# Enterovirus type 71-immunized chicken egg yolk immunoglobulin has cross antiviral activity against coxsackievirus A16 *in vitro*

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**Abstract.** To exploit a cross passive immunotherapy for enterovirus-induced hand-foot-and-mouth disease (HFMD), the cross antiviral activity of a neutralizing antibody against enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) was investigated *in vitro*. White Leghorn specific-pathogen-free chickens were immunized with EV71 antigens and a specific isolated immunoglobulin (IgY) was prepared from the chicken egg yolk. IgY was further purified and characterized by SDS-PAGE, ELISA, western blotting and bidirectional immune agar diffusion testing. The antiviral activity and dose-response of the IgY were determined by assessing the cytopathic effect in rhabdomyosarcoma (RD) cells *in vitro*. It was indicated that the levels of IgY were increased at day 7, peaked at week 7 and were maintained at a higher level for 4 weeks following immunization when compared with the negative control. The results of western blotting and bidirectional immune agar diffusion testing revealed that the IgY had cross-binding properties in EV71 and CVA16 strains through targeting the envelope proteins (VP0, VP1 and VP3) of EV71 and CVA16. Neutralization assay results indicated that the infectivity of EV71 and CVA16 strains in RD cells was cross-blocked by IgY in a dose-dependent manner. To conclude, these findings indicate that IgY has cross antiviral activity against EV71 and CVA16 *in vitro*, and could potentially be developed as a passive immunotherapy for EV71- and CVA16-induced HFMD.

## Introduction

Hand-foot-and-mouth disease (HFMD) is an infantile disease characterized by herpes on the hands, feet and mouth, and associated neurological syndrome (1,2). The major viruses that cause HFMD are EV71 and CVA16. EV71-induced HFMD is more serious out of the two because it causes a neurological syndrome of the central nervous system and may lead to mortality (3). CVA16-induced HFMD usually leads to milder symptoms, and the morbidity and mortality are lower compared with EV71-induced HFMD (4). However, EV71 is not the only major cause of HFMD outbreak. Zhu *et al* (5) conducted a 12-month follow-up of 1,704 patients with clinically confirmed HFMD and revealed that only 36 cases (2.1%) were identified as EV71-induced HFMD, 577 cases (33.9%) were CVA16-induced HFMD, 588 cases (34.5%) were caused by other enteroviruses and 503 cases (29.5%) were not associated with any enterovirus. Furthermore, some patients with severe and fatal CVA16-induced HFMD have been reported in the United States (6), France (7), Japan (8) and China (9,10).

The experimental treatment for HFMD includes inactivated virus vaccine (5,11), DNA vaccine (12), synthetic peptide vaccine (13,14), recombinant VP1 vaccine (15), live attenuated vaccines (16), neutralizing antibodies (17) and antiviral compounds (18). Inspired by previous inactivated polio vaccines, the development of an active immunoassay for inactivated EV71 vaccine has been making rapid progress (19). In December 2015, the China Food and Drug Administration approved two inactivated EV71 vaccines for the prevention of severe HFMD (20), and a CVA16 vaccine is presently being developed in China (21). However, these vaccines only provide protection against HFMD caused by a single enterovirus. The clinical symptoms of HFMD caused by CVA16 and EV71 strains are indistinguishable, and they may cause outbreaks alternately or simultaneously in Asian countries (22). EV71 can recombine viral genes with CVA16 and produce novel viral variants. In 2008, a large-scale outbreak of HFMD caused by EV71 and CVA16 recombinant virus occurred in the city of Fuyang, China (23). Therefore, it is necessary to develop effective therapeutic agents or therapies for treatment of EV71- and CVA16-induced HFMD.

In passive immunization, intravenous injection of human immunoglobulins has been widely used to provide immunological protection with passive immunity for immunocompromised individuals (24); however, the therapeutic

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efficacy is unstable (25), and the risk of adverse drug effects is high (26). The monoclonal antibody produced by hybridoma cells is another form of passive immunity that is effective (27). However, when the antibody is injected into humans, immune rejection typically occurs (28). Immunoglobulin Y (IgY), which is extracted from the egg yolk of immunized poultry, is an excellent antibody source for passive immunity (29). Compared with the IgG from mammals, IgY is more stable (30,31), easy to collect (32), has a high yield (33) and does not react with rheumatoid factors, complement components or mammalian Fc receptors in human serum (34). At present, the application of IgY in the diagnosis and treatment of human diseases has become a research hotspot, particularly regarding infections caused by *Helicobacter pylori* (35), *Vibrio cholerae* (36) and other bacterial infections, as well as the infections by human rotavirus (37), severe acute respiratory syndrome coronavirus (38) and other viruses. IgY can also be used for the preparation of diagnostic antibodies for immunohistochemistry, ELISA (39), western blotting and other diagnostic techniques.

In the present study, the cross antiviral activity of IgY against EV71 and CVA16 was assessed. White Leghorn specific-pathogen-free (BWEL-SPF) chickens were immunized with inactivated EV71 strains and a specific IgY was prepared from egg yolk. Inhibitory activity of the IgY against EV71 and CVA16 strains was indicated *in vitro*. Furthermore, the purity and titer of the IgY was determined by SDS-PAGE, indirect ELISA and western blotting. The long-term aim of the present project was to develop a specific IgY that could potentially act as an antiviral agent for cross-passive immunotherapy of EV71- or CVA16-induced HFMD.

## Materials and methods

*Cells and viruses*. Rhabdomyosarcoma (RD) cells were obtained from the China Center for Type Culture Collection (Wuhan, China). EV71, CVA16, coxsackievirus B1 (CVB1), coxsackievirus B2 (CVB2), coxsackievirus B3 (CVB3), coxsackievirus B4 (CVB4), coxsackievirus B5 (CVB5) and coxsackievirus B6 (CVB6) were purchased from the State Key Laboratory of Virology (Wuhan, China) and diluted (1:5) in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Corning Inc., Corning, NY, USA), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 $\mu$g/ml streptomycin (Sigma-Aldrich; Merck KGaA). RD cells were cultured in virus-containing medium at 37˚C in a 5% $CO_2$-humidified incubator. When RD cells containing the viruses reached ~80% confluence, cells were frozen-thawed at -80˚C, centrifuged at 2,000 x g for 5 min at 4˚C and filtered on a 0.22-$\mu$M filter for storage.

