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Infection with PRRSv Induces the Formation of Filopodia and Rearrangement of Actin Filaments in MARC-145 Cells

Victoria E. Collia-Acosta[1], Carlos G. García-Tovar[1], Víctor Quintero-Ramírez[2], Susana E. Mendoza-Elvira[2], Samantha Jardon-Xicotencatl[1*]

[1]Multidisciplinary Research Unit L4 (Veterinary Morphology and Cell Biology), UNAM-FESC. Campus 4, Cuautitlán Izcalli, México
[2]Laboratory of Virology and Microbiology of Respiratory Diseases of the Pig, UNAM-FESC. Campus 1, Cuautitlán Izcalli, México
Email: doctora.jardon@cuautitlan.unam.mx

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Abstract

Porcine Reproductive and Respiratory Syndrome (PRRS) is considered one of the diseases causing the greatest economic losses in swine production. In Mexico, various commercial vaccines are used to mitigate the severity of the infection. However, the effect of these biologicals on the morphology of cell lines permissive to infection by the virus (PRRSv) has not been evaluated. This study assessed the effect of three commercial vaccines available in Mexico by evaluating actin cytoskeleton rearrangements in MARC-145 cells. The infection altered the morphology of the cells, inducing the formation of filopodia and the loss of stress fibers. Quantitative analysis of the filopodia revealed an increase in their number and length, showing significant differences in both characteristics depending on the vaccine strain of the virus. These findings suggest that PRRSv manipulates the host's actin cytoskeleton through the formation of filopodia, which may play a critical role in viral propagation. These results open a promising avenue for research aimed at developing potential therapeutic strategies targeting cytoskeletal structures to reduce PRRSv infection and its impact on swine health.

Keywords

Cytoskeleton, Filopodia, PRRSv, Viral Infection

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is a viral disease found worldwide. It is caused by Betaarterivirus suid (PRRSv), which is an RNA virus

enclosed in an envelope [1]. This virus leads to severe reproductive issues in pregnant sows, poor semen quality in boars, and respiratory problems in pigs of all ages, particularly in piglets [2] [3]. Additionally, it is linked to or exacerbates the symptoms of other respiratory diseases [4] [5]. PRRSv is transmitted in permissive cell clones of non-porcine origin such as MARC-145 [6] and CL2621, which are derived from the green monkey kidney MA104 line [7] [8]. Cytopathic effects, such as cell rounding, agglutination, and lysis with marked granulation following monolayer destruction, are observed from 36 to 48 hours post-infection [9].

The cytoskeleton is a complex network of proteins that enables cells to take on various shapes, organize components, maintain volume, and carry out movement [3] [10]. It is composed of actin filaments, microtubules, and intermediate filaments [11] [12]. Actin filaments are helical polymers of the globular protein actin (G-actin) that form parallel bundles, contractile bundles, mesh-like networks, or dendritic networks. From these structures, higher-order structures such as the cell cortex, lamellipodia, filopodia, and stress fibers are formed [13]-[16].

Filopodia are essential structures that act as sensors in cell communication events. They consist of thin, dynamic plasma membrane protrusions supported by parallel bundles of actin filaments. These structures depend on cycles of actin filament polymerization and depolymerization and undergo phases of formation and retraction [13] [14] [17] [18]. Filopodia can grow and extend into contact with other cells, forming cytonemes (nanotubules) that are related to paracrine communication and trafficking of signaling proteins [18] [19].

The cytoskeleton plays a crucial role in virus entry, replication, and survival. Some viruses exploit the host cell's cytoskeleton for processes such as entry, replication, transport, egress, and intercellular diffusion. Virus infection can lead to diverse changes in cell morphology and cytoskeleton rearrangements. Studying these alterations can provide important insights into PRRSV infection. Rearrangements of actin filaments are studied as a marker of virus-induced cell damage. It has been observed that actin filament rearrangements caused by viruses such as SARS-CoV-2, influenza virus, picornavirus, and vesicular stomatitis virus can trigger the immune response. In contrast, inhibiting these rearrangements has been found to promote viral infection [20]. In the case of dengue virus, infected cells exhibited disorganized actin filaments, while neighboring uninfected cells showed increased stress fibers [21].

This study aimed to evaluate changes in the number and length of filopodia during the infection process of MARC-145 cells with PRRSv to determine whether these findings suggest that the virus modifies the actin cytoskeleton to infect the cells.

2. Methodology

2.1. MARC-145 Cell Culture

The MARC-145 cells were grown in RPMI culture medium with 10% fetal bovine

serum and antibiotics, specifically Penicillin (5000 IU/ml) and Streptomycin (5 μg/ml). The cells were maintained in a humidified incubator with a 95:5 air/CO_2 mixture at 37˚C. They were cultured in 60 mm culture dishes, with three coverslips attached to each dish beforehand.

