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Isolation of infectious chikungunya virus and dengue virus using anionic polymer-coated magnetic beads☆

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A B S T R A C T

Mosquitoes-borne viruses are a major threat for human populations. Among them, chikungunya virus (CHIKV) and dengue virus (DENV) cause thousands of cases worldwide. The recent propagation of mosquito vectors competent to transmit these viruses to temperate areas increases their potential impact on susceptible human populations. The development of sensitive methods allowing the detection and isolation of infectious viruses is of crucial interest for determination of virus contamination in humans and in competent mosquito vectors. However, simple and rapid method allowing the capture of infectious CHIKV and DENV from samples with low viral titers useful for further genetic and functional characterization of circulating strains is lacking. The present study reports a fast and sensitive isolation technique based on viral particles adsorption on magnetic beads coated with anionic polymer, poly(methyl vinyl ether-maleic anhydrate) and suitable for isolation of infectious CHIKV and DENV from the four serotypes. Starting from quite reduced biological material, this method was accurate to combine with conventional detection techniques, including qRT-PCR and immunoblotting and allowed isolation of infectious particles without resorting to a step of cultivation. The use of polymer-coated magnetic beads is therefore of high interest for rapid detection and isolation of CHIKV and DENV from samples with reduced viral loads and represents an accurate approach for the surveillance of mosquito vector in area at risk for arbovirus outbreaks.

1. Introduction

Chikungunya virus (CHIKV) and dengue virus (DENV) are arboviruses transmitted to humans by *Aedes* sp. mosquitoes. Dengue fever caused by DENV is the most common arboviral disease in humans, with 50 million annual cases in more than 100 countries, and 2.5 billion people at risk (WHO, 2012). About 500,000 persons require hospitalization every year for dengue hemorrhagic fever and 2.5% of cases are fatal (WHO, 2012). In the recent years, CHIKV has reemerged in Africa and spread to the Indian Ocean area and to India where it caused thousands of cases (Das et al., 2007; Renault et al., 2007; Soumahoro et al., 2011). As *Aedes aegypti* and *Aedes albopictus* mosquitoes, known as competent vectors for CHIKV and DENV dissemination to human, are spreading worldwide, including to temperate areas, both viruses represent a global threat to public health (Charrel et al., 2007). The potential threat of arboviruses for countries where most individuals bear naïve immune systems has been illustrated in the recent years by the limited CHIKV outbreak in Emilie-Romagna in Italy (Angelini et al., 2008) and by the detection of autochtonous DENV and CHIKV infection in Europe (Gould et al., 2010; La Ruche et al., 2010). In this context, the early detection of arboviruses through surveillance of insect populations is critical to provide warning of potential disease incursion and for resolving the emergence of such epidemics in the future.

Classical techniques used for detection of CHIKV and DENV in biological samples include reverse transcription (RT)-polymerase

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chain reaction (PCR), enzyme-linked immunoassays, immunofluorescence assays (ELISA) and serological tests allowing detection of specific IgM or IgG production in exposed humans. While growth in cell culture represents the "gold-standard" for viral isolation and allows the detection of any virus that replicates in the cells, the procedure requires time for the virus to grow and for subsequent identification. To the opposite, nucleic acid-based techniques, especially RT-PCR, have the advantage of speed, specificity and sensitivity for detection of the viral RNA. Nevertheless, these methods do not enable to further isolate infectious particles, because some reagents used for protein or nucleic acids extraction, including detergents and phenol-chloroform, are well known to inactivate enveloped viruses. The development of a really sensitive method accurate to isolate low amounts of infectious particles will allow the rapid access to circulating strains of viruses and is therefore of interest for researchers, including virologists and entomologists interested in the isolation and functional characterization of arthropod-borne viruses from circulating mosquitoes.

In this study, magnetic beads coated with an anionic polymer, poly(methyl vinyl ether-maleic anhydrate)[poly(MVE-MA)] were used to capture arbovirus particles including DENV from the four serotypes and CHIKV in the medium of infected cell cultures. This method therefore represents a new rapid and efficient method for arboviruses capture, purification and concentration that is of interest for isolation and further phenotypic characterization of infectious particles from a reduced amount of infected biological material.

2. Materials and methods

2.1. Cells

The C6/36 cell line derived from *A. albopictus* was grown in Minimum essential Medium (MEM) (Invitrogen, France), supplemented with 10% fetal calf serum (FCS, Lonza, Basel, Switzerland) at 28 °C. The CCL-125 cell line derived from *A. aegypti* was cultured in Eagle's Minimal Essential Medium (EMEM), supplemented with 20% FCS and 1% glutamine at 28 °C.

