Title	Characterisation of animal angiotensin-converting enzyme 2 receptors and use of pseudotyped virus to correlate receptor binding with susceptibility of SARS-CoV infection
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Characterisation of animal angiotensin-converting enzyme 2 receptors and use of pseudotyped virus to correlate receptor binding with susceptibility of SARS-CoV infection

Key Messages

1. Comprehensive surveillance of civets, mice, cats, Golden Syrian hamsters, and horseshoe bats is suggested when SARS or SARS-like CoVs re-emerge in the human population in the future.
2. Rabbits and horseshoe bats are animal carriers of SARS-CoV.
3. Investigation of the genetic diversity of CoV in two bat species (*Rhinolophus sinicus* and *Rhinolophus pearsonii*) should provide insights into the direct ancestor of human SARS-CoV.

Introduction

Civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*) were thought to be the direct zoonotic sources of the severe acute respiratory syndrome (SARS) epidemic in 2003, because the coronaviruses (CoVs) isolated from these mammals were almost identical to the human SARS-CoV.[1] However, these mammals might not be the natural reservoir of SARS-CoV owing to the lack of widespread infections. A diverse group of CoVs were identified in various species of horseshoe bats, which were thus proposed to be the natural reservoir of SARS-CoV.

In experimental infection, a broad range of animals was demonstrated to support *in vivo* replication of SARS-CoV to different extents. Some of these species showed observable pathological signs after inoculation, whereas others showed rapid viral clearance. Based on their ability to support SARS-CoV replication, they may have potential roles in the transmission of SARS-CoV. Susceptibility of host cells to CoVs is mainly determined by spike (S) protein–induced receptor binding and internalisation. Angiotensin I-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV, acting as a major determinant that restricts the tropism and host range.[2] In particular, the differences among the susceptibility of humans, mice, and rats to SARS-CoV have been correlated to the differential efficiency of interaction between their ACE2 and S protein.[3]

Methods

This study was conducted from January 2007 to December 2008. The ACE2s of 15 different species were prepared. Bagg Albino mouse, Dunkin-Hartley guinea pig, Sprague-Dawley rat, and New Zealand White rabbit were obtained from the Laboratory Animal Unit of the University of Hong Kong. Common domestic cat (*Felis domesticus*), dog (*Canis lupus familiaris*), pig (*Sus scrofa domestica*), and chicken (*Gallus gallus domesticus*) were obtained from the Agriculture, Fisheries and Conservation Department of Hong Kong. Tissues from the small intestines of these animals were harvested after terminal anaesthetisation. Fresh small intestine tissues of the Chinese rufous horseshoe bat (*Rhinolophus sinicus*) and Japanese house bat (*Pipistrellus abramus*) were provided by Dr Susanna PK Lau from the Department of Microbiology of the University of Hong Kong. Fresh small intestine tissues of masked palm civet (*Paguma larvata*), Golden Syrian hamster (*Mesocricetus auratus*), Russian dwarf hamster (*Phodopus campbelli*), and long-tailed chinchilla (*Chinchilla lanigera*) were provided by Prof JD Chen from the South China Agricultural University in Guangzhou, China. Human intestine cDNA was provided from the Department of Medicine, The University of Hong Kong.

The ACE2 of these species was expressed on the surface of the AD293 cell line, which is non-susceptible to SARS-CoV. ACE2-expressing cells were infected by vesicular stomatitis virus (VSV) pseudotyped with the S protein of

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SARS-CoV (VSV-Sh). The relative expression level of ACE2 and the relative susceptibility of ACE2 to VSV-Sh were determined by flow cytometry.

Full-length ACE2-coding sequences were cloned into a mammalian expression vector pCI with a C-terminal V5 epitope-6×His (V5H) tag. These plasmids were transfected and expressed in AD293 cells. Human ACE1 was also cloned and expressed as a negative control.

After transfection, ACE2-expressing cells were harvested and the membrane fraction in 1% Triton X-100 was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The relative molecular sizes of the ACE2s were revealed in Western blot using AP-anti-V5 antibody after deglycosylation. The percentage of cells expressing ACE2 was quantified using flow cytometry with anti-V5–fluorescein isothiocyanate antibody after fixation and permeabilisation.

