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Viral culture and immunofluorescence for the detection of SARS-CoV-2 infectivity in RT-PCR positive respiratory samples

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Highlights:

- Viral isolation was successful in 58% of respiratory samples with positive RT-PCR.
- SARS-CoV-2 isolation is correlated with days of symptoms and RT-PCR Ct value.
- SARS-Co-2 infectivity lasts no more than 14 days in immunocompetent individuals.
- Ct value <22 always indicates infectivity.

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Abstract

Background: Knowing how long SARS-CoV-2-positive individuals can remain infective is crucial for the design of infection prevention and control strategies. Viral culture is the gold standard for detecting an active-replicative virus and evaluating its infectious potential.

Objective: To assess the correlation of SARS-CoV-2 infectivity with the number of days from symptom onset and the Ct value, using culture as a reference method. Also, to describe a detailed protocol for SARS-CoV-2 culture and immunofluorescence confirmation based on our experience with other respiratory viruses.

Study design: 100 consecutive respiratory samples positive for SARS-CoV-2 by RT-PCR from different subjects were inoculated into VERO E6 cells.

Results: Viral isolation was successful in 58% of samples. The median number of days from symptom onset for culture-positive samples was 2, and 15 for culture-negative samples. Six positive cultures were obtained in patients ≥14 days after symptom onset, all of whom were immunocompromised or with severe COVID-19. The mean Ct value was 12.64 units higher in culture-negative than in culture-positive samples. The probability of successfully isolating SARS-CoV-2 in samples with a Ct value <22 was 100%, decreasing to 3.1% when >27.

Conclusions: Our findings show a significant positive correlation between the probability of isolating SARS-CoV-2 in culture, fewer days of symptoms and a lower RT-PCR Ct value. SARS-Co-2 infectivity lasts no more than 14 days from symptom onset in immunocompetent individuals. In contrast, in immunocompromised patients or those with severe COVID-19 infectivity may remain after 14 days. Ct value <22 always indicates infectivity.

Key words: SARS-CoV-2, COVID-19, Viral Culture, Viral isolation, Infectivity, VERO E6 cells.

54 **1. Background:**

55 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a zoonotic enveloped
56 RNA virus, responsible for coronavirus infectious disease 2019 (COVID-19), which emerged
57 in Wuhan, China, in late 2019 and quickly spread worldwide, causing a global pandemic
58 [1]. Vaccination, early diagnosis, contact tracing and isolation of suspected and confirmed
59 cases are crucial for pandemic control [2, 3].

60 Reverse transcription polymerase chain reaction (RT-PCR) in respiratory samples is the
61 most sensitive and the most frequently used method for COVID-19 diagnosis and in
62 infection control precautions [4, 5]. Long-term shedding of viral RNA (≥14 days from
63 symptom onset) has been reported in COVID-19 patients [6, 7, 8]. SARS-CoV-2 RT-PCR can
64 remain positive for weeks, as it cannot distinguish between infective virus and viral
65 fragments without infectious potential, leading to prolonged periods of isolation or work
66 leave [6 ,9 ,10]. Determining how long SARS-CoV-2-positive individuals remain infective is
67 thus crucial for the design of effective infection prevention and control strategies [11].

68 The cycle threshold (Ct) value obtained in the RT-PCR is inversely related to the viral load
69 and has been employed as a semi-quantitative marker of infectivity and for clinical
70 decision-making [12, 13, 14]. However, the use of Ct values to infer SARS-CoV-2
71 transmissibility has many limitations, as they can be influenced by a multitude of factors,
72 including sample type, the adequacy of sample collection, transport and storage, or the
73 variety of platforms for RNA extraction and amplification [15, 16].

