Original Article

Skeletal muscle cells of chicken embryos generating the Yellow Fever vaccine virus

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ABSTRACT

The yellow fever vaccine is produced by inoculation of the YF17DD virus in embryonated chicken eggs on the ninth day of development. Full embryos are collected on the twelfth day of development for vaccine formulation. The skeletal muscle tissue is the main site where biosynthesis of viral particles occurs, thus displaying a myoblast-like morphology when first cells are infected. The present work phenotypically characterizes these cells as myogenic precursors expressing the Pax7 transcription factor in some cases. We demonstrate that skeletal chicken embryo muscle cells are susceptible to *in vitro* infection in different MOI´s thus reproducing the same infection pattern observed *in vivo.* Furthermore, myogenic precursors and myoblasts are preferred infection targets, but settlement of infection does not depend on the presence of these cells. The peak of viral production occurs in 48 hpi, with decay occurring in 72 hpi, when the cytopathic effect can be observed. These findings allow us to conclude that the primary culture of chicken

skeletal muscle cells is a good model for studying muscle cells infected with YF17DD virus, due to its satisfactory emulation of the *in vitro* phenomenon observed thus contributing to understand virus infection dynamics and leading to the development of alternative methods of vaccine production.

Key words: Yellow Fever; Vaccine; 17DD, Chicken embryos; Muscle cell; Cell culture.

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Introduction

The Yellow Fever virus (YFV) is a prototype of the *Flaviviridae* family that also includes Dengue, West Nile, Japanese Encephalitis and Zika virus among other viruses. Many flaviviruses are pathogenic to humans and are transmitted by arthropods, especially mosquitoes (Pastorino et al. 2010), as an example we can point Yellow Fever (YF) affecting Sub-Saharan African and South American populations. Most affected individuals present mild symptoms, similar to influenza and rarely developing the severe form of the disease. These patients with this severe form present fever, hemorrhage, myocardial, kidney and hepatic injuries and shock, with a mortality rate of about 50% (Barrett et al. 2009). The best way to prevent YF is by mass vaccination of people who live in or who will travel to areas where the disease is endemic. The YF vaccine is highly effective, offering lifetime protection with a single dose (WHO 2015). In spite of that, YF is still an important cause of morbidity and mortality in Africa and South America. Most affected are inhabitants of endemic and epizootic areas, unvaccinated travelers, and people to whom the vaccine is contraindicated, such as those with allergies to chicken egg proteins, children younger than 9 months old, adults older than 60 years old and

immunosuppressed individuals (Monath & Cetron 2002, Khromava et al. 2005, WHO 2015).

Production of the yellow fever vaccine is made by inoculation of substrain YF17DD virus into embryonated chicken eggs according to standard protocols established by the World Health Organization (Monath 2005). Although chicken embryos have been used since 1937 as biological systems for the production of YFV (Barrett et al. 2009), tissues and cell targets responsible for the biosynthesis of viral particles have only been described very recently (Manso et al. 2015). Moreover, the skeletal muscle is the main infected tissue in chicken embryos, suggesting that muscle cells would be a major site for production of viral particles (Manso et al. 2016).

In the present manuscript we analyze the muscle skeletal tissue of chicken embryos infected by YF17DD virus to better characterize the first infected cells. Also, we evaluate the *in vitro* YF17DD infection of primary cell cultures of skeletal muscle cells of chicken embryos, defining the best conditions to the establishment of *in vitro* infection, including kinetics of viral production in the culture system as well as the peak of viral production. Both *in vivo* and *in vitro* processes of viral infection are similar in terms of early cell targets and evolution of tissue infection. Finally, the data found contribute to the understanding of infection in chicken embryos and thus supporting the development of an alternative method for producing the YF attenuated vaccine.

Material and methods

Histopathological analysis

Embryonated eggs were infected in the yolk sac with 17DD EPlow virus seed lot (1–5 x 10^3 PFU per inoculum) in the ninth day of development. Eggs were kept in IP70 brooder (Premium Ecologica, Brazil) with controlled temperature at 37.5°C and 55% relative air humidity. As negative controls, embryos kept under the same conditions were inoculated in their yolk sacs with water. All embryos were euthanized at 48 hours post infection (11 days of development). The experiment was carried out using three infected embryos and the same number of controls.

Histopathological analysis was performed under a brightfield microscope. Firstly, embryo trunks were cleaved transversely in 3 mm sequential sections and separated from the head, wings and legs. All samples were fixed in Carson`s Millonig formalin (Carson et al. 1973) for 48 hr and processed according to standard histological techniques for paraffin embedding. At least three sections (5 μm thick) from each block were stained with hematoxylin-eosin and analyzed under an Axio Observer Z1 microscope (Carl Zeiss, Germany) equipped with a mRC5 Axiocam digital camera (Carl Zeiss, Germany).

Cell culture establishment and infection

Primary cultures of myogenic cells were prepared from breast and leg muscles of non-infected 11-day-old chicken embryos. Pool of cells from 3-6 embryos was used to perform the cultures. Tissues were collected, dissected out and incubated at 37°C for 10 min in 0.05% trypsin solution (Gibco). After removal of trypsin, the resulting suspension was filtered, and cells were plated at a density of 4×10^5 cells/1.9 mm culture dishes onto coverslips previously coated with 2 % gelatin. Muscle cells were maintained

in MEM medium enriched with 10 % horse serum, 5 % chicken embryo extract, 100 U/mL penicillin and 100 µg/mL streptomycin. After culture settlement, cells were exposed to YF17DD virus extracted from infected embryos provided by the vaccine production unit (Biomanguinhos). Four points of infection were performed: at seeding and 5, 24 and 120 hr after plating, named T0, T5, T24 and T120, respectively. T0 cultures were exposed to YF17DD virus when myogenic precursors and myoblasts were just removed from tissues. T5 cultures were exposed to YF17DD virus when those myogenic precursors and myoblasts adhered to coverslips. In T24 cultures, young fibers were exposed to YF17DD virus when most muscle cells differentiated (Wakelam 1985). T120 cultures were exposed to YF17DD virus when differentiated fibers and fibroblasts were detected in the culture, with less differentiated muscle cells (Wakelam 1985). For each protocol, medium was removed, and cells were infected with virus at 0.1, 0.01 and 0.002 MOI by adsorption at 37 °C for 1 hr. Then, medium with virus was removed and exchanged for fresh complete MEM.