The full-length codon-optimised S gene, excluding its cytoplasmic tail (S_{XCT}) derived from HKU-39849, was cloned into a mammalian expression vector pCAGGS. VSV-Sh carrying a green-fluorescent protein (GFP) gene was prepared using the pCAGGS-S_{XCT} and VSV-ΔG*-G. The transfected cells were infected by an equal infectious unit of VSV-Sh after transfection. The percentage of GFP-positive cells was determined using flow cytometry.

Results

The nucleotide sequence similarities of the ACE2s of the 15 species towards human and Chinese rufous horseshoe bat ACE2s are shown in the Table. The ACE2s among the mammalian species were generally conserved with at least 85% identity.

The ACE2 of the 15 species was expressed as demonstrated by their relative molecular sizes shown in Western blot (Fig 1a). The expression level of each ACE2 was determined by flow cytometry and was adjusted by modifying the DNA amount in transfection until a comparable expression level was obtained (Figs 1a & 1b). The surface localisation of the recombinant human ACE2 was demonstrated by flow cytometry analysis on the transfected cell without permeabilisation (Fig 1c).

Efficiencies of the ACE2s as receptors for VSV-Sh were compared based on their relative susceptibility, which is defined as the percentage of VSV-Sh infected cells normalised by the percentage of ACE2-expressing cells calculated from three independent experiments (Fig 2). The 15 ACE2s were categorised into groups I to IV according to their relative susceptibility. Group IV was designated as the unsusceptible group with relative susceptibilities not significantly higher ($P>0.05$) than that of the negative control, ie human ACE1 (Fig 2). Group I included the host species of SARS-CoV, human, and civet. Group II included mouse, Golden Syrian hamster, cat, and Chinese rufous horseshoe bat, showing comparable ($P>0.05$) relative susceptibilities, but these were substantially lower than those of the group I species. Group III included all other species showing relative susceptibilities that were significantly lower ($P<0.05$) than those of group II, but significantly higher ($P<0.05$) than those of group IV.

Discussion

From the results of the infection assay, four groups of ACE2s with differentiated susceptibilities were categorised. Species from the first 2 groups (including human, civet, mouse, cat, Golden Syrian hamster, and horseshoe bat) are expected to support the infection of SARS-CoV. These findings correspond with an *in vivo* infection study, implying that the ACE2 receptor is one of the determining factors for *in vivo* susceptibility that can be extended to other animal studies in the future. Moreover, the potentially susceptible animal species should be under comprehensive

Table. Nucleotide sequence similarity of various angiotensin I-converting enzyme 2 (ACE2) compared with human and *Rhinolophus sinicus* ACE2, respectively

ACE2 (ranked by relative susceptibility to VSV-Sh)	Length of coding sequence (bp)	DNA similarity to human ACE2 (%)	DNA similarity to horseshoe bat (*R sinicus*) ACE2 (%)	GenBank Accession number
Human	2418	100	88.0	GQ262784
Civet	2418	89.0	89.3	GQ262789
Mouse	2418	90.2	85.7	GQ262785
Golden Syrian hamster	2418	89.8	85.6	GQ262794
Cat	2415	90.3	90.0	GQ262792
Horseshoe bat (*Rhinolophus sinicus*)	2418	88.0	100	GQ262791
Rabbit	2418	91.7	87.5	GQ262787
Guinea pig	2418	86.8	90.0	GQ262786
Dog	2415	90.4	89.7	GQ262793
Japanese house bat	2412	85.6	85.7	GQ262782
Pig	2418	89.4	86.9	GQ262781
Chinchilla	2415	90.2	87.2	GQ262783
Russian dwarf hamster	2418	89.5	85.9	GQ262790
Rat	2418	89.7	85.8	GQ262788
Chicken	2442	71.9	72.5	GQ262780
Rhinolophus pearsonii[4]	2418	88.0	96.7	EF569964