*Chicken immunization*. A total of 6 39-week-old single-comb BWEL-SPF chickens were purchased from the SPF Experimental Animal Center of Guangdong Emerging Dahua Agriculture Poultry Co., Ltd. (Guangzhou, China). Chickens were raised in 3 super-clean benches (Suzhou Antai Airtech Co. Ltd., Suzhou, China) and had access to food and water (pH 6.2). After 1 week, laying chickens were divided into two groups: Group A and Group B. After mixing the EV71 antigen ($10^9 TCID_{50}$/ml) with an equal amount of Freund's incomplete adjuvant and fully emulsifying, 3 chickens were randomly selected for Group A and injected intramuscularly in both sides of the chicken wings and left and right sides of the breast (0.25 ml/site). In Group B, 3 chickens were given a mixture (1 ml) of saline and Freund's incomplete adjuvant (0.25 ml/site). In Groups A and B, the injections were performed once/week for 4 weeks. Eggs were collected once a day, labeled and stored at 4˚C. Chickens were euthanized at week 31, following the first immunization.

*Isolation and purification of IgY from chicken egg yolk*. Once the fresh eggs were cleaned with 75% alcohol and cotton balls, the egg yolks were separated and the extra albumen was rolled off with filter paper. The egg yolk without the membrane was diluted with cold deionized water (1:9), mixed (adjusted to pH 5.0 with 0.1 mol HCl) and then stored at 4˚C overnight. The solution was centrifuged at 4,000 x g for 40 min at 4˚C and the supernatant was added to ammonium sulphate to make a final saturation of 45%. Following this, the solution was incubated at 4˚C for 3 h. After centrifugation at 4,000 x g for 10 min at 4˚C, the supernatant was discarded and deionized water (9 times the volume of egg yolk without the membrane) was used to dissolve the protein precipitate. Subsequently, sodium sulfate was added to make a final mass fraction of 13%. The mixture was incubated at 4˚C for 3 h and centrifuged again under the same conditions. The sediment was dissolved in phosphate-buffered saline (PBS). The solution was dialyzed for 4-5 h (water changed every hour), soaked in PBS overnight and stored at -20˚C. The IgY purified from eggs of group A after immunization were the specific IgY (S-IgY), and those from group B were the negative control IgY (C-IgY). The purified IgY was subjected to centrifugal ultrafiltration at 4,000 x g for 10 min at 4˚C using an Amicon Ultra-15 centrifugal filter unit (EMD Millipore, Billerica, MA, USA) to desalt and concentrate the antibody. The purified IgY was used in subsequent *in vitro* neutralization assays and western blotting.

*Quantitation of the purfied IgY*. The antibody titer of the S-IgY and C-IgY (purified IgY) was determined using indirect ELISA. The purified IgY from egg yolk was diluted to 1:5,000 in PBS, and 50 $\mu$l was added to the ELISA plate, which had been coated with the purified EV71 antigen. Three duplicated wells were set in the same sample, the C-IgY group or the S-IgY group. The plate was covered, incubated at 37˚C for 30 min and the wells were washed 5 times (30 min each time) with a washing buffer provided in an EV71 Ab ELISA kit (cat. no. SBJ-H2014; Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China). The plate was incubated with horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY antibody (1:2,000; Abcam, Cambridge, MA, USA) for 30 min at 37˚C, washed 5 times with a washing buffer (30 sec each time) and subsequently incubated with 100 $\mu$l freshly prepared TMB color liquid (50 $\mu$l each of color liquid A and B) at 37˚C in the dark for 15 min. Following color development, 50 $\mu$l of 2 mol $H_2SO_4$ was added to stop the reaction and the absorbance was measured immediately at 450 nm using a Bio-Tek EL 309 microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA). The concentration of the IgY was obtained according to the standard curves.

Table I. Primary and secondary antibodies.

| Protein | Primary antibody | Dilution used | Catalogue number | Secondary antibody | Dilution used | Catalogue number |
|---|---|---|---|---|---|---|
| EV71 | C-IgY | 1:20,000 | | HRP goat anti-chicken IgY | 1:5,000 | ab20572[b] |
| | S-IgY | 1:20,000 | | HRP goat anti-chicken IgY | 1:5,000 | ab20572[b] |
| | EV71 VP1 monoclonal antibody | 1:10,000 | MAB 1255-M05[a] | HRP goat anti-mouse IgG | 1:5,000 | ab97023[b] |
| CVA16 | C-IgY | 1:5,000 | | HRP goat anti-chicken IgY | 1:2,500 | ab20572[b] |
| | S-IgY | 1:5,000 | | HRP goat anti-chicken IgY | 1:2,500 | ab20572[b] |
| | EV71 VP1 monoclonal antibody | 1:10,000 | MAB 1255-M05[a] | HRP goat anti-mouse IgG | 1:5,000 | ab97023[b] |
| EV71 VP1 vaccine | C-IgY | 1:20,000 | | HRP goat anti-chicken IgY | 1:5,000 | ab20572[b] |
| | S-IgY | 1:20,000 | | HRP goat anti-chicken IgY | 1:5,000 | ab20572[b] |

[a]Abnova, Walnut, CA, USA; [b]Abcam, Cambridge, MA, USA; HRP, horseradish peroxidase; IgY, immunoglobulin Y; EV71, enterovirus 71; CVA16, coxsackievirus A16.