2.2. Infection with PRRSv

Four experimental groups were created using cell cultures grown on coverslips. Group 1 was infected with vaccine 1: V1 (modified live virus vaccine strain ATCC-VR-2332) [22], group 2 was infected with vaccine 2: V2 (PRRS disease modified live virus vaccine strain P129) [23], group 3 was infected with vaccine 3 V3 (PRRS type 2 modified live virus vaccine Nebraska strain) [24], and group 4 remained uninfected (control). For each group, 3 boxes were cultured to evaluate infection at 24-, 48-, and 72-hours post-infection (hpi). To infect the cultures, the culture medium was removed, and 5 ml of each vaccine was added and incubated for 3 hours. Then, 5 ml of each vaccine was recovered, and 5 ml of 4% RPMI medium was added and incubated to monitor the infection at 24, 48, and 72 hours.

2.3. Evaluation of Cell Monolayer Damage

The evaluation of changes in the morphology of PRRSv-infected MARC-145 cells was carried out using optical microscopy to determine the presence of morphological changes in the cell cultures and the development of cytopathic effects. Prior to fixation with aqueous formalin, the cell cultures of the four experimental groups were observed at 24, 48, and 72 hours using a Vision IV900 inverted microscope.

2.4. Evaluation of PRRSv Infection-Induced Changes in Filopodia

The PRRSv-induced changes in filopodia were assessed using direct fluorescence double-labeling. The culture medium was removed from the boxes containing coverslips seeded with MARC-145 cells from both the infected and control groups. The cells were then fixed with 10% aqueous formalin in PBS and incubated for 20 minutes. After removing the formalin, the cells were washed three times with PBS. Next, 5 ml of 0.05% Triton in PBS was added to permeabilize the cells. And they were left to incubate for 5 minutes. After incubation, the cells were washed three times with PBS. Following this, the cells were incubated for 20 minutes with rhodamine isothiocyanate-conjugated phalloidin (TRITC) (Sigma-Aldrich®), which was diluted 1:150 in PBS to label the actin filaments. After the incubation, the cells were washed three times with PBS, and a final wash was done with deionized water. The coverslips were then mounted on slides using mounting medium with 4'6-diamino-2-phenylindole (DAPI) to detect the nuclei (Ultracruz® Mounting Medium for Fluorescence, Santa Cruz, CA, USA).

2.5. Fluorescence Microscopy

To assess the changes in the number and size of filopodia caused by PRRSv, we

used fluorescence microscopy (Zeiss Axioscop 40, with an Evolution VF Cooled Color camera from Media Cybernetics). We analyzed the images captured by the microscope using Image J software. To combine the actin and DAPI fluorescence channels for image analysis, we used the "Z project" function to create channel splices or merges.

2.6. Quantification of Filopodia

For the quantification of the number of filopodia, the "multipoint" tool was used, which allows to leave a consecutive mark in each marked structure per field, evaluating 10 random fields for each vaccine, plus the evaluation of the fields of the control group.

2.7. Filopodia Length

To measure the length of the filopodia, we calibrated the software using a Neubauer camera and took photographs with 10×, 20×, and 40× objectives of the central quadrant. We took 3 images with the 10×, 20×, and 40× objectives of the fluorescence microscope to calibrate the software and establish a scale in micrometers for measuring the size. After determining the pixel/micron ratio, we input the values for the 3 microscope objectives into the program, saved the data, and used this configuration in the linear measurement tool to compare the infected and control groups.

3. Results

The MARC-145 cells display a clear pattern of actin filaments extending from the area around the nucleus to the outer edge of the cell. These structures play a role in creating various protrusions and contractions that are crucial for several important cellular functions. The labeling of these actin filaments forms distinct radial arches and stress fibers in the cell. In cultures with low cell density, the cells exhibit lamellipodia with actin-rich zones at the leading edge. On the other hand, in cultures with high cell density, the labeling is more prominent in the cell cortex and stress fibers, extending throughout the cytoplasm. The cell nuclei appear round with well-defined borders when visualized using nuclear labeling. Additionally, in low confluence cultures, there is a lower presence of filopodia, which are actin-rich linear structures connecting neighboring cells (**Figure 1** and **Figure 2**).

Once the cell morphology was evaluated by studying the actin filaments in uninfected cells, the cultures were exposed to three different viruses from commercial vaccines (**Figure 3**). The virus solutions were added to the cultures, and the course of infection was observed at 24 hours and 48 hours. We evaluated 10 random fields to determine the effect of V1, V2, and V3 on virus-induced cell morphology.

