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Review

Innovations in modeling influenza virus infections in the laboratory

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Respiratory viruses represent one of the most substantial infectious disease burdens to the human population today, and in particular, seasonal and pandemic influenza viruses pose a persistent threat to public health worldwide. In recent years, advances in techniques used in experimental research have provided the means to better understand the mechanisms of pathogenesis and transmission of respiratory viruses, and thus more accurately model these infections in the laboratory. Here, we briefly review the model systems used to study influenza virus infections, and focus particularly on recent advances that have increased our knowledge of these formidable respiratory pathogens.

Public health burden of respiratory viruses

Respiratory illnesses are the most common type of ailment reported in otherwise healthy adults and children, and respiratory viruses are often the culprit. The severity of illness displayed during acute respiratory infections can range from minor common cold symptoms to life-threatening disease, and depends greatly on the etiologic agent and the health status of the host. Unfortunately, respiratory infections with lethal outcome are familiar in developing countries, especially in children, and viral infections contribute to up to 40% of these cases [1]. By comparison, fatal respiratory infections in developed countries are less common, but the incidence of serious complications in vulnerable populations such as those with underlying health conditions, the very young, and elderly is considerable [2,3]. On average, respiratory illness is reported three to five times per adult per year and five to nine times per child per year, which results in over 94 work days lost and 182 school days missed per 100 individuals per year [4,5].

Viral infections of the respiratory tract can be restricted to the upper respiratory tract (URT) or the lower respiratory tract (LRT); alternatively, both URT and LRT may be targeted, or extrapulmonary tissues may be involved (e.g. gastrointestinal and central nervous system). Viral infections of the URT often result in LRT involvement either through direct spread of the virus or indirectly via neurogenic links or circulating immune mediators that can cause physiological changes such as asthmatic exacerbations [6]. Bacterial coinfection is common and is identified in approximately 30% of pediatric LRT infections [7]. URT infections may manifest as sinusitis, otitis media, pharyngitis or tonsillitis causing local symptoms such as rhinorrhea, nasal congestion, sneezing, cough, and sore throat. The main pathogens responsible for these common cold symptoms are rhinoviruses (RV), coronaviruses (CoV), and adenoviruses (AdV) [6]. LRT infections often result in more serious syndromes such as bronchitis, bronchiolitis, and pneumonia with symptoms including fever, weakness, coughing, wheezing, and shortness of breath [7]. In children and adults, the most frequent etiologies of LRT viral infections are respiratory syncytial virus (RSV), parainfluenza virus (PIV), and influenza virus [6,7].

Of particular concern to global public health is the emergence of highly infectious and transmissible respiratory pathogens that have the potential to overwhelm an immunologically naïve population. In recent years, at least two respiratory pathogens have emerged in humans and have presented a considerable public health threat to both healthy individuals and those in more vulnerable populations: severe acute respiratory syndrome virus (SARS-CoV) and Influenza A H1N1 pdm09 (2009 H1N1) virus. SARS-CoV caused LRT disease in an infamous outbreak that started in 2003 and within weeks spread to 25 countries, resulting in over 8000 cases with 15% mortality [8]. The impact of pandemic influenza viruses differ substantially in magnitude and severity [9–11], whereas seasonal influenza viruses may cause symptomatic respiratory illness in up to 9% of the world's population and over 300 000 related deaths annually [12,13]. The most recent pandemic virus, 2009 H1N1, spread across at least 214 countries and was considered mild compared to the 1918 H1N1 pandemic, which resulted in an estimated 20–50 million deaths worldwide [14,15]. Since 2003, highly pathogenic avian influenza (HPAI) H5N1 viruses have caused hundreds of human respiratory infections with a high fatality rate of approximately 60% (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html). Although H5N1 viruses are not readily transmissible among humans in their current form, the emergence of a highly transmissible H5N1 virus would likely cause a pandemic. This review will focus specifically on influenza viruses because of the ever-present and ever-changing public health threat of these respiratory pathogens.

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Transmission and seasonality of influenza viruses

Influenza viruses are shed from infected individuals in respiratory secretions that are expelled during breathing, talking, coughing, and sneezing [16]. Transmission of virus to susceptible hosts can occur in several ways: (i) through direct contact or indirect contact with fomites; (ii) by droplet transmission, when large particles contact a person's conjunctiva or respiratory mucosa; and (iii) by droplet nuclei transmission (also known as airborne or aerosol transmission), when fine particles that are capable of prolonged suspension in the air are inhaled. These modes of transmission are not mutually exclusive; the relative predominance of these different modes of transmission depends on several conditions including the environmental setting, such as temperature and humidity.

Infection rates of many of the viruses associated with respiratory illness display distinct seasonal variation, particularly in temperate regions of the world [17]. Although outbreak intensities vary from year to year, general trends can be predicted. This is especially true for seasonal influenza viruses, which peak predominately in the winter months in temperate climates. Pandemic influenza virus infections are an exception to this pattern, likely because there is a lack of immunity to the emerging novel strain [18,19]. Multiple theories have been proposed to explain seasonal trends in respiratory infections including changes in host susceptibility, virus stability and infectivity, and indoor crowding [20–24]. Consistent with seasonal trends, enveloped viruses, such as influenza virus, have demonstrated increased viability at low humidity levels and cooler temperatures [24–28]. However, in tropical regions of the world where climate fluctuations are minimal and a warm, humid climate is typical, influenza cases are detected year-round or in biannual peaks [29]. Thus, the factors that are thought to facilitate transmission of certain respiratory viruses in temperate regions during the winter months may not be the same as those that contribute to transmission in tropical climates, making it obvious that no one theory satisfactorily explains global respiratory infection and transmission patterns. Because there are so many unanswered questions regarding how respiratory viruses cause disease and transmit among people, it is necessary to model respiratory infections in the laboratory. Here, we briefly review influenza virus infection model systems with a focus given to recent innovations in modeling influenza virus infections in animals and how improvements in these model systems can increase our knowledge of influenza viruses that affect public health throughout the world.