*SDS-PAGE analysis*. Samples of IgY and 5X loading buffer (4:1) were heated in a metal water bath at 95˚C for 10 min and placed on ice for an additional 10 min. Samples (10 $\mu$l/lane) was separated via SDS-PAGE on a 10% gel at a constant current of 45 mA for 50 min. Following protein separation, the gel was stained with Coomassie Brilliant Blue R250 for 30 min and then de-stained with a de-staining solution (30 min each time). Coomassie-stained gels were imaged and protein bands were analyzed using BandScan 5.0 software (ProZyme., Hayward, CA, USA).

*Western blotting*. Total protein from the purified EV71 and CVA16 virus strains and the EV71 VP1 vaccine (cat. no. DAG1665; Creative Diagnostics Co., New York, NY, USA) was quantified using a bicinchoninic acid assay (Thermo Fisher Scientific, Inc.), 5X loading buffer (cat. no. P0015L; Beyotime Institute of Biotechnology, Haimen, China) was added to samples (4:1) and denatured by heating in a metal water bath at 95˚C for 10 min. Samples were placed on ice for 10 min. Samples (20 $\mu$g protein/lane) were separated via SDS-PAGE on 10 and 5% gels. The separated proteins were transferred onto polyvinylidene fluoride membranes and blocked with 5% skimmed milk at room temperature for 90 min. The membranes were washed five times with Tris-buffered saline containing 0.05% Tween-20 (TBST). The membranes were incubated with primary antibodies overnight on a shaking incubator at 4˚C. The membranes were washed five times with TBST. Following primary incubation, membranes were incubated with secondary antibodies for 90 min on a shaking incubator at room temperature and washed four times with TBST. The primary and secondary antibodies used are summarized in Table I. Protein bands were visualized using an Enhanced Chemiluminescence Western Blotting kit (Thermo Fisher Scientific, Inc.).

*Bidirectional immune agar diffusion test*. A 10 g/l agarose plate was prepared, and plum-shaped holes (aperture, 3-5 mm; hole distance, 3-4 mm) were punched out on the agarose plate. The inactivated EV71 virus was added to the central well and different dilutions (1:2, 1:4, 1:8, 1:16 and 1:32) of the purified IgY were added into the peripheral 6 wells. At the same time, a blank group (PBS only) was also assessed. Following this, the plate was placed upside down in a 60˚C-wet box and incubated for 24-48 h. The formation of the precipitation line was observed.

*50% tissue culture infective dose ($TCID_{50}$) assay*. Virus strains were serially diluted ($10^{-1}$-$10^{-10}$) with DMEM supplemented with 10% FBS and titrated 100 $\mu$l/well on RD monolayer cells that had been seeded in a 96-well plate at a density of $1x10^4$ cells/well. The virus-infected cells were incubated for 48 h at 37˚C in a 5% $CO_2$-humidified incubator before the presence of cytopathic effect (CPE) was observed under a microscope (IX73; Olympus Corporation, Tokyo, Japan). The $TCID_{50}$ of the virus strains were determined according to the Reed-Muench formula (40).

*In vitro neutralization assay*. RD cells were seeded into a 48-well plate at a concentration of $6x10^4$/well and cultured overnight at 37˚C. S-IgY and C-IgY were diluted to 1 mg/ml with PBS. Enviroxime (Purity, 98%; Toronto Research Chemicals Inc., North York, ON, Canada) was dissolved in dimethyl sulfoxide at 10 mg/ml and diluted 1:10 into culture media. The experiment was divided into 4 groups: The blank control group (PBS only), the negative control IgY (C-IgY), the S-IgY groups and the Envrioxime group. Subsequently, S-IgY and C-IgY were incubated at 56˚C for 30 min. S-IgY, C-IgY and Enviroxime were serially diluted (1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600 and 1:3,200) with culture media, and mixed with an equal volume of EV71 (200 $TCID_{50}$) or CVA16 (200$TCID_{50}$). The mixtures were shaken for 5 min and incubated at 37˚C in a 5% $CO_2$-humidified incubator for 30 min. Subsequently, RD cells were inoculated with 300 $\mu$l of the mixture and incubated $O_2$ at 37˚C in a 5% $CO_2$-humidified incubator for 48 h to promote the antibody binding to the viruses. Neutralization titers were determined as the highest dilutions of antibody that protected at least half of RD cells in one well from CPE.

Table II. Concentration of IgY from different chickens.

| Chicken | Week after immunization | Concentration of IgY (mg/ml) |
|---|---|---|
| A1 | 0 W | 3.56±0.184 |
| | 4 W | 4.73±0.159 |
| | 8 W | 7.44±0.396 |
| | 12 W | 6.85±0.283 |
| A2 | 0 W | 1.97±0.934 |
| | 4 W | 3.31±0.131 |
| | 8 W | 4.57±0.098 |
| | 12 W | 5.07±0.042 |
| A3 | 0 W | 2.261±0.013 |
| | 4 W | 2.91±0.109 |
| | 8 W | 7.11±0.117 |
| | 12 W | 6.46±0.294 |
| B1 | 0 W | 2.24±0.018 |
| | 4 W | 4.66±0.181 |
| | 8 W | 5.73±0.011 |
| | 12 W | 7.71±0.006 |
| B2 | 0 W | 5.95±0.044 |
| | 4 W | 5.26±0.024 |
| | 8 W | 8.75±0.008 |
| | 12 W | 11.16±0.109 |
| B3 | 0 W | 4.39±0.156 |
| | 4 W | 6.55±0.113 |
| | 8 W | 6.50±0.085 |
| | 12 W | 6.27±0.052 |

W, week. A1-3, Group A chicken 1-3; B1-3, Group B chicken 1-3.