Immunofluorescence assay

Sections of all paraffin blocks were submitted to immunofluorescence assay, as previously described (Manso et al. 2015). Also three coverslips from infected and control cell cultures were fixed in Carson`s formalin-Millonig (Carson et al. 1973) for 10 minutes at room temperature, permeabilized with 0.1 % Triton X-100 (Merck, USA). Slides and coverslips were incubated with a mouse polyclonal antibody (anti-yellow fever virus, Evandro Chagas Institute, Brazil). Double or triple staining were performed in some samples using anti-desmin (cat. RB-9014, Thermo Scientific, USA), anti-myosin (cat. Ab124205, abcam, USA) and anti-Pax7 antibodies (Developmental Studies Hybridoma

Bank, USA). As secondary antibodies, AlexaFluor 488-conjugated goat anti-mouse (cat. A11001, Invitrogen, USA), AlexaFluor 546-conjugated goat anti-mouse (cat. A11003, Invitrogen, USA) or AlexaFluor 635-conjugated goat anti-rabbit (cat. A31576, Invitrogen, USA) were applied at 37°C for 1 hr, followed by counterstaining with 1:5,000 DAPI (cat. 03571, Molecular Probes, USA). Negative controls were processed by omitting treatment with primary antibodies. All sections and coverslips were analyzed under fluorescence microscope Axio Observer Z1 coupled with Colibri system (Carl Zeiss, Germany). Some specific fields were analyzed under LSM 710 confocal microscope (Carl Zeiss, Germany) and Elyra PS.1 superresolution microscope (Carl Zeiss, Germany).

Viral titration

Supernatants of control and infected cultures (pool of three samples/experiment - three experiments) from each condition were submitted to plaque-forming unit assay (PFU). Vero cells were plated at a density of 1 x 10^5 cells/mL in 24-well plates and supernatants were serially diluted from 10^{-1} to 10^{-6} in Earles's 199 medium (Thermo Scientific, USA). After 1 hr incubation, the inoculum was removed and overlay Earles's 199 medium with 3.5% carboxymethylcellulose (Merck), 5% $NaHCO_2$ and 5 % bovine fetal serum (Thermo Scientific, USA) were added. Following 7 days incubation at 37°C and 5 % CO_2, cells were fixed in 10% formaldehyde and subsequently stained with 0.01% crystal violet. Results obtained were expressed in PFU/mL. The statistical analysis was performed using Kruskal-Wallis test and GraphPad software.

Polymerase Chain Reaction

RNA samples were extracted from cell culture dishes, either positive or negative to YF17DD virus, with Trizol (Thermo Fischer, USA) according to the manufacturer's recommendation. Then 0.1 mL chloroform per sample was added, followed by 100 % isopropanol, 70 % ethanol and ultrapure water, with washes between steps. Samples eluted after the procedure were amplified by reverse transcription-PCR (Thermoscript RT-PCR kit—cat. 11146016, Life Technologies, USA) with universal Flavivirus primers described by Tanaka (Tanaka 1993) (YF1–5`GGTCTCCTCTAACCTCTAG 3` and YF3-5`GAGTGGATGACCACGGAAGACATGC 3`). At the end, amplicons were submitted to electrophoresis. Samples with bands with 675 bp were considered positive.

Cell density assay

Chicken embryo skeletal muscle cells infected in culture with YF17DD virus at 0.002 MOI and non-infected (controls) were evaluated for cell density at 24, 48 and 72 hours post infection (hpi). After removing culture medium, cells were fixed for 10 minutes in Carson buffered formalin (Carson et al. 1973) 3.7% at room temperature and washed twice in PBS. Cells were then stained with 200µl of 1% crystal violet solution for 10 minutes also at room temperature. Following staining, samples were washed with several exchanges of distilled water. Dye was extracted with 200µl absolute methanol for 10 minutes. Afterwards, 100 µl of this supernatant was collected, placed in a 96-well plate and analyzed in a spectrophotometer at 595 nm.

Ethics statement

Specific pathogens-free (SPF) fertilized White Leghorn chicken eggs (*Gallus gallus domesticus*; Linnaeus, 1758) were obtained from the YF vaccine production unit

(Instituto de Tecnologia em Imunobiológicos - Biomanguinhos, Fiocruz, Rio de Janeiro, Brazil). All experiments were in accordance with the yellow fever vaccine production protocol, applied since 1937, when vaccine production started at Biomanguinhos, under ethical approval of Fiocruz. In addition, all procedures involving animal experimentation were performed as approved by the Ethics Committee (CEUA/IOC), under license numbers: L-025/2017 and L-028/2017.

Results

Immunophenotyping of skeletal muscle cells at initial stage of infection

Immunofluorescence assay of skeletal muscle at 48 hpi showed that the first cells to appear infected were $Pax7^+$ (Figs 1A-G, orange arrows). These cells were observed between infected (Figs 1A, D and F, white arrows) and non-infected (Fig 1G, blue arrow) muscle fibers, sometimes apparently fused with them (Figs 2A-F). Also, viral particles accompanying fiber striation pattern were observed (Fig 1H).

In vitro exposure of skeletal muscle cells to Yellow Fever virus in different stages of differentiation

Primary muscle chicken embryo cell cultures were susceptible to YF17DD virus in all infection protocols (T0, T5, T24 and T120). For these experiments, cells were infected with 0.1 MOI. Under all conditions some infected myoblasts (Fig 3A, pink arrow), myocytes (Fig 3D, yellow arrow) and new fibers (Fig 3G, white arrow) at 24 hpi were observed. A marked increase in the number of myocytes (Fig 3B yellow arrow) and infected fibers (Fig E, white arrow), with these infected myocytes trying to fuse to non-infected (Fig 3E, yellow and blue arrows) and infected muscle fibers (Fig 3H, yellow and

white arrow) were observed at 48 hpi. With 72 hpi, some infected muscle fibers (Figs 3C, F and I, white arrows) thinner then controls (Fig 3L, blue arrow), and uninfected fibroblasts (Figs 3C and I, green arrows) accompanied of few uninfected muscle fibers (Figs 3C and I, blue arrows) were observed.

