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Susceptibility of different eukaryotic cell lines to SARS-coronavirus

Brief Report

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Summary. In order to define and characterize target cells of SARS-coronavirus (SARS-CoV) we studied the susceptibility of 23 different permanent and primary eukaryotic cell lines to SARS-coronavirus. Beneath Vero E6 cells SARS-Coronavirus infection could also be demonstrated in two pig cell lines (POEK, PS) and one human cell line (Huh-7) using the indirect immunofluorescence assay and a newly established quantitative real-time PCR. In all susceptible cell lines mRNA of the Angiotensin-converting enzyme 2 (ACE2), the functional receptor for SARS-CoV infection, could be detected by RT-PCR. Our results show that there is a correlation between the abundance of ACE2 mRNA and SARS-CoV susceptibility.

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A novel coronavirus with a positive single-stranded RNA genome of approximately 30.000 bp has been identified as the causative agent of severe acute respiratory syndrome (SARS) [3, 8]. The SARS-coronavirus (SARS-CoV) infects the lower respiratory tract with patients presenting fever, dry cough, dyspnoea, headache and hypoxemia [7]. The disease spread rapidly from Southern China to more than 30 countries within a few weeks resulting in about 8.400 cases and 800 deaths [13]. The functional cellular receptor for SARS-CoV could recently be identified as the Angiotensin-converting enzyme 2 (ACE2) [9].

So far the reservoir of SARS-CoV has not yet been precisely clarified. The virus could be isolated out of palm civets (*Paguma larvata*), raccoon dogs (*Nycereutes procyonoides*) and other mammals that are for sale on live animal

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markets and sometimes eaten in China [2]. However, these species are not necessarily the natural hosts.

In our study we investigated if SARS-CoV could infect and replicate in permanent cell lines and primary cells of different species in order to i) define and characterize potential target cells of SARS-CoV, ii) to understand the mechanism of virus transmission and the nature and range of target cells and organisms. We furthermore investigated SARS-CoV susceptible and not-susceptible cell lines for ACE2 mRNA.

To study the kinetic of SARS-CoV infection, various cell lines were infected with SARS-CoV strain Hong Kong at multiplicities of infection (M.O.I.) of approximately 30. The production of SARS-CoV was determined in the supernatant and in the infected cells at definite time points post infection using a quantitative real-time PCR [11]. In parallel, infected cells were investigated for the presence of viral protein using an indirect immunofluorescence assay (IFA) [5].

For stock production, SARS-CoV (strain 6109) isolated from a Hong Kong patient (kindly provided by Wilina Lim, Government Virus Unit Hong Kong) was added to Vero E6 cells (American Type Culture Collection, ATCC, CRL 1586). After 8 h of incubation the supernatant and the infected cells were harvested, stringently centrifuged (10 min at 6000 × g) and the supernatant was aliquoted. Afterwards the virus titre was determined ($3.25 * 10^7$ PFU/ml).

All cell lines used (Table 1) were grown in the appropriate culture medium recommended by ATCC or ECACC (European Collection of Cell Cultures). Porcine Peripheral Blood Mononuclear Cells (PBMC) were isolated from a healthy pig and grown in Roswell Park Memorial Institute 1640 Medium (RPMI 1640) (Gibco, Paisley, UK) with 10% FCS and 2 mM glutamine (ICN, Costa Mesa) and 100 µg/ml streptomycin and 100 U/ml penicillin (Biochrom, Berlin, Germany). Chicken embryo fibroblasts were prepared from 11-day-old chicken embryos and cultivated in Dulbeccos Modified Eagle Medium (D-MEM) (Gibco, Paisley, UK) supplemented with 10% FCS and 100 µg/ml streptomycin and 100 U/ml penicillin.

One day before infection adherent cells were seeded onto sterile glass slides in 12-well plates while suspension cells were cultivated in 6-well plates. Adherent cells were infected with 25 µl and suspension cells with 100 µl of infectious supernatant of SARS-CoV ($3.25 * 10^7$ PFU/ml). Vero E6 cells were used as a positive control while uninfected cells were used as negative controls.

For quantitative real-time PCR RNA from approximately $2.5 * 10^4$ cells was prepared using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany). Similarly, infected cell supernatant was centrifuged at 1.000 rpm in a Heraeus Varifuge to remove cells; RNA was extracted from 140 µl supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The amount of SARS-CoV RNA was determined in triplicate by quantitative real-time PCR as described elsewhere [11].

