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ORIGINAL ARTICLE

In vitro antiviral efficacy of pleconaril and ribavirin on foot-and-mouth disease virus replication

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Abstract Antiviral therapy is a promising strategy to control acute viral infections. FMDV causes an acute infection and the vaccination provides a protective immunity 7 days post immunization. If the infection is uncontained, then it affects the entire herd. In such circumstances, if antiviral drug is administered the infection can be checked in a herd. Ribavirin is known to cure persistently infected BHK21 cells with FMD virus. However, there have been no systematic studies on antiviral activity of ribavirin against FMDV at different time points with the application of ELISA, PCR or real-time PCR. Pleconaril is known to inhibit enteroviruses and rhinoviruses but has not been explored on FMDV. Hence, the present study evaluates the in vitro antiviral efficacy of pleconaril and ribavirin on FMDV replication. The maximum non-toxic concentrations (MNTC) of pleconaril and ribavirin for BHK21 cells respectively were 7.81 µg/50 µL and 15.62 µg/50 µL. Thus, drug concentrations below MNTC were tested for their antiviral activity against serial tenfold diluted FMDV O, A and Asia 1 serotypes. Pleconaril did not inhibit FMDV serotype O replication at 7.5 µg/50 µL based on CPE inhibition assay and this was further confirmed using sandwich ELISA, PCR/real-time PCR. On the other hand, ribavirin at 15.62 µg/50 µL inhibited the in vitro replication of FMDV O, A and Asia 1 and the inhibition was confirmed by serotype specific sandwich ELISA, PCR and real-time PCR assays. The inhibition was directly proportional to the concentration of ribavirin. Therefore, ribavirin could be explored for its in vivo efficacy as a potential therapeutic in the prevention of early spread of FMDV infection in a herd.

Keywords Cytopathic inhibition assay · Foot-and-mouth disease virus · Pleconaril · PCR/real time PCR · Ribavirin · sELISA

Introduction

Foot-and-mouth disease (FMD) is an acute, highly contagious and economically important disease that affects domestic and wild cloven-hoofed animals, such as cattle, swine, sheep, goats, bison, deer, mountain gazelle, elephant, hedge hog, porcupine, giraffe and among 70 species of wild animals [4]. The causative agent, FMD virus (FMDV), belongs to the genus *Aphthovirus* of *Picornaviridae* family [2]. There are seven immunologically distinct serotypes of FMD virus namely O, A, C, Asia 1, SAT1, SAT2 and SAT3 and are distributed globally [20, 21]. There is no cross protection across the serotypes either by natural infection or vaccination. As a result, all the antigenically matched prevalent strains of the virus must be incorporated in the vaccine preparation to attain an effective immunity [18]. Traditionally, FMDV is characterized serologically and multiple subtypes exist under each serotype [22]. Most of the FMD outbreaks in India caused by type O (83–93%) followed by Asia 1 (3–10%) and A (3–6.5%) [14]. FMD virus replicates rapidly and spreads among the infected, susceptible and in-contact animals. The disease has carrier state among buffaloes, cattle, sheep and goat with variable periods [13]. Most of the livestock rearing countries suffer from FMD. Even the FMD free countries like Taiwan and United Kingdom reported the outbreaks [13].

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The key FMD control strategies include test and slaughter, movement restrictions and vaccination [28]. FMD free countries follow either emergency vaccination or stamping out policy, whereas, most of the FMD enzootic countries follow vaccination. The current vaccine is inactivated and it is associated with various draw backs. The most important limitation is the immunity gap, that is, 7 to 10 days is required to attain complete clinical protection following vaccination [11]. Further, it is required carefully to select specific vaccine prior application due to its serotype and strain specificity [28, 29]. Therefore, some of these limitations of existing FMD vaccines can be addressed by several alternate strategies like interferon therapy [30, 38], small RNA interference therapy [3], immunostimulants [7] and antiviral drugs [12]. Alternate control strategy such as the use of broad spectrum antiviral agents may be useful in addressing these vaccine inherited demerits in a rapid and serotype independent manner [12]. There are in vitro/in vivo studies, which indicate that certain antiviral agents are effective against FMDV [23] including Ribavirin [5]. However, the studies demonstrated that the ribavirin could be used to cure persistently infected BHK-21 cell monolayer with foot-and-mouth disease virus. However, no systematic study has been carried out to demonstrate its antiviral activity during different time points post infection with application of advanced diagnostic assays like ELISA, multiplex PCR and real-time PCR. Further, the pleconaril has been a promising candidate against picornaviruses in general and rhinoviruses and enteriviruses in particular [32, 33]. Pleconaril has not been evaluated against FMDV. In view of these facts, the present study was undertaken to evaluate the in vitro antiviral efficacy of synthetic molecules like pleconaril and ribavirin on FMDV replication.

