

Short communication

## Identification of cell lines permissive for human coronavirus NL63

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### Abstract

Six cell lines routinely used in laboratories were tested for permissiveness to the infection with the newly identified human coronavirus NL63. Two monkey epithelial cell lines, LLC-MK2 and Vero-B4, showed a cytopathic effect (CPE) and clear viral replication, whereas no CPE or replication was observed in human lung fibroblasts MRC-5s. In Rhabdomyosarcoma cells, Madin–Darby–Canine-kidney cells and in an undefined monkey kidney cell line some replication was observed but massive exponential rise in virus yield lacked. The results will lead to an improved routine diagnostic algorithm for the detection of the human coronavirus NL63.

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The human coronavirus NL63 was detected in 2004 (van der Hoek et al., 2004; Fouchier et al., 2004) and meanwhile appeared to be a serious pathogen causing infection of the upper and lower airway in children and elderly (e.g. Bastien et al., 2005; van der Hoek et al., 2005). Although the NL63 infections occur frequently, the virus remained undetected until the usage of a new virus discovery strategy (VIDISCA), which led to its detection. The reason for this late detection despite the use of classical virus isolation techniques remains unclear. Difficulties to initiate an NL63-infection in the cell lines routinely used for diagnostics, or lack of CPE in these cells may be responsible. In order to address these questions with the aim of the development of a more sensitive diagnostic, we consequently tested several cell lines commonly used for routine diagnostic purposes for their ability to support coronavirus NL63 replication.

The following cell lines were cultivated with MEM Hanks'Earle's medium (Invitrogen, Breda, The Netherlands) with 5% bovine fetal calf serum (FCS) at 34 °C: Human lung fibroblasts (MRC-5: ATCC-CCL-171), rhabdomyosarcoma

(RD) cells (A-204; ATCC-ACC-250), Madin–Darby–Canine-kidney cells (MDCK: ATCC-CCL-34), monkey kidney cells (Vero-B4 cells: ATCC-ACC-33; LLC-MK2 cells: ATCC-CCL7), and a laboratory adapted non-ATCC-listed monkey kidney cell line named MS (monkey stable).

Cells were inoculated with MOI 0.1 (i.e. 10,000 TCID<sub>50</sub>/ml) in MEM Hanks'Earle's medium with 3% FCS for 8 h at 4 °C. In order to avoid subsequent detection of dead input virus or free input NL63-RNA, the inoculation medium was removed, cells were washed twice with phosphate buffered saline, and fresh pre-warmed medium (34 °C) was added. Aliquots (500 µl) from the supernatant were sampled at inoculation and then every 24 h up to day 7 post inoculation (p.i.). Cells were viewed under a microscope daily and the occurrence of CPE was recorded.

Total RNA was extracted from the supernatant by the silica-affinity based Boom extraction method (Boom et al., 1990) and eluted in 100 µl water. Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen; 200 U per reaction) and 10 ng of random hexamers (Amersham Biosciences) in 10 mM Tris pH 8.3, 50 mM KCl, 0.1% Triton-X 100, 6 mM of MgCl<sub>2</sub> and 50 µM of each dNTP at 37 °C for 90 min in a total volume of 40 µl.

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Virus yield was determined by real time PCR using the Platinum Quantitative PCR SuperMix-UDG (Invitrogen) (Fig. 1). A 10  $\mu$ l of cDNA was amplified in 50  $\mu$ l 1 $\times$  Platinum quantitative PCR Super mix-UDG (Invitrogen) with 5.5 mM of MgCl<sub>2</sub>, 10  $\mu$ M of specific probe labeled with FAM and Tamra and 45  $\mu$ M of each primer. The following primers were used for

HCoV-NL63—sense: 5'-GCGTGTTCCTACCAGAGA GGA-3'; anti-sense: 5'-GCTGTGGAAAACCTTTGGCA-3'; probe: 5'-FAM-ATGTTATTCAGTGCTTTGGTCTCTCGTGAT-TAM-RA-3'. The reaction was carried out on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems Foster City, California). Following the UDG treatment for 2 min at

