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

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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 anhydride) 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.

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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 autochthonous 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 anhydride)[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-10TM) with 2 pulses at 1.5 kV, 25 λF and αω. 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 10⁹ to 10⁰ 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. 10⁵ 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% bromophenol blue and 20% glycerol and boiled for 5 min. Proteins were separated on a 12% SDS-PAGE and transferred to a polyvinylidene 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 TriReagent 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 (Surasombattana 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

Table 1
Nucleotide sequences of primers used for PCR detection of DENV1, DENV2, DENV3, DENV4 and CHIKV.

Virus	Oligonucleotide sequence	Protein	Position	Product size
DENV1	5' TCA ATA TGC TGA AAC GCG CGA GAA ACC G 3' 5' CGT CTC AGT GAT CCG GGG (A,G)C 3'	Structural polyprotein	132–160 596–614	482 bp
DENV2	5' GCA GAA CCT CCA TTC GGA GAC AGC TAC AT 3' 5' AGC TCA CAA CGC AAC CAC TAT 3'	Envelope glycoprotein	2024–2052 2406–2427	392 bp
DENV3	5' TCA ATA TGC TGA AAC GCG CGT GAA ACC G 3' 5' GTA ACA TCA TCA TGA GAC AGA GC 3'	Structural polyprotein	117–144 385–407	290 bp
DENV4	5' TCA ATA TGC TGA AAC GCG CGA GAA ACC G 3' 5' CTC TGT TGT CTT AAA CAA GAG A 3'	Structural polyprotein	136–163 506–527	392 bp
DENV2 for qPCR	5' GGAAGGAGAAGGACTGCACA 3' 5' ATTCTTGTGTCCCATCTGCT 3'	NS5	9091–9109 9175–9195	104 bp
Probe/ROX DENV2	5' [FAM] TAAGAGACGTGAGCAAGAAAGAGGGAGGAG [TAMRA] 3'	NS5	9125–9150	
CHIKV for qPCR	5' GGCAGTGGTCCCAGATAATTCAAG 3' 5' GCTGTCTAGATCCACCCATACATG 3'	nsP2	1682–1706 1764–1789	107 bp
T7-Denv-F	5' TAATACGACTCACTATAGGGGAAGGAGAAGGACTGCACA 3'	T7 promoter-NS5	9091–9109	124 bp
Denv-R	5' ATTCTTGTGTCCCATCTGCT 3'	NS5	9175–9195	

for 2 min. The resulting fragment of 392 bp for the negative strand was evidenced by electrophoresis in agarose gels.

2.8. DENV-2 quantitative real time RT-PCR

The Maxima™ Probe/ROX qPCR Master Mix (2×) (Fermentas, Saint-Rémy Les Chevreuses, France) was used in all qPCR experiments. Each reaction of 25 µl contained 400 nM of each primer, 250 nM of specific probe and 1× Maxima™ Probe/ROX qPCR Master Mix. Primers and probe sequences were already described (Kong et al., 2006) and are listed in Table 1. Amplification was performed in an Applied Biosystem 7300 system under the following conditions: 95 °C for 10 min followed by 40 amplification cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. Real-time data were analyzed using the SDS software (Thermo Fischer Scientific, Illkirch, France). Viral RNA was quantified by comparing the sample's threshold cycle (Ct) values with a Dengue virus RNA standard curve which was obtained as follows: firstly, total viral RNA was purified from an infected culture using the QIAamp Viral RNA kit (QIAGEN, Courtaboeuf, France). Then, standard RT-PCR was carried out using a primer containing the T7-Denv-F5' and Denv-R5' primers as previously described (Luplertop et al., 2011; Surasombatpattana et al., 2012). The PCR product, containing the T7 promoter sequence was used to generate DENV RNA fragments by *in vitro* transcription using the MAXIscript kit (Ambion, Saint Aubin, France). Then, RNA was purified by precipitation in sodium acetate and absolute ethanol. The amount of RNA generated was determined by spectrophotometry and converted to molecular copies using the following formula:

$$Y_{\text{molecules}}/\mu\text{l} = \frac{X_g/\mu\text{LRNA}}{\text{transcript length}(\text{bp}) \times 340} \times 6.02 \times 10^{23}$$

RNA standards containing 1.21×10^{10} – 1.21×10^3 RNA copies were used to construct a standard curve.