Corroborating morphological data, initial production of infectious particles at 24 hpi was observed. Highest level of production was observed after 48 hpi, followed by a small decrease of viral particles production at 72 hpi. Infection in T0 presented the most efficient production when compared to T5 and T24 (Fig 4).

Role of myogenic precursors and myoblasts at in vitro infection settlement

To determine if myogenic precursors and myoblasts were essential for *in vitro* infection settlement, T120 cultures were exposed to YF17DD virus at 0.1 MOI. In this protocol, muscle cell cultures were totally differentiated and mature, with robust and large fibers, and muscle cells were also susceptible to infection, even in the absence of myogenic precursors and myoblasts (Figs 5A-C, white arrows). Viral proteins following fibers striation (Figs 5D and E, red arrows) and perinuclear deposition (Figs 5D and E, yellow arrows) were observed at 48 hpi.

Analysis of virus production by muscle cell culture when infected with different MOIs

Aiming at analyzing if muscle cells were also susceptible and permissive to infections in lower MOIs, both 0.01 and 0.002 MOI, were tested in addition to 0.1 MOI. For these analyses only T0 protocol was performed. Infection with 0.01 MOI was morphologically

similar to 0.1 MOI in 24, 48 and 72 hpi (Figs 6A-C), strongly contrasting to what was observed in 0.002 MOI (Figs 6D-F). Infection with 0.002 MOI presented the most efficient production when compared to 0.1 and 0.01 MOI (Fig 7). At 24 hpi only a few infected myoblasts (Fig 6D, pink arrow) were observed, but at 48 hpi almost all muscle cells in culture were infected (Fig 6E, green marker), followed by intense cytopathic effect at 72 hpi, when only non-infected fibroblasts survived (Fig 6F, green arrows). These results corroborate cell density observed in the culture during the course of infection. At 24 and 48 hpi about 20% decrease in cell density of infected cultures was observed compared to control cultures. Loss increased to 43 % at 72 hpi and could be associated with cytopathic effect demonstrated by morphological data (Fig 8). Infection was confirmed by detection of genomic viral RNA and intermediate replicative RNA (Supplementary data).

Corroborating morphological data, initial production of infectious particles at 24 hpi was observed. The highest level of production was observed at 48 hpi, followed by a decrease of viral particles production at 72 hpi (Fig 7).

Discussion

In the present manuscript, we demonstrate that chicken skeletal muscle cells are susceptible to *in vitro* infection by YF17DD virus and Pax7 positive cells play a central role in muscle infection both *in vivo* and *in vitro.* This model reproduces *in vivo* chicken infection, where muscle fibers and myogenic progenitors are infected thus corroborating previous data demonstrated by our group (Manso et al. 2015, 2016). In these previous papers staining of viral proteins in perinuclear clusters was observed, following muscle fiber striations, a pattern also observed here. Manso *et al.* described that the first cells to appear infected showed morphology suggestive of myoblasts (Manso et al. 2016). We

showed by Pax7 immunostaining of tissue samples that these cells are actually myogenic precursors meaning that they are committed with myogenic lineage but have not yet entered the myogenic program. Preferential infection of myogenic precursor cells was also observed in Chikungunya virus (Ozden et al. 2007). Susceptibility of $Pax7^{+}$ myogenic precursors was also observed *in vitro*, although most cells undergo differentiation into myoblasts or myocytes in this system. However, it is possible that these cells were infected at precursor stage, following differentiation program. At observation time they entered myogenic program, failing to express Pax7 factor (Bentzinger et al. 2012).

Regarding the establishment of muscle cell culture, cells that are isolated by trypsinization of skeletal striated muscle from chicken embryos have spherical morphology and quickly adhere to the substrate (Shimada 1971). In our experience this adhesion occurs between approximately 4/5 hours, considering a 2% gelatin coating. This adhesion is confirmed by shaking the plate and seeing that the cells seem static. Initially, these cells have an intense mitotic and migratory behavior and, soon after adhesion, some cells begin to present an elongated shape, which will fuse to form myotubes (Shimada 1971). The time at which fusion occurs *in vitro* may vary according to medium composition and cell density (Wakelam 1985). Usually, with 17 hours of plating, chicken embryo myoblasts enter G0 and begin to align. At 24 hours after plating, well-demarcated spindle-shaped cells can be observed, and from this point the fusion of these cells begins to form multinucleated fibers (Wakelam 1985). The fusion peak occurs within a 10 hour period, starting after 20 hours of plating, i.e. approximately between 20 - 30 hours of culture (O'Neill 1972). However, 43 hours after plating, this fusion can still be observed (Wakelam 1985). After 72 hours of plating, the cells have finished fusing and the culture is completely differentiated (O'Neill 1972, Wakelam 1985). The greatest maturation of

these newly formed fibers occurs later, around one week after plating (Inestrosa 1982). It is possible to determine each phase of cell differentiation based on the transcription factors being expressed (Bentzinger et al. 2012), cell morphology (Konigsberg 1963, Inestrosa 1982) and also by the expression of muscle-specific proteins on the cytoskeleton (Lin et al. 1994). Importantly, the primary muscle cell culture is a mixed culture composed of fibroblasts and muscle cells. Fibroblasts can be identified by their morphology and by the absence of muscle markers.

We also determined the best harvest point of viral particles production, the best MOI and best infection protocol. Analyzing *in vitro* infection kinetics independently of inoculation protocol or MOI used, the initial production of viral particles occurs in the first 24 hpi and the highest production level happens at 48 hpi, followed by a titter decrease at 72 hpi. This kinetics resemble what could be observed in Influenza A infection (Desdouits et al. 2013). Yellow fever viral particles produced by muscle cells hold infectious potential, able to infect new cultures. Cell death observed at 72 hpi corroborated decrease in cell density at this point of infection showing direct relationship between cytopathic effect, reduction of cell density and decrease of infectious viral particles. The most effective production of these particles was achieved by the lowest MOI inoculation (MOI 0.002). Since the inoculation 5x (MOI 0,01) or 50x (MOI 0,1) less viral particles represented no statistical difference, MOI 0.002 is the best relation between inoculum and viral production. It could happen due to lower virus concentrations inducing lower levels of interferon production, what may allow a more appropriate establishment of infection. Considering the moment of infection, even though there is no statistical difference between protocols of infection, T0 protocol presented slightly higher titer at 48 hpi compared with T5 and T24 at 48 hpi. Also, T0 protocol seems to be a better choice for vaccine production due to less manipulation. *In vivo* interaction between the virus and

muscle cells is best represented by the T0 infection protocol, since the cells were newly isolated from the tissue. Therefore, the slightly greatest viral titer in this protocol can be explained by the fact that the muscle cells are in their most naive state. As the culture proceeds, these cells tend to differentiate. At T5, instead, cells adhered to the culture plate and, also, in T24 muscle cells aligned, preparing to fuse (Wakelam 1985). Therefore, the slightly higher titter in T0 reinforces the hypothesis that less differentiated muscle cells may play an important role in YFV infection.