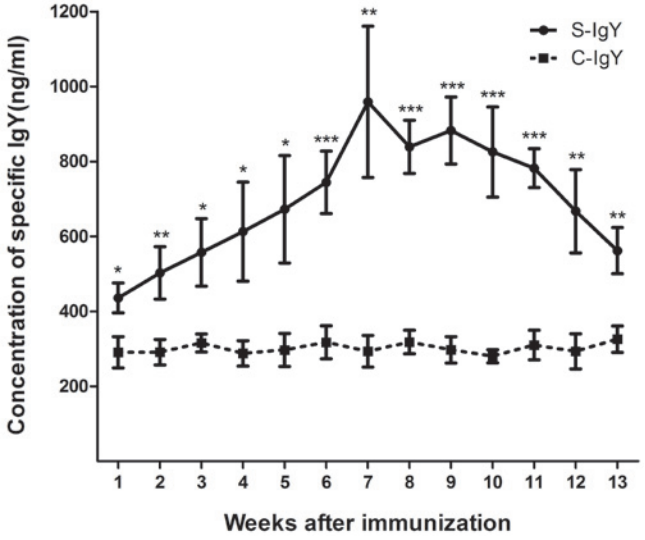


Figure 1. Levels of S-IgY in EV71-immunized chicken egg yolks. Isolation and purification of IgY from chicken egg yolk. Six 39-week-old single comb white leghorn specific-pathogen-free chickens were immunized; 3 chickens were immunized with enterovirus 71 antigens and Freund's incomplete adjuvant (group A) and the other 3 chickens were injected with saline and Freund's incomplete adjuvant (group B). Eggs were collected from immunized hens over a course of 91 days following the first immunization, and IgY was isolated and purified from the egg yolks. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 vs. C-IgY. IgY, immunoglobulin Y; S-IgY, specific IgY; C-IgY, negative control IgY.

To detect the antiviral activity of the IgY against different enteroviruses, the S-IgY, C-IgY and EV71 VP1 monoclonal antibodies (Table II) were incubated at 56˚C for 30 min. Antibodies were diluted (1:25) and mixed with an equal volume of 200 $TCID_{50}$ of EV71, CVA16, CVB1, CVB2, CVB3, CVB4, CVB5 and CVB6 strains, respectively. Oscillation and incubation were performed as mentioned above. The inhibition rate was based on the CPE.

To determine the stability of the purified IgY under different physical conditions, the IgY was diluted to 1 mg/ml with PBS. The diluted IgY was incubated at different temperatures (4˚C, room temperature, 37˚C or 60˚C) for 48 h or was frozen-thawed (frozen at -20˚C and thawed at 4˚C) five times. Serially diluted IgY (1:300, 1:600 and 1:1,200) was mixed with an equal volume of EV71 strains (200 $TCID_{50}$). The experiment was divided into 5 groups, the 4˚C group, the room temperature (RT) group, the 37˚C group, the 60˚C group and the freeze-thaw group. Following this, oscillation, incubation and calculation of inhibition rate were performed as described above.

To determine the time-dependent effect of the purified IgY on EV71 infection in RD cells, the S-IgY and C-IgY were diluted to 6 mg/ml with PBS and were incubated at 56˚C for 30 min. The culture supernatants of the RD cells in 48-well plates were replaced by 300 $\mu$l EV71 strains (100 $TCID_{50}$). A total of 1 $\mu$l IgY was added to the RD cells at 0, 1, 2, 3, 4 or 5 h post-infection. RD cells were incubated under 5% $CO_2$ at 37˚C. After 48 h of cell culture, the inhibition rate for RD cells was obtained according to CPE.

*Statistical data analysis*. All data were presented as the mean ± standard deviation. Statistical analysis was performed using Graphpad Prism software (version 7.0; GraphPad software, La Jolla, CA, USA) and SPSS 21.0 software (IBM Corp., Armonk, NY, USA). The Student's t-test was used to evaluate differences between two groups, and one-way analysis of variance followed by Dunnett's post hoc test was used to evaluate differences among different groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Levels of IgY significantly increase in EV71-immunized chicken egg yolks*. The present study was performed to isolate and purify S-IgY from EV71-immunized chicken egg yolks. As indicated in Fig. 1, the levels of S-IgY were detected at day 7, peaked at week 7 and were maintained at a higher level compared with C-IgY for a total of 4 weeks. The levels of S-IgY began to decrease gradually at week 11 following the initial immunization, whereas the levels of the C-IgY group did not change significantly over the experimental period, suggesting that the isolated IgY is specific in its response to EV71 immunization. Furthermore, the levels of IgY in group A and group B chicken egg yolks were elevated after immunization (Table II). From these findings it was concluded that the S-IgY from week 7 chicken egg yolks after EV71 antigen

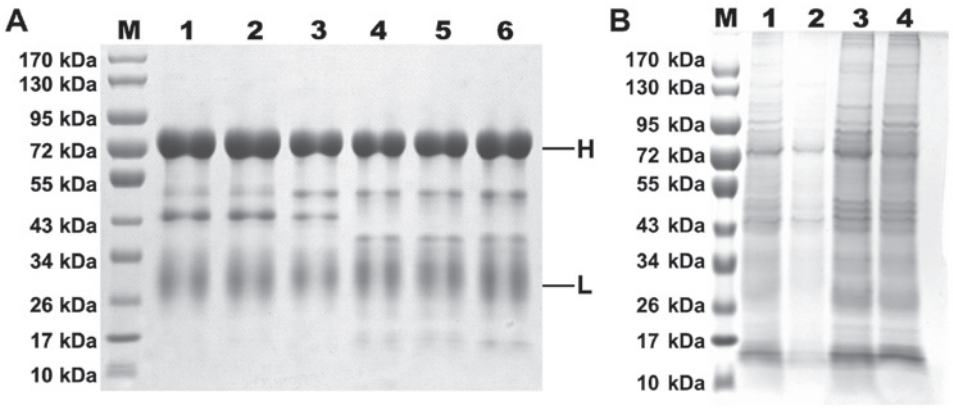


Figure 2. Characterization of the isolated IgY. (A) SDS-PAGE of the IgY antibody. Lane M: Protein markers; lanes 1, 2, and 3: IgY from the group A chickens; lanes 4, 5 and 6: IgY from the group B chickens; H and L represent the heavy chain and light chain of the IgY, respectively. (B) Viral proteins of the EV71 and CVA16 strains analyzed by SDS-PAGE. Lane 1: Protein markers; lanes 2 and 3: The CVA16 strain; lane 4 and 5: The EV71 strain. IgY, immunoglobulin Y; EV71, enterovirus 71; CVA16, coxsackievirus A16.

