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Running title: A mosquito densovirus contamination in Zika virus laboratory stocks

Detection and clearance of a mosquito densovirus contaminant from laboratory stocks of Zika virus

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Abstract

BACKGROUND: The Zika virus (ZIKV) epidemics that affected South America in 2016 raised several research questions and prompted an increase in studies in the field. The transient and low viremia observed in the course of ZIKV infection is a challenge for viral isolation from patient serum, which leads to many laboratories around the world sharing viral strains for their studies. C6/36 cells derived from *Aedes albopictus* larvae are commonly used for arbovirus isolation from clinical samples and for the preparation of viral stocks. OBJECTIVES: Here, we report the contamination of two widely used ZIKV strains by *Dipteran brevidensovirus 1* viruses, also known as the mosquito densovirus (MDV). METHODS: Molecular and immunological techniques were used to analyzed the MDV contamination of ZIKV stocks. Also, virus passages in mammalian cell line and infecting susceptible mice were used to MDV clearance from ZIKV stocks. FINDINGS: MDV contamination was confirmed by molecular and immunological techniques and likely originated from C6/36 cultures commonly used to grow viral stocks. We applied two protocols that successfully eliminated MDV contamination from ZIKV stocks, and

these protocols can be widely applied in the field. As MDV does not infect vertebrate cells, we performed serial passages of contaminated stocks using a mammalian cell line and infecting susceptible mice prior to re-isolating ZIKV from the animals' blood serum. MDV elimination was confirmed with immunostaining, PCR, and analysis of the mosquitoes that were allowed to feed on the infected mice. MAIN CONCLUSIONS: Since the putative impact of viral contaminants in ZIKV strains generally used for research purposes is unknown, researchers working in the field must be aware of potential contaminants and test viral stocks to certify sample purity.

Keywords: arbovirus isolation, contamination, C6/36 cultures, densovirus, vertebrate cells, Zika virus.

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Introduction

In the past few years, human Zika virus (ZIKV) infection has caused an increase in public health concerns due to an association with new clinical manifestations, such as *Guillain-Barré* syndrome and congenital neurological manifestations (1, 2). These concerns accelerated scientific research aimed at understanding the mechanisms by which the ZIKV interacts with its host to cause new clinical presentations.

Between 1947, when ZIKV was first reported in a Uganda forest, and 2015 (3), 124 articles were published regarding ZIKV. However, recent outbreaks and clinical manifestations associated with ZIKV infection resulted in more than 3,000 Zika-related published medical/scientific manuscripts during the 2016/2017 period. This increase in research was beneficial to the ZIKV field and added to our understanding of this new, emerging viral disease.

Arboviral isolation from clinical samples typically employs the use of mosquito cells, such as C6/36, from *Aedes albopictus* larvae (4). It is well known that mosquito cell lines can harbor contaminants including insect viruses, and the presence of contaminant viruses could induce cytopathic effects in insect cells, including syncytia formation or cell lysis, depending on the contaminant virus (5).

In this study, we identified the presence of two different *Dipteran brevidensovirus 1* viruses, also known as the mosquito densovirus (MDV), contaminating two ZIKV strains; one strain is of African origin, and the other strain is of Asian lineage. These strains were sent to our laboratory for research purposes. We also provide two simple strategies to remove MDV contamination from ZIKV strains using vertebrate cells as a bottleneck for MDV replication.

Material and Methods

Virus stocks production

Two different ZIKV strains were recently sent to our laboratory. The ZIKV strain of African origin was *strain A,* and the strain that resembled Asian origin was *strain B.* For didactic purposes, we identified both passages of these ZIKV strains as zero (P.0). Both P.0 viral supernatants were used to infect C6/36 cells (ATCC® CRL-1660™) that were cultured in L15 media supplemented with 5% FBS, 25 µg/mL gentamicin and 0,26% triptose (Thermo Fisher Scientific, Grand Island, New York, USA) at a multiplicity of infection (MOI) of 0.01 for viral stock production.

Molecular detection of Densovirus

Briefly, viral nucleic acids from C6/36 cell supernatants infected with each ZIKV strain were isolated using the RNeasy Mini kit (QIAGEN). For MDV DNA amplification (325 bp), the primers DNV3R (5'-TTTATTTCCATAGATATTGACTGTTTCGAT–3') and DNV3F (5'-AATCGAGAAACAGCATACTACACATTCGT–3') were used as previously described (6). These primers amplified a viral genomic region encompassing a small segment of the NS1 and NS2 genes of MDV. As a control for MDV amplification, a plasmid containing the same target gene from the BR/07 isolate was used.

