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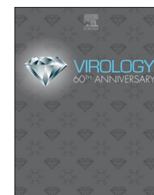

Adventitious viruses persistently infect three commonly used mosquito cell lines

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ABSTRACT

Mosquito cell lines have been used extensively in research to isolate and propagate arthropod-borne viruses and understand virus-vector interactions. Despite their utility as an in vitro tool, these cell lines are poorly defined and may harbor insect-specific viruses. Accordingly, we screened four commonly-used mosquito cell lines, C6/36 and U4.4 cells from *Aedes albopictus,* Aag2 cells from *Aedes aegypti*, and Hsu cells from *Culex quinquefasciatus*, for the presence of adventitious (i.e. exogenous) viruses. All four cell lines stained positive for double-stranded RNA, indicative of RNA virus replication. We subsequently identified viruses infecting Aag2, U4.4 and Hsu cell lines using untargeted next-generation sequencing, but not C6/36 cells. PCR confirmation revealed that these sequences stem from active viral replication and/or integration into the cellular genome. Our results show that these commonly-used mosquito cell lines are persistently-infected with several viruses. This finding may be critical to interpreting data generated in these systems.

1. Introduction

Cell culture systems have revolutionized biomedical science and provided key insights into the fundamentals of life. The tractability of these systems make it possible to perform high-throughput drug screens and gene studies (Broach and Thorner, 1996; Perrimon and Mathey-Prevot, 2007), isolate and amplify viruses and develop vaccines (Enders et al., 1949; Lloyd et al., 1936; Rivers and Ward, 1935); experiments that would otherwise be too difficult or impossible to perform in vivo. Despite their utility, it has recently been shown that many commonly used mammalian cell lines are persistently infected with a myriad of viruses, possibly confounding the results generated in these cell lineages and highlighting the need for a better understanding of cell culture systems (Hué et al., 2010; Platt et al., 2009; Uphoff et al., 2010).

Developed in the 1960s (Grace, 1966; Peleg, 1968; Singh, 1967), mosquito cell culture systems have become an indispensable tool in the study of arthropod-borne (arbo)viruses. These systems have provided insights into virus evolution and virus-vector interactions and democratized research by allowing laboratories lacking mosquito facilities to investigate arboviruses (Vasilakis et al., 2009; Walker et al., 2014). In addition, they are routinely used to isolate and amplify arboviruses, specifically *Aedes albopictus*-derived C6/36 cells which are deficient in the primary antiviral pathway, RNA interference (Brackney et al., 2010). These systems are generated by macerating whole mosquito larvae or tissues and culturing amenable cells (Walker et al., 2014). This can be problematic because the culture may be composed of one or more unknown cell types. In addition, environmental contaminants such as insect-specific viruses (ISVs) may be unknowingly co-cultured as has been reported for *Drosophila* and tick cell lines (Bell-Sakyi and Attoui, 2013, 2016; Wu et al., 2010). In fact, ISVs have been identified in many mosquito species and both cell-fusing agent virus (CFAV; *Flaviviridae*) and Phasi-charoen like virus (PCLV; *Bunyaviridae*) have been identified in *Aedes aegypti* Aag2 cells (Maringer et al., 2017; Roundy et al., 2017; Schultz et al., 2018; Stollar and Thomas, 1975). Together these data suggest that commonly used mosquito cells may be persistently infected with unknown viruses and defining the culture virome will be critical to properly interpreting data generated in these systems.

In this study, we investigated the possibility that commonly used mosquito cell lines may be persistently infected with ISVs. Using an anti-dsRNA specific antibody, we performed immunofluorescence on

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uninfected cultures of Aag2 (*Ae. aegypti*), U4.4 (*Ae. albopictus*), C6/36 (*Ae. albopictus*) and Hsu (*Culex quinquefasciatus*) cells. We observed the presence of dsRNA in each cell line indicating the presence of ostensibly viral RNA. Subsequently, we sequenced RNA from these cell lines by next-generation sequencing (NGS) in order to better characterize the origins of this signal. We taxonomically categorized non-host sequences (Fauver et al., 2016) to identify full-length or partial viral sequences in all cell lines. We further detected viral RNA by RT-PCR in cell supernatant and/or cell lysates and in some instances, DNA forms of RNA viruses. Together, these data demonstrate that many commonly used mosquito cell culture systems are persistently infected with ISVs; results which should be considered when interpreting data generated in these cell lines.

2. Materials and methods

2.1. Cell lines

The *Cx. quinquefasciatus* ovary-derived Hsu et al. (1970), *Ae. albopictus*-derived C6/36 (Singh, 1967), and *Ae. aegypti*-derived Aag2 (Lan and Fallon, 1990; Peleg, 1968) cell lines were maintained at 28 °C with 5% CO_2 in MEM supplemented with 10% fetal bovine serum (FBS), 1× nonessential amino acids (100×; ThermoFisher Scientific), 1% L-glutamine, 1% 100× antibiotic-antimycotic (10,000 mg/ml of streptomycin, 10,000 U/ml penicillin, and 25 mg/ml of amphotericin B), and 5% of a 7.5% sodium bicarbonate solution. *Ae. aegypti*-derived U4.4 cells were maintained at 28 °C with 5% CO_2 in Mitsuhashi and Maramorosch insect medium supplemented with 7% FBS, 1× nonessential amino acids, L-glutamine, and antibiotics-antimycotics (10,000 mg/ml of streptomycin, 10,000 U/ml penicillin, and 25 mg/ml of amphotericin B). RNA was sequenced from three batches of C6/36 cells (two from Colorado State University and one from the Connecticut Agricultural Experiment Station) in order to provide insight into inter-laboratory variability. All three batches were originally acquired from ATCC. Hsu cells were generously provided by Dr. Robert Tesh (UTMB; ca. 2015), Dr. Paul Turner (Yale University; ca. 2015) generously provided the Aag2 cells, and the U4.4 were originally acquired from CSU.

