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# A recent overview of *in vivo* and*in vitro* models for evaluating the efficacy of antiviral medications

**[*1]Junaid Tantray, [1]Akhilesh Patel,[3]Syed AhmedHussain,[4]Deepika Deopa, [5]Dimple Pramod,[6]Anjali Ramchandra Singh, [7]Wajihus Shams,[1]Mohseen, [1]Piyush Khandelwal. [1]Parth Vaibhav**

*[1]Dept. of Pharmacology, NIMS Institute of Pharmacy, NIMS University Rajasthan Jaipur, 303121 India junaidtantray22@gmail.com ,*

*[3]Clinical Medicine, AGA Academy Calgary Canada*

*[4]Dept. of Pharmacology, Arya College of Pharmacy, RIICO Industrial Area, Delhi Road, Kukas, Jaipur, Rajasthan 302001 deepikadeopa1@gmail.com*

*[5]Six Sigma Institute of Technology and Sciences Jafarpur – Dineshpur Road, KhanpurPurab, Rudrapur, Uttarakhand*263153*dimplenarkhede46@gmail.com*

*[6]Research Scholar Biochemistry, NIMS Institute of Allied Medical Science and Technology*

*[7]Dept. Of Medical Laboratory Technology, Nims college of Paramedical Technology*

**Corresponding author **Junaid Tantray***

*[1]Dept. of Pharmacology, NIMS Institute of Pharmacy, NIMS University Rajasthan Jaipur, 303121 India*

*junaidtantray22@gmail.com*

*+917006684447*

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**Abstract**

The swift appearance of viral pathogens and the substantial influence of viral infections require the ongoing advancement of antiviral medications. This review article offers a comprehensive analysis of *in vivo* and *in vitro* models employed for assessing the effectiveness of antiviral drugs. The text explores the essential attributes of viruses, their life cycles, and the obstacles encountered in the development of novel antiviral medications. The study emphasizes the intricacies associated with drug discovery, such as the absence of distinct targets, restricted availability of appropriate models, and the high selectivity of antiviral drugs. Additionally, it examines other cell culture systems, including Vero cells, Huh-7 cells, and other cell lines that are used for studying HCV, HBV, and influenza viruses. The significance of evaluating medication toxicity and antiviral effectiveness using cytotoxicity assays and median effective concentration (EC50) data is also highlighted. This comprehensive overview highlights the necessity for sophisticated and novel methods to improve the development of antiviral drugs and addresses the pressing global need for effective antiviral treatments.

**Keywords:** Antiviral drug development, *invivo* models, *In vitro* models, viral pathogenesis, Cytotoxicity assays

## Introduction

There are more than 100 viruses known to cause disease in humans and the need for antiviral drugs is growing rapidly as more and more new viral pathogens are discovered.Infectious diseases have been recognized throughout human history and are caused by various microorganisms, including bacteria, viruses, and fungi [1]. Viruses are relatively simple in structure, consisting of a protein coat, sometimes laminate, nucleic acid, and viral enzymes. In contrast to fungi, helminths, and protozoa the structure is less complex. Viruses are also obligate parasites, which means they cannot survive or reproduce without infecting a competent host cell to replicate their genetic material. These characteristics create problems in making drugs that are at the same time toxic to virus infestation [2]. A virus is an even small communicable agent of humans, animals, plants and other microorganisms. They possess either their genetic code in the form of DNA or RNA. The continual tug-of-war between man and virus is a series of adaptations which must be fought back with countermeasures. Antiviral drugs are developed through a multistep process that includes target identification, lead generation, clinical trials, and drug approval[3]. Given the significant impact of viral infections, which have resulted in millions of fatalities throughout human history, there is an urgent need for the continuous development of antiviral drugs. An era of antiviral medicine development commenced in June 1963 with the approval of idoxuridine, the first antiviral drug. Since then, numerous drugs with antiviral properties have been developed and utilized globally for the treatment of various viral infections affecting millions of individuals [4].

Even with the utilization of new tools and rigorous quality control measures, only a limited number of antiviral drugs receive approval for human use, often due to concerns regarding side effects or the development of drug resistance [5]. However, as understanding of viruses, their mechanisms of infection, and novel antiviral strategies and techniques increases, the pace of development for new antiviral drugs is expected to accelerate. The prevailing global situation highlights the ongoing emergence of microbial threats at an increasing rate, largely attributed to unprecedented climate change and globalization [6].

### 1.2 Viruses and their life cycles (Virology)

Viral infection begins with the introduction of viral genetic material into a host cell, where it undergoes replication before new virus particles are released. The process of viral replication typically involves six key steps: viral attachment, penetration, uncoating, replication, assembly, and release (Fig.1 and Table 1). These steps outline the virus's life cycle, emphasizing its entry into and exit from the host cell [7].

