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# Antiviral effect of amiloride on replication of foot and mouth disease virus in cell culture

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## ABSTRACT

Recently, amiloride was shown to potently suppress Coxsackievirus B3 (CVB3) replication. In the current study, we investigated whether amiloride could also exhibit antiviral activity against foot-and-mouth disease virus (FMDV), which belongs to the same family (*Picornaviridae*) as CVB3. We found that amiloride exerted antiviral activity in a dose-dependent manner against two strains of FMDV in IBRS-2 cells, with slight cytotoxicity at 1000  $\mu$ M. Besides, amiloride did not inhibit the attachment and entry of FMDV in IBRS-2 cells, but prevented early viral replication. These data implied that amiloride could be a promising candidate for further research as a potential antiviral drug against FMDV infection.

## 1. Introduction

Foot-and-mouth disease (FMD) is one of the viral diseases caused by the FMD virus (FMDV), which belongs to the family *Picornaviridae* and genus *Aphthovirus* [1]. It has become a major threat to the global livestock industry owing to its high infectivity and the presence of various FMDV serotypes [2]. Therefore, it is of considerable interest to develop effective measures to prevent and control FMD. Currently, vaccination is the primary method for the prevention of FMD. However, several factors limit the use of FMD vaccines, including slow antibody production, low antibody levels, short duration of antibody persistence, and poor immune responses [3]. The lack of effective control measures underlines the importance of research on new antivirals.

Amiloride is a known drug blocker of the cellular  $\text{Na}^+/\text{H}^+$  exchanger and epithelial  $\text{Na}^+$  channel [4]. Currently, amiloride and its derivatives are used as potassium-sparing diuretics for the treatment of hypertension and prevention of hypokalemia associated with congestive heart failure [5]. In recent years, there has been an increasing interest to explore the antiviral activities of amiloride owing to its low toxicity [6]. Previous studies have demonstrated that amiloride inhibited Coxsackievirus B3 (CVB3) replication in infected HeLa cells by directly affecting viral replication or release [7,8]. Besides, amiloride derivatives, such as 5-(N,N-hexamethylene) amiloride (HMA), have also been noted to inhibit ion channels formed by proteins of human immunodeficiency virus, hepatitis C virus, coronavirus, and dengue viruses [9–12]. It has been reported that amiloride, 5-(N-ethyl-N-

isopropyl) amiloride (EIPA), and benzamil inhibited the replication of human rhinovirus 2 in infected cells and that the antiviral activity was unlikely to be due to the inhibition of cellular  $\text{Na}^+/\text{H}^+$  exchanger or epithelial  $\text{Na}^+$  channel [13]. However, it remains unknown whether amiloride exerts inhibitory effects on FMDV infection. In the present study, we demonstrated the antiviral activity of amiloride against FMDV and investigated the effect of amiloride on different stages of FMDV infection. The results obtained indicated that amiloride might be a potential antiviral agent for reducing FMDV infection.

## 2. Methods

### 2.1. Cells, viruses and reagents

IBRS-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) provided with 100 units/mL of penicillin and 100  $\mu$ g/mL of streptomycin, and supplemented with 10% (v/v) or 2% (v/v) fetal bovine serum (FBS) as the growth medium or maintenance medium, respectively. FMDVs, including two different strains (O/MYA98/BY/2010 and A/GDMM/CHA/2013), were propagated in IBRS-2 cell culture with DMEM supplemented with 2% FBS. Amiloride (Fig. 1) was purchased from MCE (MedChemExpress), and dissolved in DMSO.

### 2.2. Cell viability assay

To investigate the cytotoxicity of amiloride, IBRS-2 cells were

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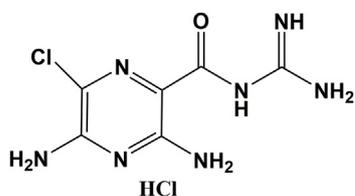


Fig. 1. The chemical structures of amiloride used in this study.

