



Replication of SARS-CoV-2 in cell lines used in public health surveillance programmes with special emphasis on biosafety

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Background & objectives: Polio, measles, rubella, influenza and rotavirus surveillance programmes are of great public health importance globally. Virus isolation using cell culture is an integral part of such programmes. Possibility of unintended isolation of SARS-CoV-2 from clinical specimens processed in biosafety level-2 (BSL-2) laboratories during the above-mentioned surveillance programmes, cannot be ruled out. The present study was conducted to assess the susceptibility of different cell lines to SARS-CoV-2 used in these programmes.

Methods: Replication of SARS-CoV-2 was studied in RD and L20B, Vero/hSLAM, MA-104 and Madin-Darby Canine Kidney (MDCK) cell lines, used for the isolation of polio, measles, rubella, rotavirus and influenza viruses, respectively. SARS-CoV-2 at 0.01 multiplicity of infection was inoculated and the viral growth was assessed by observation of cytopathic effects followed by real-time reverse transcription-polymerase chain reaction (qRT-PCR). Vero CCL-81 cell line was used as a positive control.

Results: SARS-CoV-2 replicated in Vero/hSLAM, and MA-104 cells, whereas it did not replicate in L20B, RD and MDCK cells. Vero/hSLAM, and Vero CCL-81 showed rounding, degeneration and detachment of cells; MA-104 cells also showed syncytia formation. In qRT-PCR, Vero/hSLAM and MA-104 showed 10^6 and Vero CCL-81 showed 10^7 viral RNA copies per μl . The 50 per cent tissue culture infectious dose titres of Vero/hSLAM, MA-104 and Vero CCL-81 were $10^{5.54}$, $10^{5.29}$ and $10^{6.45}$ /ml, respectively.

Interpretation & conclusions: Replication of SARS-CoV-2 in Vero/hSLAM and MA-104 underscores the possibility of its unintended isolation during surveillance procedures aiming to isolate measles, rubella and rotavirus. This could result in accidental exposure to high titres of SARS-CoV-2, which can result in laboratory acquired infections and community risk, highlighting the need for revisiting biosafety measures in public health laboratories.

Key words Biosafety - cell lines - COVID-19 - SARS-CoV-2 - surveillance - virus replication

Virological surveillance programmes provide information on prevalence of diseases such as poliomyelitis, measles, rubella, rotavirus and influenza, which are monitored through national,

clinical and laboratory surveillance. Virus isolation using cell culture is an integral part of the above mentioned surveillance programmes. During the routine poliovirus surveillance programme, stool specimens or sewage samples are processed for virus isolation in L20B (recombinant murine cells) and RD (human rhabdomyosarcoma) cell lines¹. Respiratory specimens such as nasal or throat swabs are processed for isolation of measles, rubella and influenza viruses in the Vero/hSLAM (human Signalling Lymphocytic Activation Molecule, African green monkey kidney cells transfected with measles and rubella's viral receptors) and Madin–Darby Canine Kidney (MDCK) cell lines, respectively^{2,3}. For isolation of rotaviruses, stool specimens are processed and inoculated in MA104 (African green monkey kidney cells) cell line⁴. The SARS-CoV-2 is mainly transmitted via respiratory droplets from an infected person. Throat or nasal swabs are the preferred specimens for carrying out the diagnosis of COVID-19 using real-time reverse transcription–polymerase chain reaction (qRT-PCR). Virus isolation is carried out using the Vero-CCL81 cell line. However, it has also been reported that the SARS-CoV-2 is also shed in the stool of infected individuals, and found in sewage samples⁵. Thus, there are increasing concerns regarding unintended isolation of SARS-CoV-2 from respiratory and stool specimens which are collected and processed for virus isolation during polio, measles, rubella, influenza and rotavirus surveillance.