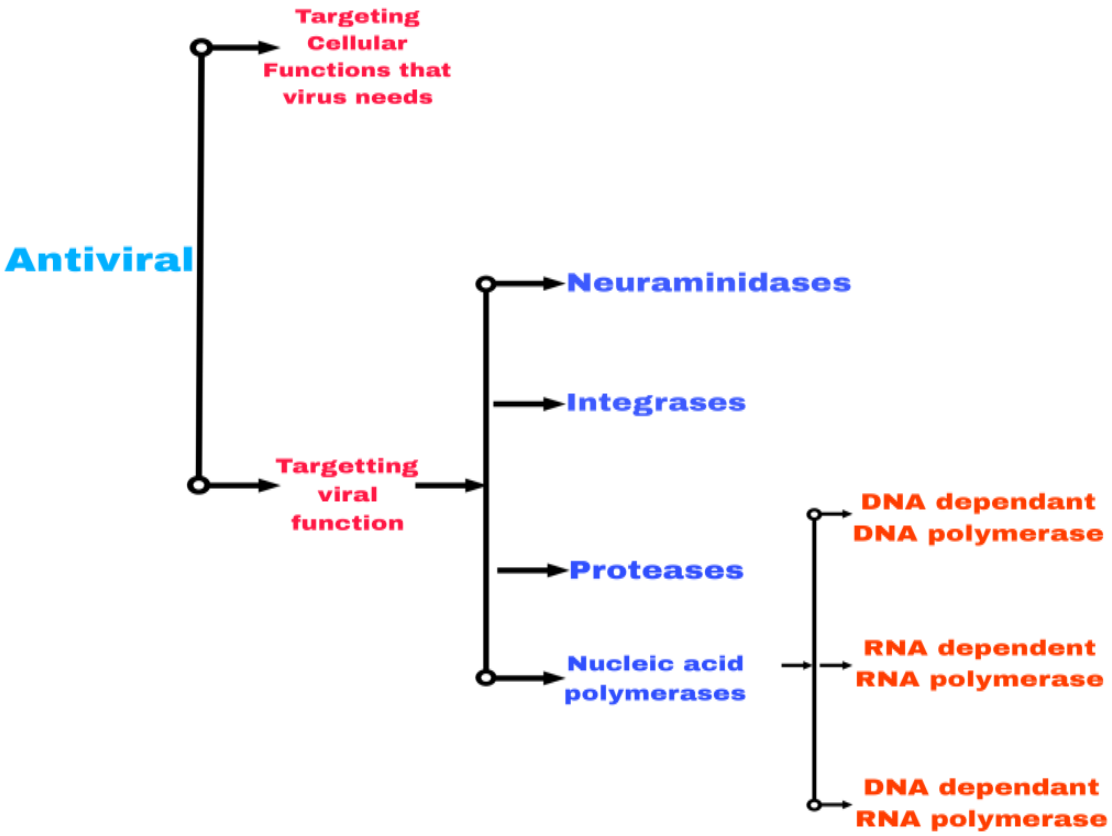


Figure 1. Standard antiviral drug inhibitory effects.

Table 1. COVID-19 therapy antiviral medication mechanism of action

| Group | Drugs | Mechanism of Action |
|---|---|---|
| Group 1 | Remdesivir | Inhibits viral RNA polymerase, disrupting viral replication |
| | Favipiravir | Inhibits viral RNA-dependent RNA polymerase (RdRp) |
| | Sofosbuvir | Inhibits viral RNA polymerase, interfering with viral replication |
| | Tenofovir disoproxil | Inhibits viral RNA-dependent DNA polymerase |
| | EIDD-2801 | Acts as a viral RNA polymerase inhibitor, impeding replication |
| Group 2 | Lopinavir/Ritonavir | Inhibits viral protease, blocking viral replication |
| | Darunavir | Inhibits viral protease, preventing viral replication |
| Group 3 | Interferons | Stimulate host immune response against viral infection |
| | Ribavirin | Inhibits viral RNA synthesis and viral mRNA capping |
| | Monoclonal antibodies | Neutralize the virus, preventing its entry into host cells |

The virus attaches to a host cell and inserts its genetic material during the process of attachment and penetration stage. The host cell then replicates the viral genome as the viral DNA or RNA is incorporated into its genetic material. Within the virus life cycle, this process includes uncoating, replication, and assembly. The host cell extrudes the freshly generated viruses through the cell membrane, ruptures the cell, or undergoes programmed cell death during the release phase [7-8].

