**RESEARCH ARTICLE**

# Antiviral Activity of Chloroquine Against Pseudorabies Virus *in Vitro* and *in Vivo*

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## ABSTRACT

Pseudorabies virus (PRV) causes Aujeszky's disease characterized by neurological disorders and reproductive failure, resulting in significant economic losses to the global pig industry. Various PRV variants in China have compromised the efficacy of existing vaccines, necessitating for the development of alternative control measures. Chloroquine (CQ), originally developed for malaria treatment, exhibits broad-spectrum antiviral activity. Aim of the present study was to evaluate anti-PRV efficacy of CQ *in vitro* and *in vivo*. PRV-infected PK-15 cells were treated with CQ at various treatment timepoints (pre-, co-, post-infection), and the impact of CQ on viral adsorption, internalization and replication *in vitro* was assessed via Western blot, qPCR, and immunofluorescence analysis. To assess *in vivo* anti-PRV efficacy of CQ, 6-8 weeks old female SPF C57BL/6 mice (n=32) were divided into three groups: Mock (n=10; mice received neither PRV infection nor CQ); PRV (n=11; PRV-infected mice without CQ treatment), and PRV+CQ (n=11; PRV-infected mice received CQ treatment at 100mg/kg body weight, i.p.). The results demonstrated that CQ (50 and 100µM) significantly suppressed PRV infection in PK-15 cells. Notably, 100µM CQ exhibited antiviral activity whether it was added co-, post-, or pre-PRV infection. Mechanistically, viral adsorption assay revealed that co-incubation or pre-treatment with CQ impeded viral adsorption, while immunofluorescence results of the early endosome marker Rab5 showed that post-treatment with CQ blocked viral internalization. *In vivo* experiments showed that CQ failed to improve survival rate or extend mean survival duration in PRV-infected mice, although it provided limited symptomatic relief in PRV-infected mice. Collectively, these findings indicated that CQ effectively suppressed PRV infection *in vitro*, which provided a theoretical foundation for development of agents against PRV.

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## INTRODUCTION

Pseudorabies virus (PRV), also known as Aujeszky's disease virus or Suid herpesvirus-1, is a member of the *Alphaherpesviridae* subfamily within the *Herpesviridae* family (Pomeranz *et al.*, 2005). It primarily infects pigs (Zheng *et al.*, 2022), but has also been detected in humans, causing encephalitis and vision impairment (Chen *et al.*, 2025). Although vaccination remains the primary control measure for preventing PRV infection (Papageorgiou *et al.*, 2022), the emergence of PRV variants has compromised the effectiveness of existing vaccines (Ren *et al.*, 2020). Therefore, novel vaccines and targeted treatment strategies are urgently needed for the control of PRV infection in clinical practice.

Recent studies have identified several potentially effective drug molecules with anti-PRV activity *in vitro* and *in vivo*. For example, polysaccharides such as those derived from *hippophae rhamnoides* and glycyrrhiza exert anti-PRV effects via immunomodulation and oxidative stress reduction (Huan *et al.*, 2022a; Huan *et al.*, 2022b). Similarly, resveratrol, a phenolic molecule, enhances host immunity by up-regulating nuclear factor kappa-B (NF-κB) and c-Jun N-terminal kinase (JNK) pathways and reduces viral load (Zhao *et al.*, 2018; Chen *et al.*, 2019). In addition, antimicrobial peptides like cathelicidin B1 (CATH-B1) and piscidin-1 also inhibit PRV by suppressing the viral infection process and modulating host defense responses ( Hu *et al.*, 2019; Ye *et al.*, 2023).

Chloroquine (CQ), a 4-aminoquinoline compound, is renowned for its antimalarial and autophagy-inhibitory properties (Krafts *et al.*, 2012). Recently, chloroquine has been found to exhibit broad-spectrum antiviral activity against diverse viruses (Wei *et al.*, 2020). Mechanistic

studies indicate that CQ interferes with pH-dependent endocytic pathways, thereby inhibiting cellular entry of multiple viruses, including human immunodeficiency virus (Chauhan and Tikoo, 2015), Zika virus (Shiryaev *et al.*, 2017), herpes simplex virus (McClain *et al.*, 2015), channel catfish virus (Chen *et al.*, 2022) and white spot syndrome virus (Huang *et al.*, 2015). Besides viral entry inhibition, CQ also inhibits the lytic replication of Kaposi's sarcoma-associated herpesvirus and Epstein Barr virus (Yang *et al.*, 2016). As an autophagy inhibitor, CQ also inhibits the replication of Seneca valley virus (Bai *et al.*, 2023) and hepatitis-B virus (Yang *et al.*, 2024) by suppressing the autophagy process. Furthermore, CQ also exhibits antiviral activity against severe acute respiratory syndrome coronavirus (SARS-CoV) by inhibiting glycosylation of the host cellular receptor (angiotensin-converting enzyme-2 (ACE-2) (Vincent *et al.*, 2005). However, the antiviral activity and mechanism of CQ on PRV infection remains to be fully elucidated.

Previous studies have indicated that CQ showed broad-spectrum antiviral activity against different viruses. Therefore, aim of the present study was to evaluate the antiviral activity of CQ against PRV by using *in vitro* and *in vivo* experimental models, and to explore the potential mechanism of CQ against PRV infection.

## MATERIALS AND METHODS

This study was carried out at the Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Southwest University, Chongqing, China, during the period from January 2024 to January 2025 to evaluate the antiviral activity of CQ against PRV by using *in vitro* and *in vivo* experimental models, and to explore the potential mechanism of CQ against PRV infection. All animal procedures were conducted in strict compliance with institutional guidelines and were formally approved by Institutional Animal Care and Use Committee of Southwest University, Chongqing, China (IACUC-20241017-02).

**Cells and the virus:** The porcine kidney (PK)-15 and African green monkey kidney (Vero) cell lines were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) under standard culture conditions (37°C, 5% $CO_2$). The PRV variant strain JS-2012 used in this study was maintained in our laboratory, which was propagated and titrated in Vero cells.

**Cell viability:** Following 24h incubation at 37°C with varying concentrations (6.25, 12.5, 25.0, 50.0, 100.0, 200.0µM) of CQ (MedChemExpress, Shanghai, China), cellular viability of PK-15 cells was assessed using the WST-8 assay. Briefly, the WST-8 reagent was introduced to cultured cells for 3h, after which absorbance was measured at 450nm using a Bio-Rad microplate reader (Japan). CQ-untreated cell populations served as control. Cell viability was determined using the following formula: Cell viability (%) = (OD$_{450 \text{ (treated sample)}}$/OD$_{450 \text{ (Control)}}$) ×100.