In light of these results, we sought to understand whether undifferentiated muscle cells are necessary for the establishment of infection, or mature muscle fibers become infected independently of immature cells. To achieve this goal, we infected muscle cell cultures after 120 hr of plating (T120), when these cultures are composed of fibroblasts and muscle fibers but myoblasts, myocytes and myogenic progenitors are no longer observed (Wakelam 1985). We then discussed that since infected cells were found after 48 hpi in this condition, more differentiated muscle cells (fibers) are also susceptible to the infection. Then, the success of infection in this protocol suggests that muscle cells are susceptible of infection in all stages of differentiation; i.e. myogenic progenitors and myoblasts seem not to be essential for establishment of infection, although it starts preferentially by very undifferentiated cells.

Besides a better understanding of the YF17DD virus infection in muscle chicken embryos, our data could be a keystone for the development of an alternative method to produce a Yellow Fever vaccine, since demonstration of susceptibility and permissiveness of chicken embryos skeletal muscle cells *in vitro* by YF17DD virus is described here for the first time. Some attempts based on a cell culture system, were unsuccessfully tested using chicken embryo fibroblasts (CEF) and Vero cells (Freire et

al. 2005, Gaspar et al. 2008, Monath et al. 2011, Pereira et al. 2015). Regarding aspects of vaccine production, chicken muscle cell lineage is not suitable for vaccine production once it was immortalized with the simian vacuolating virus 40 (AcceGen Biotech cat.#ABI-TC300D). Primary culture from chicken embryo muscle cells are then an interesting model due to the maintenance of the animal model used in current production thus reproducing *in vitro* what occurs *in vivo.* The production of Yellow Fever vaccine in cell culture system presents advantages over production in embryonated eggs since it would decrease production costs, reduce the number of animals euthanized and thus lowering the amount of chicken protein per dose (Freire et al. 2005). Altogether, it is possible that skeletal muscle chicken embryo cells could sustain the production of yellow fever virus, initially on a small scale. Nevertheless, improvements of this production must be performed to expand the capacity of viral particle production by muscle cells in culture.

Conclusion

The skeletal muscle cell culture model seems to reproduce the pattern of infection observed *in vivo*, suggesting that this would be a satisfactory infection model to study yellow fever virus and a potential model for developing a vaccine candidate.

Conflict of interest statement

The authors have declared that no competing interests exist.

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Author's contribution:

All authors contributed equally to this manuscript.

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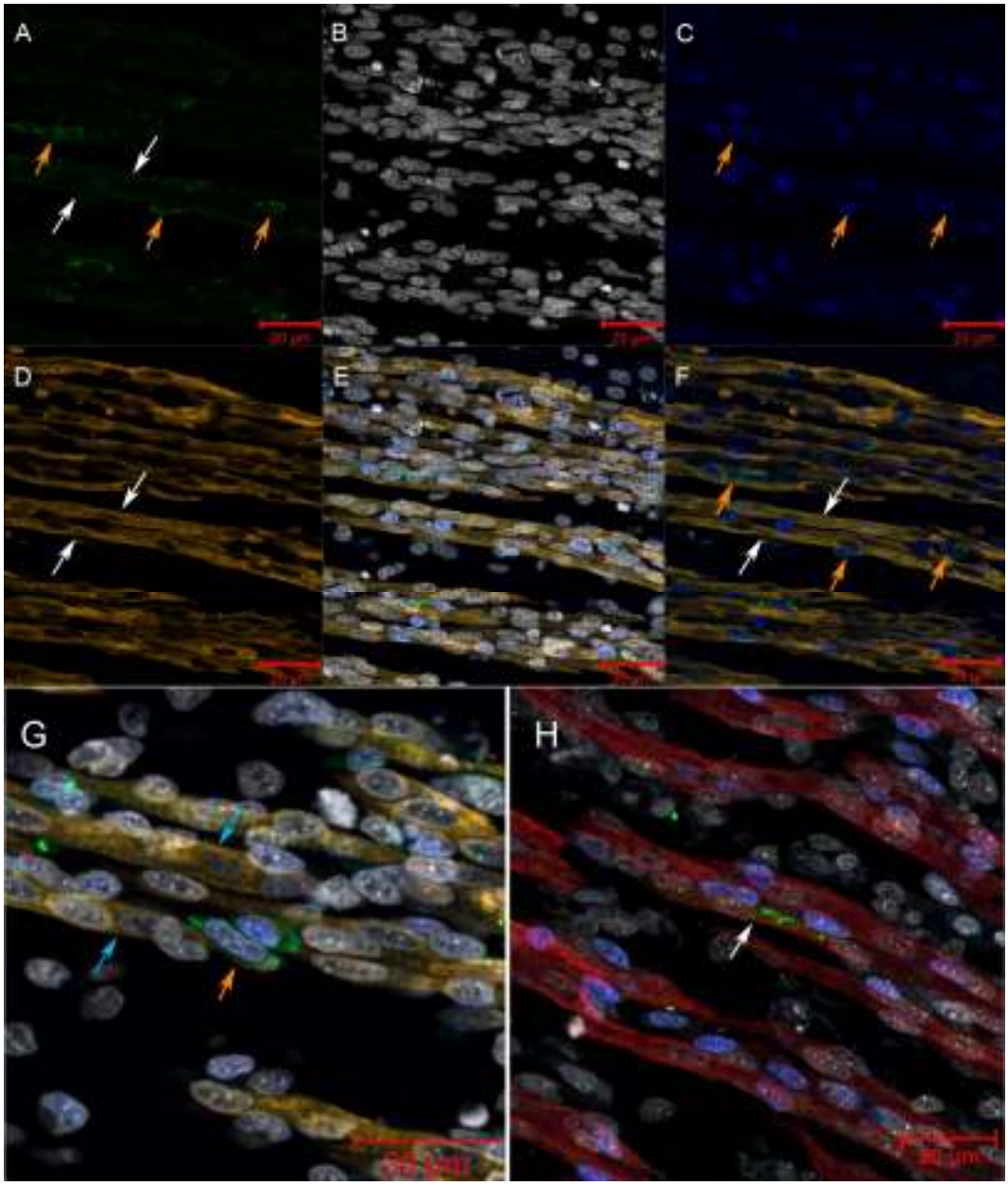


Fig 1. Myogenic precursors infected with the YF17DD virus were observed close to infected and non-infected muscle fibers.