For RT-PCR analysis total RNA was isolated using RNeasy Protect Mini Kit (Qiagen) and was twice digested with DNase I (AMBION, Huntingdon, UK) as described in the manual instructions. For the detection of ACE2 mRNA, cDNA was produced from total RNA of all cell lines susceptible for SARS-CoV

Table 1. List of cell lines used for the experiments

Designation	Tissue	Source	Organism	Susceptibility
Huh-7D12	liver	*ECACC 01042712*	human	+++
Hep2	liver	*ATCC HB-8065*	human	+
293	fetal kidney	*ATCC CRL-1573*	human	−
RH	kidney	*RKI*[1]	human	−
Ma23	lungfibroblast	*RKI*	human	−
Chang Liver	hela contaminant	*ATCC CCl-13*	human	−
RD	mouthcarcinoma	*RKI*	human	−
Wil2.NS.6TG	spleen	*ECACC 93031001*	human	−
C8166	t-lymphocyte	*ECACC 88051601*	human	−
U937	monocyte	*ATCC CRL-1593.2*	human	−
H9	t-lymphocyte	*ATCC HTB-176*	human	−
Vero E6	kidney	*ATCC CRL-1586*	monkey	+++
PBMC[2]		*Charité*[3]	porcine	−
POEK	fetal kidney	*RKI*	porcine	++
PS	kidney	*RKI*	porcine	+
PK	kidney	*ATCC CCL-33*	porcine	−
MDBK	kidney	*ATCC CCL-22*	bovine	−
PG-4	fibroblasts	*ATCC CRL-2032*	feline	−
AK-D	lung	*ATCC CCL-150*	feline	−
FeT-J	t-lymphocyte	*ATCC CRL-11967*	feline	−
CTL-6	fibroblasts	*RKI*	murine	−
RAT-2	fibroblasts	*RKI*	murine	−
Embryo Fibroblasts	11 day old embryo	*RKI*	chicken	−

[1]RKI Robert Koch-Institut, Berlin, Germany
[2]PBMC Peripheral Blood Mononuclear Cells
[3]Charité, Berlin, Germany
$^{+++}$High infection rate; 50–100% of positive cells 31 h after infection determined by IFA
$^{++}$Moderate infection rate; less than 50% of positive cells 31 h after infection determined by IFA
$^{+}$Low infection rate; isolated cell infection 31 h after infection determined by IFA
$^{-}$Negative cells 31 h after infection determined by IFA

and from some not-susceptible cell lines using the following protocol: 250 ng of total RNA was reverse transcribed in 20 µl volume with 250 ng oligo dT primer (Fermentas, St. Leon-Rot, Germany), 20 nmol dNTPs (Eppendorf, Hamburg, Germany), 40 U RNasin (Promega, Mannheim, Germany), 4 µl 5 × buffer, 100 nmol $MgCl_2$, 200 nmol DTT and 200 U SuperScript II RT (Invitrogen, Karlsruhe, Germany). Before adding the enzymes the reaction mixture was heated to 65 °C for 5 min and placed on ice for 1 min. The program for the reverse transcription was 42 °C for 50 min and 70 °C for 15 min. To check if the transcription has been successful and the transcribed RNA has been free of PCR inhibitors all human and simian cDNA samples were tested in a control PCR using primers detecting the glycerylaldehyde 3-phosphate dehydrogenase (GAPDH)