Additionally, RT-PCR was used for the molecular detection of MDV. Briefly, total RNA from the supernatants and pellets of C6/36 cells was extracted using TRIzol reagent (Invitrogen). Blood samples from ZIKV infected mice were collected 1 to 4 days post inoculation, and RNA was extracted using TRIzol reagent (Invitrogen). A total of 500 ng of RNA was reverse transcribed using 300 ng of random primers. The resulting cDNA was used as a template for PCR with the primers DensoBR07_F (5'-ATTGTTGGGAGCATGACGGA–3') and DensoBR07_R (5'–CAACGGTTTGACCAGCGAAA–3') resulting in 212 bp of amplification. To test for the presence of Densovirus in the mosquitoes that fed on ZIKV infected mice, the total RNA from individual mosquitoes was extracted and pooled to prepare cDNA.

Zika virus detection by RT-PCR

ZIKV genomic RNA was detected by reverse transcription polymerase chain reaction (RT-PCR) (364 bp) using the primer set ZIKVENVF (5'–GCTGGDGCRGACACHGGRACT-3') and ZIKVENVR (5'–RTCYACYGCCATYTGGRCTG–3') as previously described (7, 8). RNA from the ZIKV strain ZV BR2015/15261 isolate (South Brazil, 2016) was used as a control for ZIKV E gene amplification.

Immunofluorescent virus detection

C6/36 cells ($2x10^4$ cells/well) were seeded in a 96-well plate and infected (in triplicate) with P.0 of ZIKV *strain A* and *strain B* at an MOI of 1. The MOI was based on the titration of ZIKV *strain A* and *strain B* in C6/36 using a pan-flavivirus monoclonal antibody that recognizes the E protein (4G2; ATCC® HB-112™). After 72 h, the cells were fixed and permeabilized with methanol:acetone (v/v) as previously described (6). For immunostaining, three different antibodies were used—an anti-flavivirus envelope (E) protein (4G2), an *in-house* mouse polyclonal antibody anti-MDV and an anti-MDV monoclonal antibody (clone 94DL1; IgG2a kappa) (6). A goat anti-mouse IgG Alexa Fluor 488 conjugate was used as secondary antibody, and digital images were taken with a fluorescence microscope (Leica DMI6000B) using LAS AF (Leica) software. As an MDV positive control, C6/36 cells were infected with MDV BR/07 (GenBank: GU452720) with a multiplicity of genome (MOG) of 0.01 for 48 h.

Densovirus nucleic acid sequencing

PCR fragments (325 bp) from MDV detection were purified using the High Pure PCR Product Purification Kit (Roche), and nucleotide sequencing was performed with primers for DNV3R and DNV3F by dideoxynucleotide termination sequencing at Macrogen Inc. (Seoul, South Korea). The sequences were assembled using the Assembler tool (http://www.hpabioinformatics.org.uk/cgibin/assembly_tool/seq_assemble.cgi?no=2) and aligned using ClustalW (9) as implemented in BioEdit software v.7.2.5 (10). The consensus sequence of densovirus *strain A* and *B* were deposited in GenBank under accession numbers, MH720336 and MH720337, respectively.

MDV removal from ZIKV samples

As MDV does not infect vertebrate cells, we performed serial passages of ZIKV *strain A* P.0 and *strain B* P.0 in A549 cells (lung epithelial cells; ATCC: CCL185). Briefly, A549 cells ($1x10^5$ cells/well in 24 well plates) were infected with 100 µL of ZIKV *strain A* P.0 or *strain B* P.0 for 90 minutes. After infection, cell monolayers were washed three times with 1X PBS and incubated in culture medium (DMEM-F12, 7% FCS, 100 IU/µg/mL of penicillin/streptomycin) for 72 h. The cell culture supernatants were collected and used (100 µL) to infect a new set of A549 cell cultures (second passage). An additional passage in A549 cells was performed as previously described (third passage). To confirm the exclusion of MDV after three passages in A549 cells, the cell culture supernatant of ZIKV *strain A* P.3/A549 and *strain B* P.3/A549 was passaged three additional times in the C6/36 mosquito cell line. All A549 and C6/36 cell passages were performed as described above. Nucleic acid was extracted from cell supernatants, and RT-PCR and PCR were performed for ZIKV and MDV, respectively.