**Western blot:** Cellular protein lysates from PK-15 cells were obtained using SDS lysis buffer supplemented with protease inhibitors for 10 min incubation on ice. Proteins were then resolved by SDS-PAGE (12% gel) and electro-transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% (w/v) non-fat milk for 2h at room temperature (RT), followed by overnight incubation at 4°C with an in-house produced anti-PRV gE monoclonal antibody and a commercial monoclonal anti-β-actin antibody (Beyotime, China). After three washes using Tris-buffered Saline with Tween 20 (TBST), membranes were incubated with HRP-labeled goat anti-mouse IgG (H+L) (Beyotime, China) for 1h at room temperature. Following five rigorous TBST washes, immunoreactive protein bands were visualized using Enhanced Chemiluminescence (ECL) substrate (Biosharp, China) and imaged with a ChemiDoc XRS+ System (Bio-Rad, USA).

**Quantitative PCR (qPCR):** Viral genomic DNA was extracted from cells or tissues using the TIANamp Virus DNA/RNA Kit (TIANGEN Biotech, China). Pseudorabies virus DNA copies were quantified via an absolute qPCR assay with Premix Ex Taq™ (Probe qPCR，Takara), as described earlier (Niu *et al.*, 2025). The qPCR primer and probe sequences used in this study were as follows: forward primer: 5'-GAGTTCAGCAGCGACGAG-3'; reverse primer: 5'-CGCCATAGTTGGGTCC ATT-3', probe: FAM-5'-CGTCACTTCCGGTTTCTCCGGATC-3'-BHQ1.

**Immunofluorescence assay (IFA):** The porcine kidney (PK)-15 cells ($10^4$ cells/well) were fixed in 4% paraformaldehyde (30min), permeabilized with 0.1% Triton X-100 (5min, RT) and blocked in 5% BSA (1h, RT). After PBS washes, the corresponding primary antibodies (anti-PRV gE monoclonal antibody (produced in-house) or anti-Rab5 antibody (Cell Signaling Technology, USA)) were employed overnight at 4°C. Subsequently, the corresponding secondary antibodies including goat anti-mouse IgG (H&L) labeled with Alexa Fluor 488 (Abcam, UK) for PRV gE detection and goat anti-rabbit IgG (H&L) conjugated with Alexa Fluor 594 (Abcam, UK) for Rab5 detection were incubated with the cell samples (1h, RT). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) staining (Biosharp, China). Images were acquired using an Olympus inverted fluorescence microscope (Olympus, Japan).

**Antiviral activity of chloroquine *in vitro*:** Prior to treatment with CQ, PK-15 cells were cultured in 12-well plates to form an 80% monolayer. For co-incubation experiments, CQ (100µM) and PRV (Multiplicity of Infection-MOI=1) were either pre-mixed or kept as separate components for 2h at 37°C before application to cell monolayers for 24h infection. In pre-treatment studies, cells were exposed to CQ (100µM) for varying durations (2, 4, 8, 16 and 24h), followed by PBS washing and PRV infection (MOI=1) for 24h. For post-treatment evaluation, PRV-infected cells (MOI=1) were treated with CQ (100µM) for 4h after different infection intervals (1, 2, 4, 8 and 12h) with cultures maintained until 24h post infection (hpi) before sampling. After samples collection, the level of PRV gE protein in the cell lysate was detected by Western blot and viral DNA copies in the cellular supernatant were calculated by the qPCR method.

**Viral adsorption assays:** To assess the effect of CQ on viral adsorption in co-incubation experiments, CQ (100μM) and PRV (MOI=5) were either pre-incubated or kept as separate components at 37°C for 2h. Subsequently, the mixture of CQ and PRV in each group was added to PK-15 monolayers, and viral adsorption was performed at 4°C for 60min. To evaluate the effect of CQ on viral adsorption in pre-treatment experiments, CQ (100μM) was inoculated to PK-15 monolayers for 16 or 24h, and then PRV (MOI=5) was used to infect the cells at 4°C for 60min. Next, non-adherent virions were removed by three successive washes with chilled PBS. Subsequently, cells were subjected to three freeze-thaw cycles, then the viral DNA was extracted from cell lysates and subjected to qPCR detection, as described above.

**Electron microscopy:** To investigate potential structural modifications of PRV virions induced by CQ treatment, purified PRV virions were prepared according to our previous study (Ye *et al.*, 2023). Then, an equal amount of CQ (100μM) and PBS were mixed with the purified virions and incubated at 37°C for 2h. Subsequently, both 10μL of the PRV/PBS and PRV/CQ mixtures were adsorbed onto 300-mesh copper grids coated with formvar-carbon support film for 10 min. After full rinsing with distilled water, the grids were stained with 2% phosphotungstic acid for 20s, followed by complete air-drying at room temperature. Then, the stained specimens of PRV/PBS suspension and PRV/CQ mixture were examined using a transmission electron microscope (HT7800, Japan) at 80 kV.

**Animals and experimental design:** In the animal experiments, 6-8 weeks old Specific Pathogen-Free (SPF) female C57BL/6 mice (n=32) were obtained commercially from Home-SPF Biotechnology Co., Ltd (Beijing, China). All mice were housed under controlled conditions (25°C, 12h light/dark cycle) with free access to sterilized standard diet and clean drinking water. Before the start of the experiment, these mice underwent a one-week adaptation period.

Animals were randomly allocated into three experimental groups: Mock, PRV, and PRV+CQ. Mice in both PRV-infected groups (PRV and PRV+CQ) were intraperitoneally injected with 100μL PRV JS-2012 ($1 \times 10^5$ $TCID_{50}$), while mice in the Mock group were injected with 100μL sterile PBS. Subsequently, mice in the PRV+CQ group were intraperitoneally injected with 100μL CQ (100mg/kg) at 24 and 48 hpi, and mice in the PRV and Mock groups were injected with 100μL sterile PBS. Clinical symptoms and survival conditions in mice from the Mock (n=5), PRV (n=5), and PRV+CQ (n=5) groups were monitored at 6h intervals, starting from 60 hpi till 96 hpi. Clinical manifestations of mice were scored based on the methods described previously (Niu *et al.*, 2025). In a parallel experiment, mice were randomly allocated into three groups: Mock (n=5), PRV (n=6), and PRV+CQ (n=6). brain tissues of mice were collected at 72 hpi and homogenized, followed by viral DNA extraction from supernatants using the TIANamp Virus DNA/RNA Kit (TIANGEN Biotech, China). Viral loads were then quantified by the qPCR assay described above.

