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SARS-CoV-2 Isolation from Cuban COVID-19 Patients -

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ABSTRACT

Due to the urgent need for vaccine and anti-viral drug for the treatment of COVID-19 patients, isolation of the SARS-CoV-2 virus is crucial. SARS-CoV-2 from the nasopharynges sample of three Cubans asymptomatic patients in Vero E6 cell lines was isolated and confirmed by cytopathic effect from rRT-PCR assay and electron microscopy. Cytopathic effect of SARS-CoV-2 in Vero E6 cell cultures was confluent sixth days after the first pass of the sample and fourth days in the second one. The Cycle threshold (Ct) levels detection in the supernatant of cultures was related by morphology change in the Vero E6 culture. The Ct values in the supernatant were lower than in the pre-inoculated samples. Virus particles and extracellular virions ranging from 80 to 125 nm in diameter were observed by electron microscopy. This study provides an isolation and replication methodology to obtain amounts of SARS-CoV-2 virus for further studies, such as experimental trials for the development of new antiviral drugs and vaccines.

Keywords: SARS-CoV-2; Isolation; Culture; Microscopy electron; Cuba

INTRODUCTION

The novel coronavirus pneumonia, which was named later as Coronavirus Disease 2019 (COVID-19), is caused by the Severe Acute Respiratory Syndrome Coronavirus 2, namely SARS-CoV-2 [1]. It is a positive strand RNA virus (family: Coronaviridae), showing high homology with SARS-CoV and bat coronavirus. SARS-CoV-2 is the seventh coronavirus known which infect humans; SARS-CoV, MERS-CoV, and SARS-CoV-2 may cause severe disease, whereas HKU1, NL63, OC43, and 229E are associated with mild symptoms [2,3].

The SARS-CoV-2 receptor is the Angiotensin-Converting Enzyme 2 (ACE2). The spike proteins of virus bind to ACE2. Transmission of SARS-CoV-2 occurs through respiratory droplets and fomites. The virus can be detected in upper respiratory tract sample, implicating the nasopharynx as site of replication [3-5]. The COVID-19 outbreak presents enormous challenges for global health after the pandemic outbreak [2,6]. The first diagnosed patient in Cuba has been reported by Ministry of Health of Cuba Republic on March 11, 2020. Today, over two thousand cases in Cuba, and more than fourteen million cases around the world have been declared [7]. Due to urgent need for vaccine and anti-viral drug, isolation of the virus is crucial.

Here, we report the first isolation of SARS-CoV-2 from nasopharyngeal specimens of diagnosed patients in Cuba. This study provides an isolation and replication methodology, and cell culture of the virus that will be available to the research communities.

METHODS

Collection and transportation of specimen

Samples were collected from the nasopharyngeal cavity of fourth COVID-19 positive diagnosed patients, according to their Real-Time PCR (rRT-PCR) analysis, in General Military Hospital Dr. Luis Diaz Soto, Habana. The patients were asymptomatic and naive of treatments.

Swabs were put into the transportation medium, placed in a tertiary container and transported at 4°C to the Laboratory of Civilian Defense Scientific Research Center. The samples were transferred at Biosafety Level 3 (BSL3) of the research center for processed and viral isolation. The transportation medium from viral isolation contains Minimal Essential Medium (MEM, Gibco, UK), 1% Fetal Bovine Serum (Capricorn, Germany), 80 µg/mL Gentamicin (Sigma, USA), and 5 µg/mL Amphotericin (AICA Laboratory, Cuba).

Virus propagation

Vero E6 cells were used for isolation and initial pass; the cells were cultured in MEM supplemented with fetal bovine serum (4%) and antibiotic/antifungal (Gentamicin and Amphotericin).