Additionally, ZIKV *strain A* was used to infect A129 mice using a dose of $4x10^6$ PFU per individual by the intraperitoneal route. Blood samples were collected daily from 1 to 4 dpi, and the presence of MDV was tested as previously described. To certify that MDV was eliminated in mouse blood, 3 dpi *Aedes aegypti* females (5-7-day-old) were allowed to feed on ZIKV infected animals. MDV PCR was performed on mosquitoes at 4 days post feeding. A total number of 10 fed mosquitoes were used to test for the presence of MDV.

ZIKV infection to confirm MDV elimination

C6/36 cells were infected with a low (0.01) to high (10) MOI of ZIKV *strain A* P.3/C6/36 and ZIKV *strain B* P.3/C6/36. After 72 h, the supernatants and cell pellets were tested for the presence of MDV using a PCR assay as previously described.

Ethics

Experiments involving A129 mice were approved by the ethics committee at UFMG (CEUA 337/2016).

Results

Two different ZIKV strains (referred to as *strain A* and *strain B*) were recently sent to our laboratory from two different sources for research purposes. During the preparation of

viral stocks, visual inspection of C6/36 cell cultures revealed an atypical cytopathic phenotype that raised suspicion of potential contamination with an additional virus/microorganism (Figure 1A). Our previous experience with mosquito densovirus (MDV) contamination (6) showed similar cytopathic effects, so we performed molecular and immunological assays to check for possible contamination.

Using PCR, we amplified a segment of the MDV genome in ZIKV *strains A* and *B* to confirm coinfection with MDV (Figure 1B); an immunofluorescence assay was also performed. The immunofluorescence assay confirmed the presence of the ZIKV E-antigen in the cytoplasm and coinfection of both ZIKV *strains A* and *B* with MDV (Figure 1C). Furthermore, the inability of an anti-MDV monoclonal antibody to recognize *strain B* P.0 suggests that different MDV strains were coinfecting the ZIKV strains. To address this, we determined the nucleotide sequence of the MDV present in both ZIKV strains using PCR. Despite the short viral genomic region analyzed (325 bp), the nucleotide identity was 95.4% between the MDVs present in each ZIKV strain, which confirmed different viral strains while also suggesting that the contamination originated from different sources (Table 1); this could be explained by the different passage history of the two ZIKV strains. A comparison of the new MDV isolates with the MDV previously reported by our group (BR/07; GenBank: GU452720) shows a nucleotide identity of 98.4% with the sequence amplified from *strain A* and an identity of 96.2% with the one amplified from *strain B* P.0 (Table I). It is important to note that the C6/36 cell cultures in our laboratory are routinely checked for insect viral contaminations, including MDV, due to our reference laboratories activities for the Brazilian Ministry of Health.

MDV belongs to the Parvoviridae family and the *Brevidensovirus* genus (11). This nonenveloped virus presents a 4kB negative-polarity, single-stranded DNA genome (12). MDV is considered nonpathogenic for humans; however, MDV may be detrimental to mosquitoes (13, 14). Once we confirmed contamination of each ZIKV strain with MDV, we focused on strategies to eliminate it from the ZIKV samples to prevent interference with future experiments. It was previously demonstrated that MDV does not infect vertebrate cells (15, 16), so we performed serial passages of the ZIKV *strains A* and *B* using a ZIKV-susceptible A549 lung epithelial cell line (16, 17). After three passages of ZIKV *strains A* and *B* in A549 cells, the MDV coinfection was no longer detected in cell culture supernatants using PCR, while detection of a ZIKV envelope gene was successful (Figure 2A). Additionally, PCR for MDV and RT-PCR for ZIKV were performed after each passage (P1, P2 and P3) using nucleic acids extracted from the supernatants, and the results demonstrated that fragments of the NS1 and NS2 genes of MDV were not detected for *strain A* after the first passage (P1) in A549 cells or at the second passage (P2) for *strain B* (data not shown).

Additionally, to confirm the exclusion of MDV from ZIKV strains, we performed three additional passages of ZIKV *strain A* P.3/A549 and *strain B* P.3/A549 using the C6/36 mosquito cell line, as this cell line is susceptible and permissive to MDV. After the third passage in C6/36 cells, nucleic acid was extracted from the supernatants, and RT-PCR and PCR for ZIKV and MDV, respectively, were performed (Figure 2B). These results demonstrated that successive passages of MDV-contaminated ZIKV strains in A549 cells are effective for removing MDV contamination from ZIKV samples (Figure 2). An immunofluorescence assay was also used to confirm MDV exclusion from each ZIKV-

strain (Figure 2C). After passages in A549 and C6/36 cells, the cytopathic effects observed in C6/36 cells were no longer apparent compared to previous infections prior to the removal of MDV (Figure 1A and 2D). As infection with ZIKV induces cytopathic effects on C6/36 even after the elimination of MDV, some damage on C6/36 cells could be observed when compared to mock-infected cells (Figure 2D).