2.9. Real time RT-PCR amplification of CHIKV RNA

0.5 µg of RNA were converted to cDNA with M-MLV Reverse Transcriptase (Promega, Charbonnières-Les-Bains, France) with an oligodT (12:18) primer (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. PCR amplification was carried out on 100 ng cDNA in a reaction mix containing 0.4 µM of each primer, and 2 µl SYBR Green master amplification mix (Fast start DNA Master plus SYBR Green I amplification kit, Roche Diagnostics, Meylan, France). For each amplification, a control reaction was performed

in which DNA sample was replaced by water. Reactions were subjected to a first cycle of 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C; 15 s at 65 °C and 20 s at 72 °C on the RotorGene system (Labgene Scientific, Châtel-St-Denis, France). Fluorescence signal was recorded at the end of each cycle. A standard curve was generated from 10^1 to 10^5 copies of pCHIKic plasmid. Primers used for amplification are shown in Table 1.

3. Results

Anionic polymer, poly(methyl vinyl ether-maleic anhydride) (Poly(MVE-MA)) has been reported to bind the surface of some virus particles (Sakudo and Ikuta, 2008, 2012; Sakudo et al., 2011). The present study was designed to investigate whether [poly(MVE-MA)]-coated magnetic beads are applicable for capture and isolation of infectious CHIKV and DENV from the four serotypes. To this end, culture medium of CHIKV- or DENV-infected mosquito cells or medium from mock-infected cells used as control sample were subjected to incubation with Viro-Adembeads following the manufacturer's protocol (see schematic representation in Fig. 1A). Briefly, viral preparations were incubated with magnetic beads under gentle agitation and then separated from the supernatant by applying a magnetic field. Complexes recovered from samples by anionic magnetic beads were resuspended in lysis buffer, subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred onto membranes and revealed with antibodies directed either to CHIKV capsid or to DENV envelope proteins. For each dilution, a sample corresponding to the native viral preparation used in the isolation assay was run in parallel. While CHIKV or DENV proteins were undetectable from the native samples (Fig. 1B and C), a 31 kDa band was revealed from CHIKV-positive samples incubated with Virobeads, using anti-capsid mAbs (Fig. 1B). Similarly, a 50 kDa band corresponding to DENV envelope was detected from magnetic beads isolated complexes (Fig. 1C). Accordingly, in our conditions, proteins from CHIKV and from DENV could be successfully isolated from virus-containing suspensions. Moreover, envelope glycoproteins from DENV1 and DENV2 serotypes were successfully isolated.

Next, RT-PCR amplification was used to determine the capacity of magnetic beads to enrich the sample in viral genomic RNA (Fig. 2). Isolation experiments were performed starting from serial dilution of viral suspensions containing CHIKV or DENV particles of each of the four serotypes normalized according to their infectious titer determined by plaque forming assays. After isolation, the virus-beads complexes were subjected to RNA extraction and