Vero E6 monolayers were inoculated (ATCC CRL-1586), in 24-well plate, with two- hundred µL of clinical specimens and cultured at 37°C in 5% carbon dioxide atmosphere. Cytopathic Effect (CPE) was recorded each day under inverted microscope and after its detection; the cultures were freezes (-85°C) and thawed (37°C), the cells were scraped from the well and 100 µL of virus lysate was used to inoculate to other 24-well plate with the Vero E6 cell line. The monolayers cells were scrapped when CPE was observed. The cultured was centrifugated and supernatant of infected Vero E6 cells were aliquoted and frozen. From the wells in which cytopathic effect were observed, confirmatory testing was performed using rRT-PCR assay with specific primers and probes against SARS-CoV-2. Were included supernatant of non-infected Vero E6 cells.

RNA extraction and quantitative RT-PCR

Viral RNA extraction was performed from 140 µL of sample according to the instructions for use of the QIAamp Viral RNA MiniKit kit (QIAGEN, Valencia, CA, USA) and using the QIACube platform (QIAGEN). Prior to extraction, 10 µL of an RNA extraction control corresponding to a genomic fragment of Equine Arthritis Virus (EAV) was added to each sample [8]. The extracted RNAs were immediately used as template in reverse- transcription-real-time PCR (rRT-PCR).

Reverse-transcription-real-time PCR for detection of SARS-CoV-2 RNA

Primers and probes designed and distributed by TIB MOLBIOL (Roche, Germany) were used to detect SARS-CoV-2 RNA by rRT-PCR [9]. The LightMix Modular Sarbecovirus E- gene was used for first-line detection of SARS and SARS-CoV-2, as well as other related viruses (Sarbecovirus), and the LightMix Modular SARS-CoV-2 (COVID-19) RdRP (RNA- dependent RNA polymerase in the ORF1ab region), as a confirmatory assay for SARS-CoV-2 infection. The primers and probes specific for the genes to be detected and the RNA extraction control, were gently resuspended in 50 µL of nuclease- free water and stored according to the manufacturer's instructions. The Super ScriptTM III PlatinumTM One Step qRT-PCR Kit (Invitrogen) and the Rotor Gene Q-5 plex real- time PCR platform (QIAGEN) were used. The cycling conditions were: 50°C-30 min, 55°C, 95°C 2 min, 45 cycles: 95°C 15 sec, 60°C 30 sec (acquisition at 530 nm for gene E and RdRP and 660 nm for EAV Control).

The interpretation of the results was carried out according to the specifications described by the manufacturer. All samples with typical PCR curves (sigmoid curve) and Cycle threshold value [Ct] less than or equal to 36 cycles for the E gene and Ct values less than or equal to 40 cycles for the RdRP gene were considered positive, as well as the necessary positivity for the EAV extraction control (Ct of approximately 36 cycles). The quality control of each run included the analysis of the negative controls (without amplification signal)

and positive, as well as the internal controls for the acceptance of the results [9,10].

Transmission electron microscopy

Collected cells were first fixed in 3.2% glutaraldehyde diluted in 0.1 M sodium cacodylate (pH 7.4) for 1 hour at 4 °C and then overnight in cacodylate buffer pH 7.2 at 4°C. Afterwards, samples were post-fixed in 1% osmium tetroxide for 1 hour and dehydrated in increasing concentrations of ethanol and then embedded in Spurr (Sigma-Aldrich Co., USA) as previously described [11]. Ultrathin sections (400-500 A) made with an ultra-microtome (RMC, Boeckeler) were placed on 400 mesh grids, stained with saturated uranyl acetate and lead citrate, and examined with a MIRA3-TESCAN Scanning Electron Microscope (TESCAN, Czech Republic) at 25.0 kV using a transmission electron detector. Particles sizes were measured using the Image J 1.42 software (http://imagej.nih.gov/ij).

RESULTS

The swab specimens collected from COVID-19 diagnosed patients were transferred in the same day to biosafety level 3 facility. A sample was taken to determine the SARS-CoV-2 RNA from nasopharyngeal swabs and inoculated with Vero E6 cell in 24-well plate as mentioned in the method.

Figure 1 shows the results of detection of viral RNA in samples of nasopharyngeal exudates from patients included in this work. All four presented a sigmoid PCR curve and the Ct values for the E and RdRP genes met the positivity criteria of less than 36 cycles (Gene E) and 40 cycles (Gene RdRP) (Table 1); which corroborates that these patients are infected with the SARS-CoV-2 virus.