To confirm the elimination of MDV from each ZIKV strain, C6/36 cells were infected with a different MOI of ZIKV *strain A* P.3/C6/36 and ZIKV *strain B* P.3/C6/36. After 72 hs, the supernatants and cell pellets were tested for MDV using PCR. Even after infection with a high MOI (14), MDV was not detected in these ZIKV stocks. These results confirmed the efficiency of this protocol in the removal of MDV contamination from ZIKV strains (Figure 2E).

We also hypothesized that passing ZIKV isolates in susceptible mice would eliminate MDV. To test this hypothesis, type I IFN receptor KO (A129) mice were infected with contaminated stocks of ZIKV *strain B* (Figure 3A). Blood was collected from one to four days postinfection (dpi) and tested for MDV. As early as 1 dpi and throughout the kinetics, blood samples were negative for MDV. We also allowed *Aedes aegypti* to feed on the blood of infected mice at 3 dpi to further test for the successful elimination of MDV (since it would be amplified in mosquitoes even if present at low amounts). Mosquitoes that fed on infected mice were negative for MDV (Figure 3B). These results indicate that the passage of MDV-contaminated ZIKV-stocks in mice is also a suitable method to eliminate contamination. For all time points, the presence of MDV was tested in mice and mosquitoes, and we confirmed ZIKV RNA using RT-PCR (data not shown).

Discussion

The exchange of biological samples, such as viral isolates and cell lines, often occurs between research laboratories. Although this is important for scientific development, the certification of the microorganism strains or cell lines shared between laboratories is essential to avoid contamination problems. One of the most notorious cases of biological contamination in research laboratories is the contamination of cell lines with HeLa cells (18). Contamination of cell lines with MDV is not unusual, as this has already been demonstrated in the mosquito cell lines C6/36 and AP-61 (19). Although MDV infection can result in the development of cytopathic effects, the virus can also be unnoticed due to its ability to establish persistent infections without any clear cytopathic effects (16, 19, 20). Multiple authors have also demonstrated the ability of MDV to affect cell growth that is likely due to arrest of the cell cycle at the G2 phase (6, 21). Thus, it has been suggested that MDV could be used to control the mosquito population and have implications for the transmission of arboviruses (14, 22).

Years ago, contamination with MDV was verified in a Yellow fever virus isolate sent to our laboratory for diagnosis purposes (6). Although this was difficult to track, we suspect that the contamination origin began in contaminated cell cultures used for virus passages prior to both ZIKV strains being exhaustively shared between laboratories.

There are no studies addressing the effects of coinfections with MDV and ZIKV, and the outcome of such a coinfection is unknown; however, the negative impact of MDV in

dengue virus infection and replication was demonstrated *in vitro* and *in vivo*, further reinforcing the potential use of MDV for the biological control of arboviral infections (6, 22, 23). Given the similarities between DENV and ZIKV, it is plausible that MDV and ZIKV coinfection may affect *in vitro* and *in vivo* ZIKV infections. Conversely, studies using the C6/36 cell line and *Aedes aegypti* mosquito models have shown that coinfections with the chikungunya and densonucleosis viruses do not impact the infection and replication of either virus (24). Furthermore, MDV could induce the production of antibodies in BALB/C mice after immunization with Freund's complete adjuvant (first dose) and Alu-S-Gel (doses 2 to 4) (6). The potential impact of infecting mice with flaviviruses (dengue or ZIKV) contaminated with MDV is still an open question.

Since coinfections are not limited to ZIKV strains, researchers who work in the arbovirology field should check their cell lines and viral stocks periodically to avoid contamination with arthropod viruses such as MDV. The main purpose of reporting these findings is to call the attention of the scientific community of the potential presence of mosquito virus contaminants in ZIKV strains/stocks. We also suggest two simple strategies to efficiently eliminate MDV contamination from ZIKV strains/stocks and *in vitro* and *in vivo* passages in vertebrate cell lines or mice models, respectively. Finally, the potential interference of MDV contamination in ZIKV isolates needs further analysis.

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Author contributions

AC and DK performed all *in vitro* assays; AM performed the sequence analysis; ES, AF and JM performed *in vivo* assays; and PW, CS and JB participated in the experimental design. All of the authors helped to draft the manuscript and approved the final text.

Conflict of interest statement

All authors declare that they have no conflict of interest.

References

(1) Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, et al. Zika Virus Associated with Microcephaly. N Engl J Med. 2016; 374: 951–958.