Skeletal muscle tissue of chicken embryos infected with YF17DD virus at 9 dd and collected at 48 hpi were submitted to immunofluorescence assay and analyzed by a confocal laser microscope. Pax-7^{+} mononuclear infected cells fusing to infected fibers were observed in infected embryos. Yellow fever virus antigens (green - A), nucleus (whitte - B), Pax7 (blue - C), desmin (yellow - D), merge YFV, nucleus, Pax7 and desmin (E) and merge YFV, Pax7 and desmin (F). Infected Pax7 positive mononuclear cells were observed near to non-infected muscle fibers. Desmin (yellow), YFV (green), Pax7 (blue) and nucleus (white) (G). Infected muscle fibers present virus staining accompanying fiber

striation. Myosin (red), YFV (green), Pax7 (blue) and nucleus (white). Pax7 cells - orange arrows, infected fibers – white arrows, non-infected cells – blue arrows.

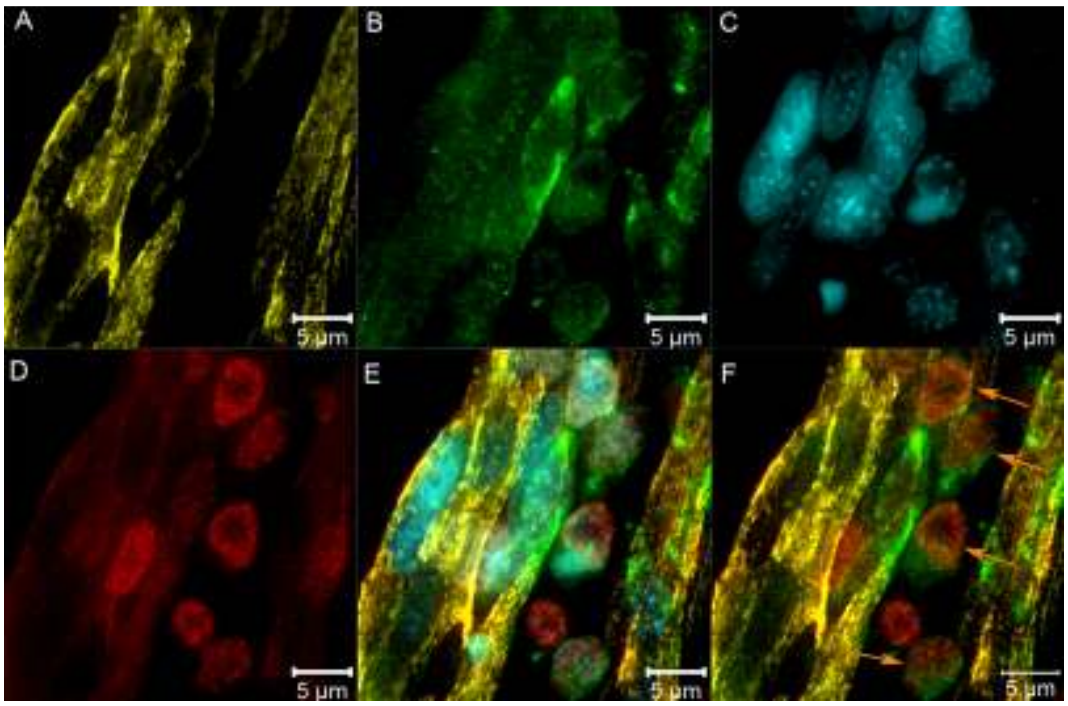


Fig 2. Infected mononucleated cells are Pax7^{+}.
Skeletal muscle tissue from chicken embryos infected with YF17DD virus at 9 dd and collected at 48 hpi, were submitted to immunofluorescence assay and analyzed by SIM super resolution microscopy. Detail of infected mononuclear cell fusing to the muscular fiber. Desmin (yellow -A), YFV (green -B), nucleus (blue - C), Pax7 (red - D), merge YFV, nucleus, Pax7 and desmin (E) and merge YFV, Pax7 and desmin (F). Pax7 infected cells – orange arrows.