Materials and methods

Cell line, virus and antiviral drugs

Baby hamster kidney (BHK21 clone 13) cell line monolayer at low passage levels maintained at the FMD Research Centre, IVRI, Bengaluru was used. FMDV serotypes namely, FMDV O (O/IND/R2/1975), A (A/IND/40/2000) and Asia 1 (Asia 1/IND/63/1972) maintained in BHK21 cell line at the authors' laboratory were used. Pleconaril and ribavirin were procured commercially (M/s Sigma-Aldrich) and used.

Cytotoxicity testing and determination of maximum nontoxic concentration (MNTC)

Cytotoxicity of pleconaril and ribavirin against BHK-21 fibroblast cells were analyzed based on cellular and morphological changes in a monolayer of cells under light microscope and viable cell count by trypan blue dye exclusion method [37]. In brief, different concentrations of the drugs were prepared in Glasgow minimum essential medium (GMEM). BHK 21 cells (1×10^4 cells/well) were propagated in 96 well cell culture plates in the presence of twofold serially diluted pleconaril (starting from 200 µg/50 µL) and ribavirin (starting from 500 µg/50 µL) by cocultivation method and incubated at 37 °C for 48 h under 5% CO_2 with appropriate cell control. Cells were observed after 24 h interval for visible morphological changes under inverted microscope and the treated wells were compared with the untreated wells. The total viable cell and viable cell (%) was calculated in comparison to cell control (untreated group) using hemocytometer at different time interval. Highest concentration of the drugs evincing no cellular or morphological alterations and viable cell count $\geq$ 50% as assessed by trypan blue staining was considered as its respective MNTC. Concentrations of drugs exhibiting toxicity to cells were not considered and the concentrations below MNTC were employed to study the antiviral activity.

Cytopathic effect (CPE) inhibition assay

Based on the results of cell cytotoxicity assay, the pleconaril at concentrations 7.5, 5 and 2.5 µg/50 µL and, ribavirin at 15, 10 and 5 µg/50 µL were tested for their antiviral activities. Each concentration of the drugs was tested in triplicate with logarithmic dilutions of FMDV O, A and Asia 1 serotypes. For pleconaril treatment, two 96 well cell culture plates, one each for 24 and 48 h were maintained and for ribavirin, separate plates were maintained to harvest the samples at 2, 4, 6, 12, 24 and 48 h intervals. The drug, virus and healthy cell control wells were also set up in each plate to validate the assay. At the end of each incubation period, plates were visualized under light microscope (20x) for the appearance of virus induced CPE, if any, and the virus titers were calculated as median tissue culture infective dose ($TCID_{50}$) [35] for each concentration of drugs at different time periods for both pleconaril and ribavirin. Then the cells were frozen at − 80 °C. After 24 h, the plates were thawed at room temperature and the contents of each well of the plates was harvested and mixed with 350µL of maintenance media and the samples were used for sandwich ELISA, PCR and real time PCR. The antiviral activity of pleconaril and ribavirin was expressed as percentage inhibition (PI) using the formula, Percent Inhibition (PI) = [1 − (Antilog of test titer/Antilog of control titer)] × 100. A drug is considered as biologically effective, when the PI is > 80%, at its MNTC [27]. Fold change values were considered as significant, if it is more than tenfold on account of differences between dilutions.

Detection of FMDV O viral antigen in pleconaril and ribavirin treated timed samples using sandwich ELISA

The contents of each well of timed samples which had shown no visible CPE in cell culture were harvested after a cycle of freeze-thawing at − 80 °C. The samples were further tested for the presence of viral antigen using sandwich ELISA [36]. Each sample was tested in duplicate in 96 well ELISA plate (M/s Nunc, Germany). The samples which gave more OD (at 492 nm) value than the negative cutoff were treated as positive. The titer equivalent of the viral antigen was calculated as 50% end point [35].