74 Viral culture is the gold standard for the detection of an active-replicative virus and the
75 assessment of its infectious potential [17, 18, 19]. As this technique requires laboratory
76 biosafety level 3 facilities (BSL3), experienced staff and a longer turnaround time than RT-
77 PCR, it is not used in routine diagnostic algorithms. Nevertheless, the culture of SARS-CoV-
78 2 plays an important role in providing a more complete understanding of its

79 transmissibility and duration of infectivity. As well as guiding recommendations for
80 infection prevention and control, this information is essential for the development and
81 validation of therapeutic agents and vaccines, and to assess the sensitivity and specificity
82 of molecular detection methods [20]. Monitoring the behaviour of SARS-CoV-2 has
83 become even more urgent with the emergence of new variants, as the impact of each
84 mutation needs to be understood [21].

85

86 ## 2. Objective:

87 To gain new insights into the behaviour of SARS-CoV-2 by comparing the results obtained
88 by culture (gold standard) with those of RT-PCR, and to establish the relationship between
89 the Ct value, the number of days from symptom onset, and the infectious potential of the
90 virus. Based on our group's experience in the diagnosis of respiratory viruses by
91 conventional techniques, another objective was to describe a protocol for SARS-CoV-2
92 culture and confirmation by immunofluorescence.

93

94 ## 3. Material and methods:

95 ### 3.1 Samples:

96 A total of 100 consecutive respiratory samples positive for SARS-CoV-2 by RT-PCR
97 (nasopharyngeal aspirates and swabs, bronchoalveolar lavage), collected in a viral
98 transport medium from different subjects between November 6, 2020 and May 25, 2021
99 in the Hospital de la Santa Creu i Sant Pau (HSCSP), were selected. Diagnostic RT-PCR in our
100 hospital is performed as soon as the sample is collected using different commercial
101 platforms. All samples were stored at 4°C until inoculation, which was always performed
102 within 48 hours of collection.

103 3.2 Culture:

104 Sample handling and cell culture procedures were performed in BSL3.

105 VERO E6 cells (Vircell, Spain) were used for SARS-CoV-2 culture. Before inoculation, each
106 sample was pre-treated with 10% of a mixture of antibiotics (vancomycin-gentamicin) and
107 amphotericin B for 30 minutes. 300µL of pre-treated sample was inoculated and incubated
108 at 37°C for up to 10 days.

109 Cell monolayers were examined daily with an inverted microscope (x40). A positive
110 culture was suspected when a characteristic cytopathic effect (CPE) was observed. Every
111 CPE (clear or doubtful) was confirmed by indirect immunofluorescence (IFI) using a specific
112 monoclonal antibody AntiSARS-CoV-2 (CertTest-BIOTEC, Spain). Culture was considered
113 negative when there was no CPE 10 days after the inoculation or a CPE was not confirmed
114 by IFI.

115 The complete protocol is provided in the Supplementary Material (S1).

116 3.3 Statistical analysis

117 The results are given as number of cases and percentage for categorical data and as
118 median and the other two quartiles for ordinal one. The comparison of Ct values and days
119 of symptoms between positive and negative cultures was done with the nonparametric
120 Mann-Whitney test. The statistical significance level was 5% (a=0.05), and two-tailed tests
121 were used throughout. All analysis was performed using IBM-SPSS software (version26;
122 SPSS. Inc. Armonk, NY).

123 3.4 Ethical approval

124 The study protocol was evaluated and approved by HSCSP Ethics Committee (IIBSP-VIR-
125 2014-41).

126

4. **Results**

A total of 100 consecutive RT-PCR positive SARS-CoV-2 respiratory samples from 100 patients were processed by culture; 46 of them were from paediatric subjects (<18 years) and 54 from adults (≥18 years). Eleven samples were from asymptomatic subjects, 59 corresponded to patients with <14 days from symptom onset and 30 to patients with ≥14 days from symptom onset. The median number of days (first quartile [Q1]; third quartile [Q3]) from symptom onset was 4 (1; 15). The presence of symptomatology and the number of days from symptom onset refer to the time the samples were collected for RT-PCR.