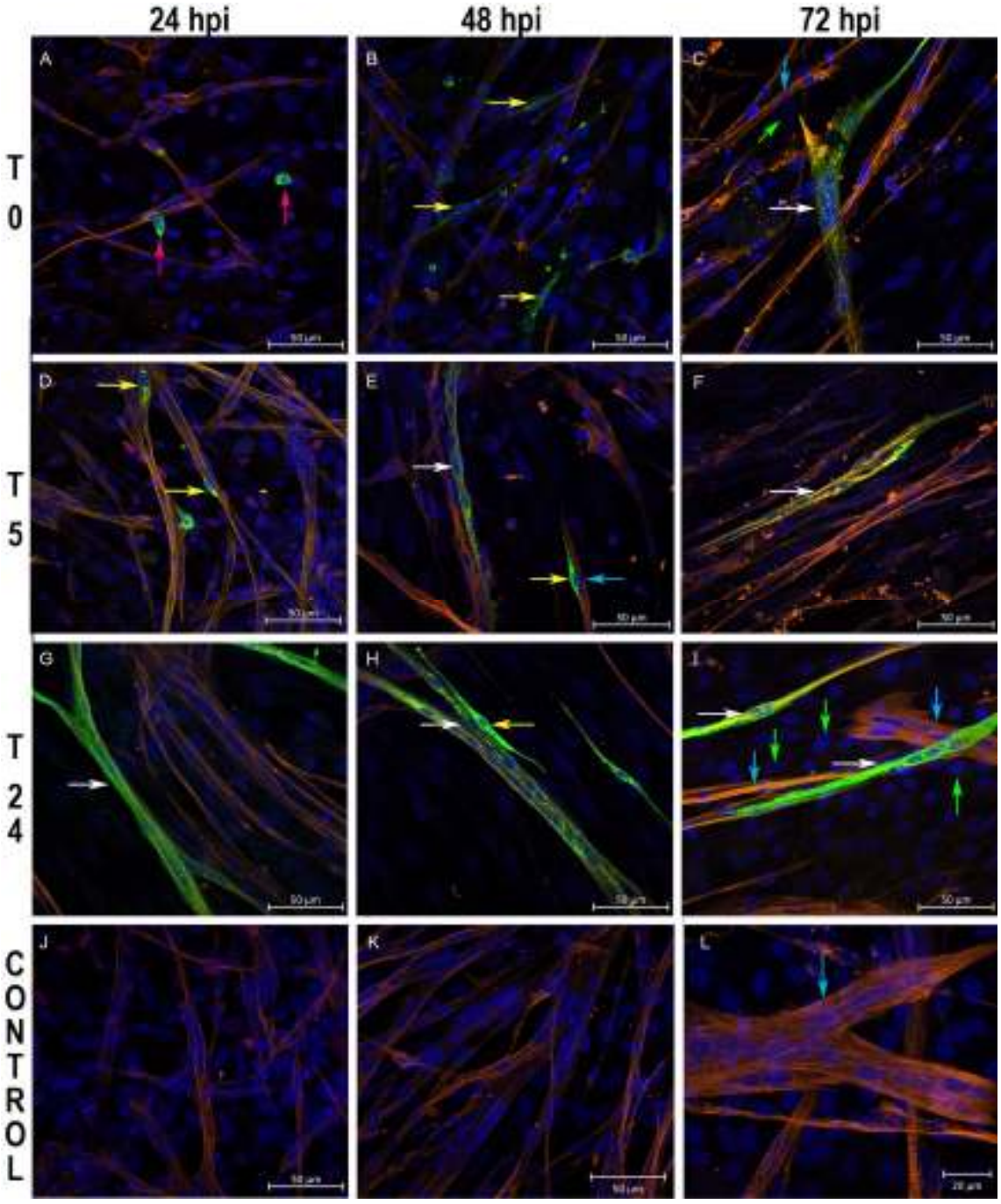


Fig 3. Muscle chicken cells are susceptible to *in vitro* infection in different stages of differentiation.
Skeletal muscle cells cultured and infected in vitro by the yellow fever virus 17DD 0,1 MOI, were submitted to immunofluorescence assay and analyzed by a confocal laser microscope. Cells infected at plated moment (T0) with 24 (A), 48 (B) and 72 hpi (C). Cells infected 5 hr post plated (T5) with 24 (D), 48 (E) and 72 hpi (F). Cells infected 24 hr post plated (24) with 24 (G), 48 (H) and 72 hpi (I). Control culture non-infected with 24 (J), 48 (K) and 72 hpi (L). Desmin (orange), YFV (green) and nucleus (blue). Myoblasts – pink arrows, Myocytes – yellow arrows, infected fibers – white arrows, non-infected fibers – blue arrows, fibroblast – green arrows.

Fig 4. Muscle cells in culture infected at different stages of differentiation produce infectious viral particles.

Chicken embryo muscle cells infected with 0.1 MOI at plating stage [T0], after five hr (T5), and after 24 hr (T24) were evaluated 24, 48 and 72 hpi titrated by the PFU technique.

Each bar represents a poll of three samples in triplicate.