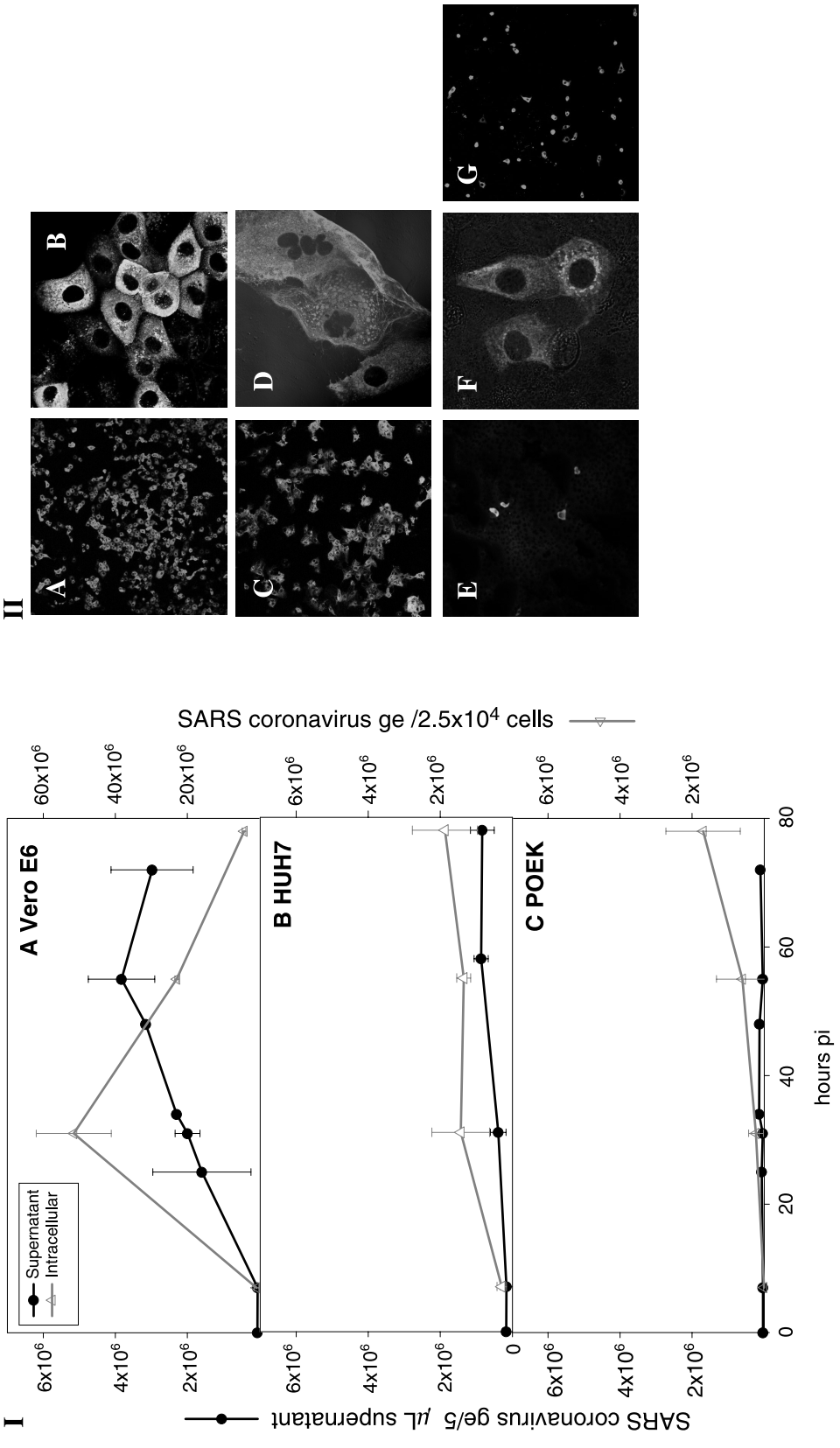
gene [12]. Amplification was performed in a 25 µl reaction volume using 1 µl of cDNA, 2.5 µl 10 ∗ buffer, 5 nmol dNTPs, 75 nmol $MgCl_2$, and 1 U Taq Polymerase (Invitrogen, Karlsruhe, Germany) (94 °C, 2 min; 35 ∗ [94 °C, 30 sec; 59 °C, 30 sec; 72 °C, 1 min] 72 °C, 3 min). The resulting PCR product had a length of 225 bp. Same PCR conditions were used for the amplification of ACE2 cDNA with primers ACE2 F 5′-$_2$GCCCAACCCAAGTTCAAAG$_{20}$-3′ and ACE2 R 5′-$_{170}$CCTCAATGGTGGACTGAGCA$_{151}$-3′. Nucleotide positions refer to NCBI sequence GenBank accession no. **NM021804** (human ACE2). The resultant PCR product of 168 bp was sequenced and analyzed in BLAST.

SARS-CoV infected cells were cultivated for a total of 78 h. At 0, 7, 31, 55 and 78 h after infection i) cell morphology was assessed by inspection with a light microscope for CPE diagnosis, ii) supernatant and cells were investigated for viral RNA load using the quantitative real time PCR, iii) cells were fixed and investigated for viral protein with the indirect immunofluorescence assay and analyzed by confocal laser scanning microscopy. Furthermore SARS-CoV susceptible and not-susceptible human cell lines were analyzed for mRNA of ACE2.

Analysis with a light microscope revealed CPE in Vero E6 cells starting 7 h after infection and in Huh-7 cells starting 31 h after infection. Vero E6 cells formed syncytia or progressed from typical elongated morphology to round dead cells with cell debris in the supernatant. By 55 h after infection almost all Vero E6 cells were detached from their support, whereas Huh-7 cells were still growing in monolayer and tended to syncytia formation. In contrast, no visible changes were observed in the porcine cells (data not shown).