(2) Santos T dos, Rodriguez A, Almiron M, Sanhueza A, Ramon P, Oliveira WK de, et al. Zika Virus and the Guillain – Barré Syndrome — Case Series from Seven Countries. N Engl J Med. 2016; 375: 1598–1601.

(3) Dick GWA, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952; 46: 509–520.

(4) Tesh RB. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am J Trop Med Hyg. 1979; 28: 1053-9.

(5) Hirumi H, Hirumi K, Speyer G, Yunker CE, Thomas LA, Cory J, et al. Viral contamination of a mosquito cell line, *Aedes albopictus*, associated with syncytium formation. In Vitro. 1976; 12: 83-97.

(6) Mosimann ALP, Bordignon J, Mazzarotto GC, Motta MC, Hoffmann F, Santos CN. Genetic and biological characterization of a densovirus isolate that affects dengue virus infection. Mem Inst Oswaldo Cruz. 2011; 106: 285-92.

(7) Faye O, Faye O, Dupressoir A, Weidmann M, Ndiaye M, Sall AA. One-step RT-PCR for detection of Zika virus. J Clin Virol. 2008; 43: 96-101.

(8) Zanluca C, Melo VC, Mosimann AL, Santos GI, Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015; 110: 569-72.

(9) Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 1994; 22: 4673-80.

(10) Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symp Ser. 1999; 41: 95-98.

(11) Bergoin M, Tijssen P. Molecular biology of Densoviriniae. *In*: Faisst S, Rommelare J, Parvoviruses from molecular biology to pathology and therapeutic uses. Basel: Karger; 2000. 12-32.

(12) Afanasiev BN, Galyov EE, Buchatsky LP, Kozlov YV. Nucleotide sequence and genomic organization of *Aedes* densonucleosisi virus. Virology. 1991; 185: 323-336.

(13) Suchman EL, Kononko A, Plake E, Doehling M, Kleber, B, Black WC IV, et al. Effects of AeDNV infection on *Aedes aegypti* lifespan and reproduction. Biol Control. 2006; 39: 465-473.

(14) Carlson J, Suchman E, Buchatsky L. Densovirus for control and genetic manipulation of mosquitoes. Adv Virus Res. 2006; 68: 361-392.

(15) El-Far M, Li Y, Fédiére G, Abol-Ela S, Tijssen P. Lack of infection of vertebrate cells by the densovirus from the maize worm *Mythimna loreyi* (MDNV). Virus Res. 2004; 99: 17-24.

(16) Jousset FX, Barreau C, Boublik Y, Cornet M. A parvo-like virus persistently infecting a C6/36 clone of *Aedes albopictus* mosquito cell line and pathogenic for *Aedes aegypti* larvae. Virus Res. 1993; 29: 99-114.

(17) Frumence E, Roche M, Krejbich-Trotot P, El-Kalamouni C, Nativel B, Rondeau P, et al. The South Pacific epidemic strain of Zika virus replicates efficiently in human epithelial A549 cells leading to IFN-β production and apoptosis induction. Virology. 2016; 493: 217-26.

(18) Horbach SPJM, Halffman W. The ghosts of HeLa: How cell line misidentification contaminates the scientific literature. PLoS One. 2017; 12: e0186281.

(19) O'Neill SL, Kittayapong P, Braig HR, Andreadis TG, Gonzalez JP, Tesh RB. Insect densoviruses may be widespread in mosquito cell lines. J Gen Virol. 1995; 8: 2067-74.

(20) Gorziglia M, Botero L, Gil F, Esparza J. Preliminary characterization of virus-like particles in a mosquito (*Aedes pseudoscutellaris*) cell line (Mos. 61). Intervirology. 1980; 13: 232-40.

(21) Paterson A, Robinson E, Suchman E, Afanasiev B, Carlson J. Mosquito densonucleosis viruses cause dramatically different infection phenotypes in the C6/36 *Aedes albopictus* cell line. Virol. 2005; 337: 253-61.

(22) Wei W, Shao D, Huang X, Li J, Chen H, Qinfen Zhang, et al. The Pathogenicity of Mosquito Densovirus (C6/36DNV) and Its Interaction With Dengue Virus Type II in *Aedes albopictus*. Am Soc Trop Med Hyg. 2006; 75: 1118–1126.

(23) Burivong P, Pattanakitsakul SN, Thongrungkiat S, Malasit P, Flegel TW. Markedly reduced severity of Dengue virus infection in mosquito cell cultures persistently infected with *Aedes albopictus* densovirus (AalDNV). Virology. 2004; 329: 261–269.