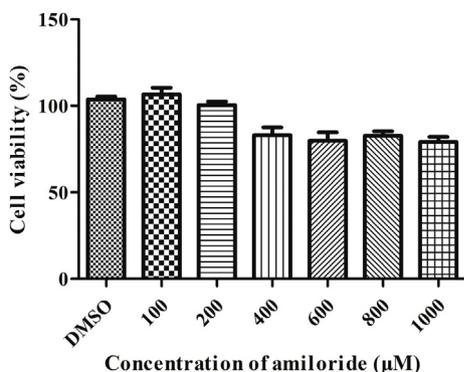


Fig. 2. Cytotoxic effects of amiloride against IBRS-2 cells. IBRS-2 cells were treated with amiloride at concentrations of 100, 200, 400, 600, 800 and 1000 µM. At 72 h after treatment, cell viability was determined using MTS assay. Cells treated with DMSO served as a control. Data are represented as means  $\pm$  SD of three independent experiments. The relative cell viability rate was calculated as (mean OD490 drug)/(mean OD490nm control)  $\times$  100%. Data are expressed as the mean  $\pm$  S.D. of three independent experiments.

seeded into 96-well culture plates and incubated. On the following day, the medium was removed and the 96-well plates were replaced with medium containing serially diluted amiloride ranging from 1000 to 100 µM. The cytotoxicity was determined after 72 h of treatment using MTS assay (Abcam). The absorbance of the formazan product was measured at 490 nm using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

### 2.3. Antiviral assays

The antiviral activity of amiloride was determined by MTS assay based on cytopathic effect (CPE) induced by FMDV infection, as previously described [14]. One day before infection,  $2 \times 10^4$  IBRS-2 cells/well were seeded into a 96-well culture plate and incubated at 37 °C in 5% CO<sub>2</sub> atmosphere. Subsequently, the cells were infected with FMDV O/MYA98/BY/2010 (MOI = 1) at 37 °C for 1 h and the unbound viruses were removed by washing with DMEM. Then, serially diluted amiloride at concentrations ranging from 1000 to 100 µM were added to the confluent IBRS-2 cells, and the culture plate was incubated at 37 °C in 5% CO<sub>2</sub> atmosphere for 24 h until appropriate CPE was

achieved, and the absorbance was determined at 490 nm by using an ELISA reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The antiviral activity was presented as a percentage of control and was determined using the following formula: %Antiviral activity = (OD-testsample-ODviruscontrol)-(ODcellcontrol-ODvirus control)  $\times$  100. Where, (ODtestsample) absorbance measured with a concentration of compound in FMDV infected cells, (ODviruscontrol) absorbance measured for the control untreated FMDV-infected cells, and (ODcellcontrol) absorbance measured for the control untreated and uninfected cells.

The concentration required to reduce virus-induced cytopathogenicity by 50% of the control value (EC<sub>50</sub>) value was calculated using GraphPad Prism5 (GraphPad Software). To evaluate the antiviral efficacy of amiloride, the culture supernatant was collected and the viral mRNA and protein expression levels were analyzed. To test the broad-spectrum antiviral effect of amiloride, the subconfluent cells were infected with another FMDV strain A/GDMM/CHA/2013 and then incubated with the drug as described earlier.

### 2.4. Effect of amiloride on viral attachment and viral entry

To assess the effect of amiloride on attachment of FMDV to IBRS-2 cells, the cells were treated with a mixture of amiloride (400, 600, 800, and 1000 µM) and FMDV O/MYA98/BY/2010 (MOI = 1) at 4 °C for 1 h, which allowed the viruses to bind to the surface of the cells but not enter the cells. As a control, infected cells without amiloride treatment were employed. After removing amiloride and unbound viruses by washing with DMEM, the IBRS-2 cells were subjected to three freeze-thaw cycles for measurement of viral mRNA levels. To evaluate whether amiloride affected entry of FMDV into IBRS-2 cells, the IBRS-2 cells were infected with FMDV O/MYA98/BY/2010 (MOI = 1) at 4 °C for 1 h. After removing the unbound viruses by washing with DMEM, the cells were incubated with amiloride (400, 600, 800, and 1000 µM) at 37 °C for 24 h, and the antiviral efficacy was determined by analyzing the viral mRNA levels. As a control, infected cells without amiloride treatment were employed.