**1.3 Challenges in the Development of New Antiviral Drugs**

The latest virus outbreaks, either new or resurgent, have brought attention to how urgently preventive or therapeutic measures are needed. Even while vaccinations are seen as a viable line of defence, their development is complex and fraught with difficulties, particularly when

infections show great genetic variability (like HIV) or unexpected identities (like SARS-CoV-2 or ZIKV). This has led to unprecedented demands for antiviral medications that can be swiftly deployed in clinical settings. The success in treating the hepatitis C virus with direct-acting antivirals, potentially leading to its elimination, highlights the promising role of antiviral drugs in combating viral infections, especially in the absence of readily available vaccines [9].

The research in the field of anti-viral drug development has increased over the past few decades. Besides high costs, the discovery of new antiviral drugs is associated with some challenges and limitations.

1. Lack of unique targets: As stated above, viruses consist of only the genetic material and protein coatand they completely lack their metabolic system. It leads to insufficient viral specific targets for antiviral drugs and hence the possibility of their low efficacy and high toxicity.

Drug discovery procedures start with the molecule and its property that the potential drug attempts to target, which is referred to as the therapeutic target. The selection is based on fundamental principles of biology and virology, with an emphasis on identifying key stages in the development of the virus and the genes and proteins necessary for replication. By focusing on viral components, medications can be developed that have fewer side effects and more efficacy. This is because they are more likely to hinder viral replication without disrupting the normal functions of host cells.The desired target may not be preserved across similar viruses, even within subtypes or genetic variations of the same virus, hence restricting the range of effectiveness of the medicine. This constraint has been apparent in numerous advanced antivirals for HCV [10]. Another concern is the possibility of resistant mutants as a result of the virus's great genetic adaptability, as shown with oseltamivir in treating seasonal H1N1 influenza. This could reduce the effectiveness of the medicine over time [11, 12].

2. Limited availability of *in vitro and in vivo* models: Some of the viruses do not naturally infect animals and cannot replicate in non-human cells. Hence the availability of enabled models and *in vitro* systems is limited making it difficult to investigate newer drugs using multiple models.

HIV research poses significant challenges due to its complexity and resource-intensive nature, requiring substantial manpower, funding, and time. Among the hurdles faced in HIV research are various factors like variability and the absence of suitablemodels that fully replicate theprocess of HIV in humans. To overcome this issue, scientists have resorted to creating and using advanced surrogate models forresearch. Although no animal model precisely replicates HIV infections in people, a variety of models can mutually enhance each other. Researchers can expand their understanding of HIV pathogenesis and advance potential therapeutic efforts by incorporating insights learned from each model and carefully matching findings with data from human clinical trials [13].

3. High specificity of antiviral drugs: Most of the compounds can target only a single infectious agent and their use for more than one viral infection remains limited [14].

The utilization of small-animal models represents a crucial step in evaluating drug efficacy. Nevertheless, variations in genetic makeup and the way genes are activated in different species can create difficulties when analyzing data, especially when comparing the biology of genetically diverse humans with genetically uniform animal models. Although there are many difficulties involved, extensive and compact modelsare still important for the preparation of therapies. These models allow us to evaluate how organisms respond to infection [14, 15]. Laboratory mice are used preferentially and considered a priority over guinea pigs, and other animal models but are also frequently used, especially for researching viral pathogenesis and transmission or meeting FDA requirements for testing in two different animal species. It is crucial that it accurately reproduces high levels of virus and illness characteristics reported in

people. Nevertheless, certain viruses do not possess the inherent ability to reproduce well or invade laboratory animals, thus requiring the creation of innovative models. When it comes to mice,and inbred populations like the Collaborative Cross are used. This allows for the inclusion of a wide range of genetic variations from Mus musculus, which can result in the development of severe diseases that resemble human disorders [15]. Specific viral receptor thatexpresses the human receptor can be created using viral transduction or genetic alteration using CRISPR-Cas9 technology [16]. Knock-in models have the benefit and ensure the correct expression patterns of host genes. In contrast, transgenic mice may have receptor expression in unrelated tissues, which can make data interpretation more difficult[17].

***IN VITRO MODELS***

*In vitro,*models are generally used before*in vivo* testing for the screening of potential compounds in terms of their cytotoxicity and antiviral efficacy. Usage of *In vitro*models is less time-consuming and more cost-effective compared to the *in vivo* models. Moreover, for some of the viral infections *in vivo* models are not available or require a particular animal species, for example, chimpanzees or other primates, and could be limited by ethical issues. Most of the *In vitro*antiviral drug studies are based on virus-induced cytopathic effects and include the detection of median cytotoxic concentration ($CC_{50}$), median effective concentration ($EC_{50}$) And selectivity index (SI).The selection of the type of cells for viral propagation and experimental drug treatment depends on the virus itself. Some viruses, for example, HSV, and influenza viruses can replicate in different cell lines while for others such as HVB and HVC, replication in cell linesis very slow and variable and requires the development of culture systems [18].

