INTRODUCTION

Infectious bronchitis virus (IBV), the causative agent of infectious bronchitis (IB), has been effectively controlled by the extensive use of vaccines but still remains a major economic problem since first reported in 1931. IBV has already caused major health concerns in the poultry industry around the world (Cavanagh, 2003). Like many other coronaviruses, IBV is an enveloped virus with a large, positive stranded RNA genome about 27–32 kilobases long (Lai and Cavanagh, 1997). The IBV virion is built from four structural proteins, including the nucleocapsid (N) protein with which the genomic RNA is packed, the spike (S) protein that forms the prominent coronavirus spikes, the M protein which is the most abundant component of coronavirus, and the envelope (E) protein which is a minor but yet critical component in virion assembly (de Haan et al., 1998). In addition, N protein, combined with the viral RNA with high affinity (Spencer and Hiscox, 2006), is sufficient to control IB (Gelb et al., 2005; Jackwood et al., 2005; Liu et al., 2006; Bochkov et al., 2006). In China, the government prohibits the use of antiviral drugs in food animals, so more attention is being paid to traditional antiviral herbs, and many reports are confirming the potential use of Chinese traditional antiviral herbs in inhibiting the virus (Yamasaki et al., 1993; Chen et al., 2003; Mori et al., 1999; Li et al., 2009).

The fruits of Forsythia suspensa Vahl. (Oleaceae) are widely used in traditional Chinese medicine to treat inflammation, pyrexia or emesis. Forsythoside A is the active ingredient of Forsythia suspensa. Endo et al. (1981) first extracted forsythoside, and Nishibe et al. (1982) successfully obtained forsythoside from weeping forsythia, and found it strongly inhibited pathogenic bacteria such as Staphylococcus aureus. Forsythoside also inhibited syncytial virus and coxsackie virus in vitro (Hu et al., 2001).

Currently, the detection and serotype analysis of IBV is performed by RT-PCR and restriction fragment length polymorphism analysis (Kwon et al., 1993) or by sequencing RT-PCR products of the S1 gene (Kingham et al., 2000). In this study, real-time fluorescence quantitative PCR was used to analyse the IBV N gene. Real-time fluorescence quantitative PCR is sensitive and accurate in the determination of copy numbers of DNA or cDNA (Zhang and Shen, 2003) and was therefore used to investigate the inhibiting effects of forsythoside A.

MATERIALS AND METHODS

Virus and cells. IBV strain M41 (China Institute of Veterinary Drug Control) was adapted and propagated in chicken embryo kidney (CEK) cells. Primary cultures of CEK cells from 19-day-old chick embryo were prepared with standard techniques. The CEK cell monolayers were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37 °C in CO2 incubator.

Cytotoxic assay of forsythoside A. The CEK cells suspension was added to 96-well culture plates

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(Costar/Corning USA) at a density of 0.1 mL per well and cultured to allow the formation of cell monolayers at 37°C in CO₂ incubator. After 48 h, it was replaced with DMEM containing 3% fetal bovine serum (FBS) and culture continued for 24 h. After washing with PBS three times, forsythoside A (Biomedical R&D Center in Liaoning Province) was dissolved in DMEM and the diluted forsythoside A was put in wells (0.2 mL per well) at eight repeats of each concentration. A normal control was set. Morphological changes of cells were observed by light microscope.

**Treatment of CEK cells prior to virus infection.** The CEK cells in 96-well plates or 24-well plates (Costar/Corning USA) were treated for 1 h with serially diluted forsythoside A (0.16 μM, 0.32 μM, 0.64 μM). Eight replicates were used for each concentration. The cells were infected with IBV at a multiplicity of infection of 0.001 for 1 h, and after washing with PBS three times, DMEM was applied. Mock and infected cells were prepared for subsequent experiments. Cytopathic effects (CPE) were observed in the infected cells.

**Treatment of infected cells.** To analyse the effect of forsythoside A on infected cells, the CEK cell monolayers were infected with IBV at a multiplicity of infection of 0.001 at 37°C for 1 h. The cell monolayers were then washed with PBS three times and the infected cells treated with serially diluted forsythoside A.

**Treatment with forsythoside A.** To analyse the effect of forsythoside A, IBV cultures were incubated with serially diluted forsythoside A at 37°C for 1 h. The CEK cell monolayers were infected with IBV at a multiplicity of infection of 0.001 at 37°C for 1 h. After washing with PBS three times, DMEM with 3% FBS was added cultured at 37°C.

**Preparation of total cellular RNA.** Total cellular RNA in the three groups was extracted using a rapid RNA extracting kit (centrifugation column type, Promega), according to the manufacturer’s instructions.