The four patients included in this study did not show COVID-19 symptoms at diagnosis (asymptomatic) and reside in Havana; three are of the female gender and one of the male gender (C2015) and three were included in the age group of 60 or more years and one in the age group of 30 to 40 years (C2018) (Table 1). C2015 and C2016, marriage, were epidemiologically related to a local transmission event.

The Vero E6 cells culture inoculated with the nasopharyngeal exudate's samples from three patients (C2016, C2017 and C2018), did not show morphology change that evidences SARS-CoV-2 replication until the sixth day after inoculation. Cytopathic Effect (CPE) was characterized by cell monolayer destruction, cell rounding, and multinucleated giant cell (syncytium) formation. The C2015 culture did not show these morphological changes, only slight detachment of the cells was observed.

The cultures inoculated with the viral lysate from the first pass of each patient (second pass), developed characteristic CPE of SARS-CoV-2 between the fourth and sixth day; without morphological differences between the C2016, C2017 and C2018 cultures. The C2015 culture was maintained for 10 days, it did not show a characteristic CPE of the virus.

Figure 2 presents images of the cytopathic effect observed in the cultures of Cuban isolates at different days after inoculation in Vero E6 cells. (Figure 2A) corresponds to the culture of the uninfected Vero E6 cells (seven-day culture). In (Figure 2B), corresponding to three days after inoculation, the formation of spaces in the cell monolayer and rounding of the cells are observed. (Figure 2C) corresponds to the fourth day of culture, where the formation of cell clusters and a greater destruction of cell monolayer are observed. On the fifth day of culture (Figure 2D), a complete destruction of the cell monolayer and the formation of multinucleated giant cells were observed.

The replication of SARS-CoV-2 in all four cultures (first and second passage) was confirmed by rRT-PCR assay using specific primers and probes (gene E and RdRP). Controls on Vero E6 cells, from different culture days, were all negative (Figure 3).

The late detection with the highest Ct for C2015 culture

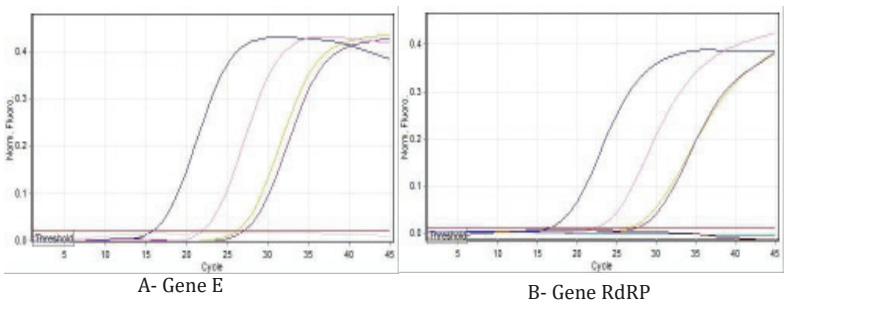


Figure 1: Real-time RT-PCR amplification plot of patients' nasopharyngeal samples, corresponding to the data in C. RFU, relative fluorescence units.

Table 1: Epidemiological clinical data of the patients.

Patients	Sex	Age (years)	Clinical state	Treatment	Ct nasopharyngeal sample	
					Gene E	Gene RdRP
C2015	M	62	Asymptomatic	No	26.27	26.67
C2016	F	61	Asymptomatic	No	15.94	16.63
C2017	F	60	Asymptomatic	No	27.18	27.64
C2018	F	34	Asymptomatic	No	22.03	22.57

M- Male; F- Female; Ct- Nasopharynges swabs cycle threshold value.