The induction of filopodia formation in PRRSv-infected cultures was evaluated qualitatively and quantitatively (**Figure 4**). The analysis of microphotographs

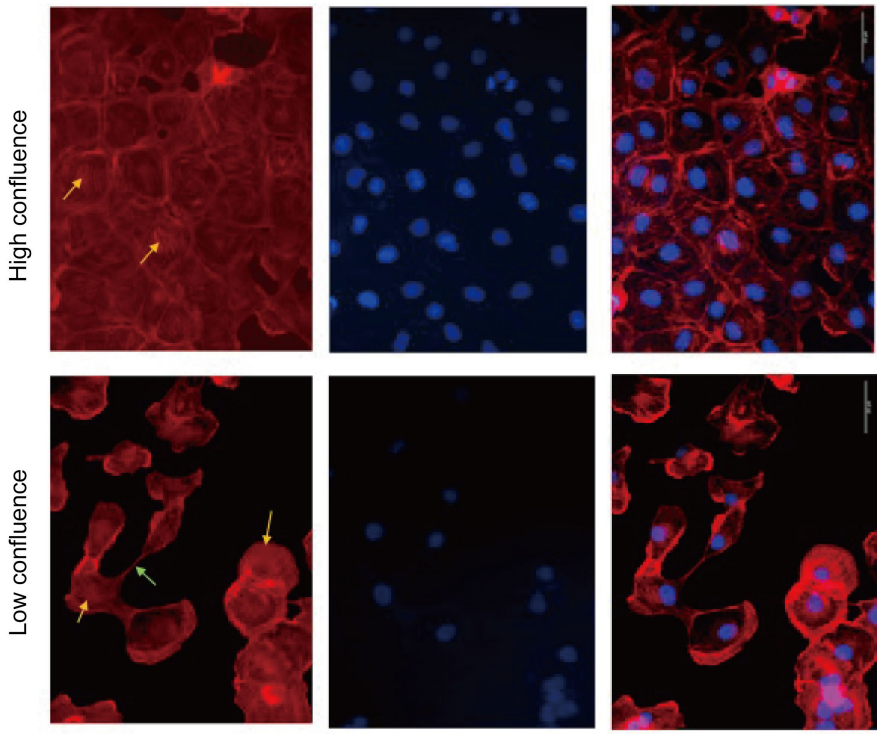

Figure 1. Control cultures with low and high confluence. The morphology of actin filaments and cell nuclei was evaluated in control cultures with low and high confluence. Photomicrographs showed homogeneous sizes with central nuclei (DAPI). In low-confluence cultures, few filopodia were observed (green arrow). In both low- and high-confluence cultures, the morphological pattern of tension fibers was homogeneous and well-defined (yellow arrows). Technique: direct fluorescence with double staining, 20×.

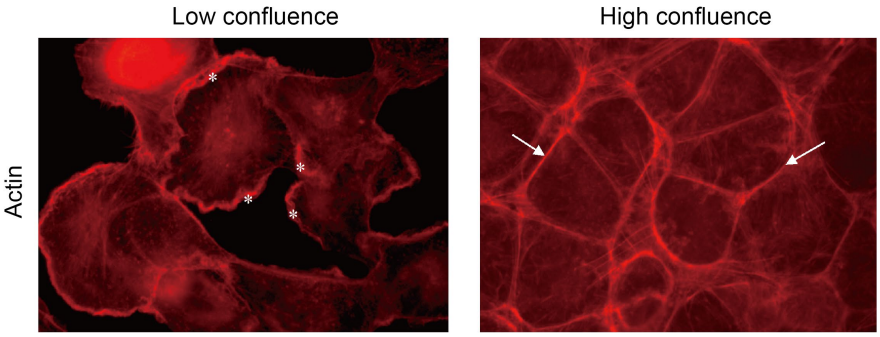

Figure 2. Control cultures with low and high confluence. MARC-145 cells were seeded at low and high confluence to establish the structure of the actin cytoskeleton and evaluate the presence of filopodia in the study fields. In low-confluence cultures, cortical staining revealed the presence of abundant lamellipodia (asterisks), while in high-confluence cultures, the staining showed a linear definition forming cell cortex (white arrows). Technique: direct fluorescence with double staining, 40×.

revealed changes in cell morphology and rearrangements of the actin cytoskeleton, such as the loss of tension fibers and the formation of filopodia (**Figure 4(a)**). Quantitative analysis showed an increase in the number of filopodia compared to the control group (**Figure 4(b)**). However, only group V2 exhibited a statistically