Processing of specimens during the routine surveillance programmes is conducted in biosafety level-2 (BSL-2) laboratories. Stool specimens and sewage samples are processed using chloroform for isolation of poliovirus⁶. A 10 per cent stool suspension is prepared and treated with antibiotics for isolation of rotavirus⁴. Respiratory specimens, collected in viral transport medium with antibiotics and centrifuged to remove debris, are inoculated onto MDCK and Vero/hSLAM cells for isolation of influenza measles and rubella viruses, respectively². A BSL-3 containment laboratory is recommended by the WHO for carrying out the isolation of SARS-CoV-2⁷. During a raging pandemic, unintended isolation of SARS-CoV-2 in a BSL-2 laboratory could result in a major breach in biosafety and might pose a high risk to the laboratory workers, as well as the community.

In view of the use of these cell lines in various virological surveillance programmes, the susceptibility of RD, L20B, MA-104, Vero/hSLAM and MDCK cell

lines to SARS-CoV-2 infection was assessed to explore the possibility of its unintended isolation.

Material & Methods

The study was conducted at the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), Pune, India after approval by the Institutional Biosafety Committee.

Biosafety considerations: All the experiments using SARS-CoV-2 virus, including inoculation of virus in cell lines and lysis of the cell culture before RNA extraction, were performed in a BSL-3 laboratory. Procedures such as cell line maintenance and qRT-PCR were performed in a BSL-2 laboratory, using class II A2 biosafety cabinets.

Virus strains: SARS-CoV-2 virus strain NIV-2020-770 from the ICMR-NIV, Pune, India⁸, Passage-3, isolated in Vero CCL-81 cells, with 50 per cent tissue culture infectious dose (TCID₅₀) titre of 10^{6.19}/ml was used for inoculation of cell lines. The study was done between August and October 2020.

Cell lines: Six different established cell lines, namely RD, MA-104, L20B (kindly provided by the Centers for Disease Control and Prevention, Atlanta, USA), Vero/hSLAM, MDCK and Vero CCL-81 (ICMR-NIV repository), at the recommended passage levels, were used in the study. Vero CCL-81 cell line was used as a positive control.

The cells were maintained in 25 cm² flasks (Corning Incorporated, USA) containing 5 ml minimal essential medium (MEM) supplemented with foetal bovine serum (FBS) (HiMedia Laboratories, Mumbai) at different concentrations, 100 U/ml penicillin and 100 µg/ml Mumbai streptomycin, and kept in humidified incubators with five per cent CO₂ at 37°C.

RD, L20B, MA-104, Vero h/SLAM and Vero CCL-81 cell lines were all maintained in Modified Eagle Medium (HiMedia Laboratories, India) supplemented with 10 per cent FBS. MDCK cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10 per cent FBS (HiMedia Laboratories). Confluent monolayers of the cells were used for preparation of 24 well plates (Nunc, Denmark) for virus inoculation. For all cell lines, the plates were seeded with 1.5×10⁵ cells/well, to be 90 per cent confluent within 24 h. Tissue culture grade 96 well microtitre plates (Nunc, Denmark) were seeded

with 1.5×10^4 cells/well, of MA-104, Vero/hSLAM and Vero CCL-81 cells for virus titration experiments to determine the $TCID_{50}$.

Virus infection with 0.1, 0.01 and 0.001 multiplicity of infection (MOI) in cell lines: RD, L20B, MA-104, Vero h/SLAM and Vero CCL-81 cell lines were infected with 0.1, 0.01 and 0.001 MOI with SARS-CoV-2 to assess the susceptibility of these cell lines.