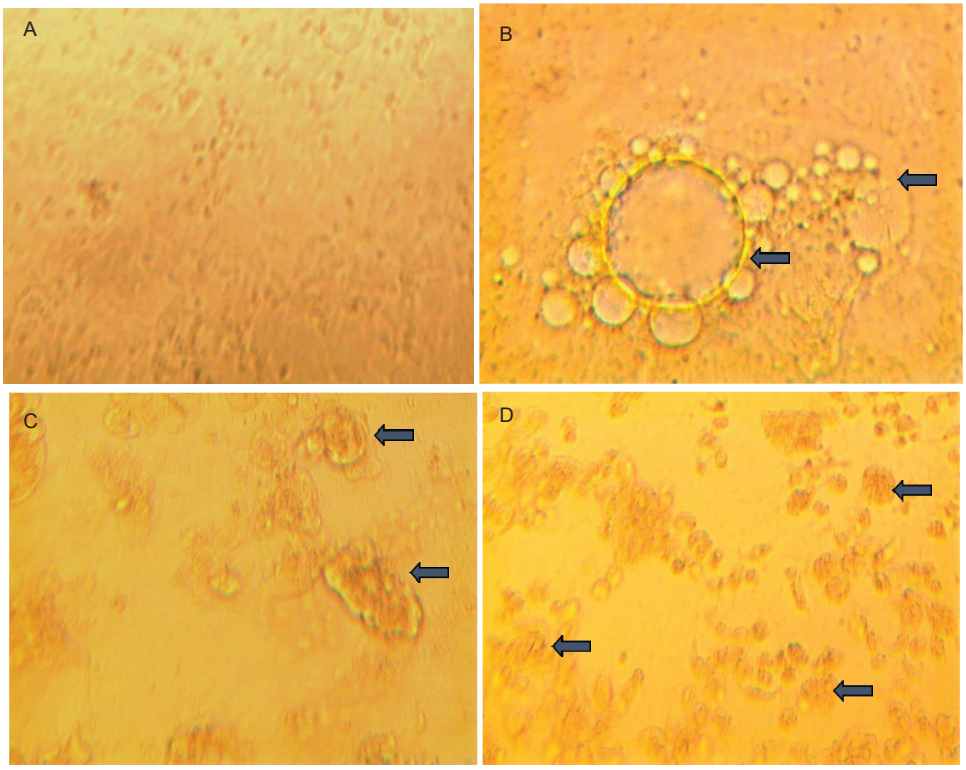


Figure 2: Cytopathic effects of SARS-CoV-2 isolation in Vero E6 cell cultures from patients C2017. Arrows show the cytopathic effects in Vero E6 cell at different days post inoculation.
(A) Vero E6 cell cultures no infected.
(B) Three-day culture post inoculation
(C) Four-day post-inoculation culture
(D) Five-day post-inoculation culture

supernatant (Gene E: 32.48 and Gene RdRP: 33.97) only for first pass (Figure 3), corresponds to the observed in the culture of the original sample of this patient, which showed only slight detachment of the cells and in the second pass no morphological changes were observed in the culture. The Ct value in the supernatant was higher than the value in the pre-inoculated sample from this patient.

Detection of the viral RNA in the supernatant of the cultures of C2016, C2017 and C2018 no showed difference between them, both for the E gene and the RdRP gene, for the first and second pass in Vero E6 cells (Figure 3). It is noteworthy that the determination of ARN viral in the first pass was made on the sixth day after inoculation and in the second pass it was performed on the fourth day after inoculation, coinciding with the days of maximum cytopathic effect.

Cells infected with the isolates were analyzed by Transmission Electron Microscopy (TEM) 72 hours after infection. Although infected cells showed extensive damage or cell death, virus particles and extracellular virions, ranging from 80 to 125 nm in diameter could be observed in those samples (Figure 4). Particularly, viral particles were shown in intracellular smooth vesicles and extracellular virions in close contact with the plasma membrane and filo podia protrusions (Figure 4B,4D). Damaged infected cells contained abundant viral particles located near double membrane vesicles (Figure 4C). These particles may be found in various vesicles (Figure 4C) that could be detected in the extracellular milieu.