Detection of viral nucleic acid in pleconaril and ribavirin treated timed samples using specific primer for FMDV O in PCR

The 24 and 48 h plates for pleconaril; and 6, 12, 24 and 48 h plates for ribavirin samples, which were negative in sandwich ELISA, were further tested by PCR [9]. Briefly, total RNA was extracted from 250 µL of each timed sample using TRIZOL reagent (M/s Invitrogen). From total RNA, FMDV O viral cDNA was prepared using RevertAid First Strand cDNA synthesis kit (M/s Thermo Fischer) using FMDV genus specific reverse primer (NK-61). The cDNA was either used immediately in PCR or stored at − 80 °C for future use in PCR or in real-time PCR. For conventional PCR, cDNA from each sample was amplified using DHP-13 and NK-61 primers (Table 1) using Dream Taq PCR master mix (M/s Thermo Fischer) in a PCR thermocycler (M/s Applied Biosystem). The amplified PCR product was checked in 1.5% agarose gel by visualizing under gel documentation system (M/s Syngene). Known positive and negative controls were included in the test to authenticate the results.

Quantification of FMDV nucleic acid in pleconaril and ribavirin treated timed sample by SYBR green based quantitative-PCR (Q-PCR)

The 24 and 48 h old timed samples treated either with pleconaril or ribavirin and were found negative in PCR, were further subjected to SYBR green based real time PCR for the detection and quantification of FMDV RNA [36]. Real time-PCR was performed using a two-step RT-PCR kit [Maxima SYBR Green/ROX qPCR Master Mix (2x), Cat # K0221, Thermo Scientific; USA] in 7500 ABI real time system (M/s Applied Biosystems). RNA extraction and cDNA synthesis were carried out as described earlier, from a FMD virus infected untreated and drug treated timed samples at different intervals. The real time PCR reaction was set up in a total volume of 20 µL using 10 µL of SYBR Green PCR Master mix, 2 µL of cDNA template, 0.6 µL (10 pmol/µL) each of gene specific forward (DHP-13) and reverse primers (NK-61) and volume was adjusted up to 20 µL with nuclease free water. Real-time PCR was done with initial denaturation at 95 °C for 10 min followed by 40 cycles of amplification with denaturation at 95 °C for 15 s., annealing at 60 °C and extension at 72 °C for 30 s. Specificity of the amplified product was assessed by dissociation curve generated at temperature 55 °C through 95 °C with 1 °C increment. The standard dilution series of FMDV O was used to construct standard curves from which the quantity of viral RNA (as viral RNA $TCID_{50}$ equivalent) of each timed sample treated with pleconaril, ribavirin and untreated virus control cell culture supernatants were estimated. The threshold was adjusted manually by inspecting the amplification plot and *Ct* values were calculated as the cycle at which the amplification curve crosses the threshold line.

Results

Determination of maximum non toxic concentrations (MNTC) of pleconaril and ribavirin in BHK 21 cell culture

Visualization of BHK-21 cell monolayer in 96 well cell culture plates revealed that there were differences in the susceptibility of BHK-21 monolayer cells to test drugs compared to untreated cells. In the wells treated with higher concentrations of the pleconaril and ribavirin, observations included were pronounced cytotoxicity in the form of clumping, disintegration, degeneration, swelling, and floating of cells in the medium and inhibition of cell growth after 48 h post drug treatment. Percentage of cell

Table 1 Sequence of primers and probes used for real-time RT-PCR

Gene	Sequence	Sequences (5′–3′)	Serotype	Fragment length	References
VP1	DHP13	GTGACTGAACTGCTTTACCGCAT	O-1D-517-539 F	249 bps	Giridharan et al. [9]
VP1	DHP9	GACCTGGAGGTYGCGCTTGT	Asia1-1D-219-239F	537 bps	Giridharan et al. [9]
VP1	DHP15	CAACGGGACGARCAAGTACTC	A-1D-390-410F	376 bps	Giridharan et al. [9]
VP1	NK61	GACATGTCCTCCTGCATCTG	FMDV-2B-80-58R		Knowles et al. [22]

viability assessed by trypan blue dye exclusion method after 48 h of drug treatment indicated an inverse linear relationship between cell viability and concentration of drug used (Data not shown).