**Replicon Cell Culture Model for HCV**

In the last 10 years, the search for new treatments for HCV infection has encountered difficulties because the virus cannot be grown in tissue culture and there are no good small animal models available. Nevertheless, the situation has been enhanced by recent breakthroughs through the implementation of a selective HCV replicon cell culture system. This advancement enables drug development researchers to analyze extensive compound collections utilizing the replicon cell culture technique, facilitating the detection of tiny compounds that can hinder HCV RNA replication (as shown in Fig.2). Furthermore, the replicon system has played a crucial role in understanding the antiviral mechanism of action of inhibitors and enhancing their effectiveness against HCV [19]. The creation of the HCV replicon cell culture model has represented a substantial advancement in HCV investigation and the identification of antiviral treatments in recent years. This model facilitates the reproduction of HCV RNA at significant levels in human hepatoma cells, enabling the assessment of antiviral drugs that target the machinery responsible for HCV RNA replication. As a result, therapeutic candidates with potential can be carefully evaluated and improved before moving on to clinical development, especially as there is no reliable small animal model for HCV [20]. While the replicon cell culture technique has proven to be very valuable for structure determination, it lacks many of the stages of the virus life cycle (cell entry, uncoating, RNA packaging, veron assembly, or release). Such stages represent possible ANV targets. The development of a real cell culture system to maintain the life cycle of HCV will be necessary for advanced study and control of HCV propagation beyond RNA replication. Accordingly, future research should focus on establishing an *in vitro* cell culture model that allows efficient replication of all stages of the HCV life cycle[19].

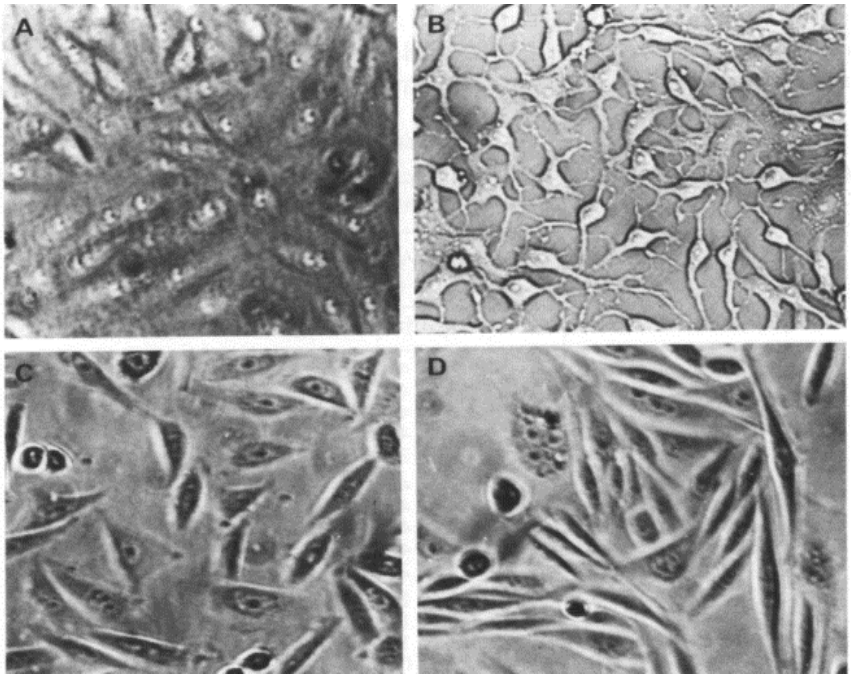


Fig.2 African green-monkey kidney (Vero) cells subjected to the same incubation conditions [28].

**Cell Culture Systems**

***Vero cells***

Vero cells are a continuous cell line originally isolated from kidney epithelial cells of African green monkey (Ceropithecusaethipus) by Yasumura and Kawakita [21]. Vero cells are very easily maintained and are extensively used in virology studies as host cells for viral propagation.They are also used for the screening of cytotoxic and therapeutic effects of drugs. One of the most important characteristics of Vero cells making them extremely attractive for virology and screening of antiviral compounds is their inability to produce interferon [22-23]. However, Vero cells do have interferon-alpha/beta receptors and can respond to interferon treatment.Itsupportsthe replication of rabies virus, reovirus, Japanese encephalitis virus, dengue fever virus, and influenza A&B viruses [24-25].