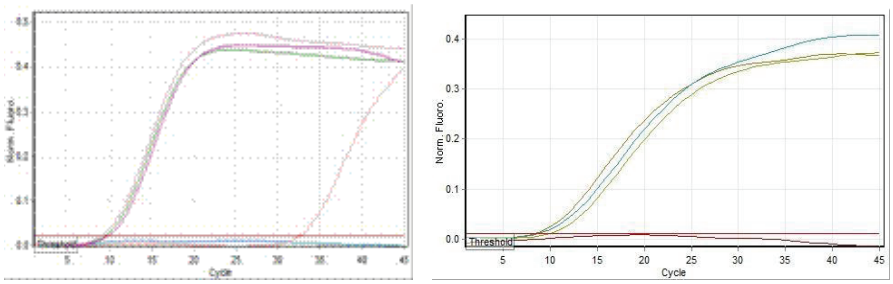
In the current study, using a system based on cell culture in Vero E6 cells and rRT-PCR to assess infectivity, allowed isolated the virus from nasopharyngeal exudate of three of the four patients, characterized because of the Ct value of the supernatant culture was less than the value in the pre inoculation sample and corroborated by visual multiple viral particles in the electronic microscopy with morphology characteristic of coronavirus.

DISCUSSION

Many basic virologic questions of SARS-CoV-2 remain unanswered. In this study, we were able to isolate the SARS-CoV-2 virus for the first time in Cuba from the nasopharyngeal exudates of COVID-19 patients in Vero E6 cell line, using the rRT-PCR and electron microscopic as techniques to confirm of cytopathic effect observed in the culture.

Using this culture system, the isolation of SARS-CoV-2 was achieved in three of the four patients, showing a CPE characterized by the degeneration of the cell monolayer with the formation of spaces and rounding of the cells and multinucleated giant cells. The cytopathic effect became more visible on the second pass (between the third and fifth day) compared to the first pass (sixth to seventh day) and the Ct values in the supernatant of the cultured were lower than the values in the pre-inoculated samples respectively.

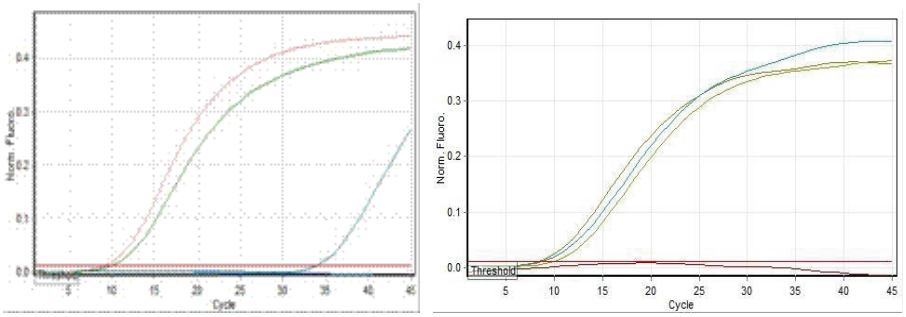
The observation of the cytopathic effect for these cultures on the sixth day is compatible with previous observations from other groups of researchers, who did not find the appearance of the ECP until after six days of inoculation of the original sample of the patients [5,12-14].



A

Color	First pass (6 dpi)	Ct (Gen E)
	C 2015	32.48
	C 2016	9.42
	C 2017	9.84
	C 2018	9.00
	Vero E6 cell line	0.00

Color	Second pass (4 dpi)	Ct (Gen E)
	C 2015	0.00
	C 2016	8.07
	C 2017	9.79
	C 2018	9.14
	Vero E6 cell line	0.00



B

Color	First pass (6 dpi)	Ct (Gen dRP)
	C 2015	**33.97**
	C 2016	**9.20**
	C 2017	**9.98**
	C 2018	**9.29**
	Vero E6 cell line	**0.00**

Color	Second pass (4 dpi)	Ct Gen RdRP)
	C 2015	**0.00**
	C 2016	**8.52**
	C 2017	**10.12**
	C 2018	**8.91**
	Vero E6 cell line	**0.00**

Figure 3: Real-time RT-PCR amplification plot of supernatant cultures using the E and RdRP primer/probe sets, corresponding to the data in C. RFU, relative fluorescence units.