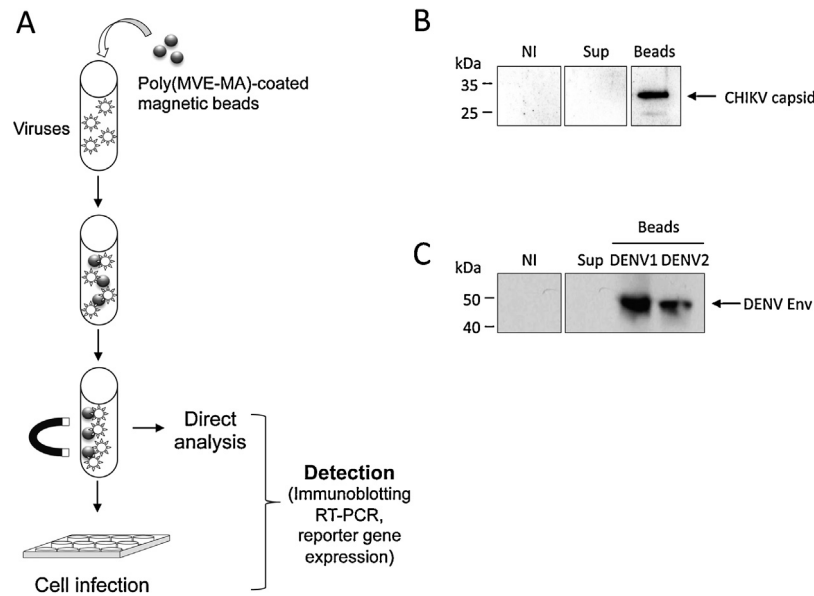


Fig. 1. Capture of CHIKV and DENV antigens by anionic polymer-coated beads. (A) Schematic representation of experimental procedures used for capture of CHIKV or DENV particles from culture supernatant. (B and C) Detection of CHIKV or DENV antigens in virus-beads complexes. Beads fraction recovered after incubation with CHIKV-positive (B) or DENV1- or DENV2-infected samples (C) were subjected to immunoblot and revealed with mAbs specific for CHIKV capsid or for DENV envelope glycoproteins. In each experiment, the supernatant of mock-infected culture (NI) and the non-captured virus-positive sample (Sup) were run in parallel. Molecular weight markers are shown on the left. Data are representative of three independent experiments.

to RT-PCR amplification using primers specific for CHIKV or DENV serotypes (see Table 1 for oligonucleotide sequences). The expected amplification products sizes were 107 bp for CHIKV and 482 bp, 392 bp, 290 bp and 392 bp for DENV1, DENV2, DENV3 and DENV4, respectively. Starting from CHIKV or DENV suspensions, RT-PCR analysis allowed the detection of a single band with the expected size. DENV RNA was efficiently isolated starting from DENV from the four serotypes (Fig. 2B). Therefore, the bead fraction where viral antigens were detected also contained the RNA genome. Next to evaluate the efficiency of magnetic beads purification method genomic RNA contained in virus-beads complexes were compared with that present in the starting sample. To this end, viral suspensions containing increasing amounts of CHIKV or DENV2 particles ranging from 10^2 to 10^6 pfu were diluted in 400 μ l serum free medium and incubated with Virobeads in

conditions described in Section 2. After extraction of beads-associated nucleic acids, real time RT-PCR amplification was carried out and RNA genomes copies were quantified in the bead fraction and in the initial sample. As shown in Fig. 3, RNA copy numbers detected from CHIKV sample corresponded to 19–27% of the initial input. For DENV efficiency of virus recovery determined according to genomic RNA copy numbers present in the starting sample and in the virus-beads preparation evidenced was comprised between 97% and 56%. The reasons accounting for the discrepancies between the two viruses remain unknown.

CHIKV and DENV are propagated through alternate replication in mosquitoes and human hosts. As polymer-coated magnetic beads may be of interest for isolation of viral particles from human blood samples, the capacity of Viro-Ademeads to capture CHIKV capture and DENV particles in serum containing medium was

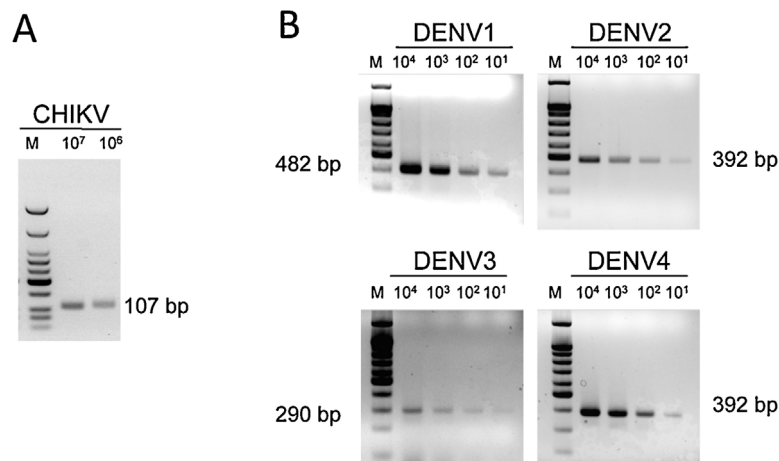


Fig. 2. Detection of genomic CHIKV or DENV RNA captured on anionic polymer-coated magnetic beads. Supernatant of cell cultures infected with CHIKV (A) or with DENV1, DENV2, DENV3 or DENV4 (B) were subjected to serial dilutions to generate suspensions containing 10^7 to 10^1 pfu/ml and incubated with anionic polymer-coated beads. After separation, total RNA was extracted and analyzed by RT-PCR using oligonucleotide primers specific for each viral serotype. The size of PCR products is indicated. Amplification products obtained either from the initial supernatant or following incubation of Virobeads with mock-infected supernatants are shown as controls. All data presented are representative of three independent experiments.