Virus inoculation and determination of $TCID_{50}$: The cells were infected with SARS-CoV-2 virus, with 0.01 MOI in triplicates, and were incubated at 37°C. The virus was removed after an incubation of two hours. The wells were washed gently three times with 500 µl medium per wash per well for 24-well plates, and 150 µl medium per well for 96-well plates. Mock-infected wells with medium only were treated as cell controls. The plates were incubated at 37°C for 72 h. The virus culture media and all the experimental conditions for all the cell lines were similar. All the cell lines were observed daily for cytopathic effects (CPE)⁸. The viral growth kinetics study of SARS-CoV-2 in Vero-CCL81 cell line showed that infectious virus was detected seven hours post-infection. Therefore, cell lines were incubated for 72 h post-infection⁹. The supernatants were collected upon completion of 72 h incubation for virus detection and quantification by qRT-PCR. Second passage of the virus was performed in cell lines negative in the first passage for viral replication, and cells were observed for 72 h post-infection for CPE. The supernatants were collected for virus detection and quantification by qRT-PCR. In cases where no CPE was observed, the cell culture fluids were still processed in a similar manner.

$TCID_{50}$ was calculated based on the results of qRT-PCR as well as by CPE method. For the estimation of the $TCID_{50}$, serial ten-fold dilutions of the virus stock were performed and four wells of the 96-well microtitre plates with monolayers of the MA-104, Vero/hSLAM and Vero CCL-81 cell lines were infected with each dilution, using the similar protocol as mentioned above. The plates were incubated for 72 h with daily observation. After incubation, the tissue culture supernatants were collected and qRT-PCR was performed to determine the presence of viral RNA with Ct value ≤ 35 as the criterion for positive results. The values of four readings for each dilution were used for calculating $TCID_{50}$ using the Reed and Muench method¹⁰.

RNA extraction: Viral RNA was extracted from the supernatants using the QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, 140 µl tissue culture supernatant was added in 560 µl of lysis buffer. In the end, viral RNA was eluted in 60 µl of the elution buffer.

Real-time reverse transcription (qRT) PCR: One-step qRT-PCR assay was performed for the detection of the RNA-dependent RNA polymerase gene of SARS-CoV-2, using the Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific, USA) on the 7300 real-time PCR system (Applied Biosystems, USA)¹¹. Following was the composition of each 25 µl qRT-PCR reaction: 5.5 µl of nuclease-free water, 12.5 µl of 2× RT-PCR buffer, 1.5 µl of primer-probe mix, 0.5 µl of SuperScript™ III enzyme and 5 µl nucleic acid template¹¹. Thermal cycling conditions were as follows: 55°C for 15 min for reverse transcription and initial denaturation at 95°C for three minutes, followed by 45 cycles of 95°C for 15 sec and 58°C for 30 sec during which fluorescence data were collected. Appropriate, negative and positive controls were used in the assays.

Statistical analysis: The virus titres using qRT-PCR and CPE method were compared using the Student's t test function in Microsoft Excel (Microsoft Corporation, United States, 2016). $P \leq 0.05$ were considered as significantly different amongst the tested groups.

Results

Virus infection with 0.1, 0.01 and 0.001 MOI in cell lines: CPE was evident in Vero/hSLAM, MA-104 and Vero CCL-81 cell lines 48 h post-infection with 0.1, 0.01 and 0.001 MOI. The characteristics of CPE were rounding, degeneration and detachment of cells (Fig. A-F). In addition, MA-104 cells also showed multinucleated giant cell formation (Fig. A); 0.01 MOI of SARS-CoV-2 was used in further experiments. No CPE was observed in L20B, RD and MDCK cell lines, even after 72 h incubation and two serial passages.

qRT-PCR findings: In qRT-PCR, Vero h/SLAM and MA-104 showed 10^6 and Vero CCL-81 showed 10^7 viral RNA copies per µl. The cycle threshold (Ct) values ranged from 17.82 to 22.37, *i.e.* under the positivity threshold. Thus, SARS-CoV-2 replicated in Vero/hSLAM, MA-104 and Vero CCL-81 cells;