Viral isolation was successful in 58% samples. The percentage of positivity in persons <18 years was 60.9%, and ≥18 years, 55.6%. The median number of days (Q1; Q3) from the inoculation of the sample to positive culture was 3 (2; 4). The median (Q1; Q3) RT-PCR Ct value was 23.08 (17.2; 29.45). The overall results are summarised in Table 1.

Correlation between culture-days of symptoms:

Out of the culture-positive samples, 6 (10.34%) were from asymptomatic subjects, 46 (79.31%) corresponded to patients with <14 days from symptom onset and 6 (10.35%) to patients with ≥14 days from symptom onset. Out of the culture-negative samples, 5 (11.91%) were from asymptomatic subjects, 13 (30.95%) corresponded to patients with <14 days from symptom onset and 24 (57.14%) to patients with ≥14 days from symptom onset. The median number of days from symptom onset (Q1; Q3) for culture-positive samples was 2 (1; 5.75), and for culture-negative samples, 15 (4.5; 22).

Long-term viral shedding was detected by RT-PCR in 30 samples, but only 6 (20%) of them were culture-positive. Three of these culture-positive samples were from patients who had presented symptoms for more than 30 days (33, 41, 44 days).

Correlation between culture-RT-PCR Ct value:

The median RT-PCR Ct value (Q1; Q3) in culture-positive samples was 18.15 (15.88; 21.26), and in culture-negative samples, 30.79 (26.79; 34), being 12.64 units higher in the latter.

The probability of successfully isolating SARS-CoV-2 in samples with a Ct value <22 was 100%. This probability decreased to 3.1% in samples with a Ct value >27, as virus isolation was only achieved in one sample with a Ct value of 31.4. The probability of SARS-CoV-2 isolation according to RT-PCR Ct value is shown in Table 2.

In Table 3, the Ct values are broken down by the number of days with symptoms and culture. The correlation between culture and days of symptoms is shown in Figure 1, and between culture, Ct value and days of symptoms in Figure 2.

5. **Discussion**:

VERO E6 cells were selected for the present work as they have been previously found optimal for SARS-CoV-2 multiplication [22, 23]. These cells provide a versatile medium for the recovery of most viruses, including those that are difficult to isolate [24]. They were used to isolate SARS-CoV-2 from the first patients admitted to a Wuhan hospital for COVID-19 pneumonia in December 2019 [1], and for isolation and investigation of SARS-CoV in 2003-2004 [25, 26].

The cytopathic effect of SARS-CoV-2 in VERO E6 cells is easily recognisable and appears relatively quickly. In most cases, small syncytia begin to be observed in the monolayer in the first 48-72 hours post-inoculation. As the days pass, the number of syncytia and their size increases, until the entire monolayer is affected, and the cells are destroyed.

One of the main limitations in drawing conclusions from published studies on SARS-CoV-2 culture is the lack of standardisation and the great variability in culture protocols [27],

175 which makes it difficult to compare the results obtained. When implementing SARS-CoV-2
176 culture, we rely on our own established protocols; the reference technique for the
177 diagnosis of respiratory viruses in our laboratory has been based on culture and
178 immunofluorescence for more than 40 years.

179 In this study, SARS-CoV-2 was successfully isolated from 58% of the inoculated samples,
180 notably higher than the percentage of culture positivity reported in other studies [28, 29]
181 which ranges mainly from 20 to 40%. An explanation for this higher percentage of
182 positivity is the long experience of our laboratory in the study of respiratory viruses by
183 culture, which has allowed us to optimise this technique and successfully implement it in
184 our diagnostic routine.