**Statistical analysis:** All statistical analyses were performed using GraphPad Prism 9.0. Data were expressed as mean±standard deviation (SD). For in-vitro studies, statistical significance between the PRV positive control and each treatment groups were analyzed by unpaired Student's *t*-test. The survival curve comparison between PRV+CQ and PRV groups was analyzed by the log-rank test, with statistical significance was set at $P<0.05$, while $P<0.01$ was considered highly significant.

**RESULTS**

**Antiviral activity of Chloroquine against PRV:** The cytotoxicity analysis of CQ on PK-15 monolayers indicated that cell viability remained stable at concentrations ranging from 0 to 100μM, while a significant reduction ($P<0.01$) in cell viability was observed at 200μM (Fig. 1A). When different concentrations of CQ were added to PRV-infected PK-15 cells for 24h, it was found that CQ treatment could inhibit PRV infection as evidenced by reduced gE expression (Fig. 1B), with significant suppression ($P<0.01$) was seen at 100μM CQ concentration (Fig. 1C). Furthermore, qPCR showed that both 50μM ($P<0.05$) and 100μM ($P<0.01$) CQ treatments significantly decreased the PRV DNA copies in the supernatant (Fig. 1D) compared to the PRV-infected control group. Immunofluorescence (IFA) experiments also revealed observable reductions in PRV infection in PK-15 cells following treatment with 50 and 100μM CQ (Fig. 1E).

**Chloroquine co-incubation inhibits PRV adsorption:** Western blot results in the present study showed that co-incubation of PRV with CQ inhibited the PRV infection (Fig. 2A), and this inhibitory effect was significantly enhanced ($P<0.01$) when CQ was pre-incubated with PRV for 2h (PRV+CQ-co) compared to separate treatment of CQ and PRV (PRV+CQ-sep), as indicated by results of PRV gE expression (Fig. 2B) and viral DNA copies (Fig. 2C). The use of electron microscopy to observe the possible destructive effect of CQ on PRV virion structure showed that co-incubation with CQ did not disrupt the structure of PRV (Fig. 2D), indicating that CQ had an indirect inhibitory effect on PRV infection. Application of viral adsorption assay to investigate the effect of CQ on viral life cycle revealed that co-incubation of PRV with CQ significantly inhibited the adsorption step of PRV, and the 2h pre-incubation of CQ with PRV (PRV+CQ-co) was more effective ($P<0.01$) than separate treatment ($P<0.05$) of CQ and PRV (PRV+CQ-sep) (Fig. 2E).