Low magnification TEM analysis of Vero E6 cells with the isolates of SARS-Cov-2 illustrated the presence of virus particles and extracellular virions similar to those previously described for SARS-Cov and SARS-Cov-2 [2,6,13]. They ranged from 80 to 125 nm in diameter and were shown in smooth intracellular vesicles. These vesicles have been previously shown to be related to Golgi compartments where SARS-CoV and SARS-CoV-2 bud, mature and secreted [3,5]. Accumulations of viral particles were also seen near double membrane vesicles which have been shown to be induced in cells infected with different coronaviruses. These coronavirus related structures have been implicated in virus replication or viral RNA storage [2,5,11]. Extracellular virions were frequently observed close to plasma membrane and around filo podial protrusions. This feature seems to be important for SARS-Cov-2 life cycle suggesting the involvement of virus-related filo podia in cell to cell transmission [15]. It is interesting to note the presence of viral particles in vesicles from damaged or dead cells that may be released to the extracellular milieu.

Our data show biological characteristics of SARS-CoV-2 that might provide insights into understanding this virus's unique clinical manifestations and transmissibility and rationalizing laboratory diagnostics.

In the process of specimen collection from patient and transfer to the laboratory, the process should be started and virus cultured immediately because lots of virus became inactivate. Adequate specimen collection is important for the diagnosis and isolation of respiratory viral infections [10,15]. At present, the sample collection for viral nucleic acid detection of suspected patients with COVID-19 is mostly upper respiratory tract samples (mainly throat swabs) [12].

Nasopharyngeal swabs generally contain a low number of cells, so sampling conditions, the means of transport for viral isolation, and time for sampling are vital to achieving isolation of SARS-CoV-2.

The maximum time to process a sample for isolation of respiratory viruses is 24 to 48 hours maximum for the sample to be useful for viral isolation [16,17]. Another vital aspect to isolate the SARS-CoV-2

