



## Letter

## Fish ACE2 is not susceptible to SARS-CoV-2



Dear Editor,

The ongoing pandemic of coronavirus disease 2019 (COVID-19) has reshaped our daily life and caused >4 million deaths worldwide (<https://covid19.who.int/>). Although lockdown and vaccination have improved the situation in many countries, imported cases and sporadic outbreaks pose a constant stress to the prevention and control of COVID-19. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent responsible for COVID-19, has a positive-sense single-stranded RNA genome of 30 kb ([Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020](#)). We and other groups have demonstrated that the SARS-CoV-2 could use the angiotensin-converting enzyme 2 (ACE2) as cell receptor, including orthologs of a broad range of animal species such as human, bats, ferrets, pigs, cats, and dogs ([Hoffmann et al., 2020](#); [Zhou et al., 2020](#); [Liu et al., 2021](#)). Although the evolutionary origin of SARS-CoV-2 can be linked to the discoveries of diverse coronaviruses related to SARS-CoV-2 in wild animals such as bats ([Zhou et al., 2020](#); [Wacharapluesadee et al., 2021](#)) and pangolins ([Liu et al., 2019](#); [Lam et al., 2020](#)), the direct origin of SARS-CoV-2 in humans remains unknown. In China, several sporadic outbreaks of COVID-19 in 2020 were linked to food in cold chain sold at trade markets, including salmon meat ([http://www.nhc.gov.cn/xcs/yqtb/list\\_gzbd.shtml](http://www.nhc.gov.cn/xcs/yqtb/list_gzbd.shtml)) ([Yang et al., 2020](#)). The detection of SARS-CoV-2 RNA on the surface of frozen meat for as long as 20 days has also been reported ([Feng et al., 2021](#)). A concern regarding the potential role of fish in SARS-CoV-2 transmission has also been raised. Therefore, we investigated the susceptibility of fish ACE2 to SARS-CoV-2.

First, we infected the following three fish cell lines with SARS-CoV-2 (IVCAS 6.7512) at a multiplicity of infection (MOI) of 1 in 24-well plates ([Zhou et al., 2020](#)): *Pimphales promelas* skin (EPC), *Ctenopharyngodon idellus* kidney (CIK), and *Lepomis macrochirus* caudal trunk (BF-2). The Vero (*Chlorocephalus aethiops* kidney) and HeLa (*Homo sapiens* cervix) cell lines were used as positive and negative controls ([Chu et al., 2020](#)), respectively. After adsorption for 1 h, the supernatant was removed and the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM, Invitrogen). The control cell lines were cultured at 37 °C and maintained with DMEM containing 2% fetal bovine serum (FBS, Life Technologies), while the fish cell lines were cultured at 24 °C and maintained with MEM containing 2% FBS. Cell susceptibility and viral replication were determined by immunofluorescence assay (IFA) and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Briefly, infected cells were fixed with 4% paraformaldehyde and subsequently permeabilized with 0.1% TritonX-100 at 24 hours post-infection (hpi). Next, the treated cells were incubated with viral nucleocapsid N protein antibody (rabbit anti-SARS-CoV-2 N protein polyclonal antibody,

1:1000, made in-house). After washing with PBS, cells were incubated with Cy3-conjugated goat anti-rabbit IgG (1:200, Abcam, ab6939) antibody for 1 h followed by nuclei staining with DAPI (1:100, Beyotime). Fluorescent images were obtained using an AMF4300 EVOS FL cell Imaging System (Life Technologies, Carlsbad, CA, USA). Concurrently, the infected supernatants were collected at 0, 24, 48, and 72 hpi; viral RNA was extracted from the supernatant using the QIAamp 96 Virus QIAcube HT Kit (Qiagen, Germany). A real-time one-step qRT-PCR assay was used for detection of SARS-CoV-2 spike gene, using the HiScript One-Step qRT-PCR SYBR Green kit (Vazyme, China) as described previously ([Zhou et al., 2020](#)). Specific primers for the S gene of SARS-CoV-2 (forward, 5'-CAATGGTTTAAACAGGCACAGG-3' and reverse, 5'-CTCAAGTGTCTGTGGATCAGC-3') were designed according to a reference sequence (Genbank No. MN996528). Each time point had three replicates. According to the IFA, no viral antigen was detected in the three fish cell lines, whereas the Vero cells were efficiently infected ([Supplementary Fig. S1A](#)). The mean viral load in the supernatant did not increase significantly in the inoculated fish cells; conversely, a million-fold increase was detected in the virus-infected Vero cells ([Supplementary Fig. S1B](#)). The IFA and qRT-PCR results revealed that these three fish cells were not susceptible to SARS-CoV-2 infection.