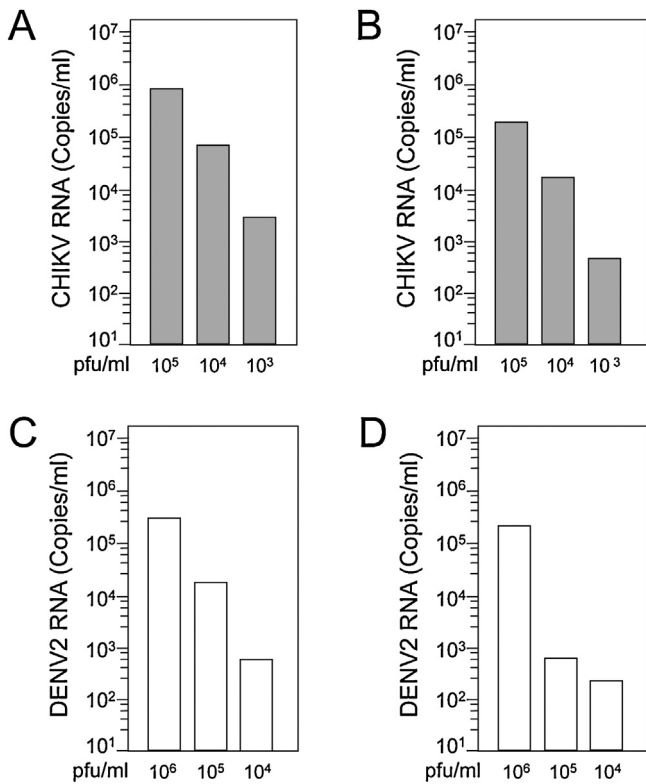


Fig. 3. Quantitative analysis of RNA in CHIKV and DENV2 captured on anionic polymer-coated magnetic beads. Serial dilutions of CHIKV (A) or DENV (C) preparations containing 10^6 to 10^3 pfu/ml and virus-beads complexes isolated from the corresponding preparations (B) and (D) were subjected to qRT-PCR quantification of genomic RNA levels. Values are representative of two separate experiments.

investigated. Viral suspension containing 10^6 to 10^2 pfu CHIKV or DENV2 were supplemented with human serum collected from an uninfected patient and processed for magnetic beads isolation as described previously. Then, viral genomic RNA present in the virus-beads complexes was detected by real time RT-PCR. In these conditions, no RNA could be detected in any sample regardless of the concentration of DENV or CHIKV present in the initial viral suspension (Fig. 4). Viral suspensions used in this experiment correspond to those used in Fig. 3. Therefore, the presence of human serum abolished the capacity of polymer-coated beads to concentrate viral particles.

Finally, the infectivity of the fraction captured with Viro-Ademebeads from CHIKV or DENV suspensions was determined. Complexes recovered from CHIKV samples were incubated with the C6/36 cell line, an *A. albopictus* cell line susceptible to CHIKV

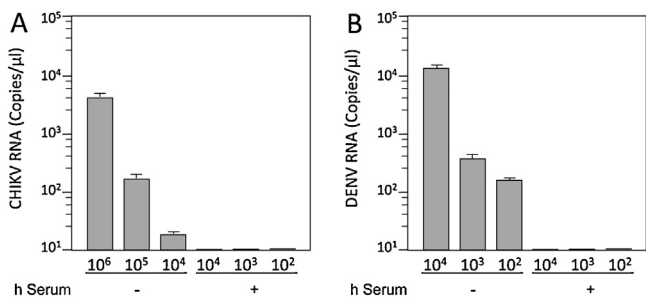


Fig. 4. Viral particles recovery is inhibited by human serum. The capacity of Viro-Ademebeads to isolate CHIKV (A) or DENV2 (B) particles in the presence of normal human serum was determined starting from serial dilutions of viral particles. Viral RNA contained in the complexes was quantified by real time RT-PCR. Values are the mean of duplicate experiments.