Multiple strains of Vero cells, including Vero, Vero 76, Vero E6, and Vero F6, are accessible. However, it is important to note that all of these strains originate from the same source and adhere to the same procedure [26]. Vero cells are cultivated in Dulbecco's Modification of Eagle Medium (DMEM) which is enriched with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Cells proliferate on conventional culture plates and should be placed in an incubator at 37°C with a 5% concentration of carbon dioxide. Vero cells are produced from normal kidney cells and hence retain their contact inhibition. View cell culture exhibits exponential growth, with the population doubling nearly every 24 hours. However, as the cells reach confluence, their growth ceases and they undergo cell death. To prevent this and maintain cells in a single layer, it is necessary to pass the cells 2-3 times/week, depending on the flask size and number of cells seeded[27].

***Huh-7 Cells***

The culture system employed for HCV is the Huh-7 human hepatoma cell line. It is a highly specialized cancer cell line produced from hepatocytes, which are liver cells. It was initially acquired from a liver tumor in a 57-year-old Japanese guy. It also includes Huh-7.5, Huh-7.5.1, and Huh-7-Lunet, which exhibit enhanced viral replication and generation in comparison to the original cell line[29].While Huh-7 cells support a high level of HCV replication and virus production, they are not normal hepatocytesand have lost some essential

properties and characteristics of normal cells. Thus, there are some limitations in the assessment of the antiviral effects of the investigational drugs such as their effect on immune response, expression of certain markers etc. [30]. However, Huh-7 cells and their subclones are still considered the best available *in vitro*model for HCV.

***Other cell culture systems used for HCV***

Recently, other non-Huh-7 cell lines (Huh-6, HepG2, IMY-N9, LH86 etc.) and primary human, mouse and chimpanzee hepatocytes have shown the ability to maintain HCV replication. However, viral replication in these culture systems was found to be leastin comparison to that in Huh-7 cells [30].

**Huh-6**

It is derived from hepatoblastoma and is characterized by low production of Claudine-1, an integral membrane protein responsible for the formation of tight junction strands between cells. Initially, Huh-6 cells were not susceptible to HCV but the addition of ectopic claudin 1 made them support HCV replicon. It is also highly resistant to interferon ¥ treatment making them potentially attractive for antiviral drug screening [31].

***Cell Culture systems used for HBV***

The HBVcan be replicated by primary hepatocytes. Nevertheless, this cell culture paradigm is limited by insufficient viral replication, low viral output, and inadequate reproducibility.

**HepG2**

Itis derived from the well-differentiated hepatocellular carcinoma of a 15-year-oldAmerican male. Since HepG2 cells are well differentiated secrete a large range of plasma proteins, such as albumin, transferring, and the acute phase proteins like fibrinogen, alpha1- antitrypsin, transferrin, plasminogen etc.They have been grown successfully in different cultivation systems and will support the replication of HBV and HCV replicon [32].HepG2 Cells especially2.2.15-line better support replication of HBV and are widely used to evaluate potential active anti-hepatitis B virus compounds [33-34].The other cell culture systems used for HBV include HepAD38, HepAD79, YMDD, and PDH.

***Cell Culture System Used for Influenza Viruses***

Influenza viruses A and B are not as in demand asHCV and HBV and may be cultivated in many cell culture systems including Vero, mink lung, and human respiratory epithelial cells [35].

**Determination of the toxicity of the Drug**

Assessment of the toxicity of the antiviral drug is based on Detection of median cytotoxic concentration ($CC_{50}$). It is the concentration of the drug required to reduce the cell number by 50% compared to untreated control. It could be identified using the number of cell viability tests Fig. 2.

There are several tests available to study about cytotoxicity of the investigational compound. The assays rely on several cellular activities, including cell membrane permeability, ATP generation, nucleotide absorption activity etc. Among the other tests enzyme-based calorimetric tetrazolium salts tests are the most commonly used because of their reproducibility,safety and easy methodology.

***MIT assay***

The reduction of tetrazolium dyes such as 3-4, 5- dimethyl thiazole -2-yl s 25 diphenyl tetrazolium bromide in bracket MIT by NAD (P) H-dependent oxidoreductase leads to the appearance of formazan products that have intense blue or purple colour [36]. Oxidoreductase is largely present in the mitochondria and cytoplasm of actively metabolizing cells. Therefore, non-viable or dead cells have low levels of oxidoreductase and lose their ability to convert MTT into forms and to be dissolved in DMSO or any other solubilizer. The absorbance of the dye is measured spectrophotometrically at a certain wavelength usually (570 nm) using an automated microplate reader. The rate of absorbance is found to be 100%

of that of untreated control cells. The percentage of viable cells in the treated group is as follows:

| | |
|---|---|
| **% Cell viability** | **Absorbance of treated cell** / **The absorbance of untreated cell×100** **X 100** |

***MTS, XTT and WST Assays***

Thetetrazolium reagents used in the XTT (2,3-BIS-(2-methoxy-4-nitro-5-sulfophenyl)2H-Terazolium -5-carboxanilide) and Water-soluble Tetrazolium Salts tests can dissolve in water and generate formazan products when exposed to living cells. This improves the assay procedure because a second addition of a solubilizing reagent is not required. Measurement of absorbance is usually done at 490nm.