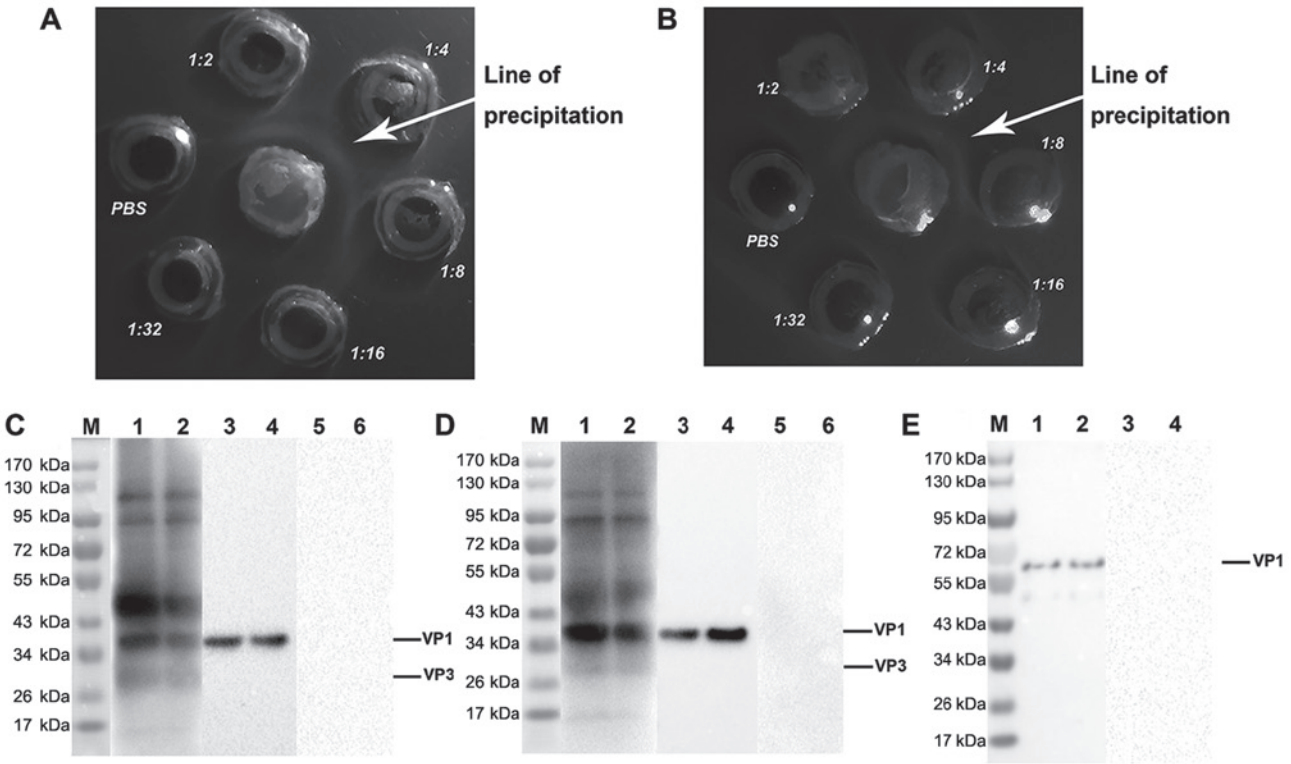


Figure 3. Characterization of the specific IgY against EV71 and CVA16 strains. (A) Bidirectional immune agar diffusion test for EV71. (B) Bidirectional immune agar diffusion test for CVA16. (C and D) Western blotting of the immunoreactivity of the specific IgY. (C) EV71 and (D) CVA16 were assessed. The EV71 and CVA16 viral extracts were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes and detected with the specific IgY, VP1 monoclonal antibody, or the negative control IgY. Lanes 1 and 2, the isolated IgY; lanes 3 and 4, VP1 monoclonal antibody; lanes 5 and 6, negative control IgY. (E) The VP1 recombinant protein was subjected to SDS-PAGE, transferred to a polyvinylidene fluoride membrane and detected with the specific IgY or negative control IgY. Lane M, protein markers; Lanes 1 and 2, the specific IgY; lanes 3 and 4, the negative control IgY. IgY, immunoglobulin Y; EV71, enterovirus 71; CVA16, coxsackievirus A16.

immunization would be purified and used for the following experiments.

*Characterization of the isolated IgY.* The titers of the viruses were determined using the $TCID_{50}$ assay. Results indicated that the $TCID_{50}$ for the EV71 strain was $10^{7.1}$ $TCID_{50}$/ml, whereas the $TCID_{50}$ for CVA16 strain was $10^{6.3}$ $TCID_{50}$/ml. As indicated in Fig. 2, the results of SDS-PAGE demonstrated that the disulfide bond of the target protein was opened under reduction conditions, and the presence of the two dominant bands, a 70-kDa-sized species that represents the H chain and a 30-kDa-sized species corresponding to the L chain, was noted.

As the water-soluble fraction (WSF) contains a large amount of heteroprotein after egg yolk acid-isolation, water was used to dissolve the egg yolk, which was then salted out