### 2.5. Time-of-addition assay

Time-of-addition (TOA) assay was designed to determine the mechanism of action of amiloride. In brief, amiloride (at a final concentration of 800 µM) was added to the cells at 0 h or after 2, 4, 8, and 16 h of FMDV infection. After incubation for 24 h incubation, Q-PCR was performed using SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Dalian, China) to ascertain the level of FMDV 2B mRNA.

### 2.6. RNA extraction and Q-PCR

The total RNA was extracted using TRIZOL reagent (Invitrogen), in accordance with the manufacturer's instructions. An RT reaction was performed with 1 µg of total RNA using PrimeScript™ RT reagent kit

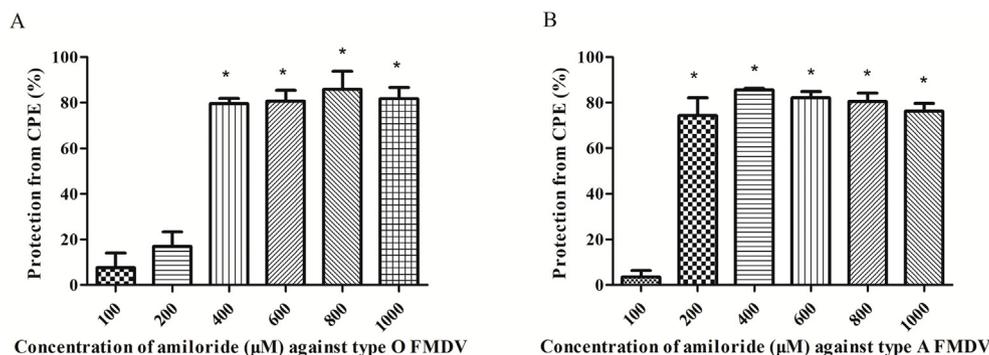
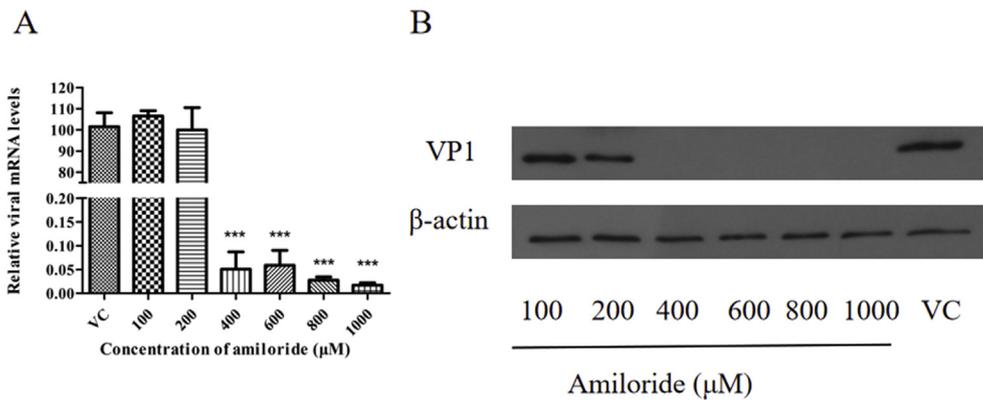
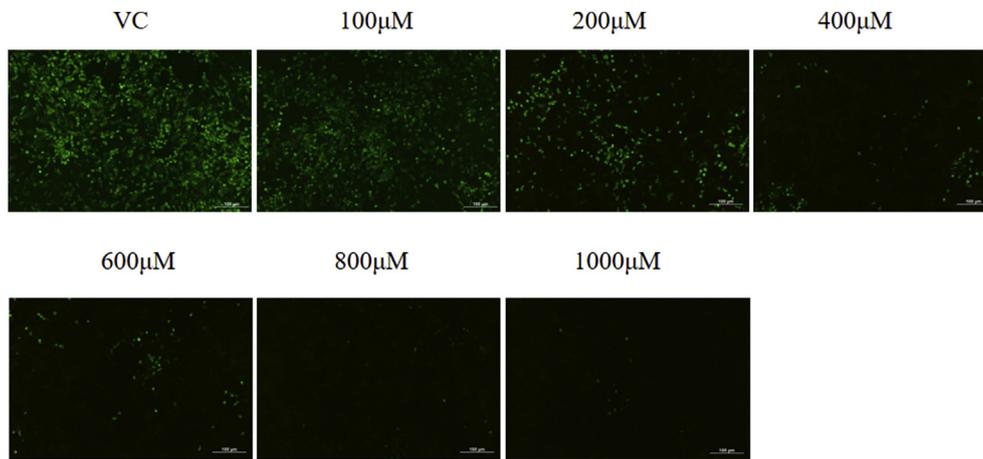