**Determination of Antiviral Activity of the Drug**

The median effective concentration (EC 50) is the amount of the medication needed to stop virus multiplication by 50% and it represents the antiviral activity of the medication. Fig. 2. Reduction, cytopathic, inhibition, binding and diffusion, and other assays are among the numerous ways to evaluate antiviral activity. Based on virus-induced cytopathic effects, CPE, plaque formation, and hemagglutination are some of the most often utilized antiviral tests. These techniques' time- and labor-intensive natures restrict their use in screening. Furthermore, these methods are less acceptable because different virus strains may differ in their ability to cause cytopathic effects or hemagglutination.

In contrast to cytopathic effect-based viral essay, tests of polymerase chain reaction (PCR) on quantitative real-time PCR (q RT-PCR) are characterized by much greater sensitivity, specificity and reproducibility. They are currently used for the detection of viral DNA or RNA and measurement of viral load (q RT-PCR).

***PCR***

The basis of PCR is the amplification, over multiple cycles, of a single copy of a specific DNA sequence, ultimately producing thousands of copies of the primary DNA sequence.Heat-stable DNA polymerase, such as Taq polymerase; has an amplifier assembling new DNA strands from a single-stranded DNA sample (DNA target). DNA oligonucleotides that are complementary to the particular DNA template are used for the initiation of DNA synthesis. PCR consists of a series of repeated temperature cycles. Each of them usually includes a few steps with different temperatures set up as required for the initiation and synthesis of new DNA copies. The number, level and time of temperature step depend on a wide range of parameters, such as the type of DNA polymerase, the concentration of ions, deoxyribonucleotides, etc.PCR is a sensitive, specific and reproducible method however it does not allow quantitation of DNA or RNA and detection of viral load that is essential for determining the efficacy of antiviral drugs [37].

***Quantitative Real-Time PCR***

qRT-PCR distinguishes itself from regular PCR by enabling the measurement of DNA or RNA levels produced after each cycle using fluorescently labelled oligonucleotide probes. An augmentation in DNA or other resulting substances during PCR, consequently, results in an augmentation in fluorescence intensity, enabling the quantification of DNA concentrations. In real-time PCR, each sample is given a distinct value known as the cycle threshold (Ct). This value is the point or cycle number at which the Florence curb, for instance, surpasses the background fluorescence, indicating that the observations are now significant. Samples with the greatest initial target quantity also exhibit the highest values of the amplified target at a given PCR cycle number. The values obtained from q RT-PCR do not have absolute units associated with them but express the amount of measured DNA or RNA in the sample as a fraction of the standard. The relative DNA or RNA level of each target nucleic acid is expressed as a change inthe number offoods relative to the value of the corresponding

control. Thereby q RT-PCR allows quantitation of nucleic acid and detection of viral load to identify $EC_{50}$ [38-39].

***IN VIVO MODELS***

*In vivo* studies are an essential part of antiviral drug discovery. Different parameters may be considered forthe effectiveness of antiviral drugs in animal models. The choice of these parameters depends on the type of viral infection and animal model used however, the most commonly used parameters include viral infection-associated death, mean time to death, change in water and food intake, change in height, hyper and hypothermia, blood cell count, the population parameters, biochemical parameters, viral titer and histopathological changes in the relevant organs and tissues etc.Unfortunately,*in vivo* models available for antiviral drug development studies are very limited Table 2. This can be explained by a few reasons (i) some viruses cannot replicate in non-human species are require adaptation by multiple passes through the animal model, (ii) high cost and ethical issues associated with particular animal models. All animal models used in the *in vivo* studies could be divided into three groups

1. Chimpanzees or other great apes
2. Non-human primates
3. Other animal models.

Table 2: Common animal models for various viral infections

| **Viral Infection** | **Animal Model** | **Reference** |
|---|---|---|
| Influenza | Mice, Ferrets, Guinea Pigs | Belser, J. A., &Tumpey, T. M. (2013) [40] |
| Human Immunodeficiency Virus (HIV) | Humanized Mice | Marsden, M. D., & Zack, J. A. (2017) [41] |
| Hepatitis B Virus (HBV) | Chimpanzees (historically), Transgenic Mice | Lucifora, J., &Protzer, U. (2016) [42]. |
| Hepatitis C Virus (HCV) | Chimpanzees (historically), Humanized Mice | Dorner, M., Horwitz, J. A., Robbins, J. B., &Barry, W. T. (2011) 00[43]. |
| Herpes Simplex Virus (HSV) | Mice, Rabbits, Guinea Pigs | Szpara, M. L., & Enquist, L. W. (2013) [44]. |
| Zika Virus | Mice, Non-Human Primates | Dowall, S. D. et al [45]. |