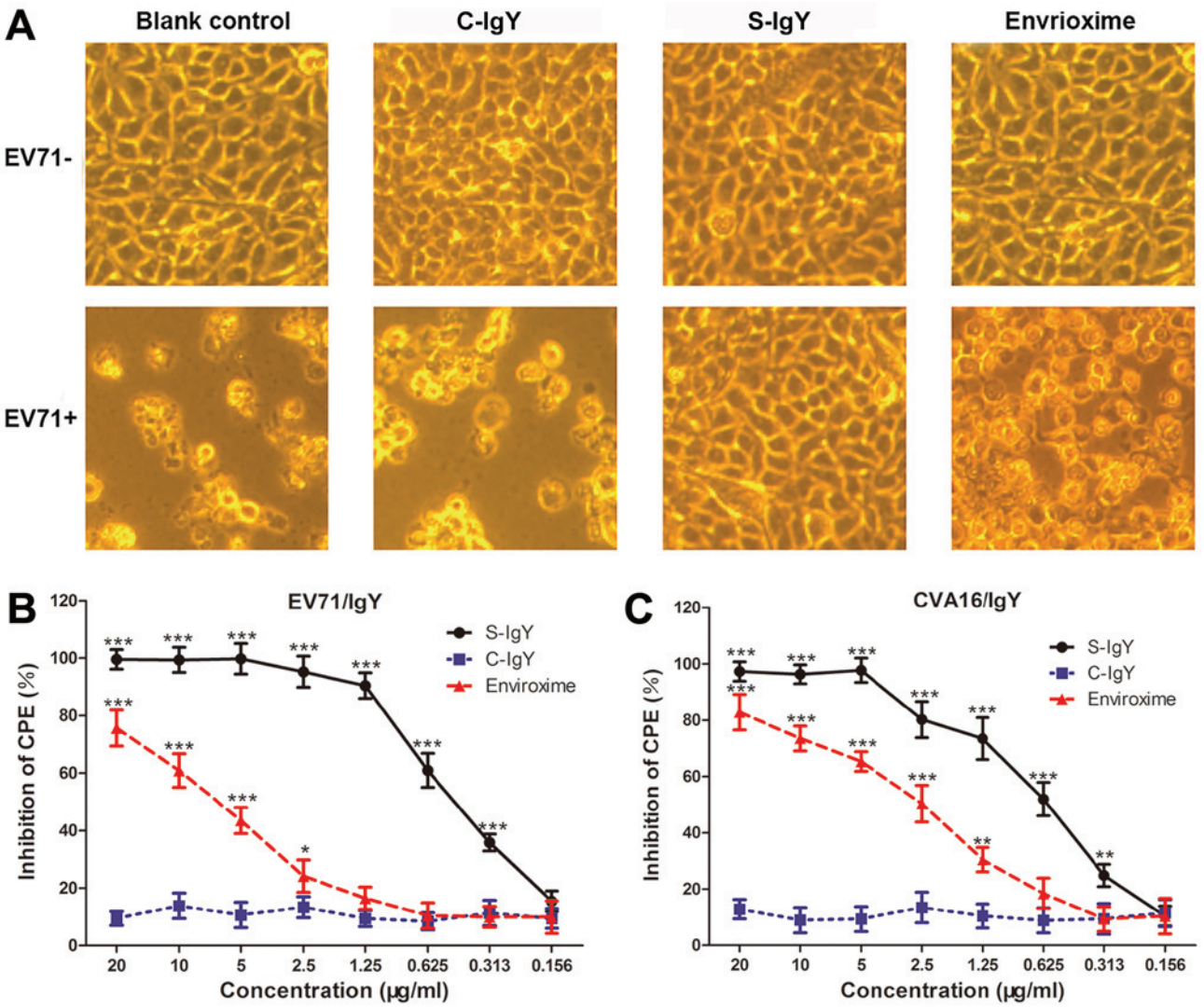


Figure 4. *In vitro* neutralization assays in RD cells. (A) RD cells were infected with or without EV71, and the cells were treated with PBS (blank control), negative control IgY (C-IgY), S-IgY or Envrioxime. A representative image from each treatment group is indicated (magnification, x400). (B and C) Dose-response inhibitory effect of IgY on CPE in RD cells. (B) EV71-infected RD cells and (C) CVA16-infected RD cells were treated with different concentrations of the C-IgY, the isolated S-IgY or Envrioxime as described in (A). The ability of the antibodies to inhibit CPE was determined using the neutralization assay. CPE values were expressed relative to those for cells with no antibody treatment (control CPE value, 0%). Data were presented as the mean ± standard deviation of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 vs. C-IgY. RD, rhabdomyosarcoma; IgY, immunoglobulin Y; EV71, enterovirus 71; CVA16, coxsackievirus A16; C-IgY, negative control IgY; S-IgY, specific IgY; CPE, cytopathic effect.

twice by 45% saturation of $(NH_4)_2SO_4$ and 13% mass fraction of $Na_2SO_4$. This markedly improved the purity of IgY, however, the SDS-PAGE results indicated that some impurity bands were still present in the samples, which may include some low density lipoproteins and active proteins. The purity of the IgY was determined to be over 85% according to gel system software Bandscan 5.0.

*IgY cross binds to the structural proteins VP0, VP1 and VP3 of EV71 and CVA16*. The titer of the purified IgY was measured with a bidirectional immune agar diffusion test. As indicated in Fig. 3A and B, a white precipitation line was identified between the antigen hole in the center and the antibody wells with dilution ratios of 1:2, 1:4, and 1:8, but no precipitation line was indicated between the PBS wells and the antigen wells. These data suggested that the purified S-IgY has the ability to specifically cross bind to the antigens of EV71 and CVA16.

The results of western blotting further confirmed that the S-IgY exhibited a good immunological binding reaction with the viral proteins of EV71 and CVA16, whereas neither EV71 nor CVA16 had immunoreactivity with C-IgY (Fig. 3C and D), suggesting that the purified IgY specifically recognizes the proteins of EV71 and CVA16 strains.

The proteins of EV71 and CVA16 viruses in the samples were identified to be 36 kDa for VP1 and 28 kDa for VP3. The predicted molecular weights of the VP1 protein is 35 kDa and 26 kDa for VP3 (41,42). VP1 protein of EV71 and CVA16 was verified with VP1 monoclonal antibody (Fig. 3C and D, lanes 3 and 4), and the purified IgY was verified with a commercial VP1 protein (Fig. 3E). Taken together, these data indicate that the IgY isolated from the egg yolks of EV71-immunized BWEL-SPF chickens specifically cross binds to EV71 and CVA16 viruses, and these data are consistent with the results of ELISA.