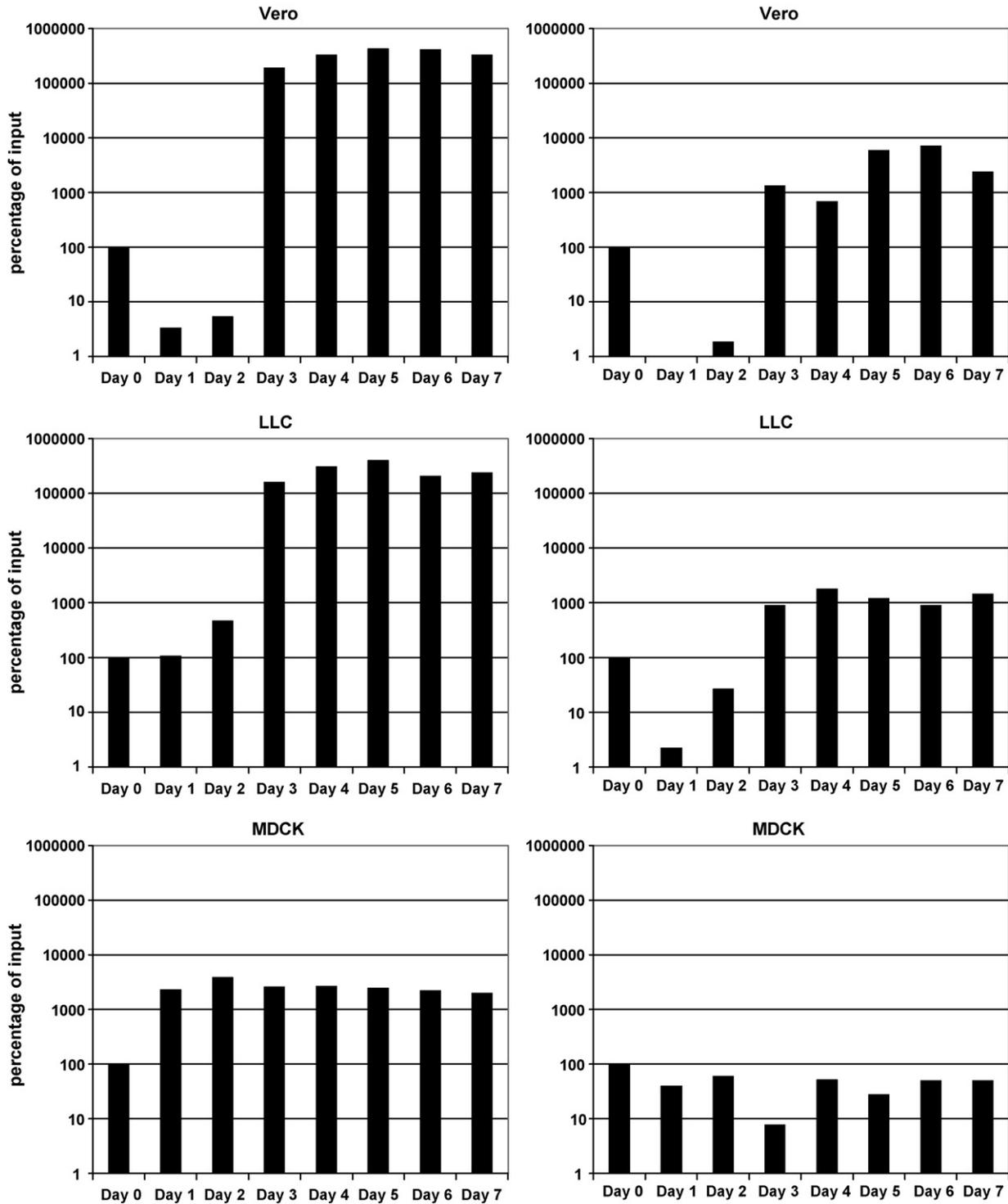


Fig. 1. Two representative quantifications of coronavirus NL63-RNA in culture supernatant of different cell lines. Viral RNA was measured by quantitative real time RT-PCR and is represented as percentage compared to the inoculated virus before washing (day 0). Six indicated cell lines were used of which only the LLC-MK2 and Vero-B4 cells displayed clear NL63 replication.