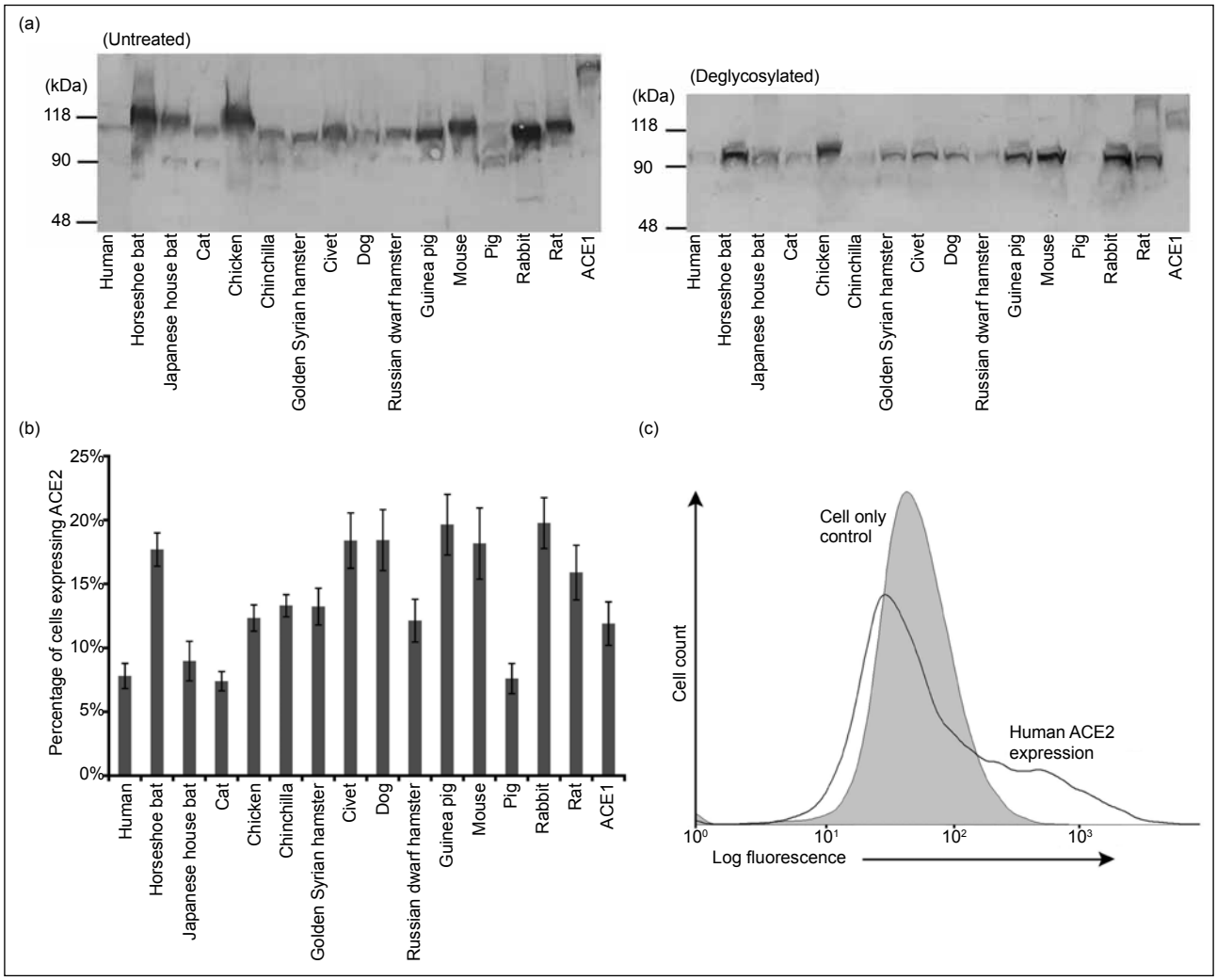

Fig 1. Expression of various angiotensin I-converting enzyme 2 (ACE2) in AD293
(a) Membrane fraction of ACE2-expressing cells analysed in Western blot. (b) Percentage of cells expressing various ACE2s detected in flow cytometry analyses. (c) Cell surface transient expression of human ACE2