185 When optimizing viral culture, an important point is to store the sample correctly and
186 inoculate it as soon as possible after collection. The longer the period between collection
187 and inoculation, the lower the chances of recovering the virus, due to degradation,
188 especially if optimal storage conditions are not maintained [30]. In our laboratory, all
189 samples were stored at 4°C and inoculated within 48 hours of collection. Another
190 significant aspect is how long the sample should be incubated before the culture can be
191 considered negative. In this work, all samples were incubated for 10 days; although most
192 CPE were observed within 72 hours post-inoculation, in some cases positive cultures were
193 obtained after 6 days. Some studies have classified cultures as negative before 6 days [6,
194 31], which entails a risk of missing positive cultures. A notable difference between our
195 approach and those of other SARS-CoV-2 culture studies is that, instead of confirming CPE
196 with an RT-PCR of the supernatant, the verification was done by immunofluorescence,
197 using a specific monoclonal antibody. RT-PCR of the supernatant can detect RNA from the
198 inoculated sample without the need for virus multiplication, but IFI allows the direct
199 observation of virus-infected cells. The last point to note about this work is that it includes

200 samples taken over a long period of time (more than 6 months), in contrast with 2 months
201 or less in other studies [28, 29].

202 Besides their differences in viral culture techniques, the studies are also quite diverse in
203 the type of individuals involved (age, symptomatology, days of evolution), which is a
204 further hindrance when attempting to draw conclusions. The majority include only adult
205 and symptomatic patients, with only a few small-scale studies of SARS-CoV-2 in paediatric
206 patients [13, 32]. In the present work, 46% of samples were taken from a paediatric age
207 group and like Singanayagam et al., a higher percentage of culture positivity was found in
208 paediatric individuals (60.9%) compared to adults (55.6%), although the difference was not
209 significant.

210 Likewise, there are very few published studies involving individuals who were
211 asymptomatic when testing positive for RT-PCR [13, 33]. We included 11 samples from
212 asymptomatic individuals at the time of sample collection, 6 (54.54%) of which were
213 culture-positive. These individuals were tested because of close contact with positive cases
214 or for hospital pre-admission screening. In agreement with the literature [13, 32, 33], our
215 results show that a high percentage of asymptomatic and paediatric individuals with a
216 positive SARS-CoV-2 RT-PCR are infective, which probably plays an important role in the
217 spread of the virus.

218 Also in agreement with the literature [28], a significant positive correlation was found
219 between the likelihood of isolating SARS-CoV-2 in culture, fewer days of symptoms and a
220 lower RT-PCR Ct value. A significant difference (13 days) was observed in the median
221 number of symptom days between culture-negative and culture-positive samples (15 days
222 vs. 2 days, respectively). The highest percentage of culture positivity was obtained in
223 samples from people with 1-2 days of symptoms (82.05%). In samples collected within the
224 first 7 days of symptoms, successful virus isolation was achieved in 77.8% of cases, but this

225 percentage decreased to 20% at 14 days or more of symptom onset, with positive cultures
226 obtained in only 6 out of 30 inoculated samples. All 6 were immunocompromised patients
227 who required admission to the Critical Care Unit due to severe complications of COVID-19
228 (3 patients with haematological malignancies, an untreated HIV patient, a morbidly obese
229 diabetic patient, and a 101-year-old man). Our data are in agreement with most studies
230 [28, 29], which could not detect an infectious virus after 10 days of symptom onset, except
231 in immunosuppressed individuals [34] or those with severe COVID-19 [35]. In these cases,
232 virus isolation was achieved 70 days and 32 days after symptom onset, respectively.

233 It is well established that the Ct value is inversely related to the probability of obtaining a
234 positive culture, which decreases by 32% for each Ct unit from a Ct of 24 [31]. The mean Ct
235 value was 12.64 units higher in culture-negative than in culture-positive samples, which
236 represents a significant increase. Virus isolation was successful in all samples with a Ct
237 value <22, but not achieved when the Ct value was >27, except in one case with a Ct value
238 of 31.4. This sample was from a 2-year-old girl with acute lymphoid leukaemia who was
239 tested by RT-PCR for pre-admission screening. However, another study [13] found that up
240 to 25% of samples with a Ct>30 corresponded to a potentially infectious virus. Despite
241 these results, the use of the Ct value as an indicator of infectivity is not recommended due
242 to its high variability, mainly conditioned by sample and technical factors [15, 16]. The
243 correlation between the Ct value and culture observed here is specific to our laboratory,
244 and therefore cannot be extrapolated elsewhere.