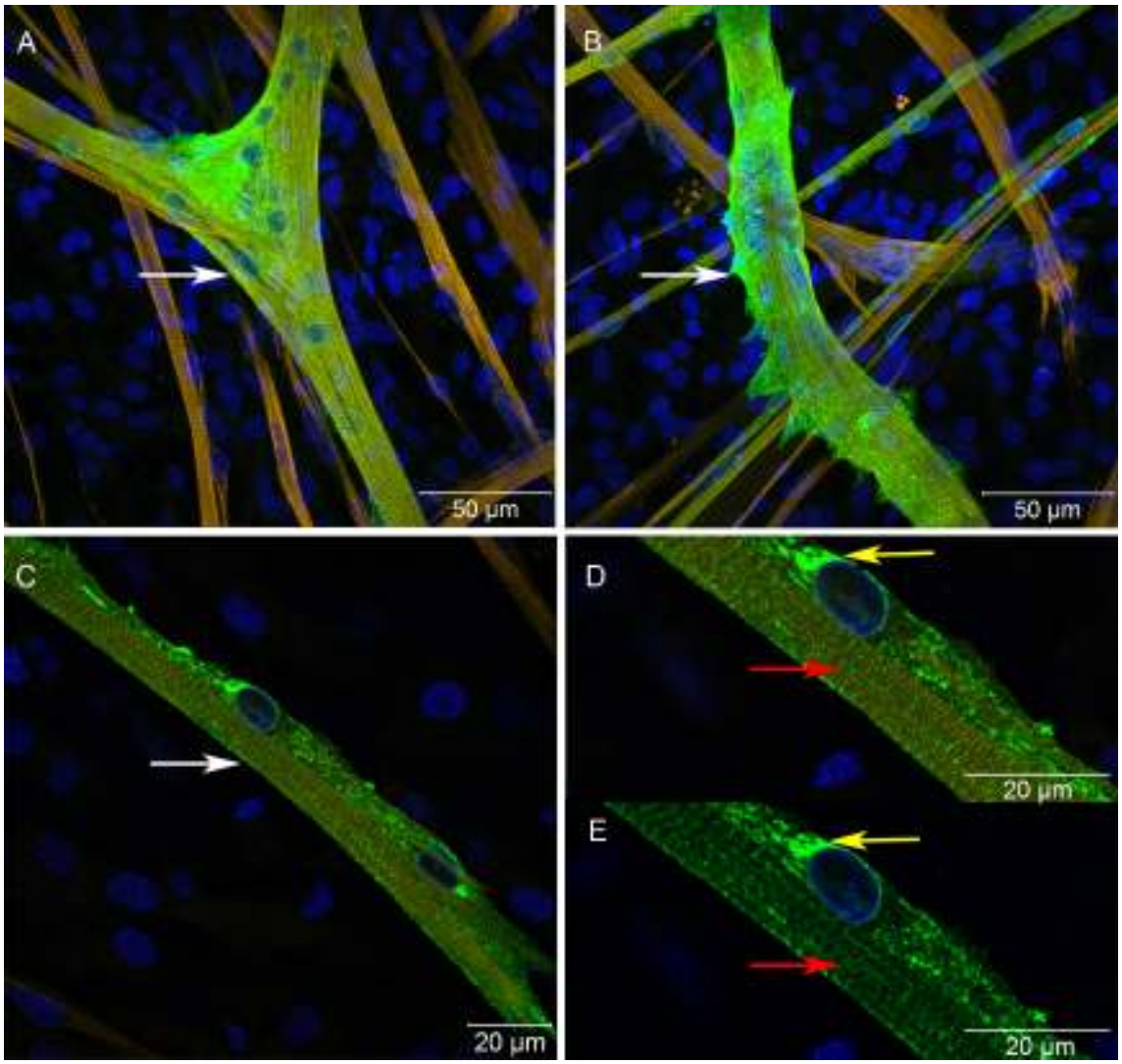


Fig 5. Myofibers were susceptible to infection by the YF17DD virus and viral proteins intercalate with myosin labeling.

Skeletal muscle cells from chicken embryos cultured and infected *in vitro* by the yellow fever virus 17DD 0,1 MOI 168 hours after plating [T120] / 48 hpi, were submitted to immunofluorescence assay and analyzed by a confocal laser microscope. Huge myosin positive fibers were strongly infected by yellow fever virus (A-C). Detail of viral proteins following fibers striation, with perinuclear deposition (D and E). Myosin (yellow), nucleus (blue) and YFV (green). Muscle fibers – white arrows, YFV following fiber striation – red arrows, YFV perinuclear deposition – yellow arrows.

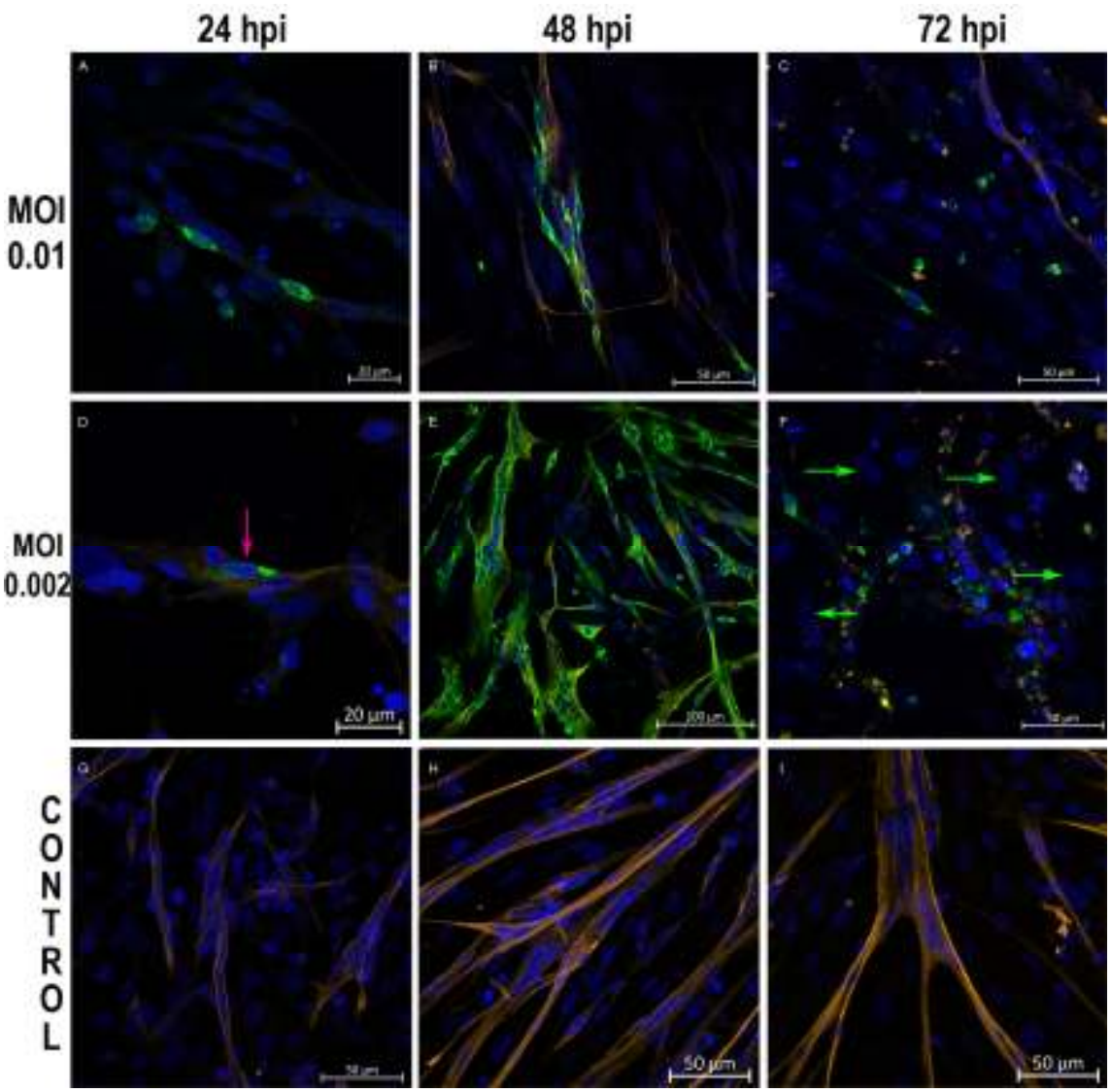


Fig 6. *In vitro* Infection of chicken muscle cells were more intense with 0,002 MOI.

Skeletal muscle cells cultured and infected *in vitro* at different MOI´s by the yellow fever virus 17DD at seeding moment (T0) were submitted to immunofluorescence assay and analyzed by a confocal laser microscope. Infection performed with: 0,01 MOI at 24 (A), 48 (B) and 72 hpi (C); 0,002 MOI at 24 (D), 48 (E) and 72 hpi (F). Control culture non-infected at 24 (G), 48 (H) and 72 hpi (I). Desmin (orange), YFV (green) and nucleus (blue). Myoblast – pink arrow, fibroblasts – green arrows.