IFA revealed that 7 h after infection viral protein could be detected in approximately 50% (data not shown) and 31 h after infection in approximately 100% of the Vero E6 cells (Fig. 1/II A, B). In contrast, 50% of the Huh-7 cells were positive for viral antigen not until 31 h after infection (Fig. 1/II C, D). At that time, the first presence of viral antigen could also be detected in porcine POEK (Fig. 1/II E, F) and in porcine PS cells (data not shown) but not in any of the other cell lines. However, compared to Vero E6 and Huh-7 cells (Fig. 1/II A and C) the number

Fig. 1. Detection of SARS-CoV RNA in supernatant and cell lysate of SARS-CoV infected cells and results of the indirect immunofluorescence assay. Detection of intracellular SARS-CoV RNA (**1/I A–C**, grey line) and in the corresponding supernatant (**1/I A–C**, black line) of SARS-CoV-infected Vero E6 (**1/I A**), Huh-7 (**1/I B**) and POEK (**1/I C**) cells at different time points post infection (pi) using the quantitative real time PCR. Genome equivalents (ge) are given per 25,000 cells and per 5 µl supernatant. Consider different scaling for the individual figures. Results of the indirect immunofluorescence assay 31 h after infection of Vero E6 (**1/II A**, **B**), Huh-7 (**1/II C**, **D**) and POEK cells (**1/II E**, **F**) with SARS-CoV Hong Kong ($3.25 * 10^7$ PFU/ml). The picture shown in **1/II G** was taken after a 4-week incubation of POEK cells with SARS-CoV Hong Kong ($3.25 * 10^7$ PFU/ml). Cells were analyzed with a confocal laser scanning microscope at a 100-fold magnification (**1/II A**, **C**, **E**, **G**) and a 630-fold magnification (**1/II B**, **D**, **F**) respectively


I
A Vero E6
B HUH7
C POEK
Supernatant
Intracellular
SARS coronavirus ge /2.5x10^4 cells
SARS coronavirus ge/5 μL supernatant
hours pi
II
A
B
C
D
E
F
G

of SARS-CoV-positive porcine cells was low, despite of the same M.O.I. that had been used for the infection of all adherent cell lines.

The quantification of SARS-CoV RNA by quantitative real-time PCR revealed a significant increase of intracellular viral RNA in Vero E6, Huh-7, POEK, (Fig. 1/I A–C) and PS cells (data not shown). To determine whether extracellular virus particles had been produced by SARS-CoV infected human and porcine cells, cell-free supernatants were tested by quantitative real-time PCR at different times post infection. An increase of SARS-CoV RNA was detected in the supernatant of infected Vero E6, Huh-7, POEK (Fig. 1/I A–C) and PS cells (data not shown).

As expected, the investigation of all SARS-CoV susceptible cell lines (Vero E6, Huh-7, POEK and PS) for mRNA of ACE2 was positive in all cases though we failed to detect ACE2 expression by IFA, Western Blot and FACS analysis using commercially available monoclonal and polyclonal antibodies (ALPHA DIAGNOSTICS, San Antonio, USA) against human ACE2 (data not shown).

Although same amounts of cDNA were used and the experiments were repeated three times the signals of the porcine cell lines maintained much weaker (Fig. 2A lane 3 and 4). After sequencing, BLAST analysis of the resultant PCR products of SARS-CoV susceptible Vero E6 and Huh-7 cells showed a 98% homology to the mRNA of the human ACE2 (GenBank accession no. **NM021804**), while porcine POEK and PS cells showed 87% homology to the mRNA of the human ACE2 (GenBank accession no. **NM021804**).

No mRNA of ACE2 could be detected in the not-susceptible cell lines (Fig. 2B). All cDNA samples used in the ACE2 PCR have been tested positive in the control PCR (Fig. 2C).

To examine whether the infection efficiency in porcine POEK could be increased by viral adaptation cells were infected with SARS-CoV Hong Kong as mentioned above and cultivated for 4 weeks. After this period of time the indirect immunofluorescence assay was carried out and SARS-CoV positive cells were counted. An adaptive effect resulting in a 10-fold higher infection rate could be observed in POEK cells (Fig. 1/II G).

