ABSTRACT

Feline Infectious Peritonitis (FIP) is a prevalent and devastating viral disease of cats, with no effective therapy currently available. The project aim is to develop an assay to screen for drugs with antiviral efficacy and to isolate the viral polymerase as a potential drug target.

INTRODUCTION

Feline coronaviruses are a subject of much interest due to the intra-host conversion of feline enteric coronavirus (FECV) to pathogenic feline infectious peritonitis virus (FIPV). While the enteric form of FECV is widespread throughout populations of domestic cats worldwide, it is not particularly pathogenic (1). However, genetic mutation of the virus within an individual animal can lead to development of the disease feline infectious peritonitis (FIP). FIPV infection in cats is associated with a >90% mortality, and trace currently is no effective treatment. Identifying drug compounds with antiviral activity against FIP would be an important step forward in managing the disease. This project has two distinct aims:

1. To develop a high throughput, quantitative in vitro assay to screen for compounds with anti-viral efficacy, and
2. To clone and express the viral RNA polymerase (NSP12 gene). This enzyme would be an excellent candidate for drug targeting. The NSP12 sequence is well-conserved, essential to viral replication, and its inhibition is unlikely to lead to host cytotoxicity.

METHODS AND MATERIALS

Viral characterization: FIP virus was obtained from the Pedersen laboratory at UC Davis. Virus was propagated in CRFK cells in vitro and cell-free viral RNA (FIPV) is a +RNA virus was isolated using a QIAamp Viral RNA MiniKit (Qiagen). The RNA was then transcribed to cDNA (Oligene First Strand cDNA Synthesis Kit). Quantification of virus was performed using QRT-PCR (Applied BioSystems), amplifying a small and well-conserved 110 base pair (bp) region of the viral 7b gene (2).

In vitro assay of virus-induced cytopathic effects: CRFK cells were plated on a 96-well plate and allowed to replicate and attach for 24 hrs, such that the cells were 80% confluent. All experiments were performed in quadruplicate. 100 uL of various dilutions of virus were then added to the wells, and the plate was incubated for 24 or 48 hrs. Media was then removed and the remaining cell monolayer methanol-fixed, then stained with crystal violet. Absorbance at 540 and 595 nm was determined with an ELISA plate reader.

Cloning of viral polymerase: A multi-sequence alignment of 15 different complete FECV/FIP viral genomes was performed with Clustal (www.clustal.org). In order to amplify ~3000 bp NSP12 region it was first necessary to sequence the 5’ and 3’ NSP12 regions of our FIPV strain. This was accomplished by searching for highly conserved regions of ~30 bp upstream and downstream of both ends of NSP12, and creating primers for those regions. The amplification products bracketing the start and end of NSP12 were then sequenced (UC Davis sequencing), and the consensus sequences used to construct primers for 5’ and 3’ ends of NSP12. Q5 polymerase (New England Biolabs) was used to amplify NSP12 using these strain-specific primers.

RESULTS

Viral characterization: Real-time PCR for CRFK-passaged FIPV generated a CT-value of 15.8, equivalent to about 4 x 10^9 copies of viral RNA/mL media. Virus-induced cellular synovia were abundant after 24 hours of in vitro infection, along with generalized destruction of the monolayer (Figures 1-3). Cytopathic effects were most evident at 48 hours.

50% Tissue Culture Infective Dose (TCID50): The Reed-Muench equation was used in conjunction with the in vitro results to give an estimate of 1 x 10^6 infective virions/mL. After fixation and staining, absorbance at 540nm showed a biphasic relationship: viral dilutions of 10^4 or less led to complete obliteration of the monolayer, while dilutions greater than 10^6 led to no detectable cytopathic injury (Figure 4).

Cloning of polymerase: Sequencing for 5’ and 3’ regions of NSP resulted in two 200bp regions of strong consensus, with no ambiguous reads in the primer regions. Amplification of NSP12 proved too difficult to perform with Taq polymerase, likely due to secondary pseudoknot structure of viral cDNA associated with a ribosomal frameshifting element (3), but was performed successfully with Q5 polymerase (Figure 5). The amplified NSP12 was digested with Sal I and Eag I to verify the lack of restriction sites within the polymerase (Figure 6), and no additional restriction products were identified.

Amplification of a modified NSP12 with restriction sites and TTT-clamps appended to both ends was again accomplished using Q5 polymerase (Figure 7), and a reaction schematic with two stages of amplification.

DISCUSSION

The crystal violet staining of FIP-infected CRFK cells yielded consistent TCID50 estimates of infective virion concentration, which were reasonable in light of the viral copy number from QRT-PCR. However, this method is not sensitive to small variations in viral concentration, above a certain threshold. This is likely a consequence of the very high in vitro virulence of this strain of FIP, but it complicates identification of potential antivirals, which would need to exhibit very strong effects in order to be identified with this assay.

The cloning of the viral polymerase presents a very promising avenue for drug therapy. The viral polymerase gene is long and complex (~3000 bp), but it has been successfully amplified and isolated and will soon be inserted into a plasmid vector for bacterial expression. Restriction sites have been identified and successfully engineered onto the viral polymerase for plasmid insertion. Viral polymerase production may be induced once the gene is cloned into the plasmid, and this protein will form the basis of future assays.

CONCLUSIONS

The tissue culture assay presents a relatively high-throughput method for assessing drug efficacy against FIP, but the system is relatively insensitive and only capable of distinguishing between large differences in viral concentration. However, it may still be useful to validate antiviral properties.

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REFERENCES