245 In summary, culture remains the gold standard for determining the infectious capacity of
246 viruses [17], but its laboriousness, turnaround time and the need for special biosafety
247 facilities make it unsuitable for routine COVID-19 diagnosis. However, specialized
248 laboratories equipped for viral culture and with the relevant expertise are needed to i)
249 increase our knowledge of virus transmissibility and duration of infectivity; ii) monitor

changes in the behaviour of emerging variants; and iii) conduct research on treatments, vaccines, or diagnostic techniques. Also required is a single defined protocol for SARS-CoV-2 culture, based on experience of culturing other respiratory viruses, and preferably including confirmation by visual identification via immunofluorescence [17]. Our findings show that the probability of isolating SARS-CoV-2 in culture is significantly and positively correlated with fewer days of symptoms and a lower RT-PCR Ct value. However, due to its high variability, use of the Ct value as a general marker of infectivity is not recommended, and the number of symptom days is a more reliable indicator.

Based on the results, it can be concluded that in immunocompetent individuals with mild-moderate COVID-19, SARS-CoV-2 infectivity does not last more than 14 days from symptom onset. In contrast, in immunocompromised patients or those with severe COVID-19, infectivity may remain beyond 14 days, after which the Ct value can be taken into consideration. A Ct value <22 always indicates infectivity, but when Ct value ≥22 it is inconclusive and culture is recommended.

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Declaration of competing interests

The authors declare that they have no financial interests or personal relationships that could have appeared to influence the work presented in this paper.

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275
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278 **Table 1: Comparative association of SARS-CoV-2 viral culture results with RT-PCR Ct**
279 **values and days from symptom onset.**

	Total	Positive culture	Negative culture	P-value
N	100	58	42	
Median RT-PCR Ct value (Q1; Q3)	23.08 (17.2; 29.45)	18.15 (15.88; 21.26)	30.79 (26.79; 34)	<0.001
Asymptomatic (n)	11	6	5	
Days of symptoms (median)	4	2	15	<0.001
• 1-2 days (n)	39	32	7	
• 3-7 days (n)	15	10	5	
• 8-13 days (n)	5	4	1	
• 14-30 days (n)	22	3	19	
• > 30 days (n)	8	3	5	

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281

282 **Table 2: Probability of isolating SARS-CoV-2 in culture in function of RT-PCR Ct value.**

RT-PCT Ct value (n)	Positive culture (%)	Negative culture (%)
<22 (45)	45 (100%)	0
22-27 (23)	12 (52.2%)	11 (47.8%)
>27 (32)	1 (3.1%)	31 (96.9%)

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285 **Table 3. Ct value according to days after symptom onset and the viral culture result**

Days of symptoms	Global Ct value	Ct value culture positive	Ct value culture negative
Asymptomatic	22.74 (16.48; 27.79)	16.51 (15.75; 21.19)	24.17 (23.05; 23.61)
1-2 days	19.3 (15.95; 23.08)	17.53 (15.64; 19.68)	31 (25.96; 32.95)
3-7 days	22.9 (17.37; 27.48)	18.89 (16.68; 22.61)	29 (27.86; 29.2)
8-13 days	19.7 (19.67; 21.6)	19.69 (19.67; 20.18)	32.1 (32.1; 32.1)
14-30 days	29.75(25.78; 34.88)	24.43 (20.82; 25.53)	30.58 (26.83; 35.08)
>30 days	29.95 (21.88; 33.13)	18.2 (16.7; 20.65)	32.8 (32.6; 34.1)