**Pre-treatment with chloroquine inhibits PRV adsorption:** Interestingly, pre-treatment of PRV with CQ for 16 and 24h also inhibited PRV infection (Fig. 3A), with a significant decrease ($P<0.05$) in gE protein expression compared to PRV infected positive control group (Fig. 3B). Meanwhile, qPCR analysis of PRV DNA copies showed that CQ pre-treatment at 8h ($P<0.05$), 16h, and 24h significantly suppressed ($P<0.01$) PRV infection compared to positive control group, demonstrating progressively enhanced prophylactic efficacy with longer pre-treatment durations (Fig. 3C). In viral absorption assay, the results
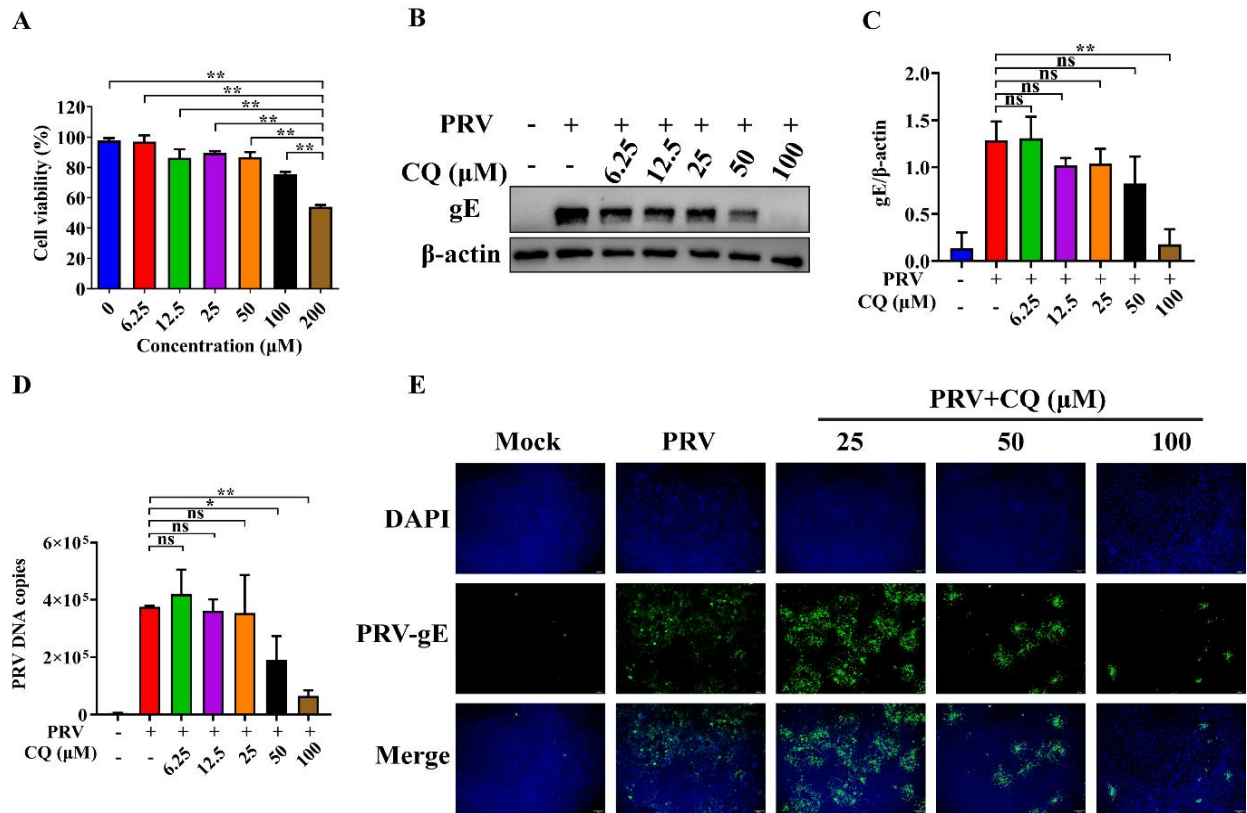
**Fig. 1:** Showing antiviral activity chloroquine against PRV. (A): cell viability of PK-15 cells treated with different concentrations (6.25, 12.5, 25.0, 50.0, 100.0, 200.0μM) of CQ. (B): Detection of PRV gE expression in PRV-infected cells treated with different concentrations (6.25, 12.5, 25.0, 50.0, 100.0μM) of CQ by Western blot analysis. (C): Quantification of PRV gE expression following CQ treatment by ImageJ. (D): PRV DNA copies detection following different concentrations of CQ (6.25, 12.5, 25.0, 50.0, 100.0μM) treatment. (E): IFA detection of PRV gE in infected cells treated with varying concentrations (25.0, 50.0, 100.0μM) of CQ (×100). ns, non-significant; *P<0.05; **P<0.01.
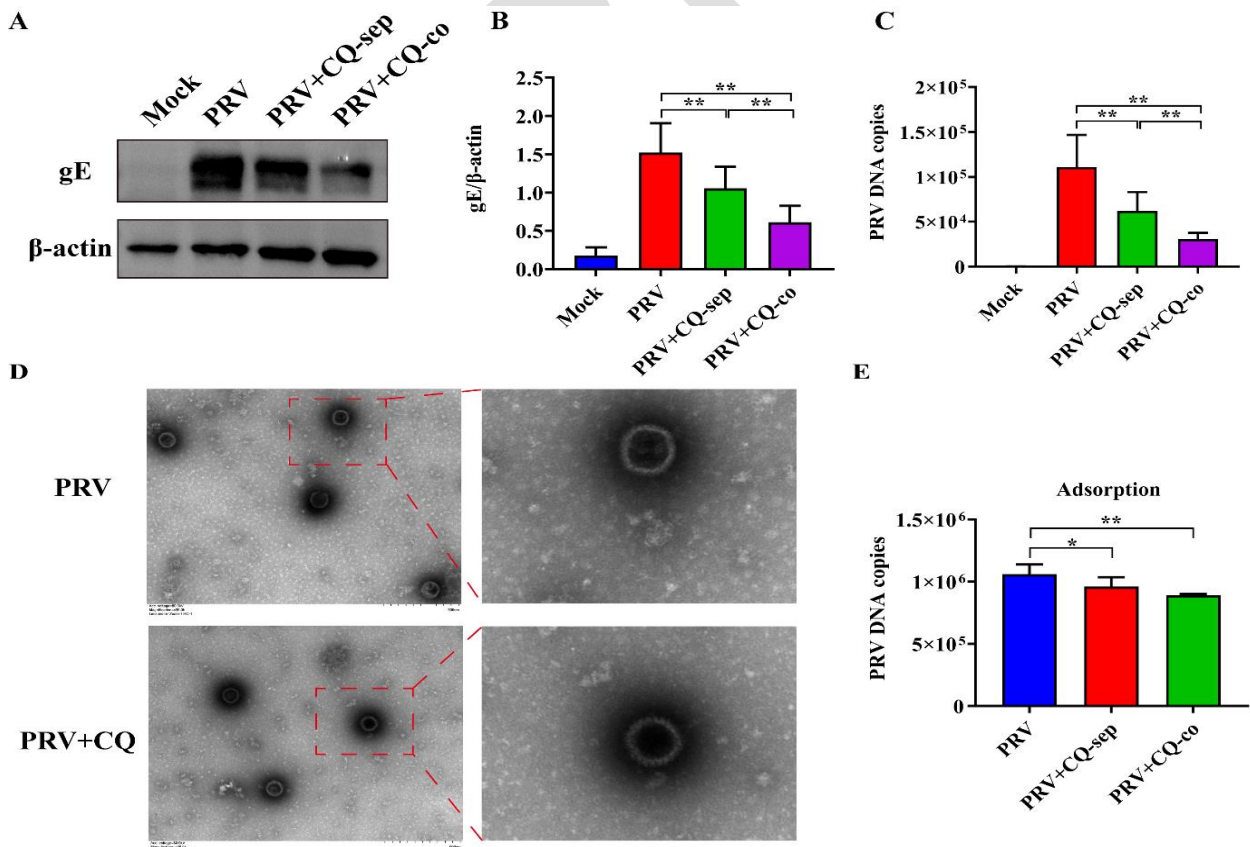


**Fig. 2:** Showing inhibition of PRV adsorption by Chloroquine co-incubation. (A): Western blot analysis of PRV gE protein expression levels in different treatment groups. (B): Quantitative analysis of gE protein levels by ImageJ. (C): PRV DNA copies in different treatment groups. (D): Electron microscopy analysis of CQ-treated PRV virions (×30,000). (E): Effects of CQ co-incubation on PRV cellular adsorption measured by qPCR. *P<0.05; **P<0.01.

showed that pre-treatment with CQ for 16 and 24h significantly (P<0.01) reduced PRV adsorption to PK-15 cell monolayers than PRV positive control group, and the inhibition effect of pre-treatment with CQ for 24h was relatively more obvious than that seen at 16h (Fig. 3D).

**Post-treatment with chloroquine inhibits PRV internalization:** Administration of PK-15 cell monolayers with CQ (100μM) at varying intervals following PRV exposure indicated that CQ post-treatment at 1 hpi modestly reduced PRV gE protein expression (Fig. 4A), although this reduction was not statistically significant (Fig. 4B). Additionally, no inhibitory effects were observed when CQ was administered at 2-12 hpi, as evidenced by both gE protein expression levels (Fig. 4A) and statistical evaluation (Fig. 4B). The qPCR analysis of viral DNA copies showed that CQ at 1 hpi significantly inhibited (P<0.01) PRV infection compared to positive control group, while post-treatment with CQ during PRV late infection (2-12h) showed non-significant inhibitory effect on the virus infection compared to PRV infected control group (Fig. 4C), although numerically there was an increase in PRV infection at 2, 8 and 12 hpi compared to positive control group. To verify whether CQ post-treatment had an inhibitory effect on PRV early infection, PK-15 cells were infected with PRV for 1h, followed by CQ treatment for 4h. Then, the expression of early endosomal marker Rab5 in PRV-infected cells was detected by IFA to reflect viral internalization. It was observed that CQ post-treatment inhibited Rab5 aggregation in PRV-infected cells (Fig. 4D), indicating that CQ interferes with early endosome formation and thus inhibits virus internalization.