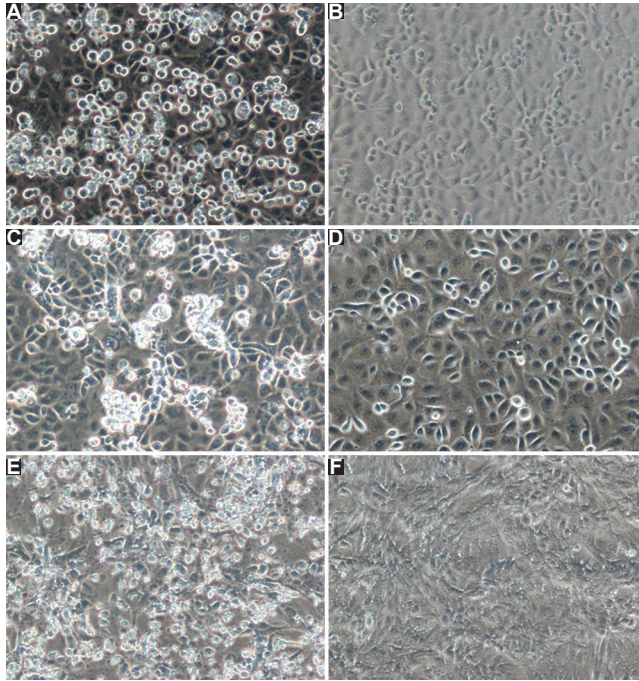


Figure. Cytopathic effects of SARS-CoV-2 in MA-104, Vero/hSLAM and Vero CCL-81 cell lines. Cytopathic effects observed 72 h post-infection in MA-104, Vero/hSLAM and Vero CCL-81 cell lines infected with SARS-CoV-2. The characteristics of CPE in all the infected cells showed rounding, degeneration and detachment of cells. (A) MA-104 infected, (B) MA-104 cell control, (C) Vero/hSLAM infected, (D) Vero/hSLAM cell control, (E) Vero CCL-81 infected, and (F) Vero CCL-81 cell control.

qRT-PCR results showed that the virus grew to high titres in these cell lines. MDCK, L20B and RD cell lines were negative for virus replication. Ct values of the supernatants of the first passage of the virus in MDCK cells ranged from 34.8 to 37.6. Of the three replicates, Ct values of two replicates were >35 . One replicate showed borderline Ct value 34.8, which could be because of the residual inoculum of the virus. All replicates in the second passage showed Ct values >35 (Table).

TCID₅₀: Since virus growth was observed in the MA-104, Vero/hSLAM and Vero CCL-81 cell lines, TCID₅₀ titres in these cells were determined using standard procedures. The TCID₅₀ titres of the virus based on qRT-PCR in MA-104, Vero/hSLAM and Vero CCL-81 cells were $10^{5.29}$, $10^{5.54}$ and $10^{6.45}$ /ml, respectively, indicating comparable growth of the virus in all these three cell lines. The TCID₅₀ titres by CPE method were $10^{4.8}$, $10^{6.4}$ and $10^{6.8}$ /ml in MA-104, Vero/hSLAM and Vero CCL-81 cells, respectively. The TCID₅₀ titres by CPE and qRT-PCR methods were compared and the difference was not significant.

Discussion

The present study showed that SARS-CoV-2 replicated in Vero/hSLAM, MA-104 and Vero CCL-81 cell lines. High viral RNA copy numbers were detected in these cell lines in spite of 0.01 MOI virus infections within 72 h. This indicated that these cell lines permitted the growth of SARS-CoV-2 even at low viral load that could be present in clinical specimens. At the time of infection, viral suspension was removed after virus adsorption by three rounds of washes to remove the non-adsorbed virus particles. Thus, any detection of viral RNA by qRT-PCR in the supernatant was considered as intracellular replication and shedding of the virus. This was confirmed by testing the washing medium from the third wash. It was found that medium from the third wash was negative for the presence of viral RNA (data not shown), indicating that detected viral RNA in all further experiments was from replicated virus in cell lines.