**Chimpanzees** (***Pan troglodytes***)

Chimpanzees are the closest similar to humans sharing about 98% genetic identity [46]. This explains the greatest suitability of chimpanzees compared to all other great apes as an animal model for vaccine development and antiviral drug discovery.Chimpanzees are naturally susceptible to most of the human viral photo genes and have the same pathogenic mechanisms, clinical symptoms and course of illness as humans. In addition,the large size of animals provides enough volume of biological materials and facilitates appropriate investigations. Chimpanzees are used as a model to study pathogenesis, vaccination and pharmacokinetic and pharmacodynamics of new candidates for the treatment of some viral infections such as norovirus [47], hepatitis B [48-49] and hepatitis C [50].However, the use

of chimpanzees as an animal model is rarely considered due to low numbers and ethical issues.

**Other Non-Human Primate (NHP) Models**

The genetic proximity of NHP to humans makes them a very attractive animal model for antiviral drug discovery. NHP such as *Cynomolgus macaque*s, *Rhesus macaques*, *green monkeys*, *Chacma baboons*, *Cottontop tamarins* and marmosets are the most commonly used models for the development of antiviral drugs and vaccines compared to chimpanzees. For example, different species are used as models for West Nile virus fever, Japanese encephalitis viral infection, FN influenza H5N1, cytomegalovirus CMV infection, dengue, Ebola, Marburg and some other virus infections [51-52].

**Tree Shrews (TupaiaBelangeri)**

The three shapes are squirrel-like mammals belonging to the primate order and sharing with them some similarities in anatomy, including brain anatomy and phylogenesis. Apparently,due to their proximity to primates, they are one of the main models used for the studies of various aspects of hepatitis B&C infection including the investigation of efficacy of new antiviral drugs [33].Many studies have demonstrated that both HBV and HCV enter and replicate in tree shrew primary hepatocyte-infected animals and develop hepatitis [53-54].Closeness to primates, small size and uncommonness make tree shrews currently a useful model for HBV and HCV research.

**Other Animal Models**

***Mice***

Mice and other rodents have traditionally been used as lab models because of many advantages such as low cost and most importantly the size which significantly reduces the amount of drugs that are needed in experiments. Another important advantage of these models is the availability of a large range of reagents, quantification assays, microarray proteome expression Luminextechnology-based quantification assays etc. Required for evaluation of disease progression, and immune response, is pathological change and viral titer.Mice are mostly used for influenza virus and HSV studies [55-56].However, most of the wild type of human viruses do not replicate and transmit efficiently and or do not produce typical clinical signs of viral infections in normal immunocompetent murine lines, requiring to be adopted by the serial passage in suckling, aged or immunocompromised mice to show clinical features and high level of viral titer [57]. Hence, models currently used for antiviral drug screening studies are mainly transgenic and humanized mice.

***Transgenic Mice***

Transgenic Mice are genetically modified mice with altered genomes. Currently, several thousand strains of transgenic mice are available. Usually, they are named for the gene which has been disrupted. Genetic engineering technology allows creating the streams of mice with different properties and increases their sensitivity to human viruses.

**Knockout mice**

These are genetically modified mice where the activity of a single gene is removed or disruptive currently a large range of knockout mice strains is available for research including pathology, immunology of viral infections and antiviral drug development.

Knockout AG 129 mice do not possess the genes for interferon-alpha beta and gamma receptors, making them more vulnerable to viruses in comparison to wild-type mice. This model is used for dengue virus infection and for conducting *in vivo* testing of certain antiviral drugs [58]. I studied KK epsilon mutant mice and observed the absence of native Kinase activity in the lung, spleen, and embryonic connective tissue fibroblasts. These mice are more likely to get infected by viruses because their interferon (IFN) signaling is faulty. They are utilized as models for studying influenza virus infection in living organisms. UPA mice possess the gene for mouse urokinase-type plasminogen activator (uPA). Excessive

expression of the uPA gene in the liver causes severe liver damage, resulting in long-term hepatic insufficiency. In addition, it induces elevated plasma uPA levels and hypofibrinogenemia, potentially leading to severe haemorrhaging.

***Genetically Humanized Mice***

Genetically humanized mice are the mice that express human host factors and usually have inactivation of the production of murine proteins in parallel. MUP-uPA/scid/Bg transgenic mice are constructed by the backcross of two murine lines. Severe combined immunodeficiency mice subclone (SCID/Bg) MUP-uPA. This line expresses the secreted form of human uPA and is used as a model of HCV infection [59].