Then the virus attachment was quantified by incubating SARS-CoV-2 at an MOI of 10 with adhered cells in 24-well plates at 4 °C. The cells were washed for three times with culture medium before and after incubation, respectively. Then the attached virus particles were quantified by qRT-PCR. No statistical differences were observed in viral RNA levels on BF-2, CIK, EPC, HeLa, and Vero cells ([Supplementary Fig. S1C](#)), which suggested that SARS-CoV-2 could attach to the fish cells but cannot enter or replicate in fish cells. We further tested whether SARS-CoV-2 was able to use fish ACE2 for cell entry or not. The sequences of ACE2 of five fish species were compared with hACE2. Among the 20 key aa sites that interact with the receptor domain of SARS-CoV-2, only one or two critical residues in each fish cell line were identical to hACE2 ([Supplementary Fig. S2](#)).

Next, HeLa cells were transfected with plasmids expressing the ACE2 orthologs of five fish species (zebrafish, tilapia, catfish, salmon, and rainbow trout) and human ACE2, including three plasmids expressing humanized fish ACE2. These transfected cells were infected with SARS-CoV-2 at an MOI of 1 in 24-well plates. Infection and viral RNA quantification were performed as described above. ACE2 expression was confirmed by the detection of the S-tag labelled at the terminus of each ACE2. Mouse anti-S-tag monoclonal antibody and FITC-labelled goat anti-mouse IgG H&L (Abcam, ab96879) were used as the primary and secondary antibodies, respectively, for IFA ([Zhou et al., 2020](#)). IFA