Trypan blue dye viability analysis of cells from pleconaril treated wells revealed that the percentage of cell viability was 54.73% at 7.81 μg/50 μL after 48 h of drug treatment compared to cell control indicating MNTC of pleconaril for BHK-21 cell was ≤ 7.81 μg/50 μL. Similarly, viable cells in ribavirin treated wells at 15.62 μg/50 μL concentrations was 66.47% at 24 h and 52.59% at 48 h of incubation in comparison to cell control, in which the cells were healthy with 100% confluency, indicating that the MNTC of ribavirin for BHK-21 cell was ≤ 15.62 μg/50 μL. Thus, pleconaril and ribavirin, respectively at ≤ 7.50 μg/50 μL and ≤ 15 μg/50 μL concentrations was selected for assaying their antiviral efficacy against FMDV serotypes O, A and Asia 1.

CPE inhibition assay for pleconaril and ribavirin against FMDV O, A and Asia 1 in BHK21 cell culture system

Three concentrations of pleconaril namely 2.5, 5 and 7.5 μg/50 μL were tested, initially against FMDV O serotype (monolayer passaged virus, MP4, with a titer of 7.80 $\log_{10}TCID_{50}$/mL) at 24 h and 48 h post drug treatment by co-cultivation method. Each concentration of the drug was tested against tenfold serially diluted virus supernatant in triplicate wells. The results showed that characteristic CPE, in the form of rounding, aggregation, detachment and floating of cells from monolayer, was prominent in all the three tested concentrations of the pleconaril treated wells against FMDV serotype O. The FMDV O titers were reduced by a maximum of 1.8-fold (PI = 43%) in 7.5 μg/50 μL pleconaril treatment group at 24 h post incubation (Table 2). However, at 48 h post incubation, there were no change in virus titer (PI = 0%) in pleconaril treated wells compared to untreated virus control at all three concentrations tested. Hence, the pleconaril was not considered for testing against FMDV serotypes A and Asia 1 further.

On the other hand, the ribavirin was tested for its inhibitory effect on FMDV using BHK21 cell culture system at 2, 4, 6, 12, 24 and 48 h following the drug treatment (Table 3). At 2 and 4 h post drug treatment, there was no visible CPE observed in virus control and ribavirin treated wells. At 6 h post drug treatment, there was no visible CPE in the wells treated with ribavirin. However, in virus control group, the CPE was visible at lower dilutions, and the titer of the virus in untreated virus control was 3.3 $\log_{10}$-$TCID_{50}$/mL (Table 3). At 12 h post treatment, titer in the untreated virus control was 5.3 $\log_{10}TCID_{50}$/mL. However, the drug control and cell control wells appeared healthy. There was an appearance of CPE even in drug treated wells at lower dilution of virus, but there was an inhibition of CPE at higher dilutions of virus. The FMDV O titers were reduced by 32 (PI = 96.84%) and 316-fold (PI = 99.68%) at ribavirin concentrations ≤ 10 μg/50 μL and 15 μg/50 μL, respectively. At 24 h post treatment, there was a drastic and dose dependent inhibition of FMDV O virus replication in test groups with higher concentration of ribavirin. FMDV O titers were reduced by 6-(PI = 82%), 100-(PI = 99%) and 1000-folds (PI = 99.9%) with three different increasing concentrations of ribavirin, respectively. At 48 h post treatment, the trend was similar to that of 24 h post treatment. At this time point, FMDV O titers were reduced by 100-fold (PI = 99%), 1000-fold (PI = 99.9%) and 5623-fold (PI = 99.98%) with three different increasing concentrations of ribavirin, respectively.

As there was a significant reduction (> tenfold) in the titer of FMDV O when treated with ribavirin, the study was extended to other FMDV serotypes namely, FMDV A and Asia 1. In case of FMDV serotype A, there was a significant decrease in titer (59-fold, PI = 98.3%) was observed compared to control at 24 h post treatment in 15 μg/50 μL treated wells, which has further decreased to 316-fold (PI = 99.68%) by 48 h. However, in case of 10 μg and 5 μg/50 μL treated wells, no significant reduction (≤ tenfold, PI = 94%) of FMDV A was observed even after 48 h.