(Gay et al., 2012). Infection of the culture was analyzed for expression of the virus-encoded GFP reporter gene at days 1, 3 and 6 post exposure (Fig. 5A). This reporter gene, located at the 3' end of the non structural proteins, is expressed upon synthesis of the CHIKV replication complex. GFP fluorescence was detected after 1 day in culture. Almost all the cells were GFP-positive after 3 days, attesting for CHIKV replication and propagation in the culture. Basal fluorescence level detected following incubation of C6/36 cells with complexes formed by beads and supernatant of mock-infected cells is shown as control. Similar experiments were repeated using the LR-OPY1 CHIKV strain isolated from the recent Reunion outbreak (Tsetsarkin et al., 2006). Data produced with this variant were strictly identical those obtained with the 37997 CHIKV reference strain (data not shown). The release of viral particles in culture supernatant was investigated. qRT-PCR analysis of supernatants collected from CHIKV-infected C6/36 cells evidenced the presence of viral RNA, attesting that viral particles are released at high levels in the culture medium (Fig. 5B). Infection assays were repeated with complexes captured from DENV2 viral suspensions (Fig. 5C). As a DENV recombinant virus expressing a GFP reporter gene was not available, infection of C6/36 cells incubated with DENV-Virobeads complexes was monitored by RT-PCR amplification of envelope glycoprotein gene starting from RNA isolated after lysis of the cell culture. A 392 bp amplicon corresponding to DENV minus-strand RNA and indicative of DENV replication was detected after 24 h in culture. Identical results were obtained using the CCL-125 cell line originating from *A. aegypti*. qRT-PCR analysis of culture supernatant attested for the capacity of the culture to release DENV2 particles (Fig. 5D). Altogether these results attest for the infectivity of complexes isolated from CHIKV and DENV2 suspension using anionic polymer-coated magnetic beads.

4. Discussion

This study reports the use of magnetic anionic polymer-coated beads is suitable for the isolation of intact CHIKV and DENV particles. According to our results, this method is applicable to the four DENV serotypes and to the prototypal African CHIKV (37997 strain) as well as to the LR-OPY1 CHIKV isolate with genetic characteristics of the strain that recently reemerged during the 2005–2006 outbreak in the Indian Ocean area. This isolation technology can be combined with classical detection methods, including immunoblot analysis of viral proteins and quantitative amplification of viral genomes. In addition, captured particles are infectious and propagate efficiently in a fresh culture. Accordingly, the combination of approaches reported herein could serve as a useful strategy not only for identification of positive samples with low viral burden, but also for simultaneous isolation of infectious particles from a single sample. Gold standard methods used to concentrate viral particles generally rely on the use of ultracentrifugation and PEG precipitation; nevertheless, these methods display several caveats. Indeed, PEG precipitation does not allow the production of ultra-purified viral particles. This may be a major limitation in some type of studies. Mainly, the presence of contaminating material, i.e. derived from serum added to the culture medium, may create a bias for transcriptomic and proteomic analysis of cellular responses elicited by the virus in target cells. In contrast, ultracentrifugation on density gradient or even on cushion generally leads to the production of ultrapure viral preparations. However, in some cases, viral particles may not support well repeated ultracentrifugation steps. Viruses collected in these protocols may show a decreased infectivity due to the loss of envelope glycoproteins or to the alteration of viral particles structure. This is specially the case for CHIKV (E. Bernard, personal communication). The use of anionic