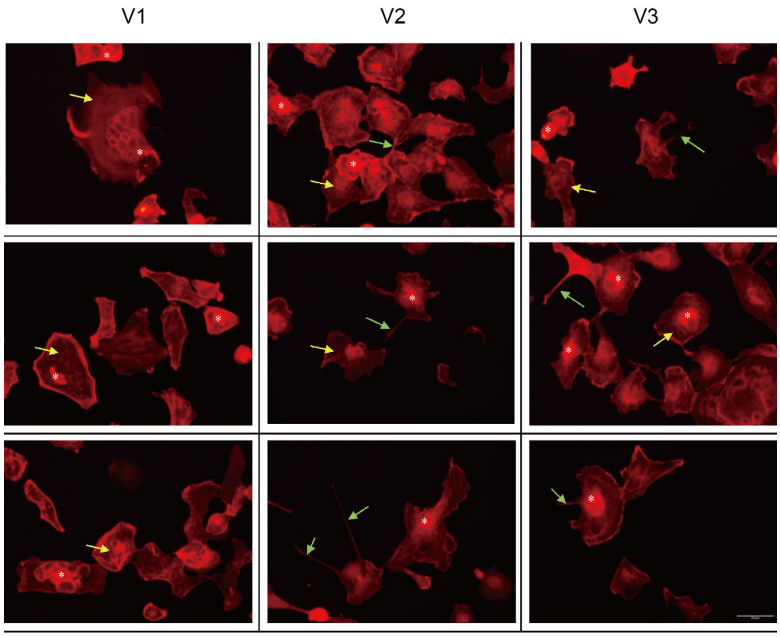

Figure 3. MARC-145 cells infected with PRRSv from three different vaccines at 24 hpi. Evaluation of morphological changes induced by PRRSv infection from three different commercial vaccines. Filopodia (green arrows), loss of tension fibers (yellow arrows), and cytoplasmic aggregates (asterisks) were observed. Technique: direct fluorescence with double staining, 20×.

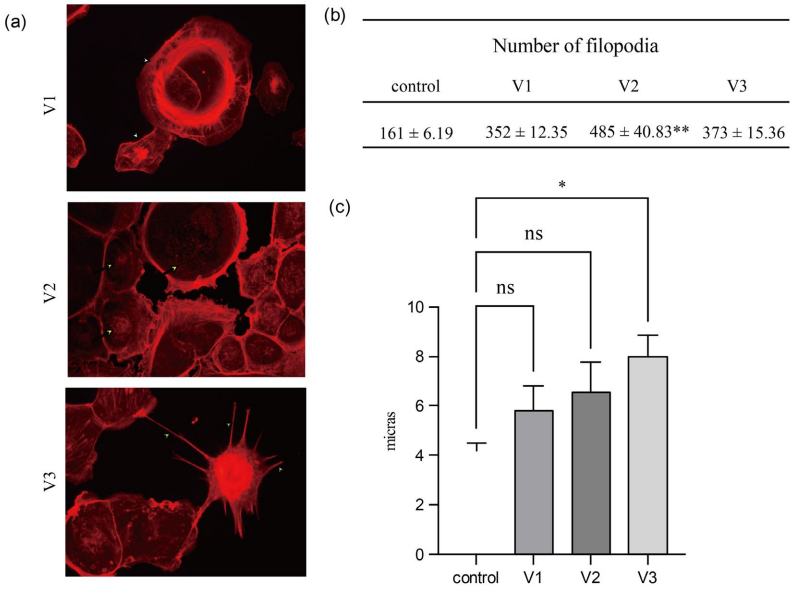

Figure 4. Comparative analysis. (a): Morphological and structural changes observed in cell cultures caused by viruses from the three different vaccines. Changes in cell size (white arrows), loss of tension fibers (yellow arrows), and formation of filopodia (green arrows) are shown. Technique: direct fluorescence, 40×. (b): Comparison of the number of filopodia between the control group and the infected groups (V1, V2, and V3). (c): Comparison of filopodia length between the infected groups (V1, V2, and V3) and the control group. Statistical analysis was performed using one-way ANOVA and Bartlett's test. (*) $0.01 < p \leq 0.05$; (**) $0.001 < p \leq 0.001$.

significant difference compared to the control, with 485 filopodia versus 161, respectively. Furthermore, the evaluation of filopodia length induced by PRRSv showed a higher average in group V3, which demonstrated a significant difference compared to the control group in the analyzed fields (**Figure 4(c)**).