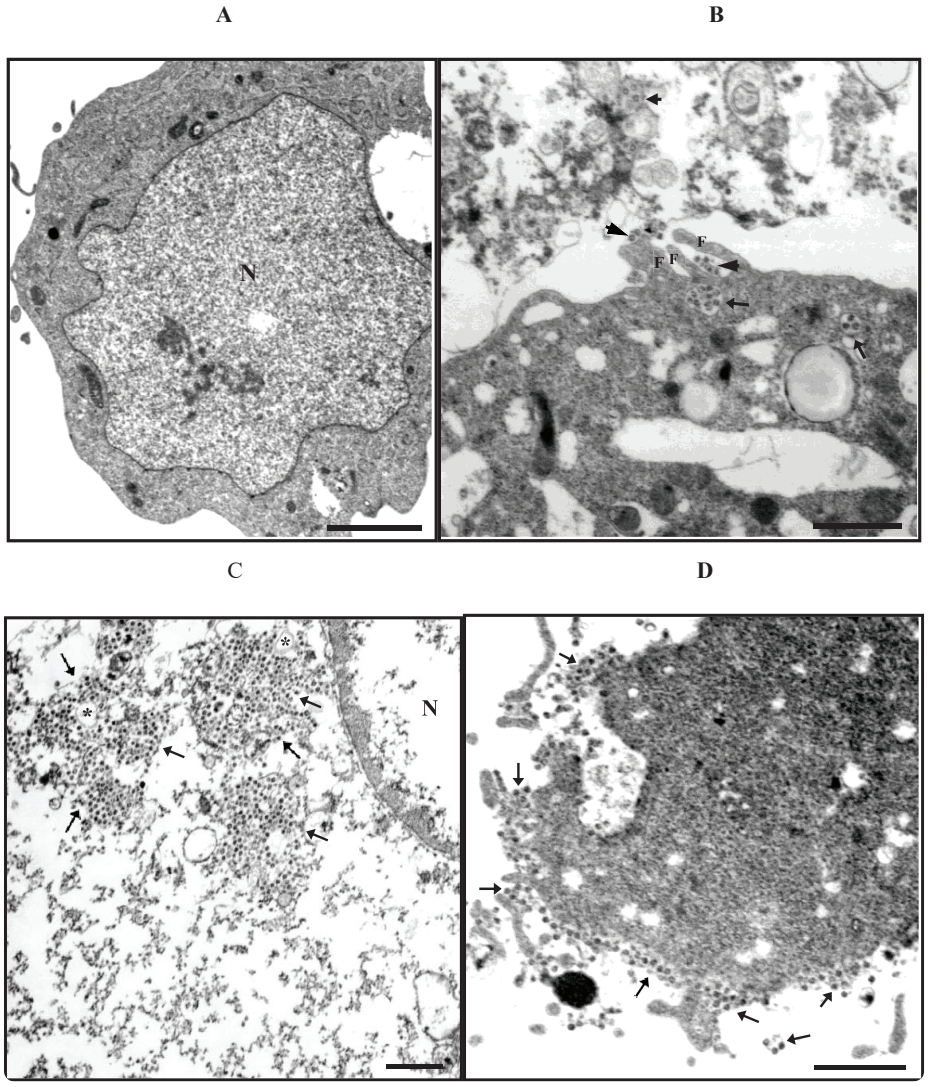


Figure 4: Electron microscopy analysis of SARS-CoV-2 infected Vero E6 cells Shown are uninfected Vero E6 cells (A), Vero E6 cells infected with SARS-CoV-2 isolates C2018 (B), C2016 (C) and C2017 (D) (72 hours post-infection).
B: Part of a Vero E6 cell (below) and rests of a damaged cell (above). Note smooth vesicles containing viral particles (arrows) and extracellular released virions (large arrow heads) near the surface of filopodia (F). Also note viral particles inside a vesicle among the rests of a damaged cell (small arrow heads).
C: Part of a damaged Vero E6 cell showing numerous viral particles (arrows) near double-membrane vesicles (*).
D: Part of a Vero E6 cell showing numerous extracellular virions in close contact with the cell membrane (arrows). N: Nucleus. (Bars: 1 µm).

is the medium of transport for the preservation of the sample, it is necessary to keep them at pH 7 and avoid drying them [16]. For the conservation and transport of the nasopharyngeal exudates of the patients included in this study, MEM culture medium supplemented with fetal bovine serum and antibiotics was used. The fetal bovine serum, as a stabilizer to protect the viral particle present in the infectious material, and gentamicin and amphotericin B as antibiotics to prevent bacterial and fungal contamination.

It must be appreciated that no matter how accurate and fast laboratory testing methods are, the diagnosis of viral pneumonias such as caused by SARS-CoV-2 involves collecting the correct specimen from the patient at the right time [5,9]. An important factor to consider is that the viral excretion period for these viruses is short, so samples must be collected within the first 72 hours from the onset of the first symptoms or after diagnosis for asymptomatic patients [16,18]. The SARS-CoV-2 incubation period after infection is generally 4-8 days. All age groups are susceptible to the virus, of which elderly patients with comorbidities are more likely to experience severe illness. Importantly, the people who are primary, asymptomatic and in incubation period are the main sources of infection, which is of critical significance to the epidemic prevention and control [2,15,19]. The patients included in this study were asymptomatic and the sample was collected within the first 72 hours after diagnosis; using a virological transport medium that favored the stability of the viral particles in the nasopharyngeal exudates, which facilitated the isolation of the SARS-CoV-2.

The isolation of the SARS-CoV-2 virus in Cuba allows conformation of a collection of autochthones primary strains. Besides, studies of phylogeny and evolutionary history of the complete SARS-CoV-2 genome in Cuba. And also provides an important tool with which to obtain considerable amounts of virus for further studies, such as clinical trials for the development of new antiviral drugs and comparative genomic analyses with the SARS-CoV-2 viral strains isolated from different populations.

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