There is only one report each for the isolation of SARS-CoV-2 in Vero/hSLAM and MA-104 cell lines and the present study corroborates their previous findings^{12,13}. The virus did not replicate in RD, L20B and MDCK cell lines. There is only one study on the susceptibility of L20B and RD cell lines to SARS-CoV-2 and it corroborates the present findings¹⁴. Since the SARS-CoV-2 has been isolated from clinical specimens using Vero CCL-81 cell line and also has been used for the propagation of SARS-CoV-2, it was used as a positive control in the present study^{8,15}. The virus showed comparable TCID₅₀ titres in MA-104, Vero/hSLAM and Vero CCL-81 cell lines highlighting its growth potential in these cell lines, in case of unintended isolation. The TCID₅₀ was also calculated by conventional CPE method and compared with TCID₅₀ by qRT-PCR as a read out. No significant difference was found between the titres by both methods. The SARS-CoV-2 isolate was used for infection in the cell lines. The limitation of the present study was that the clinical specimens were not used for inoculation in these cell lines. It would be interesting to study the infection patterns of clinical specimens in all these cell lines, though the present data indicated growth even at low viral load. As there are various strains of SARS CoV-2 in circulation, the possibility of differential growth potential of such SARS-CoV-2 variants cannot be ruled out, which needs further study¹⁶.

Regarding the isolation of polio and non-polio enteroviruses in polio laboratories, stool and sewage samples are treated with chloroform before inoculation⁶.

Table. Replication of SARS-CoV-2 virus in cell lines 72 h post-infection

| Cell line | Species of origin and cell type | Passage number (P) | CPE | Real-time PCR results Ct values (<i>RdRp</i> gene copy numbers) | | |
|------------|--|--------------------|-----|--|-------------------------------|-------------------------------|
| | | | | Replicate 1 | Replicate 2 | Replicate 3 |
| Vero | African green monkey | P1 | 4+ | 17.82 (2.88×10 ⁷) | 20.05 (9.04×10 ⁶) | 18.51 (2.02×10 ⁷) |
| CCL-81 | kidney epithelium | P2 | 4+ | ND | ND | ND |
| Vero/hSLAM | African green monkey kidney epithelium expressing measles virus receptor | P1 | 3+ | 20.97 (5.60×10 ⁶) | 20.29 (7.97×10 ⁶) | 21.14 (5.11×10 ⁶) |
| | | P2 | 3+ | ND | ND | ND |
| MA-104 | African green monkey kidney epithelium | P1 | 2+ | 21.78 (3.66×10 ⁶) | 21.55 (4.12×10 ⁶) | 22.37 (2.69×10 ⁶) |
| | | P2 | 2+ | ND | ND | ND |
| RD | Human, Rhabdomyosarcoma | P1 | - | Undetermined | 38.18 (7.07×10 ²) | Undetermined |
| | | P2 | - | Undetermined | ND | ND |
| MDCK | Canine kidney epithelium | P1 | - | 37.58 (9.66×10 ²) | 35.37 (3.06×10 ³) | 34.78 (4.18×10 ³) |
| | | P2 | - | Undetermined | Undetermined | 39.72 (3.17×10 ²) |
| L20B | Murine, expressing poliovirus receptor | P1 | - | Undetermined | ND | ND |
| | | P2 | - | Undetermined | ND | ND |

Real-time RT-PCR results and cytopathic effects gradation of SARS-CoV-2 infection in six different cell lines; Each experiment was performed in triplicates. CPE gradation 1+25; 2+50; 3+75 and 4+100 per cent cells detachment, respectively. -, indicated no CPE; CPE, cytopathic effect; Ct, cycle threshold; ND, not done; PCR, polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; MDCK, Madin–Darby Canine Kidney, RT, reverse transcription; hSLAM, human signalling lymphocytic activation molecule; RD, human rhabdomyosarcoma

As SARS-CoV-2 is a lipid enveloped virus, it is prone to chloroform inactivation¹⁷. Both L20B and RD cells did not support the replication of SARS-CoV-2 and as sewage and stool specimens are treated with chloroform, the possibility of the unintended isolation of SARS-CoV-2 during polio surveillance is non-existent. There are no reports of the isolation of SARS-CoV-2 virus from sewage samples as of now. Therefore, isolation of polio and non-polio enteroviruses can be safely carried out by polio laboratories in BSL-2 facilities. However, though L20B and RD cell lines do not support growth of SARS-CoV-2, there is a possibility of isolation of polioviruses from these cell lines. Therefore, these cell lines should be used with caution in BSL-2 laboratories during post-eradication period of polio.