(24) Sivaram A, Barde PV, Gokhale MD, Singh DK, Mourya DT. Evidence of co-infection of chikungunya and densonucleosis viruses in C6/36 cell lines and laboratory infected *Aedes aegypti* (L.) mosquitoes. Parasites & Vectors. 2010; 3: 95.

Table I: Nucleotide identity matrix comparing the two MDVs presented on ZIKV-isolates.

	GU452720	**ZIKV *strain A***	**FJ805445**	**ZIKV *strain B***
GU452720	100%	98,4%	97,3%	96,2%
ZIKV *strain A*	98,4%	100%	96,6%	95,4%
FJ805445	97,3%	96,6%	100%	98,8%
ZIKV *strain B*	96,2%	95,4%	98,8%	100%

Note: GU452720 = Mosquito densovirus BR/07; FJ805445 = Culex densovirus 0507JS11.

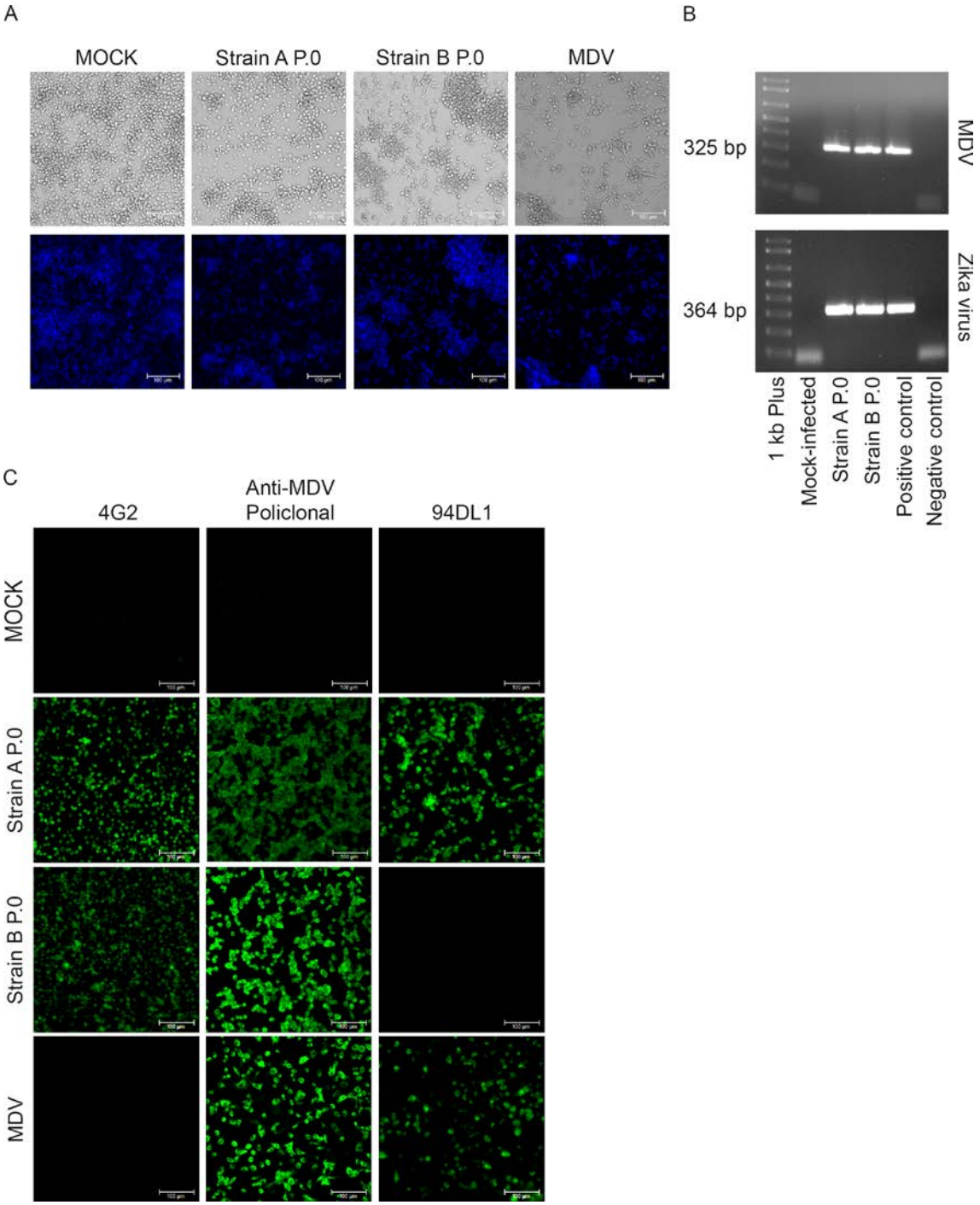

Figure 1: Mosquito densovirus (MDV) coinfection in ZIKV *strains A* P.0 and *B* P.0. (**A**) Cytopathic effects in C6/36 MDV-contaminated ZIKV *strain A* and *strain B* cells compared to mock-infected cells and C6/36 cells infected with MDV BR/07 at an MOG of 0.01 for 48 h. (**B**) Agarose gel showing PCR amplification of a 325 bp fragment from the MDV genome and RT-PCR amplification of a 364 bp fragment of the ZIKV E gene

in *strain A* and *strain B* (P.0). (**C**) Immunofluorescence assay of ZIKV *strain A* and *strain B* (P.0) infected C6/36 cells stained with a 4G2 monoclonal antibody, anti-MDV mouse polyclonal serum, and monoclonal antibodies (clone 94DL1) raised after immunization with the MDV BR/07 strain. As a positive control, C6/36 cells were infected with MDV BR/07 at an MOG of 0.01 for 48 h.