2.2. West Nile virus infections

Mosquito cells were plated in 12-well plates at concentrations between 8.1×10^5 and 1.8×10^6 cells/well on poly-L-lysine treated coverslips. Cells were infected with West Nile virus (WNV) strain 10679-06 at a multiplicity of infection (MOI) of 0.1. Mock infected cells were treated with media. The inoculated plates were incubated at 28 °C for 1 h, with manual rocking at 15 min intervals, to allow for virus adsorption. After the incubation period, 1 ml of media was added to each well and plates were placed in a 28 °C incubator with 5% CO_2. Both the experimental and control cells were harvested either 24 or 72 h post infection (h.p.i.).

2.3. Immunofluorescence

Cells were fixed in well with 4% paraformaldehyde for 20 min at room temperature. Subsequently, cells were permeabilized (PBS + 0.3% TritonX100) for 10 min at room temperature and incubated with blocking buffer (5% BSA + 0.1% TritonX100) at 4 °C overnight.

Coverslips were placed in a humid chamber, 50 µl of primary anti-dsRNA antibody (J2) diluted 1:200 in blocking buffer was added to each, and incubated at room temperature for 1 h. Coverslips were washed three times in wash buffer (PBS + 0.1% Tween 20) and incubated with 50 µl of secondary antibody (Alexa-Fluor 555 α-mouse) in the dark for 1 h at room temperature. Coverslips were washed three additional times in wash buffer and mounted on glass slides with Prolong Gold anti-fade with DAPI counterstain. Slides were visualized on a Leica SP5 confocal microscope using the 405 nm laser (DAPI; nuclei) and 561 Argon laser (Alexa-Fluor 555; dsRNA) at 63× magnification. Brightness and contrast from resultant images were adjusted manually in Adobe Illustrator. All images were adjusted equally.

2.4. Next-generation sequencing of cellular RNA

RNA from cell lines was extracted using the Qiagen viral RNA kit and prepared for sequencing as previously described (Grubaugh et al., 2016). Briefly, each sample was DNase treated using Turbo DNase (Ambion). Total RNA was then non-specifically amplified and converted into dsDNA using the NuGEN Ovation RNA-Seq System V2. dsDNA was then sheared using the Covaris S2 Focused-ultrasonicator according to the manufacturer's recommendations. Sequencing libraries were prepared from sheared cDNA using NuGEN's Ovation Ultralow Library Kit according to the manufacturer's recommendations. Agencourt RNAclean XP beads (Beckman Coulter Genomics, Pasadena, CA) were used for all purification steps. Finished libraries were analyzed for correct size distribution using the Agilent Bioanalyzer High Sensitivity DNA chips (Agilent). 100 nt paired-end reads were generated using the Illumina HiSeq. 2500 platform at Beckman Coulter Genomics.

2.5. Virus discovery pipeline

An in-house virus discovery pipeline was used to identify novel viral sequences as previously described (Fauver et al., 2016). Briefly, reads were first trimmed with cutadapt version 1.13 (Martin, 2011) and then PCR duplicates were removed with CD-HIT-EST tool, version 4.6 (Li and Godzik, 2006). Sequences that mapped to the *Ae. aegypti* (GCF_002204515.2), *Ae. albopictus* (GCF_001876365.2), *An. Gambiae* (GCF_000005575.2), or *Cx. quinquefasciatus* (GCF_000209185.1) genome assemblies were then removed by alignment with Bowtie2 (Langmead and Salzberg, 2012). Remaining reads were assembled using the SPAdes genome assembler (Bankevich et al., 2012). The contigs produced were then aligned to the NCBI nucleotide database using BLASTn (Altschul et al., 1997; Camacho et al., 2009). Contigs that did not align at the nucleotide level with an e-value less than 10^{-8} were then used for a translation-based search against protein sequences using the DIAMOND (Buchfink et al., 2015). Contigs whose highest scoring alignments were to virus sequences were manually inspected in Geneious v11 (Kearse et al., 2012), and validated by mapping reads back to assemblies using Bowtie2 as above. Even after host filtering, we identified virus-like contigs that appeared to derive from cellular EVE sequences. We used several criteria to distinguish legitimate exogenous virus sequences from virus-like sequences that likely originated from cellular EVEs. These included: presence of intact open reading frames (EVE sequences commonly contain disrupted ORFS); Coverage over the entire genome; Presence of reads mapping to all genome segments for segmented viruses, and lack of a high degree of similarity to genomic sequences in other mosquito genomes.

2.6. Viral RNA/ DNA detection by PCR

Approximately 8×10^6 cells of each cell line (Aag2, C6/36, Hsu and U4.4) were harvested by scraping, equally divided into two separate tubes (one for RNA and one for DNA), and pelleted at 10,000xg at 4 C for 5 min. Cell supernatant was removed and placed in two separate tubes. DNA was extracted from cell pellets using the Zymo Quick gDNA mini-prep. Samples for RNA extraction were all treated with DNase (Promega, Madison, WI) prior to extraction to remove cellular DNA. One of the tubes of cell supernatant was also subjected to RNase A (Thermofisher, 100 µg/ml at 37 C for one hour) treatment to remove unencapsidated RNA. RNA was extracted from cell pellets, cell supernatant, and RNase A treated RNA using the Zymo DirectZol RNA extraction kit. cDNA was produced from extracted RNA using Protoscript II RT (NEB) using random hexamers. DNA or cDNA was then used for PCR or qPCR using OneTaq DNA polymerase (NEB) or iTaq SYBR green

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