Fig. 3. Inhibition effect of amiloride on FMDV infection. IBRS-2 cells were inoculated with FMDV O/MYA98/BY/2010 (A) and A/GDMM/CHA/2013 (B) at an MOI of 1 and treated with amiloride at concentrations of 100, 200, 400, 600, 800 and 1000 µM. Cells treated with DMSO served as a control. Cell viability was measured at 24 h after inoculation by MTS assay. Data are represented as means  $\pm$  SD of three independent experiments. \* $P$  < 0.05 compared with the control group.



**Fig. 4. The effects on viral mRNA and protein in the cells treated with different concentration of amiloride.** The viral mRNA and VP1 protein levels in IBRS-2 cells treated with different concentrations of amiloride were determined by qPCR (4A) and Western Blot (4B), respectively. “VC” indicates that the cells that were not treated with amiloride. Data are represented as means ± SD of three independent experiments. \*\*\**P* < 0.001 compared with the control group.



**Fig. 5. FMDV replication in IBRS-2 cells determined by IFA.** IBRS-2 cells were inoculated with O/MYA98/BY/2010 at an MOI of 1 and treated with amiloride. At 12h after infection, the FMDV were determined by IFA with hyper-immune serum raised against O/MYA98/BY/2010. “VC” indicates that the cells that were not treated with amiloride.

**Table 1**  
Assessment of cytotoxicity and antiviral activity of amiloride in IBRS-2 cells.

Virus	Amiloride (μM)		
	CC <sub>50</sub> <sup>a</sup>	EC <sub>50</sub> <sup>b</sup>	SI <sup>c</sup>
O/MY98/BY/2010	> 1000	304.0 ± 97.1	3.28
A/GD/MM/2013	> 1000	168.8 ± 98.57	5.92

<sup>a</sup> The 50% cytotoxic concentration (CC<sub>50</sub>) for IBRS-2 cells; mean ± SD of triplicate samples, determined by cell viability assay.

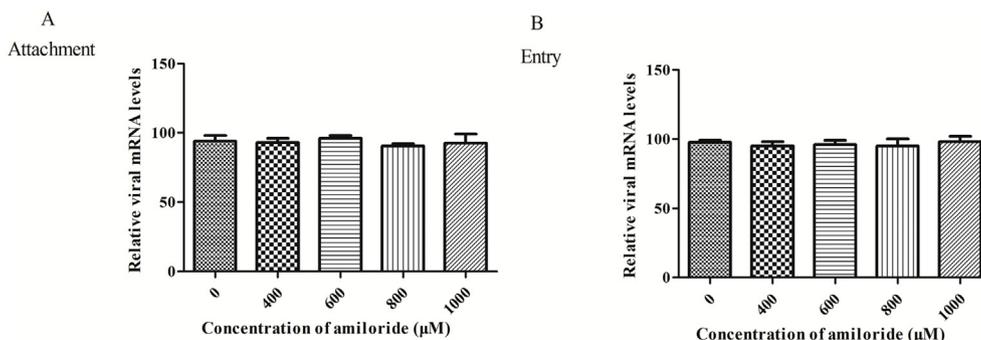
<sup>b</sup> Concentration(s) of amiloride producing 50% inhibition of virus-induced infection of three separate experiments, the result was expressed as the mean ± SD of three separate experiments.

<sup>c</sup> Selectivity index (SI), determined by the ratio of CC<sub>50</sub> to EC<sub>50</sub> (CC<sub>50</sub>/EC<sub>50</sub>).