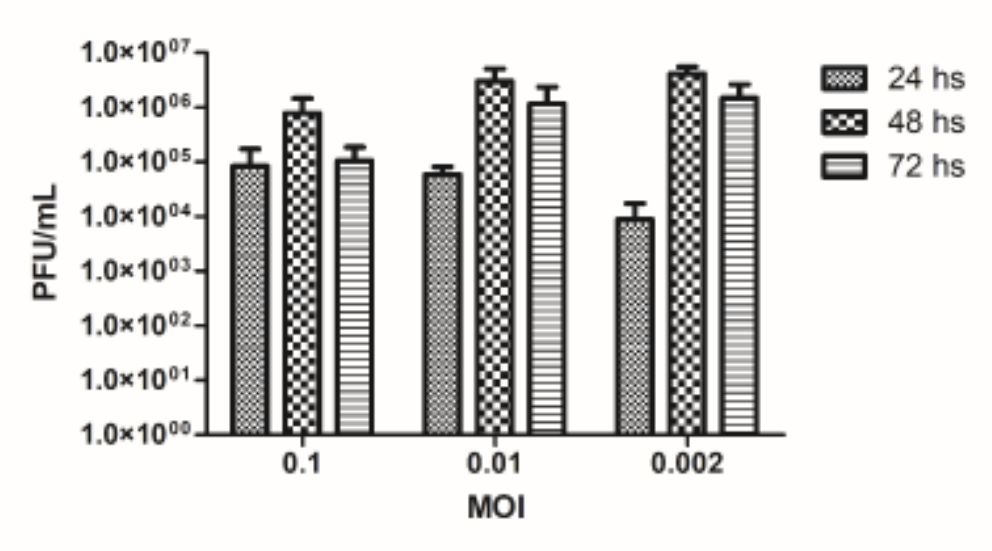


Fig 7. Production of YF infectious viral particles in the muscle cell cultures.

Chicken embryo muscle cells infected by YFV at plating stage [T0] with 0,1; 0,01; and 0,002 MOI were evaluated 24, 48 and 72 hpi titrated by the PFU technique. Each bar represents a poll of three samples in triplicate.

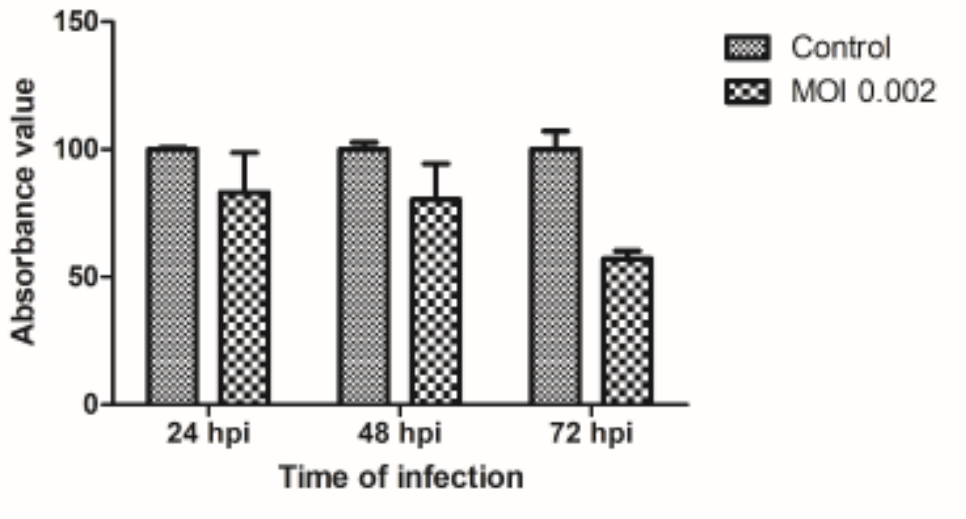
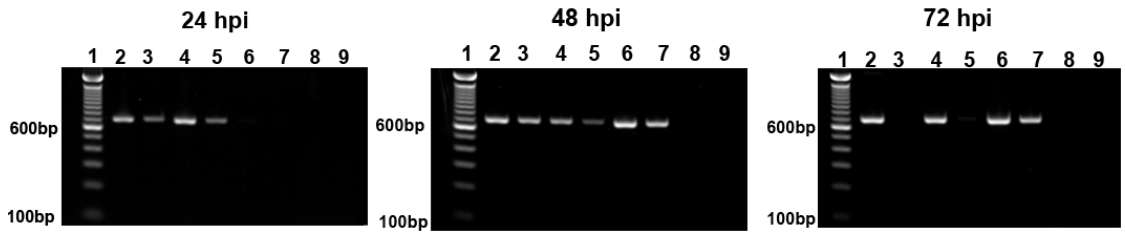


Fig 8. YF17DD infection leads to decrease of muscle cell density in culture.

Chicken embryo muscle cells infected at the time of plating [T0] with 0,002 MOI were evaluated at 24, 48 and 72 hr after infection by violet crystal staining. 24 and 48 hpi infected cultures demonstrated loss of about 20% of cells compared to controls cultures. This loss increased to 43% at 72 hpi. Absorbance reading was performed with a spectrometer at 595 nm.



Supplementary data. Susceptibility of culture to infection by YF17DD virus was confirmed by detection of viral genomic RNA and replicative intermediate by RT-PCR.
Detection of genomic and replicative intermediate RNA from 17DD yellow fever virus extracted from skeletal muscle cells of chicken embryo infected *in vitro* at plated moment (T0) at 0.1, 0.01 and 0.002 MOI. Polymerase chain reaction. 1: Standard of 100bp, 2: RNA genomic 0.1 MOI; 3: replicative intermediate 0.1 MOI; 4: RNA genomic 0.01 MOI; 5: replicative intermediate 0.01 MOI;

6: genomic RNA 0.002 MOI; 7: replicative intermediate 0.002 MOI; 8: Total RNA extracted from the control culture with genomic primers; 9: Total RNA extracted from the control culture with replicative intermediary primers.