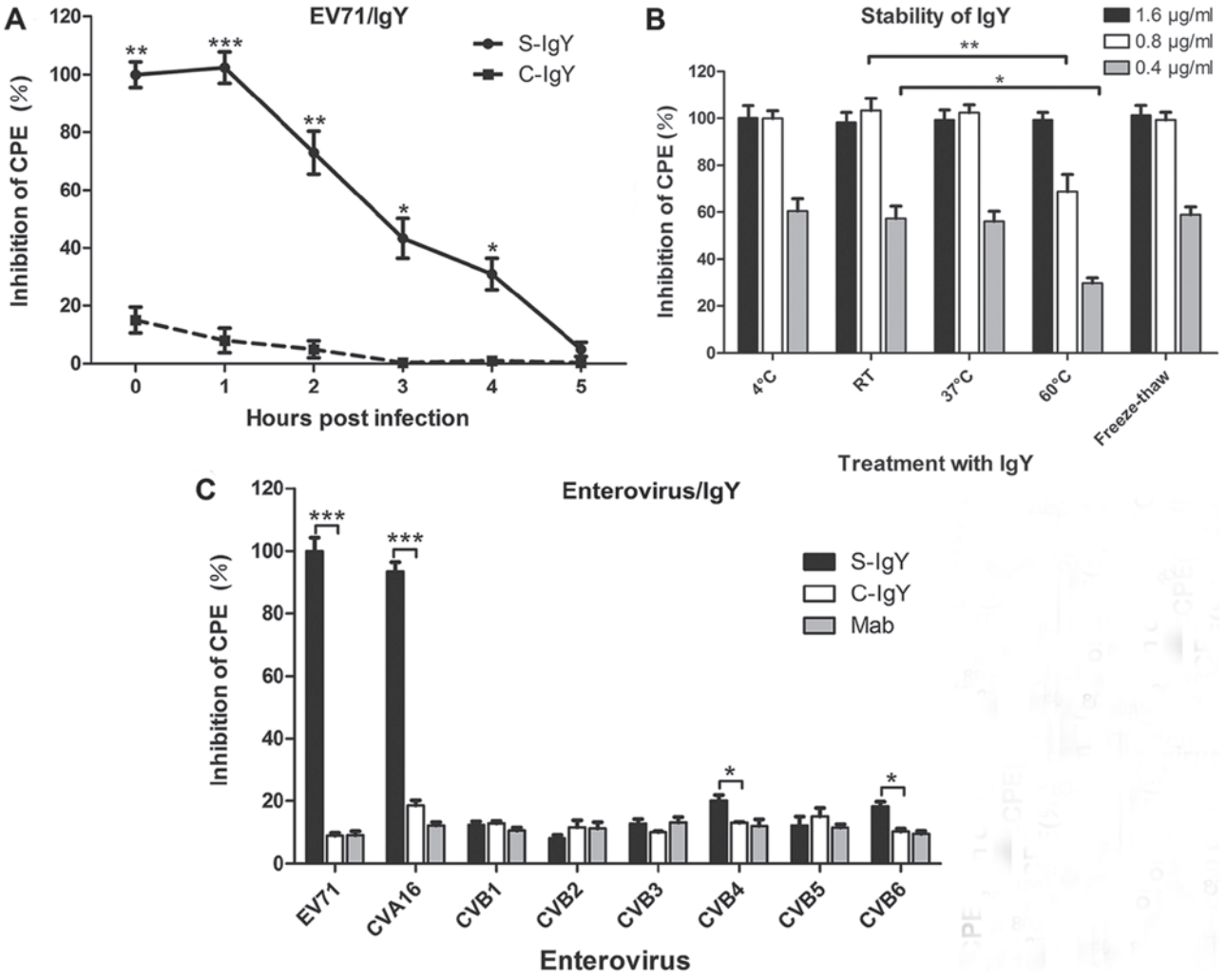


Figure 5. Characterization of the IgY bioactivity by *in vitro* neutralization assay. (A) Time-dependent inhibitory effect of the S-IgY on EV71-induced CPE in RD cells. RD cells were infected with EV71 and treated with C-IgY or S-IgY at different time points following EV71 infection. CPE values were expressed relative to those for cells with no antibody treatment (control CPE value, 0%). $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 vs. C-IgY (Student's t-test). (B) Effect of physical factors (temperature and freeze-thaw) on the S-IgY bioactivity in RD cells. RD cells were infected with EV71 and treated with IgY (0.4, 0.8, or 1.6 $\mu$g/ml) following exposure of the IgY to different temperatures for 48 h, or by freezing-thawing for five times. CPE values were expressed relative to those for cells with no antibody treatment (control CPE value, 0%). $^{*}$P<0.05 and $^{**}$P<0.01 $^{*}$ vs. RT IgY. (C) Inhibitory effect of the S-IgY on CPE induced by different enterovirus strains. RD cells were infected with eight different enterovirus strains, and the cells were treated with the C-IgY, the S-IgY or Mab. CPE values were expressed relative to those for cells with no antibody treatment (control CPE value, 0%). Data were presented as the mean ± standard deviation of three independent experiments. $^{*}$P<0.05 and $^{***}$P<0.001 as indicated. Mab, IgY monoclonal antibody; RD, rhabdomyosarcoma; IgY, immunoglobulin Y; EV71, enterovirus 71; C-IgY, negative control IgY; S-IgY, specific IgY; CPE, cytopathic effect.

*IgY cross blocks the CPE induced by EV71 and CVA16 in vitro.* The protective effect of the purified IgY on enterovirus-induced CPE in RD cells was assessed by neutralization assay *in vitro*. As indicated in Fig. 4A, RD cells in the EV71 non-infected groups were normal and healthy, indicating that the IgY and the positive control drug Envrioxime themselves had no cytotoxic effect in RD cells. Once the cells were infected with EV71 strains, different degrees of CPE (atrophied, rounded, shedding and apoptosis) appeared in the blank control group, the C-IgY group and the Envrioxime group, whereas RD cells did not exhibit CPE in the S-IgY group, suggesting a protecting effect of the specific IgY on EV71 infection in RD cells (Fig. 4A). Fig. 4B indicated that the IgY had a strong anti-EV71 activity *in vitro* at the concentrations of 1.25, 2.5, 5, 10, and 20 $\mu$g/ml. The results in Fig. 4C revealed that IgY had a strong anti-CVA16 activity *in vitro* at the concentrations of 2.5, 5, 10, and 20 $\mu$g/ml, which substantially inhibited EV71- or CVA16-induced CPE and blocked infectivity of the virus; however, lower concentrations of the IgY did not prevent from the virus infection of RD cells.