4. Discussion

In this study, researchers examined changes in the structure of actin filaments in PRRSv-infected MARC-145 cultures. In the control group, MARC-145 cells displayed a distinct pattern of actin filaments. The actin filaments formed stress fibers and were visible in lamellipodia, which are characteristic of cells growing in rigid substrates [9].

Given that previous evidence indicates that an intact cytoskeleton is essential for PRRSV infection [25], in our research, we found that cells infected with PRRSv undergo significant changes in their morphology and structure. This includes alterations in cell size and changes in the structure of actin filaments, leading to the loss of stress fibers. These observations are consistent with previous reports suggesting that these alterations may result from viral effects on actin filaments, employed as a strategy to promote cell infection [26]. For example, pseudorabies and dengue viruses are known to induce similar changes [27] [28], while Marek's virus has been observed to degrade stress fibers [29]. Furthermore, some strains of rubella virus can reduce cell stiffness by reducing stress fibers, and the SV40 virus disrupts actin filaments significantly [30]. We also observed the formation of actin aggregates in our work, which may have resulted from the dissolution and aggregation of stress fibers. This led to the formation of lamellipodia and filopodia. Similar observations have been reported in cells infected with classical swine fever virus [31].

The movement of viruses between cells has been observed in various viral families as a means of virus distribution. The processes by which viruses spread have evolved to avoid the immune response. The typical receptor-mediated spread is often hindered by the presence of antibodies. In response, viruses have developed alternative methods to protect themselves, such as modifying the actin cytoskeleton. These modifications can include the formation of syncytia, actin comets, nanotube tunnels, and filopodia [32]. It has been noted that filopodia may play a significant role in viral infection events, including initial viral binding to host cells, viral trafficking, virus internalization, budding and release, as well as spread to other cells, bypassing the host immune system [33].

Viruses can spread via cell-free transmission or through cell-to-cell interactions. In the case of the latter, the presence of filopodia is crucial during in vitro infection in cell culture. In our study, we observed an increase in the number and size of filopodia in infected cell cultures, which could be caused by the virus itself. Previous studies have shown that filopodia formation occurs in cultures infected with herpes simplex virus type 1 [34], human immunodeficiency virus (HIV) (type 1 and 2), human T-cell lymphotropic virus type 1 (HTLV-1) [35], as well as

in human and avian influenza virus infections. It has been observed that an increase in filopodia formation enhances endocytosis [36]. The virus from vaccine 3 induced the highest level of filopodia formation, leading to destruction of the cell monolayer after 48 hpi. This suggests that the increase in filopodia formation facilitates viral propagation and induces apoptosis, favoring cell-to-cell transmission by manipulation of the actin cytoskeleton. Additionally, PRRSv-induced apoptotic processes in MARC-145 cells also support viral propagation through effective replication and cell-to-cell transmission of the virus [37].

The viruses used in the 3 vaccines tested showed that they can cause changes in the cell structure, leading to an increase in the number of filopodia compared to control cultures. This begins the formation of these structures between 12 to 24 hours after infection. Studies show that the infectivity of Human Papillomavirus 16 decreases when the formation of filopodia is inhibited [38]. This suggests that the viruses cause the formation of filopodia to facilitate the spread of infection between cells, which aligns with our findings. In cultures infected with viruses from V1 and V2, the filopodia retracted at 48 hours after infection. Similar occurrences have been observed in viruses like SARS-CoV-2, where this was linked to the regulation of the timing and location of the activity of the actin cytoskeleton by proteins such as formin, Arp 2/3, and Cdc42 in the formation of filopodia, acting as "highway" structures for virus entry [39].

There are diverse actin structures that link distant cells and allow intercellular transfer of molecular information, including genetic information, proteins, lipids, and even organelles. For instance, nanotubes facilitate intercellular communication but can also be exploited by pathogens to aid in their spread [40]. Therefore, studying filopodia and similar structures could lead to the development of new antiviral treatments aimed at blocking the signaling pathways used by viruses to induce filopodia formation, creating potential antiviral therapeutic targets.

5. Conclusion

The infection by PRRSv from viruses derived from three commercial vaccines in Mexico caused significant changes in the morphology and size of MARC-145 cells, including an increase in the number and length of filopodia, along with the loss of cytoplasmic tension fibers in the in vitro infection of MARC-145 cells. These alterations in the actin cytoskeleton began at 24 hours post-infection (hpi), progressed to a 70% reduction of the monolayer at 48 hpi, and culminated in its destruction at 72 hpi. The virus from group V2 induced the greatest increase in the number of filopodia, while group V3 generated the longest filopodia. These findings suggest that PRRSv manipulates the actin cytoskeleton as part of its viral infection cycle, opening new lines of research to understand how this interaction can be leveraged to reduce viral spread.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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