In our studies we could demonstrate that the green monkey cell line Vero E6 and the human Huh-7 cells show a high susceptibility to SARS-CoV resulting in high infection rates within hours. Using the quantitative real-time PCR we could detect up to $6 * 10^7$ RNA copies in $2.5 * 10^4$ SARS-CoV infected Vero E6 cells 31 h after infection. These data could be confirmed with the IFA showing up to 100% infection of Vero E6 cells and 50% infection of Huh-7 cells 31 h after infection. Another finding in our studies was the demonstration that SARS-CoV could replicate in porcine PS and POEK cells. These observations have also been made by others [4] who demonstrated replication of SARS-CoV in the porcine cell line PK-15. However, in our experiments infection rates of the porcine cells with SARS-CoV were clearly lower.

The Angiotensin-converting enzyme 2 has been identified to play an important role in SARS-CoV entry [9]. It can be expected that the SARS-CoV susceptible cells express a SARS-CoV specific receptor. We could identify mRNA of ACE2

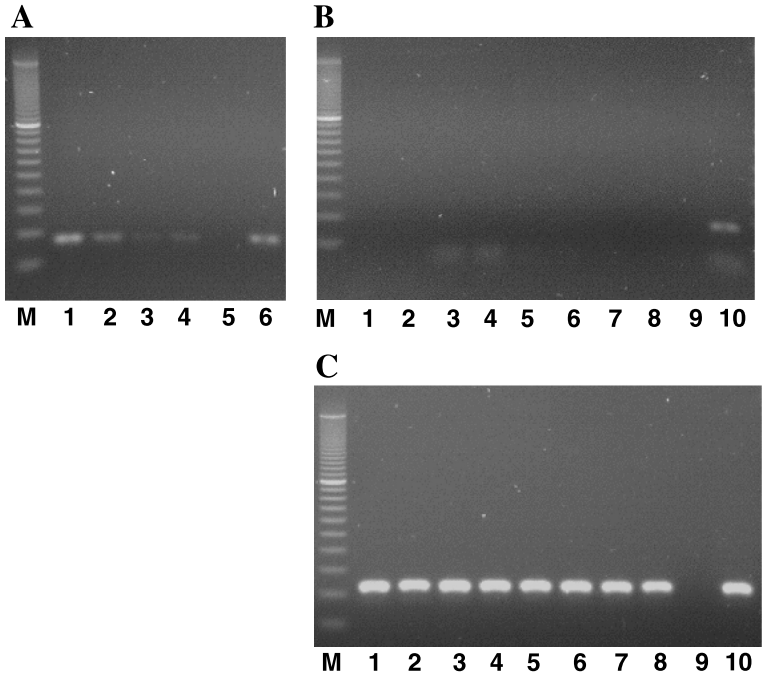


Fig. 2. Results of the ACE2 PCR (susceptible and not-susceptible cell lines). Results of the ACE2 PCR with cDNA transcribed from mRNA of SARS-CoV susceptible cells are given: *M* Marker, *1* Vero E6, *2* Huh-7, *3* POEK, *4* PS cells; *5* shows the negative control while Vero E6 cDNA was used as a positive control (*6*). **B** shows the results of the ACE2 PCR after transcription of mRNA of some not-susceptible cell lines: *M* Marker, *1* DNA of Chang liver, *2* MRC-5, *3* 293, *4* U937, *5* Wil-2, *6* H9, *7* Hep2, *8* PG-4, negative control (*9*), positive control (*10*). The size of the fragment expected after amplification with primers ACE2 F/R was 168 bp. **C** shows the results of the GAPDH PCR from transcribed cDNAs of the above mentioned not-susceptible cell lines. Huh-7 cDNA was used as positive control

in SARS-CoV susceptible Vero E6, Huh-7, porcine POEK and PS cells but ACE2 protein expression could not be verified by several methods suggesting that the expression level may be very low. Recent studies have revealed that recombinant ACE2 expressed on the cell surface does trigger viral permissiveness and that infection can be blocked by soluble ACE2 [6, 10].

The different infection rates in porcine POEK and PS cells may be due to a lower expression rate of the ACE2 which we showed on mRNA level. Moreover there could be the necessity of an accessory factor for virus adsorption and entry. This could be confirmed by Chan et al. [1] who found ACE2 expression also in cells that were not susceptible for SARS-CoV.

Another possibility for lower infection rates in porcine cells may also be that the sequence homology of the human ACE2 strongly deviates from the porcine ACE2. Interestingly, infection efficiency in POEK cells could be increased quickly by adaptation of the virus to POEK cells. Present studies will therefore clarify the

question to what extent the adapted viruses differ from the original virus stock by growth comparisons and sequencing.

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