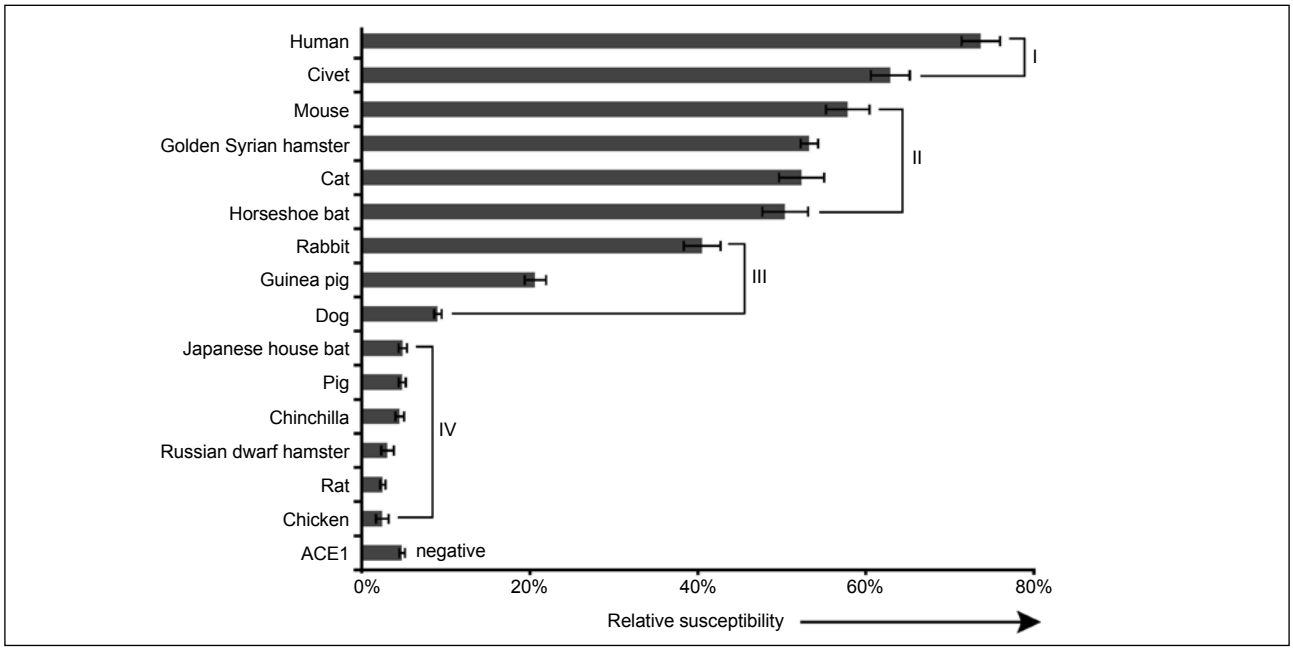

Fig 2. Percentage of VSV-Sh infected cells normalised by the percentage of cells expressing the corresponding angiotensin I-converting enzyme 2 (ACE2) to 100% expression, ie (% of green-fluorescent protein-positive) / (% of ACE2 expression) × 100%

surveillance when SARS or SARS-like CoVs appear in the human population again.

Most species investigated were common consumption and companion animals that intensively interact with humans. It is crucial to assess their potential for being animal vectors for SARS-CoV transmission. Rodents have been proposed as a possible vector for SARS-CoV transmission. The *in vivo* susceptibility of group III animals (chinchilla, Russian dwarf hamster, dog, and rabbit) has not been investigated. Based on the results, the ACE2s of these animals did not seem to support entry of VSV-Sh as efficiently as groups I and II species. Nonetheless, the relative susceptibility of rabbit ACE2 was significantly higher than that of the guinea pig, which was found to inefficiently support *in vivo* replication of SARS-CoV. Thus, SARS-CoV might replicate more efficiently in rabbits than in guinea pigs. The risk that group IV species transmit SARS-CoV may be remote, as none of these species has been shown to efficiently shed virus after experimental inculcation. Although further *in vivo* experiments are needed to confirm such speculations, this study should provide a better scope of investigation for animal vectors of SARS-CoV, particularly of rabbit and Chinese rufous horseshoe bat.

The relative susceptibility of the ACE2 of *R sinicus* was comparable to that of cat, mouse, and Golden Syrian hamster, whereas the ACE2 of Japanese house bat (in this study) and *Rhinolophus pearsonii* (in a previous study[4]) did not seem to support the entry of SARS-CoV S-pseudotyped viruses. These findings imply that the susceptibility of ACE2 towards human SARS-CoV may be species-specific in bats. Further investigations of the ACE2s of different bat species are needed to extend this observation. The susceptibility of *R sinicus* ACE2 towards VSV-Sh implies the potential existence of a bat SARS-like-CoV, which may carry an S protein that shares significant structural homologies with the human SARS-CoV S protein, and may be the direct progenitor of human SARS-CoV. Although the S protein of all currently sampled bat SARS-like-CoV in *R sinicus* is highly divergent from that of human SARS-CoV, the presence of an uncharacterised lineage in *R sinicus* is possible based on the relatively high genetic diversity of bat SARS-like-CoV. Our previous study speculated that the direct progenitor of human SARS-CoV may exist in *R pearsonii* based on the ORF1 phylogeny.[5] Interspecies transmission of CoVs between *Rhinolophus* spp appears to be a common process, suggesting the possibility of coinfection and thus recombination between bat SARS-like-CoVs.

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