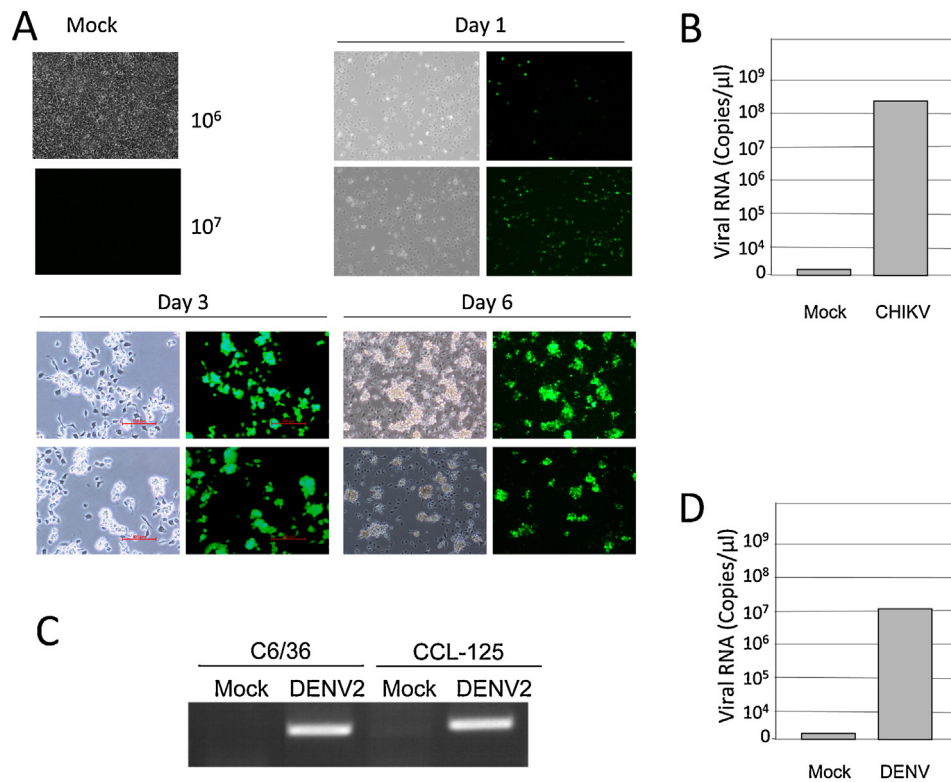


Fig. 5. Efficient recovery of infectious arbovirus particles using poly(MVE-MA)-coated magnetic beads. (A) Complexes formed by poly(MVE-MA)-coated magnetic beads and CHIKV particles were incubated with the CHIKV-permissive C6/36 cell line. Viral replication was monitored by direct visualization of GFP reporter gene expression in the cell culture at day 1, 3 and 6 post-exposure. Cultures exposed to beads incubated with supernatant from mock-infected cells are shown as control. (C) Infectivity of DENV2 particles recovered using anionic polymer-coated magnetic beads was monitored by incubating the virus-beads complexes with C6/36 or CCL-125 cultures. After 6 days in culture, cells were lysed and RNA was extracted. The replicative complexes present in the cells were evidenced by RT-PCR amplification of DENV2 RNA negative strand. Data are representative of three independent experiments. The supernatants of cell culture presented in (A) and (C) were collected at day 6 post infection. Viral RNA was extracted, reverse transcribed and quantified by real time PCR amplification using specific primers.

polymer-coated beads simultaneously allows the isolation of pure preparations of infectious particles.

Virus capture using anionic polymer-coated magnetic beads has previously been shown to be applicable to the isolation of various viruses, including respiratory syncytial virus, human and avian influenza viruses, cytomegalovirus, herpes viruses, borna disease virus and human immunodeficiency virus, vaccinia and rotavirus (Sakudo et al., 2009a, b, 2011; Hatano et al., 2010; Sakudo and Ikuta, 2012). Therefore, this technology has a broad potential for the isolation of diverse viruses. The results reported herein indicate that this technique is suitable for isolation of DENV or CHIKV two viruses with high epidemic impact in tropical countries and that are subjected to an epidemic vigilance in temperate areas. According the present study, this method is of interest for the detection of DENV and CHIKV viruses. The possibility to use polymer-coated beads purification method for diagnosis of exposed patients was also considered. Human serum was found to inhibit CHIKV and DENV isolation. This inhibitory effect may reflect the capacity of serum components such as albumin, to saturate the magnetic beads. Accordingly, when applied to human blood samples, the use of magnetic Virobeads must be combined with depletion methods capable to decrease albumin levels in the starting sample. More likely, the significant capacity of Virobeads to enrich arbovirus particles in samples containing low amounts of starting material is of particular interest to be applied to surveillance of arthropod vectors in order to provide early detection of invading viruses or for functional studies of DENV or CHIKV strains vectored by mosquitoes in various geographic areas. This non-destructive method is of major interest for a variety of

applications including genetic analysis of circulating arboviruses, especially when isolated from samples containing low amounts of viral particles. It will permit coupling the detection and functional studies of the corresponding viruses isolated from infected insects.

Recently, CHIKV and DENV-2 have been reported to be responsible for a large simultaneous outbreak centered on Gabon (Caron et al., 2012). *A. albopictus* mosquitoes, the main vector for DENV and CHIKV in recent outbreaks, can be experimentally orally coinfectd by these two viruses (Vazeille et al., 2010) and the simultaneous presence of CHIKV and DENV-2 was recently detected in a wild-caught mosquito (Caron et al., 2012). In this context, isolation with anionic polymer-coated beads appears as a useful strategy to monitor the status of circulating mosquitoes in region at risk for DENV and/or CHIKV outbreak. The reduced cost, the good sensitivity and the rapidity of this technology allowing faster isolation than traditional techniques are especially appropriate for simultaneous analysis of a large number of samples. Obviously, besides its interest for epidemiological vigilance and diagnosis, this strategy will represent, in the future, an interesting way to investigate virus-host interactions engaged during early replication steps of CHIKV and DENV in mosquitoes and in humans.

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