**Determination of IBV N gene expression by real-time reverse transcription PCR.** The RT system included 1 μg extracted RNA (4.8 mL), 0.5 mL Oligo dT primer (1 mg/mL), 1 mL dNTP mixture (10 mM each), 0.2 mL RNase inhibitor (40 U/mL), 2 mL MgCl₂ (25 mM/mL), 0.5 mL reverse transcriptase M-MLV (200 U/mL) and 1 mL 10× RT buffer. The mixture was kept at 42°C for 30 min, 99°C for 5 min and 4°C for 10 min prior to PCR.

According to the published sequence of IBV strain M41 (GenBank EU116941) and avian β-actin (GenBank L08165), the primers p1: 5′-AAACCAGTCCCAATGCT3′; p2: 5′-GAGGAATGAAATCCCAACG-3′; p3: 5′-CCACGTGCTTGCTG3′; and p4: 5′-ACATCTGCTGAAAGGTGAC-3′ were designed. While P1 and P2 were used to amplify the IBV N open reading frame, P3 and P4 amplified the avian β-actin. The PCR system included 10 μL SYBR Green PCR mix; 0.2 μL 10 pmol/μL upstream and downstream primers respectively; 0.2 μL referencing dye; 2 μL cDNA templates, and 7.4 μL water. The PCR was performed at 95°C for 10 min, and 30 cycles of 94°C for 1 min, 60°C for 1 min. The PCR products were separated by 1% agarose gel electrophoresis and detection and amplification of RNA levels measured using Stepone Applied Biosystems equipment. The results were statistically analysed according to the method of Livak and Schmittgen (2001).

**RESULTS AND DISCUSSION**

Currently, IBV causes highly contagious diseases in chickens and is a constant threat to the poultry industry. Vaccination is a key strategy for the prevention and control of IB in poultry, but is not always successful. Antiviral therapy is a possible strategy for treating IBV infection, although there are few effective antiviral drugs available. The purpose of the study was to identify whether forsythoside A can inhibit IBV infection in vitro, and if so, to investigate its mode of action.

**Cell-toxicity of forsythoside A**

The effects of forsythoside A on CEK cell viability and proliferation were determined using CEK cell monolayers. The maximum non-toxic concentrations of forsythoside A diluted in DMEM was found to be greater than 1.28 μM. Previously, it has been shown that forsythoside A has an antiviral effect on the syncytial virus when used at a concentration of 0.624 μM (Hu et al., 2001). In this study, forsythoside A showed a direct anti-IBV effect at a concentration of 0.64 μM, as shown by real-time PCR assay and observation of the cytopathic effects. Although forsythoside A did not inhibit IBV infection after virus absorption, and pretreatment of the cells with forsythoside A resulted in only partial inhibition of IBV infectivity, the sensitivity of cellular factors such as the surface receptors of IBV to the drug remains under investigation. Two approaches were used to analyse virus infection, and both real-time PCR and CPE gave rise to similar results, which confirms the feasibility of these results.

**Forsythoside A treatment of CEK cells prior to infection inhibits IBV production**

When forsythoside A was applied to the infected cells, as shown in Fig. 1a, it did not decrease IBV infectivity at any of the concentrations tested, as determined by CPE. The IBV N gene was detected and it was found that viral loads decreased with increasing concentrations of forsythoside A. The inhibiting effect of forsythoside A on virions was analysed up to concentrations of 0.64 μM. No reduction in IBV-N was observed at 0.16 μM and 0.32 μM samples, but partial inhibition of the infectivity was seen at 0.64 μM, as determined by real-time fluorescence quantitative PCR (Fig. 2). It is speculated that the result may be caused by a decrease in the number of cells.

**Forsythoside A has a direct virucidal effect on IBV**

Partial inhibition of the infectivity was seen at 0.32 μM and complete inhibition at 0.64 μM of forsy-
The antiviral effect of forsythoside A was confirmed by real-time PCR analysis. The trend in reduced viral yield was seen by a reduction in virus titre and IBV N gene expression, compared with untreated IBV infected cells. At a concentration of 0.64 mM, the production of virus progeny was abrogated (Fig. 3).
Forsythoside A treatment does not reduce progeny virus production in IBV-infected cells.

Forsythoside A, when added to the infected cells, did not decrease IBV infectivity after 36 h at any concentration analysed, as determined by observations of CPE (Fig. 1b). The lack of inhibitory effect was confirmed by real-time PCR (data not shown).

These results indicate that forsythoside A inhibits IBV infection in vitro, in a dose-dependent manner. It displayed a direct virucidal effect, but had no effect on IBV-infected cells. However, when a high dose of forsythoside A (0.64 mM) was used to treat CEK cells prior to IBV infection, it was able partially to inhibit the infectivity of IBV. These data suggest the potential use of forsythoside A as an antiviral agent against IBV, although the mechanisms remain unclear. It has effects on cell signalling that could lead to changes in virus replication, so further studies could focus on changes induced by forsythoside A on the signal pathways relevant to IBV infection.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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