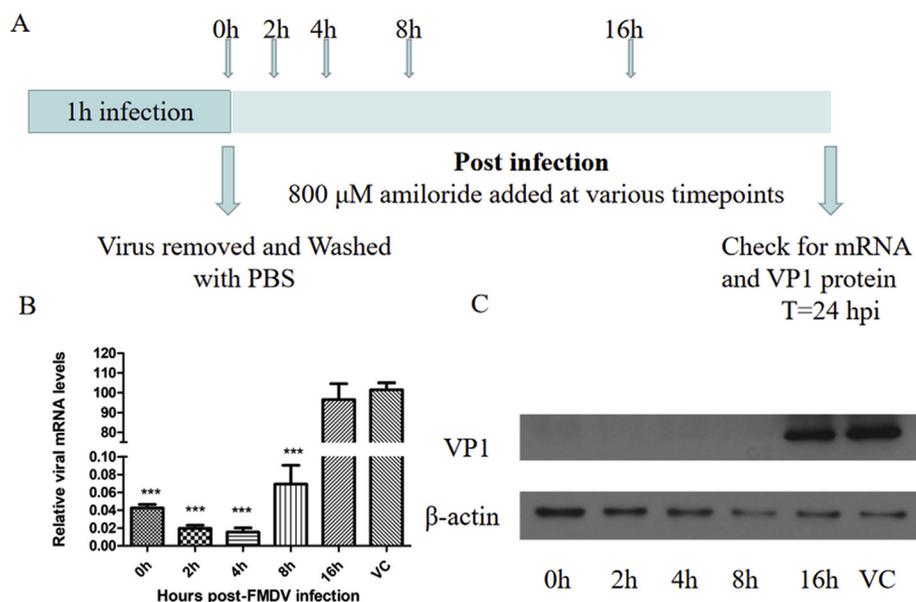
containing gDNA Eraser (Takara, Dalian, China), and real-time PCR was conducted with SYBR Premix Ex Taq™II (Tli RNaseH Plus) (TaKaRa, Dalian, China) in the presence of specific primers including FMDV 2B-for, 5'-CAACAAAACACGGACCCGAC-3'; FMDV 2B-rev, 5'-TTGTACCAGGGTTTGGCCTC-3'; and β-actin for, 5'-GACCACCTTCAACTCGATCA-3'; β-actin-rev, 5'-GTGTTGGCGTAGAGGTCCTT-3' in Agilent Technologies Stratagene Mx3005P instrument (Agilent, USA). The relative mRNA expression levels were calculated by 2<sup>-ΔΔCT</sup> method using β-actin as an internal control for normalization. The mean mRNA level of the mock-treated group was set at 100.

**2.7. Western blot analysis**

The collected cells were lysed with cold Pierce RIPA buffer (Thermo Fisher Scientific) for 15 min, and the cell lysates were further fractionated using bioruptor sonicator (1 min on, 30 s off pulses). Then, the



**Fig. 6. The virus mRNA in IBRS-2 cells treated with different concentrations amiloride at different stages of the viral cycle.** A. Viral mRNA of cells treated with the drug at the viral attachment stage. B. Viral mRNA of cells treated with the drug at the viral entry stage.



**Fig. 7.** Time course of FMDV replication in IBRS-2 cells with amiloride treatment. **A.** Schematic diagram describing the workflow for the time-of-addition studies. IBRS-2 cells were infected with FMDV O/MYA98/BY/2010 at an MOI of 1 for 1 h. 800  $\mu$ M amiloride was then added at different timepoints post-infection in the time-of-addition studies. All supernatants were harvested at 24 hpi. Viral mRNA and VP1 protein were determined at 24 h post infection by qPCR (7B) and Western Blot (7C). “VC” indicates the cells that were not treated with amiloride. Values represent the mean  $\pm$  standard deviation for three independent experiments. \*\*\* $P < 0.001$  compared with the control group.

lysates were centrifuged at  $12,000 \times g$  for 15 min at  $4^\circ\text{C}$ , and the protein concentration was confirmed using BCA assay (Thermo Fisher Scientific). Average amounts of protein were mixed with loading buffer (0.125 M Tris-HCl, pH 6.8, 10% glycerol, 2%  $\beta$ -mercaptoethanol, 2% SDS, and 0.1% bromophenol blue) and boiled for 10 min. After electrophoresis, the proteins were transferred to PVDF membrane using a standard protocol. The membrane was blocked with 5% BSA for 1 h, and the primary antibodies, anti-VP1 polyclonal antibody (gift from Hai-xue Zheng, OIE/National Foot-and-Mouth Disease Reference Laboratory), and  $\beta$ -actin (Abcam), were used to detect bands on the protein blots. Thereafter, the membranes were washed with TBST (TBS containing 0.01% Tween-20) and incubated for 1 h at  $37^\circ\text{C}$  with HRP-conjugated secondary antibodies (Abcam), and the bands were visualized by enhanced chemiluminescence (ECL) kit (Invitrogen) exposed to radiography film.