**Chloroquine shows limited therapeutic efficacy against PRV infection in mice:** Administration of mice infected with PRV at 24 and 48 hpi showed that CQ had no effect on the survival rate of mice, with both PRV-infected groups (PRV and PRV+CQ) showing 0% survival at 96 hpi, compared to 100% survival in Mock group (Fig. 5A). Although CQ prolonged the survival time of PRV-infected mice by 6h (Fig. 5A), the mean survival time did not differ significantly between the PRV and PRV+CQ groups (Fig. 5B). Additionally, mice in the PRV group began to show obvious scratching symptoms with a score of 2 at 60 hpi, while mice in the PRV+CQ group showed no obvious scratching symptoms until 66 hpi; after 66 hpi, mice in both the PRV and PRV+CQ groups exhibited obvious scratching symptoms until death (Fig. 5C). Then, qPCR results showed that CQ inoculation did not significantly reduce viral loads in brain tissues of PRV-infected mice at 72 hpi (Fig. 5D). These results indicated that CQ exhibited limited therapeutic efficacy against PRV infection in mice.
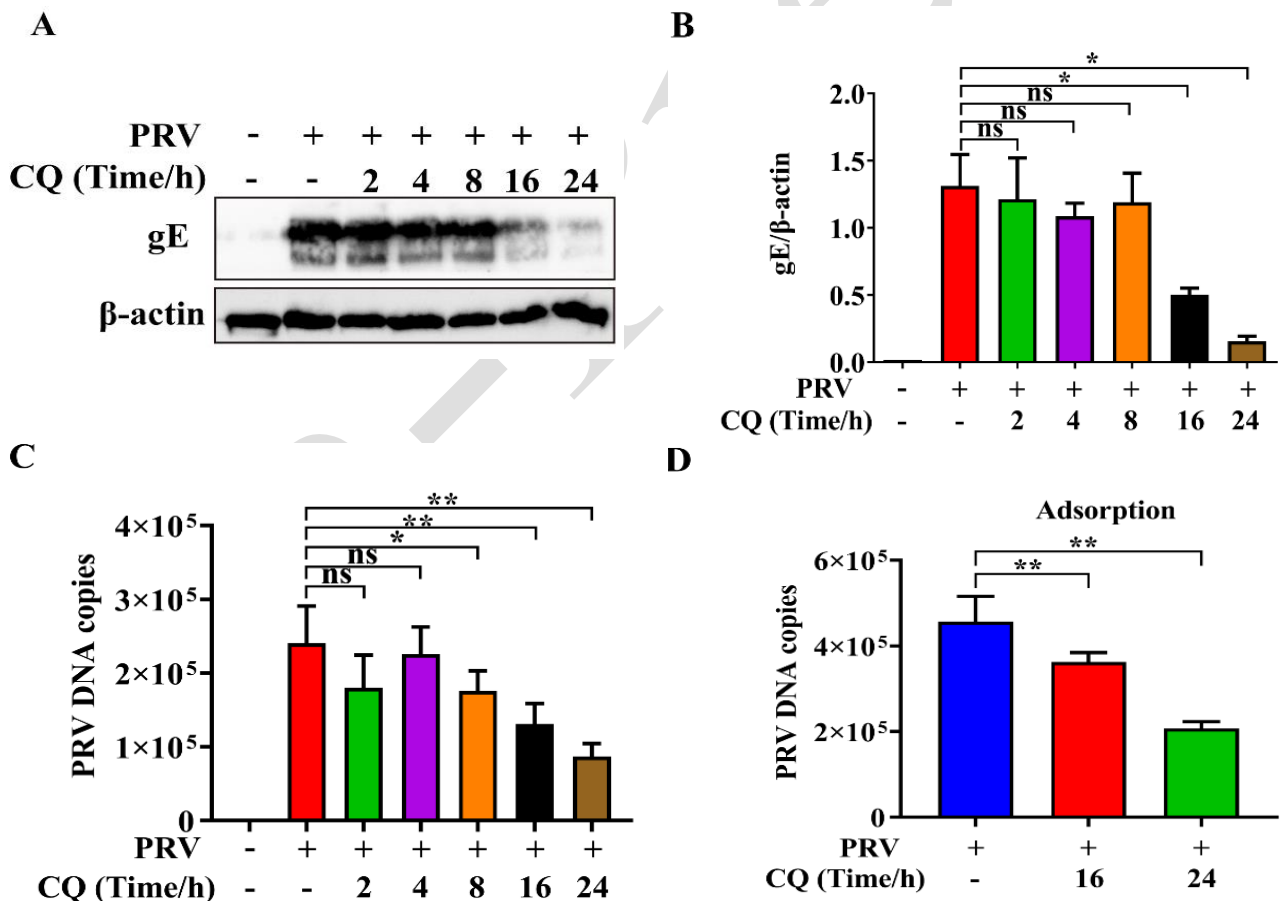