The permissiveness of Vero/hSLAM and MA-104 cell lines to SARS-CoV-2 underscores the possibility of its unintended isolation during measles, rubella and rotavirus surveillance. During rotavirus surveillance, pre-treatment of stool or sewage specimens with chloroform cannot be performed as chloroform has the ability to inactivate rotavirus¹⁸. Alternative methods for processing the stool and sewage specimens or respiratory specimens which could inactivate SARS-CoV-2 and isolate only rotaviruses or measles and rubella viruses need to be further explored. There are

a few reports of isolation of SARS-CoV-2 from stool samples⁵. The presence of SARS-CoV-2 RNA in stool samples of COVID-19 patients have been reported from India¹⁹. Viral load ranging from 5×10³ to 10^{7.6} has been reported from stool samples²⁰. However, no significant difference in the viral load in symptomatic, mildly symptomatic and severe symptomatic patients has been reported^{21,22}. This highlights the need of reviewing the biosafety measures for these programmes whenever isolation procedures are conducted.

In the present study, the MDCK cell line did not support SARS-CoV-2 replication, indicating that there is no possibility of unintended isolation of SARS-CoV-2 during influenza surveillance programmes. The absence of growth of SARS-CoV-2 in MDCK cell line has also been reported previously^{23,24}. It has been shown that angiotensin-converting enzyme 2 (ACE2) receptors present on host cells are used by the SARS-CoV-2 for viral entry²⁵. ACE2 receptors have been shown to be present on MDCK cells^{23,26}. This probably indicates that post-adsorption mechanisms and/or cell machinery of the MDCK cell line do not allow replication of SARS-CoV-2. Since embryonated chicken eggs are widely used for the isolation of influenza viruses, these have been explored for the growth of SARS-CoV-2²⁴. It was found that the

virus did not grow in eggs. Thus, there is no risk of inadvertent growth of SARS-CoV-2 in MDCK as well as embryonated chicken eggs which are used during influenza surveillance programme.

Indirect evidence indicates the presence of ACE2 receptors in the MA-104 cell line²⁷. It has been shown that ACE2 receptors are present on Vero E6 cells²⁸. However, there are no data on the presence of ACE2 receptors on Vero/hSLAM, Vero CCL-81, L20B and RD cells. L20B is originally a genetically engineered cell line of mouse origin, with predominant expression of CD155 receptors used by the poliovirus²⁹. It has been shown that SARS-CoV-2 is incapable of utilizing the murine ACE2 receptors³⁰. Therefore, it was expected that L20B would not support the growth of SARS-CoV-2.

Replication of SARS-CoV-2 in Vero/hSLAM and MA-104 underscores the possibility of its unintended isolation during surveillance procedures aiming to isolate measles, rubella and rotaviruses. This could result in accidental exposure to high titres of SARS-CoV-2, which can result in laboratory-acquired infections and community risk. For research programmes undertaking virus isolation from respiratory or stool specimens using other cell lines capable of supporting the growth of SARS-CoV-2, appropriate biosafety precautions must be followed in the times of the pandemic⁴.

To rule out the accidental isolation of SARS-CoV-2 from respiratory and stool samples, it would be helpful if all the clinical samples from which there is a possibility of isolating SARS-CoV-2 virus in Vero/hSLAM and MA-104 cell lines are screened for the presence of SARS-CoV-2 by RT-PCR before processing them for isolation of measles, rubella and rotaviruses during surveillance programmes and in research laboratories. Thus, there is a need for revisiting biosafety measures in public health laboratories undertaking virological surveillance.

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Conflicts of Interest: None.

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