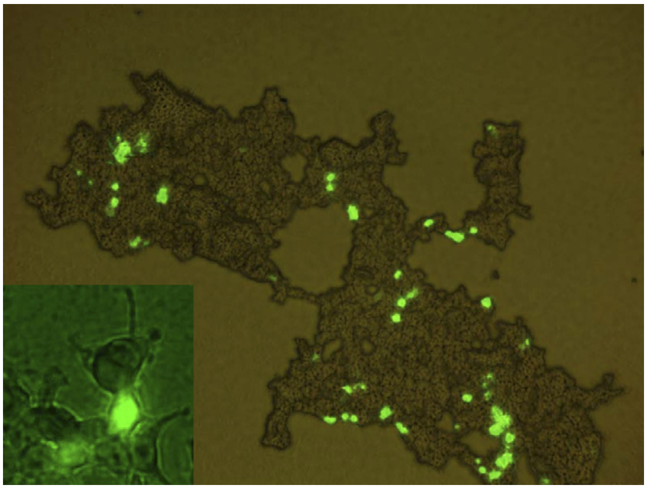

Fig. 2. Human 293T cells were infected by replication deficient lentivirus. Lentiviral virions carrying a green fluorescent protein (GFP) gene were added to human 293T cells. Forty-eight hours after adding the viral particles, infected cells began to express GFP.

3.2. Viral RNA extraction from serum

To prove the efficiency of this viral RNA extraction device, various numbers (25, 250, 7500, 12,500 and 195,000) of retrovirions were spiked into each of 300 μl of bovine serums. These serum samples represented the most common scenario of in-the-field testing, namely screening for viruses in human or animal blood samples. After denaturing viruses in serum samples with guanidine isothiocyanate (GITC)-containing buffer with a syringe, the resulting lysates were passed through the device. Viral RNA then was washed with ethanol and eluted with RNase-free water. Viral RNA was eluted into 50 μl of RNase-free water. From the eluted RNA, 2 μl was used for real-time one-step RT-PCR. As shown in Fig. 3, the GFP viral sequence was detected from all the positive samples. Since only 2 μl of

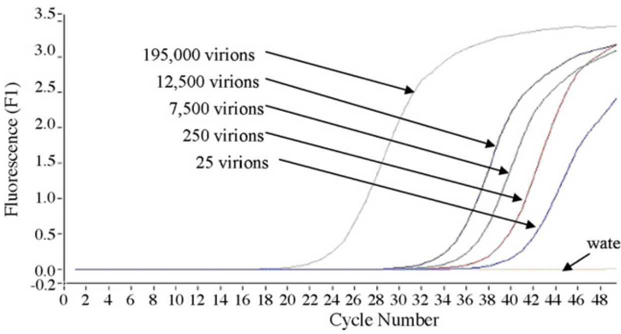

Fig. 3. One-step real-time RT-PCR detection of recombinant retrovirus. Viral RNAs extracted from 300 μl of bovine serums containing 25, 250, 7500, 12,500 and 195,000 virions respectively were used in a real-time one-step RT-PCR. The target GFP sequence carried by the recombinant retrovirus was detected at different cycle numbers corresponding to the number of virions in each sample. The viral RNA can be detected from samples containing as few as 25 infectious virions.

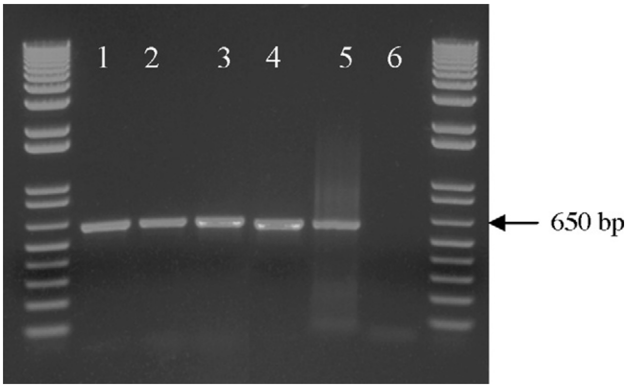

Fig. 4. Electrophoresis confirms the real-time one-step RT-PCR result. From each of the 20 μl RT-PCR reaction, 2 μl product was used for electrophoresis with a 2% agarose gel. Lane 1, sample with 25 virions; Lane 2, sample with 250 virions; Lane 3, sample with 7500 virions; Lane 4, sample with 12,500 virions; Lane 5, sample with 195,000 virions; Lane 6, negative control sample without spiked-in virion. The 609 bp PCR products were detected in lanes 1–5. All positive lanes have same intensity because the RT-PCR was carried out until plateau.

the 50 μl eluted RNA (1/25 of the total eluted RNA) from each sample were used, detection from the sample with 25 spiked virions indicates that the sensitivity of this RNA extraction device may extend in range to a single infectious virion. Given that the tested viral supernatant has 13 infectious particles in every 20 particles with viral RNA, the method described can detect 1–2 viral particles from samples with serum. Electrophoresis confirmed the real-time RT-PCR result by showing the correct size of amplified DNA fragments from each of the samples (Fig. 4).

4. Discussion

In order to perform in-the-field detection of retrovirus, a method must be developed to extract viral RNA using a simple, reliable and rapid procedure without access to standard laboratory equipment. In-the-field detection of bacterial genomic DNA by heating samples at 95 °C has been reported previously (Belgrader et al., 1999). This method cannot be used for detecting RNA virus because RNase, which rapidly destroys RNA molecules, is stable and functional at 95 °C. In addition, the reverse transcription reaction is more susceptible to the interference by the contaminated proteins than is the standard PCR reaction. To obtain suitable viral RNA for RT-PCR, a viral RNA extraction method was developed based on passing denatured viral samples through RNA-binding silicon membrane. This simplified method could be safely performed by a layperson with minimal basic training in a very short time. The minimal training requirement is important for large-scale in-the-field detection of virus in underdeveloped areas where skilled and experienced technicians are generally not available.

Attached to a regular 1 ml disposable syringe, the viral RNA extraction device reported is capable of isolating viral RNA suitable for RT-PCR from serum specimens. A level of sensitivity as high as the detection of a single infectious virion was achieved in these initial studies. It is known that defective virions may also carry RNA genomes, and extracting RNA from one infectious virion (based on titer) is not equivalent to extracting RNA from a single virion. Therefore, the ratios of non-infectious particle (with or without genetic material, RNA or DNA) to infectious particles vary in different samples. However, as a screening and early detection method, the procedure described meets the immediate need to identify early cases as well as population clusters of infected individuals for more extensive study and assessment of disease transmission risk. In the present study it was demonstrated that viral RNA can be extracted reliably and reproducibly from bovine serum containing virions in less than 5 min. Furthermore, in the present viral samples employed in this study, it proved possible to extract viral RNA suitable for RT-PCR detection from as few as one infectious virion. The sensitivity achieved in the field may be different in other samples that have a different ratio of non-infectious to infectious particles. This total RNA extraction method can also be used in the field to test animals from which specimens of body fluid can be obtained. The inexpensive, durable and simple construction of this device renders it suitable for distribution to underdeveloped areas where electricity and sophisticated laboratory methods are not easily accessed. By integrating this device with portable thermal cyclers, a simple, low cost, durable infectious agent detection system can be configured for the underdeveloped regions of the world, where early detection perhaps is the best approach to combat outbreaks of highly transmissible infectious diseases.

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