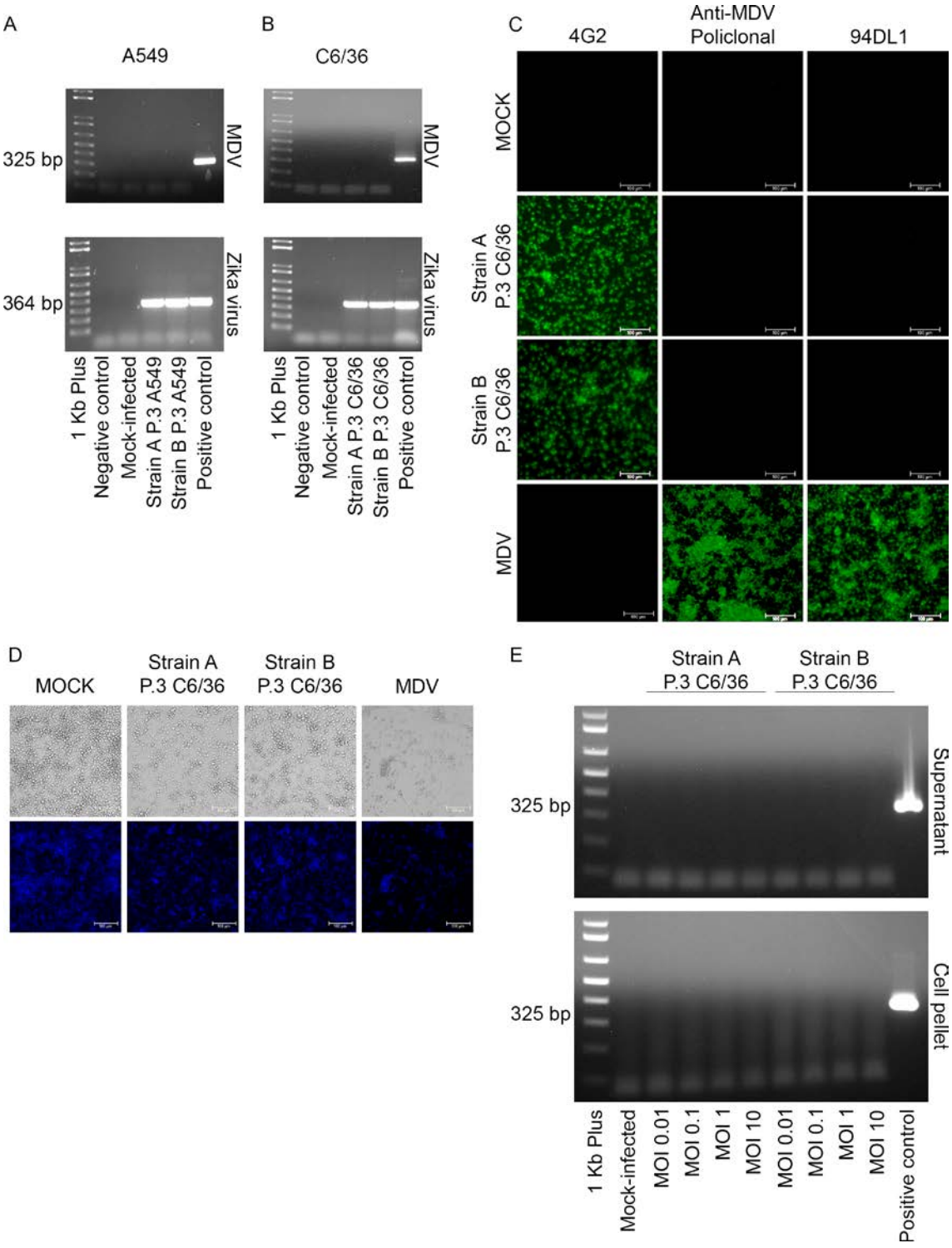

Figure 2: Mosquito densovirus (MDV) clearance from the ZIKV *strains A* and *B* (P.0). Agarose gels showing PCR amplification of a 325 bp fragment from MDV and RT-PCR amplification of a 364 bp ZIKV E gene fragment in ZIKV *strain A* and *strain B* after three passages in A549 cells (P.3/A549) (**A**) followed by three passages in C6/36 cells

(P.3/C6/36) (**B**). (**C**) Immunofluorescence assay in C6/36 cells infected with ZIKV *strain A* and *strain B* (P.3/C6/36) after three passages in C6/36 cells stained with 4G2 monoclonal antibody, anti-MDV mouse polyclonal serum, and anti-MDV monoclonal antibody (clone 94DL1). (**D**) Cytopathic effects on C6/36 cells infected with ZIKV *strain A* and *strain B* after three passages in C6/36 compared to mock-infected cells. Infection with the MDV BR/07 strain (MOG 0.01 for 48 h) was used as positive control for immunofluorescence and cytopathic effect assays. (**E**) Agarose gel showing PCR amplification of a 325 bp fragment