In case of FMDV serotype Asia 1, trend was similar to that of A. The titer was reduced by 18-fold (PI = 94.4%) compared to control at 24 h post treatment in 15 μg/50 μL treated wells. At 48 h post treatment, the more pronounced reduction of titer (1778-fold, PI = 99.94%) was observed in 15 μg/50 μL treated wells. However in contrast to serotype A, the titers were reduced from 5.6-fold to 31.6-fold in case of 10 μg/50 μL treated wells.

Confirmation of pleconaril and ribavirin induced inhibition of FMD virus serotype O using sandwich ELISA, PCR and real time PCR

The inhibition of FMDV serotype O replication by pleconaril and ribavirin was also tested and confirmed by using sandwich ELISA for 24 h post treatment timed samples. Based on sandwich ELISA, FMDV serotype O titers against pleconaril were shown to be reduced, though not significantly, by a maximum of 5.6-fold (6.55 vs 7.3 $\log_{10}$ $TCID_{50}$/mL in untreated control) at 24 h post treatment (Table 2).

Since, pleconaril was shown to be ineffective in cell culture and sandwich ELISA, the PCR was performed in order to detect viral nucleic acid taking only the group of samples where pleconaril concentration was 7.5 μg/50 μL at 24 h and 48 h incubation period. In PCR also, at 7.5 μg/50 μL treated samples at 24 h and 48 h of incubation

Table 2 Antiviral activity of different concentrations of pleconaril against FMDV O (in terms of $\log_{10}$ $TCID_{50}$/mL with 95% confidence interval) at different timed samples using (a) cell bio-assay, (b) sandwich ELISA, (c) PCR and (d) SYBR Green based real time PCR

Assays	Time of harvest (h)	Pleconaril concentration (μg/50 μL)			
		2.5 $\log_{10}TCID_{50}$/mL	5	7.5	Virus control
Cell bioassay	24	6.55 (5.61–7.49)	6.55 (5.61–7.49)	6.05 (5.11–6.99)	6.30 (5.07–7.53)
	48	6.80 (6.09–7.51)	6.80 (6.09–7.51)	6.80 (6.09–7.51)	6.80 (5.93–7.67)
Sandwich ELISA	24	6.55 (5.61–7.49)	7.05 (6.11–7.99)	6.80 (5.79–7.81)	7.30 (6.07–8.53)
	48	–	–	–	–
PCR	24	–	–	8.55 (7.61–9.49)	8.30 (7.07–9.53)
	48	–	–	9.47 (8.12–10.82)	9.80 (8.93–10.67)
Real time PCR	24	–	–	9.80 (9.09–10.51)	9.30 (8.07–10.53)
	48	–	–	9.80 (9.09–10.51)	9.80 (8.93–10.67)

Table 3 Antiviral activity of ribavirin against FMDV O (in terms of $\log_{10}TCID_{50}$/mL with 95% confidence interval) at different timed samples using (a) cell bio-assay, (b) sandwich ELISA, (c) PCR and (d) SYBR Green based real time PCR

Assays	Time of harvest (h)	Ribavirin concentration (μg/50 μL)			
		5 $\log_{10}TCID_{50}$/mL	10	15	Virus control
Cell bioassay	6	0	0	0	3.30 (2.07–4.53)
	12	3.80 (3.09–4.51)	3.80 (3.09–4.51)	2.80 (2.09–3.51)	5.30 (4.07–6.53)
	24	6.05 (5.11–6.99)	4.80 (3.79–5.81)	3.80 (3.09–4.51)	6.80 (5.93–7.67)
	48	5.80 (5.09–6.51)	4.80 (4.09–5.51)	4.05 (3.11–4.99)	7.80 (6.07–8.53)
Sandwich ELISA	6	4.80 (4.09–5.51)	4.80 (4.09–5.51)	4.55 (3.61–5.49)	4.80 (3.93–5.67)
	24	7.55 (6.61–8.49)	6.55 (5.61–7.49)	4.80 (4.09–5.51)	7.30 (6.07–8.53)
	48	6.80 (6.09–7.51)	5.80 (5.09–6.51)	5.80 (5.09–6.51)	7.30 (6.07–8.53)
PCR	6	5.80 (5.09–6.51)	5.63 (4.81–6.45)	5.55 (4.61–6.49)	5.80 (4.93–6.67)
	12	7.80 (7.09–8.51)	6.30 (5.14–7.46)	5.80 (5.09–6.51)	7.30 (6.07–8.53)
	24	7.30 (6.18–8.42)	7.55 (6.61–8.49)	6.80 (5.79–7.81)	7.30 (6.07–8.53)
	48	7.30 (6.14–8.46)	7.55 (6.61–8.49)	7.55 (6.61–8.49)	7.30 (6.07–8.53)
Real time PCR	24	7.80 (7.09–8.51)	7.05 (6.11–7.99)	6.80 (5.79–7.81)	7.30 (6.07–8.53)
	48	7.80 (6.79–8.81)	7.55 (6.61–8.49)	6.80 (6.09–7.51)	7.30 (6.07–8.53)