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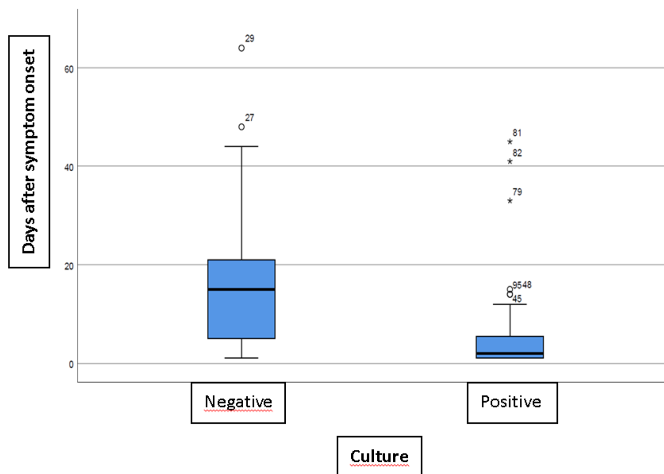

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290 **Figure 1. Viral culture results plotted by days after symptom onset:** most of the positive
291 cultures are concentrated within the first 13 days from symptom onset, but 6 outliers are
292 observed beyond 14 days, all of them patients with severe COVID-19 or immunodepressed.

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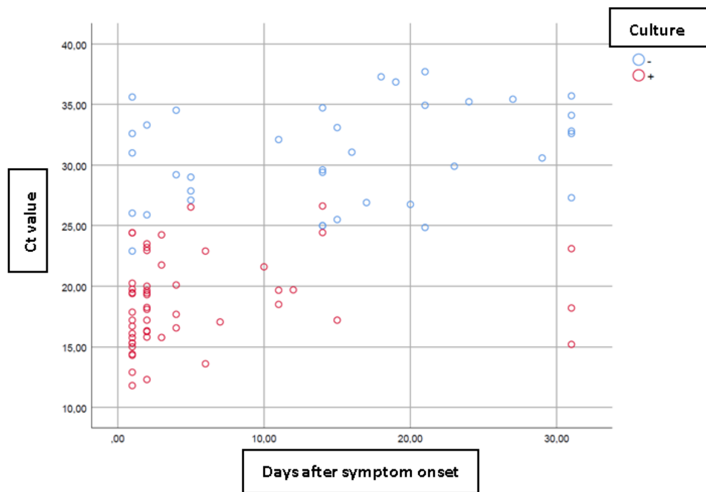

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296 **<u>Figure 2</u>. Viral culture results plotted by Ct values and days after symptom onset**. Most positive
297 cultures (red dots) are concentrated at low Ct values and within a few days of symptom onset.

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309 **S1. Complete protocol for cell preparation, sample inoculation and confirmation by**
310 **immunofluorescence.**

311 Cell preparation:

312 VERO E6 cells (ATCC CRL-1586. VIRCEL), which have an epithelial morphology and originate from
313 African monkey kidneys, were used for SARS-CoV-2 culture. Cells were maintained at 37°C in
314 cylindrical tubes with Basal Medium Eagle supplemented with 1% L-glutamine, 10% foetal calf
315 serum, 0.1% penicillin-streptomycin and 0.1% neomycin. *Mycoplasma* testing was performed,
316 and no *Mycoplasma* was detected. Tubes are ready for inoculation when a monolayer of cells is
317 fully formed (Photo 1: uninoculated monolayer).

318 Sample inoculation:

319 Sample handling and cell culture procedures were performed in biosafety level 3 facilities,
320 following all the required safety standards. Before inoculation, each sample was pre-treated
321 with 10% of a mixture of antibiotics (vancomycin and gentamicin) and an antifungal
322 (amphotericin B) for 30 minutes at 4˚C.

323 Culture inoculation protocol:

324 a. Check that the cell monolayer of the tubes selected for inoculation is in a suitable
325 condition. eave one tube from each pass uninoculated as a control.
326 b. Remove the medium from the tube of VERO E6 cells and inoculate 300 µL of the pre-
327 treated sample.
328 c. Leave for 1 hour at 37°C to facilitate virus contact with the cell monolayer.
329 d. Add 3 mL of Minimum Essential Medium + 2.5 % foetal calf serum.
330 e. Incubate the inoculated tubes at 37°C for up to 10 days.