HEK293T human epithelial cells were maintained at 37 °C in DMEM (Lonza, Basel, Switzerland) containing 10% inactivated fetal calf serum and 1% antibiotics. BHK-21 and Vero cells used for virus production and titration were cultured under similar conditions.

2.2. Production of viral stocks and titration

The pCHIKic subgenomic clone containing the entire CHIKV genome (37997 strain) and a green fluorescent protein (GFP) sequence fused to the 3′ end of the nonstructural genes was kindly provided by S. Higgs (UTMB, Galveston) (Tsetsarkin et al., 2006). The infectious clone was transcribed in vitro from the SP6 promoter using the mMESSAGE mMACHINE kit (Ambion, Saint Aubin, France) according to manufacturer's instructions. RNA (0.5 μg) was then electroporated into BHK-21 cells (5×10^6) derived from hamster kidney fibroblasts (ATCC# CCL-10^{TM}) with 2 pulses at 1.5 kV, 25 λF and $\alpha\omega$. After two days, cell culture supernatant was harvested, filtered onto 0.22 λm filters and propagated in the C6/36 cell line derived from *A. albopictus* as previously described (Gay et al., 2012). After 2 days, culture supernatant was filtered, aliquoted and stored at −80 °C. Viral stocks were tittered using plaque assay formation performed on Vero cells, as previously reported (Bernard et al., 2010). The four dengue serotypes DENV1 (Hawaii strain) (Halstead et al., 1970), DENV2 (16681 strain) (Halstead et al., 1970), DENV3 (H87 strain) (Halstead et al., 1970) and DENV4 (814669 strain) (Yao et al., 2003) were also propagated in C6/36 cells using similar culture conditions.

2.3. Virus capture

Virus capture was performed using Viro-Adembeads (Ademtech, Pessac, France) following manufacturer's instructions. Virus-containing supernatants were serially diluted from 10^9 to 10^0 pfu/ml with serum free medium. Briefly, 40 μl Viro-Adembeads were washed twice with binding buffer, mixed with 40 μl culture supernatant and 360 μl of serum free medium and incubated for 20 min at room temperature. The tubes were set in a magnetic field for 1 min using the Adem-Mag SV magnetic device (Ademtech, Pessac France). The supernatants were discarded and the beads-viruses complexes were washed three times with serum free medium. Then, the complexes were resuspended either in 25 μl serum free medium when used in infection assays and PCR experiments or diluted in 25 μl of RIPA buffer for proteins analysis by immunoblotting.

2.4. Experimental infections

The beads-viruses complexes were used directly for cell infection of C6/36 or CCL-125 cells. 10^5 cells were seeded in 24-wells plates in appropriate medium. After 24 h in culture, beads-virus complexes were added to the supernatant and the infection was allowed to proceed for the indicated time.

2.5. Western blotting

Samples in RIPA buffer were resuspended in 25 μl of gel-loading buffer containing 90 mM Tris–Cl (pH 6.8), 10% 2-mercaptoethanol, 2% SDS, 0.02% bromopheol blue and 20% glycerol and boiled for 5 min. Proteins were separated on a 12% SDS-PAGE and transferred to a polyvinyldene difluoride (PVDF) membrane (Millipore, Molsheim, France). After proteins transfer, the membrane was saturated with 5% skim milk in PBS for 1 h at room temperature and incubated with mAbs hybridizing with CHIKV capsid (Greiser-Wilke et al., 1989) or with 4E11 anti-DENV envelope mAbs (Cockburn et al., 2012). After three washes in PBS containing 0.1% Tween-20, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase and revealed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific, Illkirch, France).

2.6. RNA extraction

Viral RNA in culture supernatant was isolated using the QIAamp viral RNA mini kit (QIAGEN, Courtaboeuf, France). The RNA was resuspended in 30 μl of RNAse free distilled water and stored at −80 °C until used. RNA isolation from virus-Viro-Adembeads complexes was performed as previously described using TriReageant and phenol-chloroform extraction (Fenard et al., 2009).

2.7. RT-PCR of DENV2 negative strand

DENV2 negative strand RNA was amplified by semi quantitative RT-PCR as previously described (Surasombatpattana et al., 2011). Briefly, 0.5 μg of RNA was converted to cDNA with M-MLV Reverse Transcriptase (Promega, Charbonnières-Les-Bains, France) with a DENV-specific primer according to manufacturer's instructions. Then, PCR was carried out on the cDNA using Taq DNA Polymerase (Roche Diagnostics, Meylan, France). Each reaction of 50 μl contained 200 nM of specific primers (see Table 1). The amplification program was performed under the following condition: one denaturation cycle at 95 °C for 2 min followed by 40 cycles of 95 °C 15 s, 56 °C for 15 s and 72 °C for 30 s and one final extension step at 72 °C