### 2.8. Immunofluorescence assay

Monolayers of IBRS-2 cells grown in a 12-well plate containing 2% FBS were infected with FMDV O/MYA98/BY/2010 (MOI = 1). After 1 h of adsorption at  $37^\circ\text{C}$ , the supernatant was discarded and replaced with serial dilutions of amiloride ranging from 100 to 1000  $\mu$ M. Then, the cells were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  atmosphere for about 12 h, and immunofluorescence assay (IFA) was conducted as described previously with minor modifications [14]. The cells were fixed with 4% paraformaldehyde for about 15 min and washed with PBS. Then, rabbit hyper-immune serum against type O FMDV (O/MYA98/BY/2010) (kindly provided by Guang-qing Zhou, OIE/National Foot-and-Mouth Disease Reference Laboratory) was added. The mixture was incubated overnight at  $37^\circ\text{C}$  and washed thoroughly with PBS to eliminate unbound antibodies. Afterwards, the cells were treated with goat anti-rabbit IgG (H + L) (ZSGB, Beijing, China) at  $37^\circ\text{C}$  for 1 h, washed with PBS five times, and photographed on an inverted fluorescence microscope.

### 2.9. Statistical analyses

Significance of differences were determined by *t*-test and one-way ANOVA using SPSS software, followed by Tukey's post-hoc multiple comparison test. All data are reported as means  $\pm$  SD ( $n \geq 3$ ), and values with  $p < 0.05$  were considered statistically significant.

## 3. Results and discussion

As a highly infectious publicly and politically high-profile disease, FMD affects millions of cloven-hooved animals and has attracted global concern [15]. A major problem in the control of FMD outbreaks in normally disease-free countries is the delay in onset of immunity following emergency vaccination [16], and antiviral agents have the potential to provide vital protection during this delay.

Amiloride has been reported to exhibit antiviral activity against CVB3 [6,7]. However, to the best of our knowledge, there have been no previous definitive studies on the antiviral activity of amiloride against FMDV, which belongs to the same family of *Picornaviridae* as CVB3. Analysis of the cytotoxicity of amiloride revealed that amiloride had little effect on cell viability. Treatment of 1000  $\mu$ M amiloride induced mild cytotoxicity in the host cells at concentrations (Fig. 2). In addition, at this concentration, no differences in cell morphology were found between amiloride-treated and mock-treated IBRS-2 cells (data not shown). The  $\text{CC}_{50}$  of amiloride estimated by Graphpad software was about 1879  $\mu$ M. To investigate antiviral protection to the cells from CPE caused by FMDV infection, a serial dilution range of amiloride was used from 100 to 1000  $\mu$ M. With the treatment of 400, 600, 800, and 1000  $\mu$ M of amiloride, significant protection for cells from CPE ( $P < 0.01$ ) (Fig. 3A), other concentrations of amiloride were found to not provide effective protection to the cells infected with FMDV O/MYA98/BY/2010 (Fig. 3A). To further confirm the inhibitory effect of amiloride on FMDV replication in IBRS-2 cells, real-time PCR, Western blot analysis, and IFA were performed. The mean relative mRNA load of IBRS-2 cells treated with 100, 200, 400, 600, 800, and 1000  $\mu$ M amiloride was 106.46, 99.96, 0.050, 0.058, 0.027, and 0.017, respectively (mean relative mRNA load of mock-treated cells was set at 100) (Fig. 4A). Consistent with these results, the FMDV VP1 protein was significantly suppressed following treatment with 400, 600, 800, and 1000  $\mu$ M amiloride (Fig. 4B). With regard to IFA, both control and IBRS-2 cells treated with 100 and 200  $\mu$ M amiloride generated strong fluorescent signals at 12 h post-infection, whereas cells treated with 400, 600, 800, and 1000  $\mu$ M amiloride showed reduced fluorescent signals (Fig. 5). Therefore, amiloride represents a novel antiviral molecule against FMDV. Interestingly, amiloride exhibited antiviral activity against different strain of FMDV. The effective dose of amiloride against type A FMDV was from 200  $\mu$ M (Fig. 3B). As shown in Table 1, the  $\text{EC}_{50}$  values for O/MYA98/BY/2010 and A/GD/MM/2013 in IBRS-2 cells were  $304.0 \pm 97.1$  and  $168.8 \pm 98.57$   $\mu$ M, respectively, while the SI values