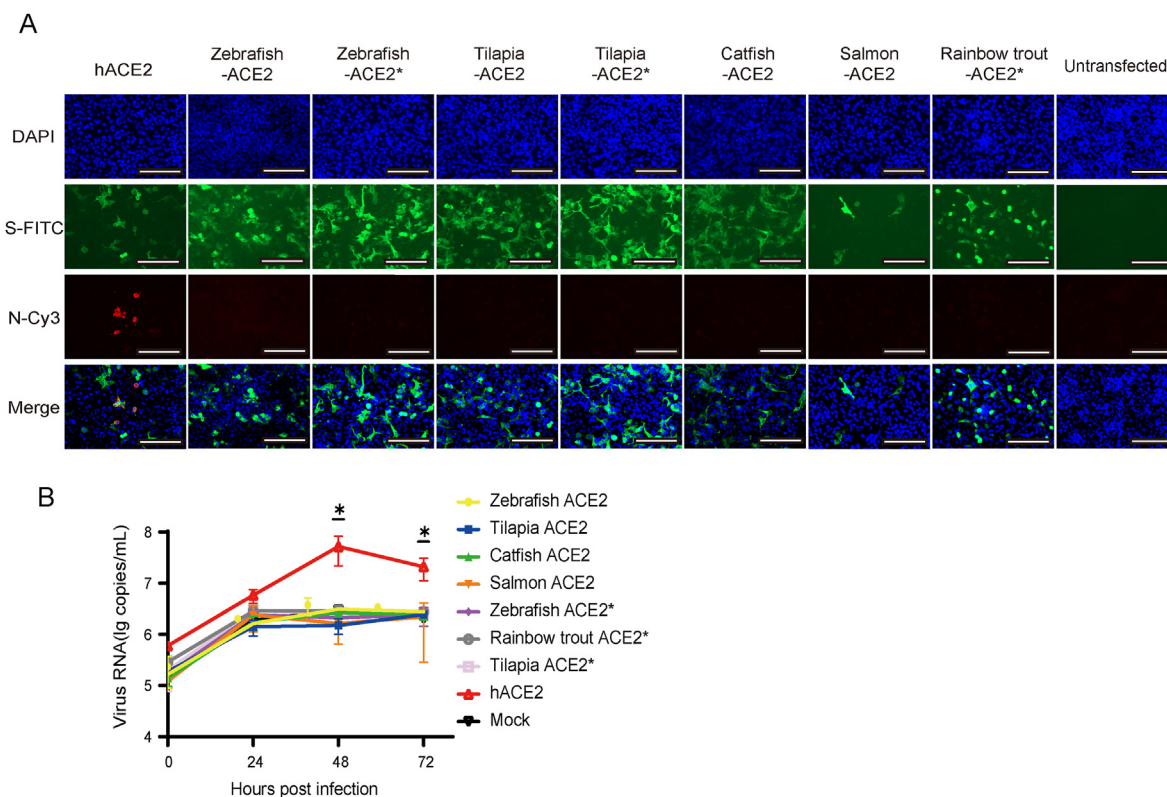
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**Fig. 1.** Susceptibility of fish ACE2 to SARS-CoV-2. **A** HeLa cells were transiently expressed with different fish ACE2 fused with S-tag. Fish ACE2 expression was detected by mouse anti-S-tag monoclonal antibody and FITC-labelled goat anti-mouse IgG. SARS-CoV-2 N protein was detected using the rabbit anti-SARS-CoV-2 N protein polyclonal antibody followed by Cy3-conjugated goat anti-rabbit IgG. Cells transiently expressing different fish ACE2 were infected with SARS-CoV-2 (MOI = 1). Scale bars, 200  $\mu$ m. \*, humanized optimization. **B** Viral RNA copies in supernatant were quantified by qRT-PCR in triplicates. The supernatant was harvested at 0, 24, 48, and 72 hours post-infection (hpi). Data represent the mean of triplicate values, and error bars represent SD. \* $P < 0.05$ .

revealed expression of ACE2 in all cells; the expression levels were regardless of codon optimization (Fig. 1A). ACE2 expression of salmon was low, probably due to a low transfection or expression efficiency. The viral antigen was detected in HeLa cells expressing human ACE2 but not those expressing fish ACE2 (Fig. 1A). qPCR results revealed that the number of copies of HeLa cells transfected with four different fish ACE2 receptors and three optimized fish ACE2 receptors did not increase significantly and exhibited a curve similar to that of HeLa cells (Fig. 1B). Conversely, the viral RNA copies were significantly increased in HeLa cells expressing human ACE2 compared to HeLa cells expressing fish ACE2. Taken together, IFA and qRT-PCR results confirm that the SARS-CoV-2 cannot use fish ACE2 to invade host cells (Fig. 1).

After the successful containment of COVID-19 in China in May 2020, more than a few sporadic localized outbreaks of COVID-19 were associated with frozen sea food in the markets in cities including Beijing, Qingdao, and Dalian. Concerns were raised regarding SARS-CoV-2 infection in fish commonly found in the cold chain transportation. Our results indicate that the SARS-CoV-2 detected in sea food were not due to infection in fishes but a contamination by SARS-CoV-2 infected workers handling the cold food productions. Recent studies have tested SARS-CoV-2 stability by simulating the cold chain transportation; viral RNA and live virus have been detected or isolated from the surface of meat samples stored under different temperatures (Fisher et al., 2020; Feng et al., 2021). Our study supports the possibility of SARS-CoV-2 transmission through cold food handling. A limitation of this study is that only three types of fish cell lines and five types of fish ACE2 were tested; more fish species need to be evaluated to establish concrete conclusions. In sum, our research indicates that SARS-CoV-2 cannot use these tested fish ACE2 to entry host cells. Therefore, more attention should be paid to the disinfection and inactivation of virus on frozen food surfaces.

## Footnotes

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.01.020>.

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