showed no significant reduction in FMDV O titers (2.1-fold, PI = 53.2%) (Table 2). The results of the SYBR green based real time PCR also corroborated with gel based PCR in respect of samples collected after 24 h and 48 h of treatment, in that there has been no significant reduction in FMDV serotype O titer due to pleconaril treatment.

But then, inhibitory effect of ribavirin against the replication of FMDV O was also confirmed by sandwich ELISA, PCR and real-time PCR. In sandwich ELISA (Table 3), the inhibitions were found to be significant (> 316-fold, PI = 99.68) only at 15 μg/50 μL concentrations at 24 h post treatment. However, in case of 48 h, both 10 and 15 μg/50 μL, showed significant reduction in virus titer (> 31.6-fold, PI = 96.84). In case of gel based PCR, the ≥ tenfold inhibition were observed only at 15 μg/50 μL concentrations at 12 h post treatment samples. The findings of the real-time PCR results have shown that though reduction in titer was observed in 15 μg/50 μL, they were less than tenfold when tested at 24 and 48 h post incubation.

Discussion

In enzootic countries like India, mass vaccination of susceptible livestock is the method of choice to build herd immunity and ultimate control and eradication of FMD. However, the conventional FMD vaccine induces complete protection after 7 days post vaccination. This ensues a wide window of susceptibility in unexposed animals and animals exposed to infection may succumb to disease. Following the acute FMDV infection in ruminants, some proportions

of them become carrier to FMDV [13]. To overcome these issues, emergency immunization with a high potency vaccine is practiced. The other possible alternatives would be the use of antiviral agents that inhibit FMDV replication. Therefore, the present study was conceptualized to explore the in vitro antiviral efficacy of pleconaril and ribavirin by their inhibition of CPE in BHK21 cell culture system at different time period against FMD virus serotypes O, A and Asia 1.

Prior the selected drugs were evaluated for their CPE inhibition against FMDV in BHK21 cells, the compounds were screened for their cytotoxicity to BHK21 cells in varying concentrations. This was a prerequisite as the FMDV serotypes used in the study have been adapted in this cell line. Both the chosen compounds were found cytotoxic at higher concentrations characterized by change in cell morphology like rounding, increased brightness, and detachment of the cells from the surface. The observed cytotoxic changes in the cells were directly proportional to the concentration of the drug. However, pleconaril and ribavirin below 7.81 and 15.62 μg/50 μL respectively were non toxic to BHK21 cells as more than 50% of cells found to be healthy after 24 and 48 h of incubation with these drugs at or below these concentrations. Hence, these concentrations of the drugs were chosen for evaluation of their antiviral activity in BHK21 cell system. The MNTC of ribavirin was found to vary depending on the cell type used. In one of the earlier studies, ribavirin at $\geq$ 50 μg/mL showed mild cytostatic effect on Vero E6 cells and inhibited cell growth up to 50% [6]. In contrast, the MNTC of ribavirin for Vero and BHK21 cell lines were 200 [8], and 500 μg/mL [26], respectively, and at this concentration the ribavirin did not induce visible cytotoxicity, though there was a reduced BHK-21 cell proliferation [16, 26, 34]. The differences in MNTC, as reported by earlier workers and this study, might be attributed to intrinsic characteristics of the cell, factors related to the drug or the methodology followed in determining the MNTC. The reported concentrations were estimated by incubating the ribavirin on preformed BHK-21 cell monolayers. At different time period post incubation indicated that the cytotoxic effects were minimal, but has definite effect on cell growth as estimated from viable cell count. The methodology followed here is of co-cultivation, wherein, the cells are allowed to grow in the medium in the presence of drug. This has resulted in MNTC levels estimated were found to be lower than that reported in other studies. This indicates that, MNTC need to be established at individual laboratory level depending on the procedure of growing the BHK-21 cells, so that the antiviral effect of compound may be established without bias.