for A/GD/MM/2013 (3.28) were approximately two-fold higher than those for O/MY98/BY/2010 (5.92), indicating that amiloride could serve as a broad-spectrum drug to treat FMDV infections.

To further investigate the action of amiloride against FMDV, viral attachment and entry assays were performed to determine which stage of viral lifecycle was affected by amiloride treatment. The results of the viral attachment tests revealed that the mean relative mRNA levels in IBRS-2 cells treated with 400, 600, 800, and 1000  $\mu$ M amiloride were 93, 96, 90.5, and 92.5, respectively (with mean relative mRNA level in mock-treated cells set at 100) (Fig. 6A). The findings of the viral entry experiments demonstrated that the mean relative mRNA levels in IBRS-2 cells treated with 400, 600, 800, and 1000  $\mu$ M amiloride were 95, 96, 95, and 98, respectively (with mean relative mRNA level in mock-treated cells set at 100) (Fig. 6B). Thus, no significant differences in the relative levels of viral mRNA were observed between drug-treated and mock-treated cells, indicating that amiloride had no effect on FMDV attachment and entry into IBRS-2 cells.

To identify which stage of viral replication was inhibited by amiloride, a TOA experiment was performed. The IBRS-2 cells were incubated with amiloride at 0 h or 2, 4, 8, and 16 h after FMDV infection, and a Q-PCR and Western blot assay were conducted at 24 h post-infection to determine the relative mRNA levels and VP1 protein in the infected and untreated cells (Fig. 7). The viral mRNA levels were noticeably inhibited by amiloride treatment at 0, 2, 4, and 8 h post-infection. The mean relative mRNA levels of cells treated with amiloride at 0, 2, 4, 8, and 16 h were 0.042, 0.019, 0.015, 0.069, and 96.52, respectively (with mean relative mRNA level of mock-treated cells set at 100) (Fig. 7B). Similarly, the viral VP1 protein expression was also low even after amiloride treatment at 8 h post-infection (Fig. 7C). However, only negligible inhibitory effects were observed after amiloride treatment at 16 h post-infection, indicating that amiloride blocked FMDV replication at the early stages of viral infection. Interestingly, previous studies have revealed that amiloride could inhibit CVB3 genome replication and act as a competitive inhibitor inhibiting the enzymatic activity of CVB3 3Dpol in cell culture, affecting VPgpUpU synthesis and RNA elongation [6,7]. In particular, amiloride could compete with nucleoside triphosphates and  $Mg^{2+}$ , and the molecular mechanism of action of amiloride against FMDV should be examined in detail. It has been reported that amiloride analogs have a different content inhibitory activity against CVB3 3Dpol [17]. Thus, it will be interesting to investigate and compare the efficacy of amiloride analogs such as EIPA, 5-(N,N-dimethyl) amiloride, and HMA against FMDV. Recently, we investigated the protective effect of amiloride in an FMDV-challenged suckling mouse model based on the anti-FMDV replication activity of amiloride in vitro. The findings revealed that 50  $\mu$ g of amiloride showed better protection (33.3%) when compared with DMSO-treated group (8.3%) after 5 days post-infection. Nevertheless, larger studies are required to determine if there is a significant difference in the survival rate.

In conclusion, FMDV infection in IBRS-2 cells was inhibited in a dose-dependent manner by amiloride treatment. These in vitro results show that amiloride could be a promising novel antiviral agent that can be used in the early stages of viral infection before a vaccine-induced immune response is triggered; however, further studies are required to determine the exact mechanism of action of amiloride.

## Competing interests

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2019.103638>.

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