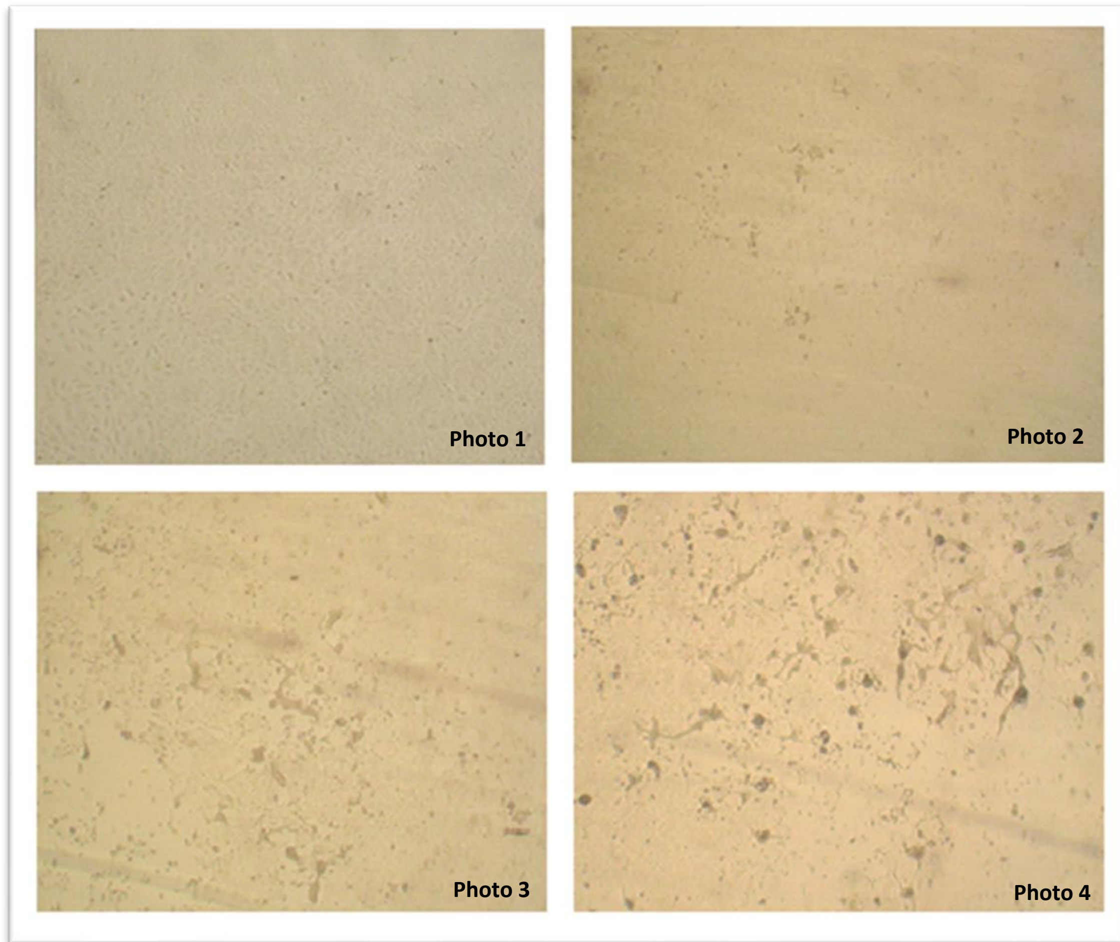
331 Virus isolation and identification

332 Cell monolayers were examined daily with an inverted microscope (x40). Tubes with
333 uninoculated VERO E6 cells were used as a cell culture control. A positive culture was
334 suspected when a characteristic cytopathic effect (CPE) was observed. Usually within 48-72
335 hours post-inoculation, small syncytia begin to be observed in the monolayer (Photo 2). As the
336 days pass, syncytia increase in size and number (Photo 3), until the entire monolayer is
337 affected, and the cells are destroyed (Photo 4).