from MDV. C6/36 cells were infected with different MOIs (0.01, 0.1, 1 and 10) of ZIKV *strain A* and *strain B* after three passages in C6/36 cells (P.3 C6/36). Cell pellets were obtained, and nucleic acid was extracted from the supernatant of both cell cultures and tested for the amplification of MDV genes by PCR. As a positive control, a plasmid containing the same target gene from the isolate BR/07 was used (325 bp).

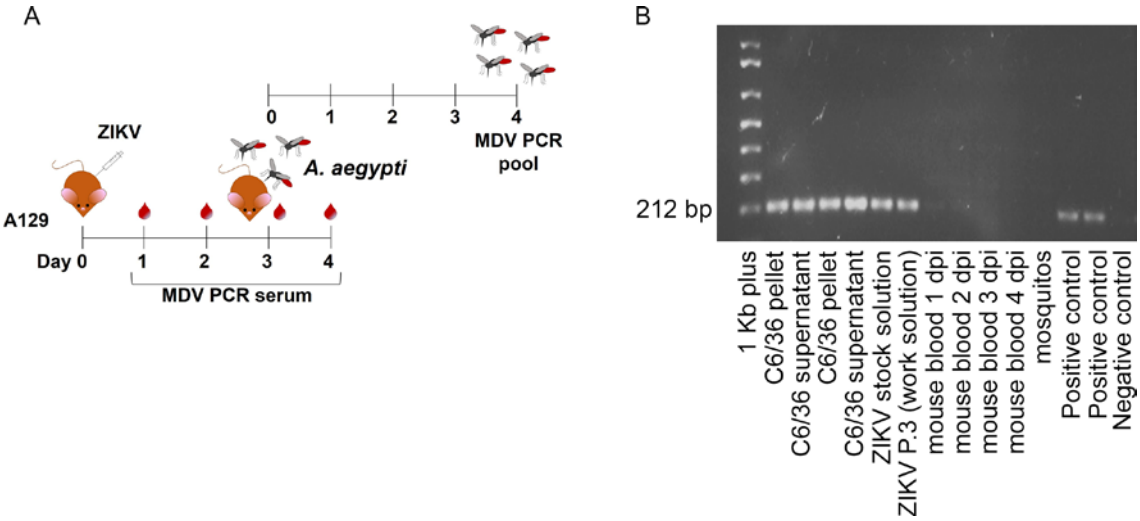

Figure 3: Infection of A129 mice provide a reliable strategy for clearance of MDV-contamination. (**A**) Experimental design for *in vivo* assays. (**B**) Agarose gel showing RT-PCR amplification of a 212 bp fragment from MDV in the blood of A129 mice infected with $4x10^6$ PFU of ZIKV *strain A* at different days post infection (dpi) and in *Aedes aegypti* mosquitoes that fed on infected animals. As a control, ZIKV *strain A* stocks and C6/36 cells (pellets and culture supernatants were used) together with a plasmid control were tested for MDV.