**Fig. 3:** Showing that pre-treatment with chloroquine inhibits PRV adsorption. (A): Western blot analysis of PRV gE protein expression in infected cells following CQ pre-treatment. (B): Quantitative analysis of gE protein levels by ImageJ. (C): PRV DNA copies following CQ pre-treatment. (D): Effects of CQ pre-treatment on PRV adsorption measured by qPCR. ns, non-significant; *P<0.05; **P<0.01.
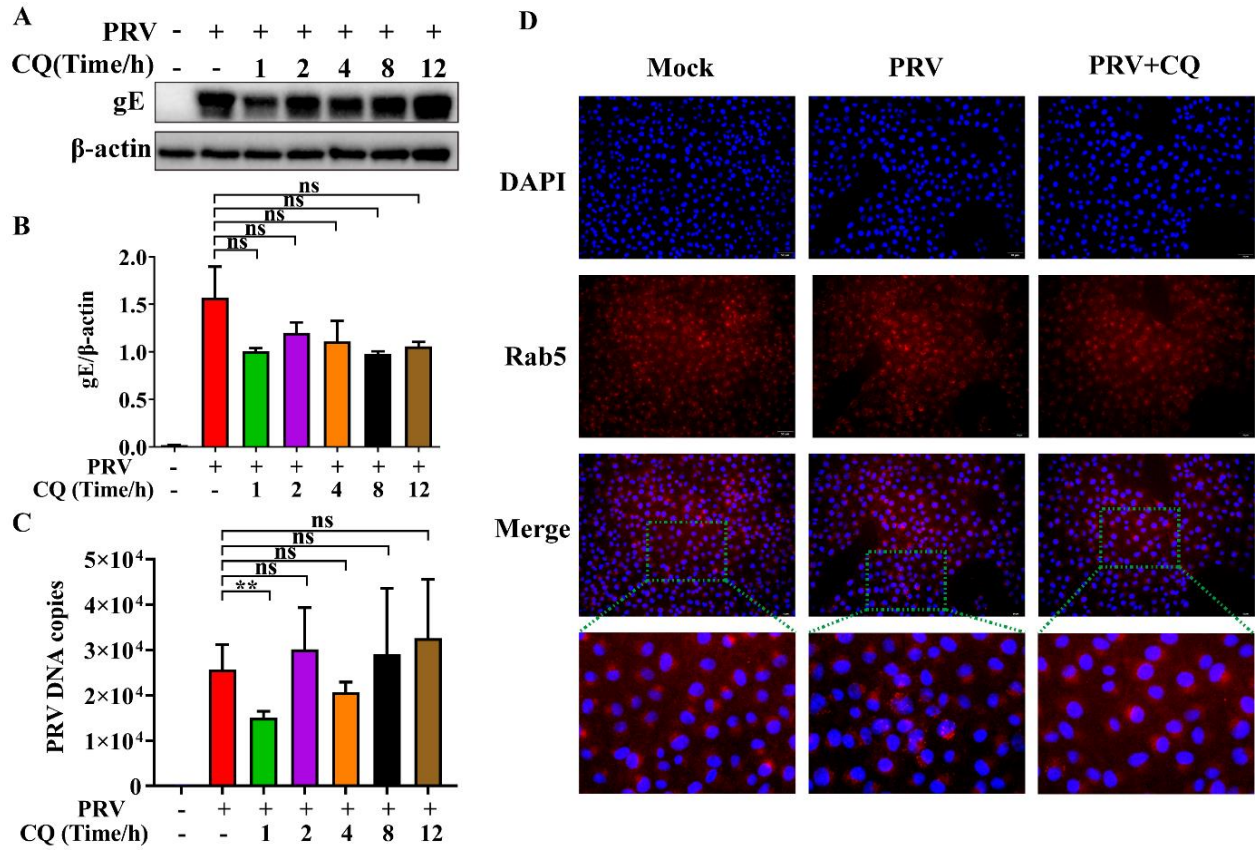
**Fig. 4:** Showing post-treatment with chloroquine inhibits PRV internalization. (A): Western blot analysis of PRV gE protein expression in infected cells following CQ post-treatment. (B): Quantitative analysis of gE protein levels by ImageJ. (C): PRV DNA copies detection following CQ post-treatment by qPCR. (D): IFA results of Rab5 expression in PK-15 cells infected with PRV for 1h followed by CQ treatment for 4h (×200). ns, non-significant; **$P<0.01$.
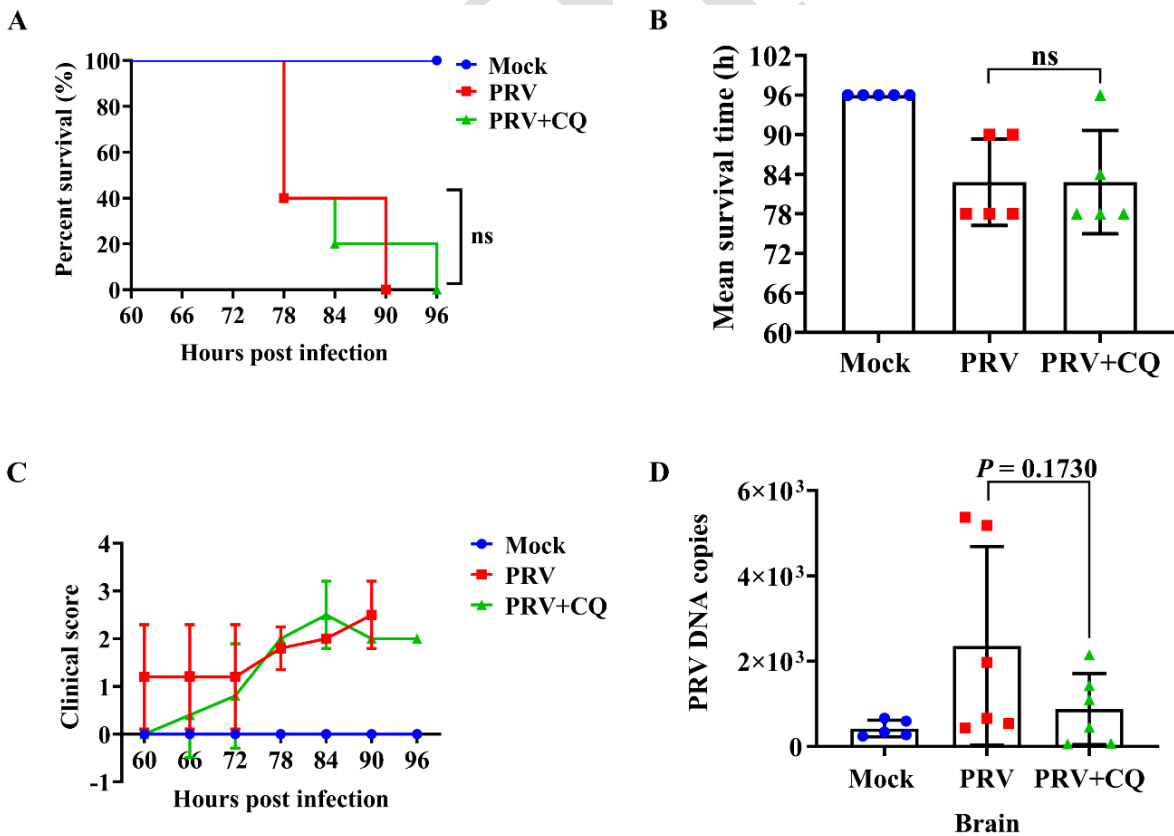


**Fig. 5:** Showing limited therapeutic efficacy of chloroquine against PRV infection in mice. (A): Survival rate of mice in different groups. (B): Mean survival time of mice in different groups during the observation period. (C): Clinical scores of mice in different groups. (D): Detection of viral load in brain tissues at 72 hpi by qPCR. ns, non-significant.