***Xenotransplantation Mice***

Transplantation of human cells or tissues to mice may be very helpful in terms of increasing replication of particular viruses and production of clinical science of the infection. Immunodeficient mice such as hello SCID NDL subclones are usually used to produce xenotransplantation mice. SCID mice have genetically repaired both the humoral and cellular immunity and thus can sustain xenografts.

Human liver-uPA/SCID mice are produced by the cross-breeding of uPA and SCID mice followed by intrasplenic injection of human hepatocytes. Transplantation of donor cells should be done within the second week of life. Donor cells injected into the spleen quickly move and enter the liver, where they promote a greater amount of HCV replication compared to mouse liver cells [60]. Hu-PBL-SCID mice are created by injecting peripheral blood lymphocytes into SCID mice. These mice are commonly employed in studies on HIV pathogenesis and the evaluation of new HIV medicines. They were initially utilized as a model for studying HIV in 1995[61].

***Rats***

Rights are not commonly used as *in vivo* models for antiviral drug research however, some of them such as cotton rats are used for the study of influenza A viruses [62].

***Guinea Pigs (Cavia Porcellus)***

These are employed as a substitute for mice in virus research. Guinea pigs are vulnerable to various viruses and are utilized as animal models for studying human viral hemorrhagic fevers, human influenza viruses, HSV, and filovirus infections. Ebola, Marburg, and other viral diseases [51-63]. However, limitations are there due to the lack of available reagents and specific essays (ELISA, Western blot, PCR, q RT- PCR etc.) to monitor host responses, including immune responses, pathological change and viral load.

***Syrian GoldenHamster (MesocrietusAuratus)***

These models are widely usedfor human infectious diseases caused by bunyaviruses (Crimean-congo hemorrhagic fever virus), flaviviruses, henipaviruses, and SARS-coronavirus [64-65-66].

***Ferrets (Mustela Putorius Furo)***

Ferrets are highly popular and widely used animal models for several viruses, including, morbillivirus, influenza viruses, and others [67]. Ferrets and humans have comparable lung physiology, and both human and avian influenza viruses demonstrate similar methods of contact, including cellular receptors found in the respiratory system [67]. Moreover, ferrets are very suitable for antiviral drug research because of their relatively small size and ability to exhibit many of the typical clinical science associated with human disease, especially about influenza virus suggesting nasal discharge, congested eyes, and fever [68].

**Conclusion**

The evaluation of antiviral medications requires a comprehensive understanding of viruses, their life cycles, and the challenges associated with developing new antiviral drugs. Both *in vivo* and*in vitro* models play crucial roles in assessing the efficacy of these medications. *In vitro* models, such as Huh-7 cells and other cell culture systems, are commonly used to study

the toxicity and antiviral activity of drugs. Techniques like PCR and quantitative real-time PCR aid in assessing viral load and drug efficacy in these models.On the other hand, *in vivo* models provide insights into the effectiveness of antiviral drugs in living organisms. Chimpanzees, tree shrews, genetically humanized mice, ferrets, and guinea pigs are among the animal models utilized for this purpose. Each model offers unique advantages and limitations.The combined use of *in vivo* and*in vitro* models enables a comprehensive evaluation of antiviral medications, aiding in the development of effective treatments for viral infections. Overall, researchers must carefully consider the strengths and limitations of each model to draw an accurate modelof drug efficacy and safety.

**Abbreviations**

**SCID - Severe Combined Immunodeficiency**
**uPA - Urokinase Plasminogen Activator**
**HCV - Hepatitis C Virus**
**HIV - Human Immunodeficiency Virus**
**HSV - Herpes Simplex Virus**
**ELISA - Enzyme-Linked Immunosorbent Assay**
**PCR - Polymerase Chain Reaction**
**qRT-PCR - Quantitative Real-Time Polymerase Chain Reaction**
**HBV - Hepatitis B Virus**
**MIT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide**
**ATP - Adenosine Triphosphate**
**DMSO - Dimethyl Sulfoxide**
**EC50 - Median Effective Concentration**
**CPE - Cytopathic Effects**
**Ct - Cycle Threshold**

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The authors declare no conflicts of interest that could influence the conduct or reporting of this review. Any potential conflicts of interest, financial or otherwise, have been disclosed and addressed in accordance with the policies ofVeterinary Integrative Sciences.

**Ethical Compliance**:

No need of ethical approval. The review article has been written according to Code of Conduct and Best Practice Guidelines set forth by the Committee on Publication Ethics (COPE). The articles cited for the review are ethically approved by their countries.

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