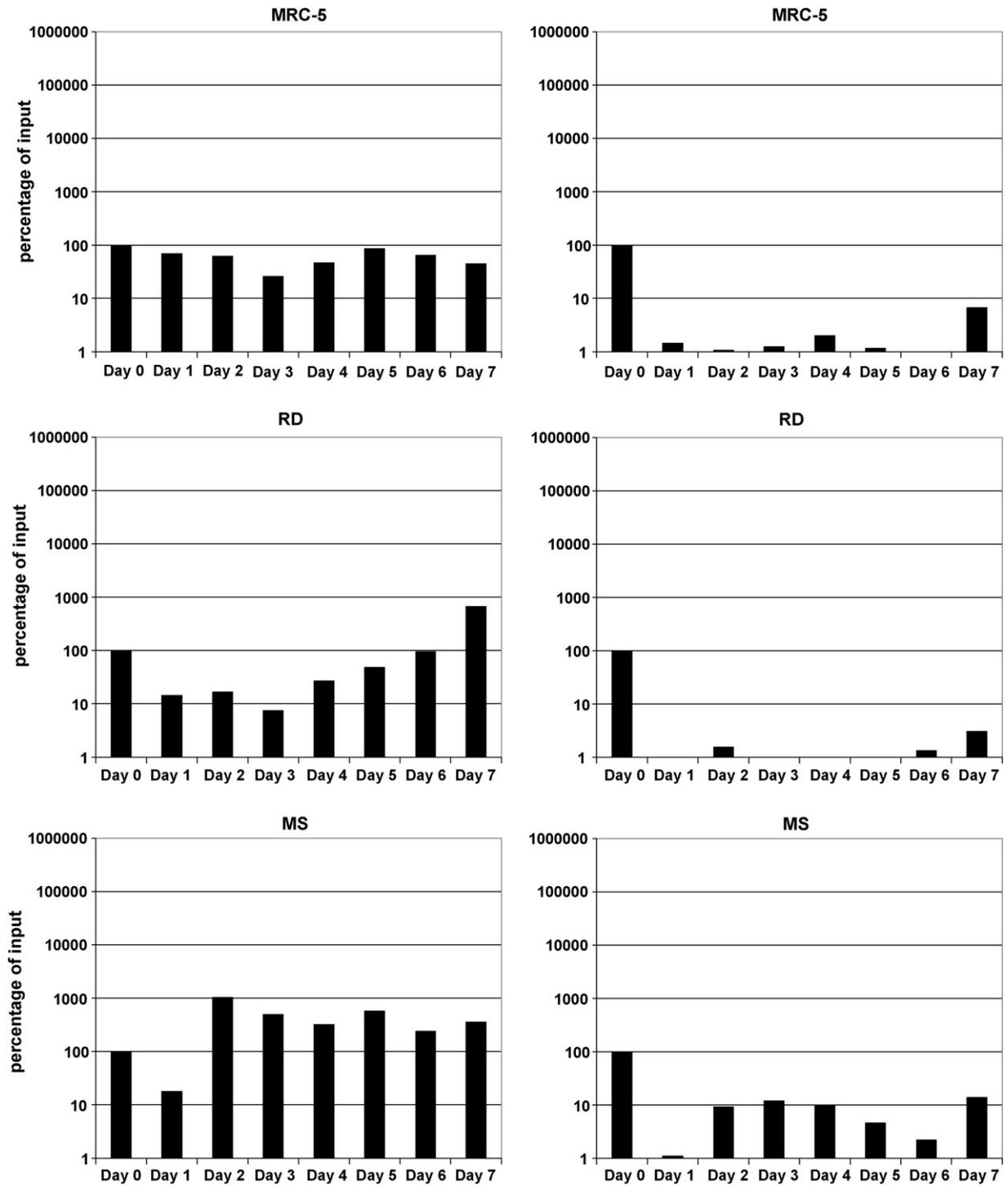


Fig. 1. (Continued).