To understand the time-dependent protection of the S-IgY against enterovirus infection, RD cells were infected with EV71 and then IgY was added to the cells at different time points after infection. The present data indicated that the IgY inhibited >70% of EV71-induced CPE in RD cells when the IgY was added 2 h post-infection, and inhibited >40% of EV71-induced CPE when the IgY was added 3 h post-infection (Fig. 5A).

The stability of antiviral activity was determined for the isolated IgY. As observed in Fig. 5B, purified IgY was stable after 48 h at room temperature at 4˚C and 37˚C, and the inhibition rate was almost 100% when the cells were treated with 1.6 and 0.8 $\mu$g/ml IgY after 48 h at 60˚C. Furthermore, there was no decrease in antiviral activity observed with S-IgY

after freeze thawing five times (Fig. 5B), indicating that the freeze-thaw cycle did not impair IgY activity.

The antiviral activity of the purified IgY in 8 different strains of enterovirus demonstrated that the S-IgY had a strong inhibitory activity against EV71 and CVA16 strains ($P<0.001$), but had only marginal or no antiviral activity in the 6 other strains of the enterovirus examined, including CVB1, CVB2, CVB3, CVB4, CVB5, and CVB6 (Fig. 5C). The results revealed that the S-IgY has a differential antiviral activity among different types of enterovirus, further suggesting that the IgY isolated from EV71-immunized chicken egg yolks has a specific protection against EV71- and CVA16-induced infections.

## Discussion

EV71 and CVA16 belong to the small RNA family and enterovirus genus and have approximately the same structure (43). Of the four structural proteins of EV71 and CVA16, VP4 is located inside the capsid and connects with RNA (44); VP1, VP2 and VP3 are located on the surface of the capsid; and VP2 and VP4 are cleaved from the VP0 protein by autocatalytic action that involves virion stability and infectivity (45,46). Previous findings suggest that the antigenic determinant is based on the surface proteins, VP1 and VP3 (47). In the treatment of HFMD, inactivated EV71 virus vaccine has been proved to have immunogenicity, but it has no cross protection against other entericviruses, such as CVA16, in phase III clinical trials (48). Lim *et al* (49) screened monoclonal antibodies that can recognize the N-terminal of VP1 by passive immunization. Their study demonstrated the cross-neutralization of monoclonal antibodies against multiple EV71 subtype strains; however, there was no effect on the CVA16 strains.

In the present study, the results of the neutralization assay revealed that S-IgY cross blocked CPE induced by EV71 and CVA16 in a dose-dependent manner *in vitro*. Furthermore, bidirectional immune agar diffusion testing and western blotting further demonstrated that the isolated IgY cross bound to the envelope proteins VP1 and VP3 of EV71 and CVA16, suggesting that the inhibitory effect of IgY on EV71- and CVA16-induced CPE is mediated through targeting VP1 and VP3 structural proteins. Therefore, it was concluded that the isolated IgY is likely to be effective in recognizing the sequential epitopes or conformational structure of VP1 and VP3, preventing EV71 and CVA16 from entering and infecting host cells, and ultimately preventing and treating EV71- and CVA16-caused infections. Notably, chicken antibodies may recognize the sequential epitopes or conformational structure.

IgY is the sum of antibodies extracted from the egg yolk of immunized BWEL-SPF chickens, of which 2-10% have antigen specificity (50). In the present study, 150 mg IgY was extracted from each egg. Notably, the total IgY produced by an immunized chicken in a year is ~20 times as much as the IgG produced by a immunized rabbit (51). The protein in egg yolk is predominantly divided into WSFs and water-insoluble fractions. In the present study, the results indicated that the levels of the IgY in the egg yolk were significantly increased following immunization, which may be due to the increase in the age of the chickens (33). The SDS-PAGE results revealed that the purified IgY was composed of a 70-kDa H chain and a 30-kDa L chain, which is consistent with the literature (52), suggesting that IgY antibody with high purity can be obtained by the water dilution combined with sulfate precipitation. The indirect ELISA for measuring the S-IgY titer and the growth-decline rule indicated that the titer of antibody peaked at week 7 after the initial immunization and was maintained at a higher level for ~ 4 weeks compared with C-IgY, the antibody levels decreased gradually after week 11. The high titer, suitable purity and long duration of the IgY in the immunized egg yolks make it possible for manufacturing plants to prepare a large quantity of IgY.

In conclusion, the present findings indicated that the levels of S-IgY were significantly increased in chicken egg yolk following immunization with EV71. It was also revealed that IgY cross blocked CPE induced by EV71 and CVA16, and that the IgY cross bound to the envelope proteins VP1 and VP3 of EV71 and CVA16, suggesting that the cross protection of IgY against EV71 and CVA16 infection may be mediated through targeting VP1 and VP3 structural proteins of the two viruses. These findings provide a scientific basis for developing IgY as a cross passive immunotherapy for EV71- or CVA16-induced HFMD. Further *in vivo* studies in preclinical animal models with this cross immunotherapy are warranted.

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## Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

JW, YY, SW and QX contributed to the conception and design of the study. EG, YZ, NT JW, YY and QX performed the experiments. JW, YY, SW, QX and EG prepared the manuscript. SW and SH performed the statistical analysis. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The procedure strictly followed the Guide for the EU Directive for animal experiments, the protocols use in the current study were approved by the Institutional Animal Care and Use Committee of Guilin Medical University (Guilin, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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