338 Every CPE (clear or doubtful) was confirmed by indirect immunofluorescence (IFI) using a
339 specific AntiSARS-CoV-2 monoclonal antibody (Photos 5 and 6: IFI negative and positive,
340 respectively). Viral culture was considered negative when there was no CPE 10 days after
341 inoculation or the CPE was not confirmed by IFI.

342 Immunofluorescence protocol:

343 a. Remove the medium from the tube of VERO E6 cells and rinse twice with saline
344 solution.
345 b. Scrape the cell monolayer from the tube wall with a pipette, homogenize and place 50
346 µL of the suspension on a slide. Leave to dry on a hotplate for 10 minutes at 90ºC.
347 c. Fix with acetone for 10 minutes and leave to dry.
348 d. Add 50 µL of the Anti-SARS-CoV-2 monoclonal antibody (MT-16CV10, CertTest BIOTEC,
349 Spain) previously diluted 1/2000. Incubate for 30 minutes at 37ºC.
350 e. Rinse first with PBS and then with distilled water.
351 f. Add 50 µL of the fluorescein-labelled conjugate (Goat Anti-Mouse IgG Antibody FITC
352 Reagent. LIGHT DIAGNOSTICS, USA). Incubate for 30 minutes at 37°C.
353 g. Rinse first with PBS and then with distilled water.
354 h. Add mounting fluid (Diagnostic HYBRIDS, USA), place a coverslip on the slide and
355 observe under a fluorescence microscope (x400).



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357 **Photos 1-4: Condition of VERO E6 monolayer observed under inverted microscope (x40).**

358 Photo 1: VERO E6 monolayer uninoculated. Photo 2: VERO E6 monolayer 2 days postinfection.

359 Photo 3: VERO E6 monolayer 4 days postinfection. Photo 4: VERO E6 monolayer 5 days

360 postinfection.

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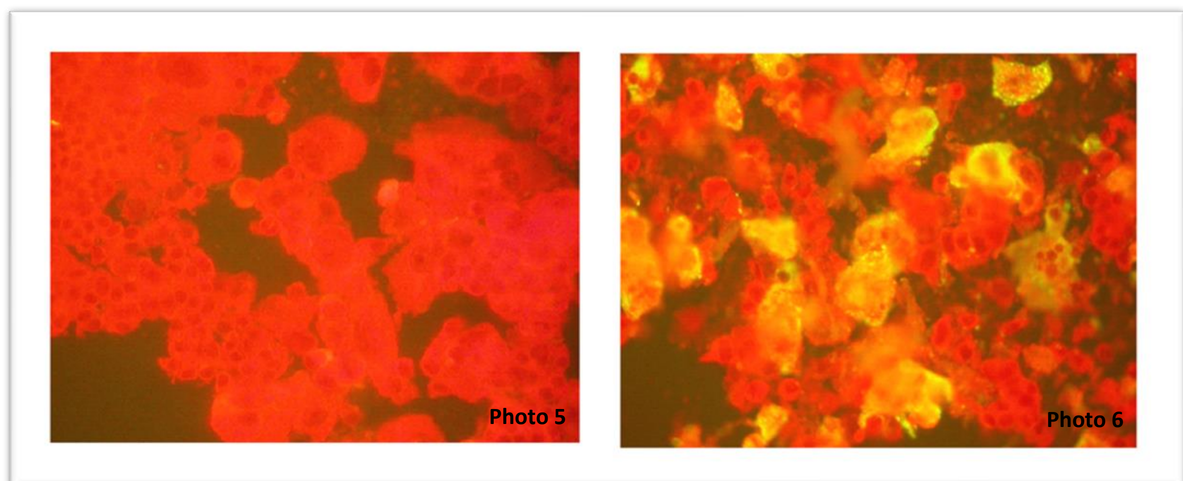

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363 **Photos 5-6**: Indirect immunoflourescence (IFI) on VERO E6 using a specific AntiSARS-CoV-2
364 monoclonal antibody, observed under a fluorescence microscope (x400) with negative an
365 positive results respectivily.

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