## DISCUSSION

The Pseudorabies virus (PRV) infection can cause a highly infectious disease in pigs, which has resulted in significant economic losses to the global pig industry (Pomeranz *et al.*, 2005). Recent studies have identified numerous bioactive compounds with anti-PRV activity, including plant-derived polysaccharides and phenols, as well as antimicrobial peptides (Zhou *et al.*, 2022; Ye *et al.*, 2023). Findings of these studies have highlighted the potential of pharmacological intervention for PRV control. Chloroquine, an FDA approved anti-malaria agent, has exhibited broad-spectrum antiviral properties against numerous medically important viruses (Rodrigo *et al.*, 2020), however, its antiviral activities and mechanisms against PRV remain incompletely understood. In this study, the antiviral efficacy of CQ against PRV was systematically evaluated using both *in vitro* and *in vivo* models. Notably, our findings reveal, for the first time, that CQ exerts potent anti-PRV activity in PK-15 cells, irrespective of its administration timing—whether pre-, co-, or post-viral infection.

Results of the present study showed that CQ exhibited minimal cellular toxicity in PK-15 cells when used at concentrations up to 100μM, while inducing marked cytotoxic effects (P<0.01) at 200μM. These findings are consistent with those of the previously reported toxicity range of CQ (Delvecchio *et al.*, 2016), where concentrations up to 200μM showed marked cellular toxicity in various cell types. Furthermore, the present study demonstrated a clear concentration-dependent antiviral effect of CQ against PRV, with significant viral inhibition was observed at both 50 (P<0.05) and 100μM concentrations (P<0.01). These findings strongly support the potential of CQ as an effective anti-PRV agent *in vitro*.

In order to analyze the inhibition mechanism of CQ against PRV, three distinct treatment patterns (co-incubation, pre-treatment, and post-treatment) were employed to evaluate its inhibitory effect on PRV infection. It was found that co-incubation with CQ significantly (P<0.01) suppressed viral infectivity, and pre-mixing CQ with PRV for 2h prior to infection significantly (P<0.01) enhanced antiviral activity of chloroquine. According to some recent studies, several bioactive compounds, such as anthranilamide peptidomimetics (Urmi *et al.*, 2023) and Carnosol (Sun *et al.*, 2025), have been reported to inactivate viruses through direct structural disruption. To investigate the direct influence of CQ on PRV virions, the viral particle integrity was examined after incubation with CQ by electron microscopy, and the results indicated that CQ had no direct disruptive effect on PRV virion structure. Subsequent adsorption assay demonstrated that co-incubation with CQ inhibited the adsorption step of PRV, and the 2h pre-mix of CQ with PRV enhanced this blocking effect, suggesting that co-incubation of CQ and PRV could interfere with the attachment stage of PRV without disrupting the integrity of PRV virions.

In the experiments exploring the preventive effect of CQ on PRV, it was found that prolonged pre-treatment of PK-15 cells with CQ showed a strong inhibitory effect on PRV infection, indicating that CQ pre-treatment could block the corresponding stages or processes of PRV infection by modulating host cell physiology. Furthermore,

viral adsorption assay confirmed that CQ pre-treatment could reduce PRV adsorption to cells. A previous investigation by Vincent *et al.* (2005) has demonstrated that CQ pre-treatment reduces SARS-CoV infectivity by impairing ACE2 glycosylation, thereby diminishing viral spike protein binding affinity. Subsequent research revealed an additional mechanism whereby CQ directly binds to ACE2, competitively blocking the binding of SARS-CoV-2 to ACE2 (Wang *et al.*, 2020). By analogy, CQ may interfere with PRV-host receptor interactions to suppress viral infection, although this precise inhibitory mechanism requires further investigation.

In the present study, the antiviral potential of CQ after PRV infection *in vitro* was also assessed. For this purpose, PK-15 cells were treated with 100μM CQ at various intervals (1-12h) following viral exposure. Our data showed that administration of CQ at early-stage of viral infection (1h) significantly suppressed PRV infection compared to positive control group (P<0.01). A recent study has suggested that PRV infects PK-15 cells via clathrin-mediated endocytosis (Andreu *et al.*, 2024). To test whether CQ post-treatment could block PRV endocytosis, levels of Rab5 (a marker of early endosomes) were examined in PRV-infected cells by IFA. Our results showed that CQ post-treatment markedly decreased both expression and aggregation of Rab5 in PRV-infected cells. These findings suggest that CQ post-treatment may inhibit PRV infection by blocking viral endocytosis.

Previous studies have shown that CQ can inhibit viral replication in relevant animal models (Takano *et al.*, 2013; Rahman *et al.*, 2021). However, conflicting evidences suggest that CQ and its hydroxylated form may lack efficacy against some viral infections *in vivo* (Falzarano *et al.*, 2015; Cochin *et al.*, 2022). To assess the therapeutic effects of CQ against PRV infection *in vivo*, mice were treated with CQ at 24 and 48 hpi. The results revealed that CQ administration delayed the onset of clinical symptoms, without improving the survival rate or extending the mean survival time in PRV-infected mice. Additionally, a modest but non-significant reduction in viral load was observed in brain tissues following CQ treatment. Collectively, these findings demonstrated that CQ showed limited therapeutic potential against PRV infection in mice. A previous report indicates that CQ requires sustained therapeutic blood concentrations for effective antiviral activity, while this relatively low-toxicity compound has also been shown to possess both acute and cumulative toxicity (Wei *et al.*, 2020). Our findings revealed limited efficacy of CQ against PRV infection in mice, most probably due to insufficient plasma drug concentrations, toxicity constraints at high doses, or limitations in the current treatment regimen. Moreover, due to the limited sample size in this study, it may not yet be appropriate to draw definitive conclusions regarding the observed effects. Further optimization studies, including dose adjustment, treatment regimen refinement and increased number of experimental animals remain crucial for improving CQ-mediated PRV suppression *in vivo*.

**Conclusions:** In this study, antiviral activity of CQ against PRV was evaluated by both *in vitro* and *in vivo* models. The results demonstrated that CQ effectively inhibited PRV infection *in vitro* regardless of whether CQ was added pre-

, co-, or post-infection. Mechanically, CQ inhibited the infection of PRV by suppressing its adsorption and endocytosis processes. *In vivo* experiments also confirmed that administration of CQ delayed the onset of PRV symptoms, but could not improve the survival rate in PRV infected mice.

**Declaration of competing interest:** There is no conflict of interest in this manuscript.

## REFERENCES

Andreu S, Agúndez C, Ripa I, *et al.*, 2024. Pseudorabies virus uses clathrin mediated endocytosis to enter PK-15 swine cell line. Front Microbiol 15:1332175.