With regard to pleconaril, the estimated MNTC was 7.81 μg/50 μL, beyond which there was a visible toxicity to cells and therefore, pleconaril was used at the concentrations below this to study its inhibitory effect on FMDV replication. The cytotoxicity of pleconaril in BHK21 cell monolayer at the concentration above MNTC was characterized by rounding, clumping, inability to form monolayer and floating of cells. The calculated cytotoxic concentration of pleconaril on BHK21 cell was comparatively higher than that observed for RD cells (0.075–0.21 μg/50 μL) [40], HeLa, WIS, LLC-MK_{2D} cell line (0.24–0.48 μg/50 μL) [33] indicating the greater tolerance of BHK21 cells for pleconaril compared to other cells.

Having estimated the MNTC for pleconaril and ribavirin, the effect on FMDV virus replication by CPE inhibition assay was carried out using three different concentrations of each drug against tenfold dilutions of FMDV. The visible CPE was expressed in terms of $TCID_{50}$. Though the CPE can be easily observed under microscope, at low virus titers and during initial hours of addition of virus and drug, evidence of CPE was less appreciable. To overcome these issues of underestimating the quantity of virus, the individual wells of 96 well cell culture plates were also subjected to sandwich ELISA, estimation of viral RNA content by multiplex and real-time PCR. Scoring of the individual well as positive and negative for FMDV antigen and RNA template, titer equivalent against different concentrations of drugs were calculated starting from serial dilutions of virus. The percentage inhibition of virus was deduced by comparing the CPE in drug treatment well with that of CPE in virus control well.

At 2 and 4 h post infection, little CPE was observed in ribavirin treated cells against all three drug concentrations tested. CPE, as early as 4 h was difficult to appreciate as one replication cycle of FMDV takes approximately 4–6 h [13]. However at 6 h, the CPE was evident in virus control only, but the titer was less compared to the original titer of the virus used for infection. The absence of virus induced CPE in ribavirin treated cells indicate that the ribavirin can reduce virus replication during early stages of virus replication and continued availability of drug may result in reduced virus secretion in infected animals and thereby can reduce the transmission events. The inhibitory effect of ribavirin could be proved authentically at 12, 24 and 48 h post drug treatment as shown by the high percent inhibition of CPE estimated by cell bio-assay, sandwich ELISA, RT-PCR and real-time PCR. Ribavirin even at a concentration of 10 μg/50 μL was found to inhibit FMDV serotype O, A and Asia 1 to an extent of 99% at 48 h post treatment. Earlier, ribavirin was used to eliminate FMDV from persistently infected cell cultures [5]. The inhibitory concentrations of ribavirin against other viruses are also shown to be in the concentrations tested against FMDV. Ribavirin showed 80% inhibition in the replication of *Takaiuma serogroup of Brazilian orthobunyavirus* at 50 μg/mL

concentration [25]. Inhibitory concentration of ribavirin in the present study was comparatively lower than that observed against mumps virus. Further, ribavirin has poor in vitro and in vivo activity against the Filoviruses like *Ebola* and *Marburg virus* [10] and the *Flaviviruses* (*Dengue, Yellow Fever, Omsk Hemorrhagic Fever, and Kyasanur Forest Disease*). However, search for new generation compounds with selective inhibition on FMDV replication and minimal adverse effect on host continues to surface with objective of treating the FMDV infection in animals. One among them is, 2′-C-Methylcytidine (2-CMC) and has been reported as inhibitor of FMDV in vitro [12]. In severe combined immunodeficient mice [23], the 2-CMC was estimated to be 100 to 140-fold more potent than ribavirin.