50 °C and the denaturation for 5 min at 95 °C, 45 cycles of amplification were performed for 15 s at 95 °C and 60 s at 60 °C.

RNA from a titrated virus culture was used as input for the standard curve. The concentration in the viral RNA stock was determined using in vitro transcribed RNA.

The viral growth kinetics examined by real time PCR revealed that LLC-MK2 cells as well as Vero-B4 cells, both monkey kidney cell lines, supported the amplification of viral genomes,

resulting in high titers with an up to 4000-fold increase in virus yield. In both cell lines an initial decrease in the number of genomes in the supernatant was observed that was most obvious for Vero-B4 cells inoculated with coronavirus NL63. The number of genomes increased from days 2 to 3 post inoculation and reached a plateau on day 4, resulting in equal kinetics. This observation confirmed and extended the earlier finding by Fouchier et al. (2004) and Kuiken et al. (2003) who found Vero118 permissive for NL63 and SARS coronavirus, respec-

tively, consequently giving rise to the assumption that Vero cells in general are permissive for NL63.

Only marginal increases in the number of genomes that were in the range of the intra-assay variability were observed for MDCK cells, MS cells, and RD cells, giving raise to the hypothesis that these cell lines were not permissive or at most not fully permissive for coronavirus NL63. Taking into account that these cell lines also displayed a CPE (see below) one may speculate that inoculation of MDCK cells, MS cells, and RD cells with coronavirus NL63 is toxic for these cell lines, probably due to the temporarily expression of viral genes of NL63 that was not earlier. However, it remains to be investigated whether there is indeed no intracellular genome amplification or whether these cell lines solely cannot excrete viral particles into the cell culture supernatant, both of which is possible in case of LLC-MK2 and Vero-B4 cells. Most interestingly, lung fibroblasts were not permissive for NL63 at all, suggesting that these cell lines have de-differentiated in cell culture. CPE were observed in all cell lines from days 4 to 5 (MDCK, MS, Vero-B4, LLC-MK2) or at days 5–6 (RD). This effect was virus-specific since negative control cells displayed no cytopathicity until day 6. The observed NL63-CPE was virtually indistinguishable from cytopathic effects caused by other viruses; thereby, from the morphological changes observed, the observed CPE resembles the CPE induced by picornaviruses. Infected cells became small and detached from the cell culture flask. Thereby, no plaque formation but a diffuse initiation of CPE formation was observed. Neither a CPE nor an increase in the number of genomes was observed for MRC-5 lung fibroblast.

The results indicate, in analogy to earlier observations with human metapneumovirus (Deffrasnes et al., 2005), that some cell lines routinely used for viral laboratory diagnostics are permissive for coronavirus NL63 infection. The lack of a specific CPE but also the limited number of fully permissive cell lines may be the reason for the “late” identification of the virus, as the CPE, if it occurred, may have been interpreted as CPE from known viruses.

This latter observation that the CPE was unspecific or may have been absent at all may explain why isolation of new NL63 isolates has not been described since the initial studies by van der Hoek et al. (2004) and Fouchier et al. (2004). As LLC-MK2 and Vero are very common cells in diagnostic facilities, the original isolation of NL63 may thus have been an exceptional event and diagnostic culture on these cells may be very unrewarding. Thus, also in case of a lacking CPE it may be worth to monitor cell culture supernatants by PCR assays in case of a given clinical evidence for a viral respiratory infection.

In summary, in order to improve the laboratory diagnosis of coronavirus NL63, we would recommend first to perform the

PCR analysis of the specimen and then to subsequently inoculate a broad spectrum of cell lines, followed by an additional PCR analysis of the cell culture supernatant. By this algorithm there would be no delay in the diagnosis, because in routine diagnostics time is often a critical issue, but additional information may be available after all procedure were finished. Although we have not compared the differences in the sensitivity of the combined PCR/culture/PCR protocol versus a PCR-only protocol, a number of earlier publications describing the identification of “new” viruses clearly demonstrate that with respect to the spectrum of viruses the combined approach appears to be superior (e.g. van den Hoogen et al., 2001; van der Hoek et al., 2004; Fouchier et al., 2004).

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