Bai L, Zhang R, Zheng H, *et al.*, 2023. Seneca Valley Virus degrades STING via PERK and ATF6-mediated reticulophagy. Viruses 15(11):2209.

Chauhan A and Tikoo A, 2015. The enigma of the clandestine association between chloroquine and HIV-1 infection. HIV Med 16(10):585-90.

Chen H, Yu F, Xu J, *et al.*, 2022. Channel catfish virus entry into host cells via clathrin-mediated endocytosis. Virus Res 315:198794.

Chen M, Chen X, Song X, *et al.*, 2019. The immune-adjuvant activity and the mechanism of resveratrol on pseudorabies virus vaccine in a mouse model. Int Immunopharmacol 76:105876.

Chen Y, Gao J, Hua R, *et al.*, 2025. Pseudorabies virus as a zoonosis: Scientific and public health implications. Virus Genes 61(1):9-25.

Cochin M, Touret F, Driouich JS, *et al.*, 2022. Hydroxychloroquine and azithromycin used alone or combined are not effective against SARS-CoV-2 *ex vivo* and in a hamster model. Antiviral Res 197:105212.

Delvecchio R, Higa LM, Pezzuto P, *et al.*, 2016. Chloroquine, an endocytosis blocking agent, inhibits Zika virus infection in different cell models. Viruses 8(12):322.

Falzarano D, Safronetz D, Prescott J, *et al.*, 2015. Lack of protection against Ebola virus from chloroquine in mice and hamsters. Emerg Infect Dis 21:1065-67.

Hu H, Guo N, Chen S, *et al.*, 2019. Antiviral activity of Piscidin 1 against pseudorabies virus both *in vitro* and *in vivo*. Virol J 16:95.

Huan CC, Xu Y, Zhang W, *et al.*, 2022a. *Hippophae rhamnoides* polysaccharides dampen pseudorabies virus infection through downregulating adsorption, entry and oxidative stress. Int J Biol Macromol 207:454-63.

Huan CC, Xu Y, Zhang W, *et al.*, 2022b. Glycyrrhiza polysaccharide inhibits Pseudorabies virus infection by interfering with virus attachment and internalization. Viruses 14(8):1772.

Huang J, Li F, Wu J, *et al.*, 2015. White spot syndrome virus enters crayfish hematopoietic tissue cells via clathrin-mediated endocytosis. Virology 486:35-43.

Krafts K, Hempelmann E and Skórska-Stania A, 2012. From methylene blue to chloroquine: A brief review of the development of an antimalarial therapy. Parasitol Res 111(1):1-6.

Mcclain L, Zhi Y, Cheng H, *et al.*, 2015. Broad-spectrum non-nucleoside inhibitors of human herpesviruses. Antiviral Res 121:16-23.

Niu J, Li G, Liu Y, *et al.*, 2025. The important role of TLR2/MyD88/JNK in regulating the pathogenesis and inflammation induced by pseudorabies virus in mice. Vet Microbiol 304:110496.

Papageorgiou KV, Michailidou M, Grivas I, *et al.*, 2022. Bartha-K61 vaccine protects nursery pigs against challenge with novel european and asian strains of Suid herpesvirus 1. Vet Res 53:47.

Pomeranz LE, Reynolds AE and Hengartner CJ, 2005. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiol Mol Biol Rev 69(3):462-500.

Rahman F, Libre C, Oleinikov A, *et al.*, 2021. Chloroquine and pyrimethamine inhibit the replication of human respiratory syncytial virus A. J Gen Virol 102(8); doi: 10.1099/jgv.0.001627.

Ren J, Wang H, Zhou L, *et al.*, 2020. Glycoproteins C and D of PRV strain HB1201 contribute individually to the escape from Bartha-K61 vaccine-induced immunity. Front Microbiol 11:323.

Rodrigo C, Fernando SD and Rajapakse S, 2020. Clinical evidence for repurposing chloroquine and hydroxychloroquine as antiviral agents: A systematic review. Clin Microbiol Infection 26(8):979-87.

Shiryaev SA, Mesci P, Pinto A, *et al.*, 2017. Repurposing of the anti-malaria drug chloroquine for Zika virus treatment and prophylaxis. Sci Rep 7(1):15771.

Sun L, Zhang Y, Xu C, *et al.*, 2025. Carnosol inhibits influenza A virus by disrupting the viral envelope and interfering with Jak2/STAT3 signaling pathway. Eur J Pharmacol 997:177606.

Takano T, Katoh Y, Doki T, *et al.*, 2013. Effect of chloroquine on feline infectious peritonitis virus infection *in vitro* and *in vivo*. Antiviral Res 99(2):100-107.

Urmi UL, Attard S, Vijay AK, *et al.*, 2023. Antiviral activity of anthranilamide peptidomimetics against herpes simplex virus 1 and a coronavirus. Antibiotics (Basel) 12(9):1436.

Vincent MJ, Bergeron E, Benjannet S, *et al.*, 2005. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. Virol J 2:69.

Wang N, Han S, Liu R, *et al.*, 2020. Chloroquine and hydroxychloroquine as ACE2 blockers to inhibit viropexis of 2019-nCoV Spike pseudotyped virus. Phytomedicine 79:153333.

Wei ZX, Tang TT and Jiang SP, 2020. The antiviral mechanisms, effects, safety and adverse effects of chloroquine. Eur Rev Med Pharmacol Sci 24(12):7164-72.

Yang J, Zheng L, Yang Z, *et al.*, 2024. 5-FU promotes HBV replication through oxidative stress-induced autophagy dysfunction. Free Radic Biol Med 213:233-47.

Yang M, Huang L, Li X, *et al.*, 2016. Chloroquine inhibits lytic replication of Kaposi's sarcoma-associated herpesvirus by disrupting mTOR and p38-MAPK activation. Antiviral Res 133:223-33.

Ye C, Wan C, Chen J, *et al.*, 2023. Cathelicidin CATH-B1 inhibits Pseudorabies virus infection via direct interaction and TLR4/JNK/IRF3-mediated interferon activation. J Virol 97(7): e00706-23.

Zhao X, Tong W, Song X, *et al.*, 2018. Antiviral effect of Resveratrol in piglets infected with virulent Pseudorabies virus. Viruses 10(9):457.

Zheng HH, Fu PF, Chen HY, *et al.*, 2022. Pseudorabies virus: From pathogenesis to prevention strategies. Viruses 14(8):1638.

Zhou M, Abid M, Cao S, *et al.*, 2022. Progress of research into novel drugs and potential drug targets against porcine Pseudorabies virus. Viruses 14(8):1753.