Apart from 2-CMC, pleconaril, a small molecule and an inhibitor of picornavirus infection, is evaluated for antiviral activity against various entero and rhinovirus infections [32, 33, 40]. In this study, pleconaril did not inhibit the replication of FMDV serotype O. The mode of action of pleconaril is the integration of the drug into hydrophobic pocket in the VP1 region of human picornaviruses and interferes with attachment of viruses to their cellular receptor and uncoating [31, 39, 41]. Pleconaril is also shown to inhibit several viruses of picornaviridae family like rhinovirus [32] and enterovirus 71 [40]. It could be attributed to the presence/absence of drug binding pockets on the capsid of the virus. In case of rhinovirus and enterovirus, the pleconaril has been shown to integrate with VP1 thereby hindering binding of virus to cellular receptor [31, 39, 41]. However, the FMDV capsid is relatively smooth and does not contain drug binding pocket/canyon/pit as it is present in other picornaviruses [1]. The cellular attachment site of FMDV capsid contains flexible GH-loop [1], which could be the reason behind lack of binding of pleconaril with FMDV and thus shown to be ineffective against FMDV at the concentrations tested. The GH-loop region has shown to be the target of antibody to FMD and hence subjected to immune pressure and resulting in continuous mutations, alteration in the structural surface of FMDV capsid. The ineffectiveness of pleconaril on other picornaviruses has been also been reported [15]. Another possible reason could be the concentration of pleconaril used in this study. Perhaps, higher doses of this compound might be required to inhibit FMDV replication in vitro in BHK21 cells. But in contrast, it has been found that in RD cells the EC_{50} range was found to be less compared to what is used in this study (0.13–0.57 μg/mL) [40].

The infection and inhibition of CPE of FMDV in BHK21 cells was estimated by four methods namely, cell culture, serotype specific sandwich ELISA, multiplex PCR and SYBR Green based real-time PCR. The infectivity titer (in cell culture $TCID_{50}$) or its equivalents ($TCID_{50}$ equivalents in sandwich ELISA, multiplex PCR and SYBR Green based real-time PCR) were estimated to support for their inter dependence.

The ability of FMDV to infect and produce CPE in BHK21 cell has been already demonstrated previously. Therefore, we applied BHK21 cells as host cells for FMDV in vitro. The $TCID_{50}$ assay is a classical method of measuring the titer of a virus, and it appears to be a convenient method for measuring virus titer due to its reliability, simplicity and sensitivity [24]. Our data showed that the titer of FMDV O, A and Asia 1 was 7.80, 6.97 and 7.2 $\log_{10}$ $TCID_{50}$/mL, respectively.

In this study, the virus titer has been shown to be reduced in cell culture at different time interval in case of ribavirin treated test group. However, we did not observe any detectable FMDV inhibition in pleconaril treated group even after 48 h post infection. The cell culture finding was further substantiated by sandwich ELISA, PCR and real time PCR to detect quantity of viral antigen or viral genomic RNA in treated and untreated control groups. The use of real time PCR for expressing and estimating infectivity titer in picornavirus has been proved to correlate with cell bio-assays [17, 19], although literature cites that the immune histochemistry assay and electron microscopy was also applied to detect presence/absence of virus in test samples [40]. ELISA, RT-PCR and real-time PCR results supported the cell culture infectivity assay findings and further reconfirmed virus inhibition at different timed samples.

In summary, we successfully used BHK21 cell model for evaluating anti-FMDV activity of pleconaril and ribavirin. The MNTC, selective index (S.I), percent inhibition (PI), of these drugs could be calculated using BHK21 cell model. We conclude that the BHK21 cell model can be used as a tool for evaluating the antiviral activity of drugs or new compounds against FMDV O, A and Asia 1 in vitro. This may also serve as valuable tool for studying the pathogenesis and mechanism of antiviral activity by analyzing changes in receptors and the distribution of virus. The concentration of ribavirin tested in this study was effective in inhibiting the in vitro replication of FMDV O, A and Asia 1. However, it is required to be tested in in vivo models like sucking mice, Guinea pigs, pigs and cattle. This would be useful in the therapeutic (curative) application of the drug in treating the FMD infected animals and also as a preventive measure to prevent the spread of the infection across the herd.

The present study can be one step forward towards the therapeutic measures taken against FMD virus. Rapid breakage of transmission cycle during the outbreak is an effective way to control FMD in enzootic areas. As the ribavirin acts on FMDV in serotype independent manner, it can be used in the field condition, though a step awaits further evaluation in laboratory and natural host models.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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