Influenza virus infection models: *in vitro, ex vivo,* and *in vivo*

Modeling infections in the laboratory is a fundamental tool of infectious disease experimental research and is critical to understanding the mechanisms of virus infection, transmission, host disease, and immune responses. Model systems can be divided into *in vitro*, *ex vivo*, and *in vivo* categories, with increasing complexity and each with particular applications and limitations (Table 1). Immortalized cell lines representative of the human airway (notably the human bronchial adenocarcinoma epithelial continuous cell line, Calu-3) can be grown on transwell membranes and become highly polarized with tight junctions separating distinct apical and basolateral surfaces providing the opportunity to assess the localization of virus attachment, entry, and release [30]. Primary human airway epithelial cells can be used for many of the same applications but also provide the additional complexity of a more heterogeneous cell population, including ciliated, nonciliated, mucin-secreting goblet cells and basal epithelial cells. Studies using primary human airway cells cultured from nasal, adenoid, tracheal, bronchial, or alveolar tissues samples and differentiated on an air–liquid interface have furthered our understanding of influenza virus infectivity, tissue tropism properties, and host immune responses that are specific to regions within the respiratory tract [31–33]. For example, human influenza viruses were shown to infect nonciliated cells expressing α2-6-linked sialic acid glycans, whereas avian influenza viruses infected ciliated cells expressing α2-3-linked sialic acid glycans in primary human tracheobronchial epithelial cultures [34]. In addition to respiratory cell cultures, primary human corneal and conjunctival cell cultures have been used for *in vitro* modeling of ocular infections and have revealed potential mechanisms of ocular tropism for influenza viruses but also how respiratory infections may develop after ocular inoculations [35].

Ex vivo human airway tissues serve as a snapshot of the airway epithelial environment within the human host and have been used to demonstrate that highly transmissible seasonal influenza viruses attach to URT tissues more readily than avian influenza viruses that are not capable of sustained transmission in humans [36,37] but that human URT tissues are capable of supporting H5N1 avian influenza virus replication [38]. Availability of human *ex vivo* tissue samples is often limited; therefore, experiments may be conducted using tissues derived from appropriate animal models. Considerable attention has been given to the distribution of receptors used by influenza viruses within the respiratory tract of various animal models and how they compare to humans. The patterns of viral attachment (PVA) observed in human *ex vivo* respiratory tract tissues is most similar to ferrets and pigs for human influenza viruses [39] and ferrets, pigs, and cats for avian influenza viruses [39,40]. Ferret and pig tracheal *ex vivo* tissues were not only used to assess PVA [41], but also replication of influenza viruses [42] and ciliary activity, linking the destruction of ciliary activity to a possible role

Table 1. Modeling influenza virus infections

	Applications	Limitations
In vitro	Independently assess viral and host parameters	May not correlate with *in vivo* data
Ex vivo	Tissue-specific virus deposition, attachment, infection, and replication patterns	May not correlate *with in vivo* data, limited tissue availability
In vivo	Virus pathogenesis, transmission, immune response, drug and vaccine efficacy	Expensive, ethical issues, may not represent human situation

in virulence [43]. Ferret nasal turbinate *ex vivo* tissues were used to compare the infectivity of influenza viruses exhibiting diverse transmissibility phenotypes in the context of assessing the inhibitor effects of nasal mucous [44]. Others identified mucin-secreting submucosal glands in the ferret respiratory tract expressing α2-6 sialylated glycans in the context of *O*-linked glycans as the predominant receptor for human influenza viruses and proposed that the proximity of virus to sites of mucous secretion as a possible mechanism of enhanced aerosol transmission of virus [45]. Additionally, *ex vivo* imaging of the entire respiratory tract of the ferret provided visualization of virus deposition patterns after inoculation [46]. Studies such as these provide insight into how influenza viruses deposit, attach, replicate, and possibly transmit in respiratory tract tissues.

In vitro and *ex vivo* model systems are attractive methodologies for addressing questions of tissue tropism, immune response, and virus replication independent of the whole host system. However, a whole-systems approach can be advantageous by allowing the assessment of virus replication and immune responses in the context of diverse virulence and transmissibility phenotypes displayed in infected hosts. Human influenza challenge studies have been conducted to improve policies for infection prevention and intervention [47] but naturally, the experimental range of these studies are limited, necessitating the need for animal models of infection. Nonhuman primates (NHPs) have been used to recapitulate influenza virus infections in humans [48] but despite the relevance of NHPs to humans, the expense and inherent ethical issues limits the broad utility of this animal model, thus, small animals are more routinely used for influenza virus research.

Although many mammalian models have been evaluated [48], the animals most often used to model influenza virus infections are mice, guinea pigs, and ferrets. Mice have been used extensively for the assessment of pathogenesis, immune responses, and vaccine efficacy, even though human influenza viruses typically require prior adaptation for this animal model [48,49]. Knockout mouse models have been particularly useful in the study of specific host responses in an *in vivo* setting, which is an advantage not shared with other models [50]. The guinea pig has proven to be a useful outbred model for studying influenza virus transmission and recent innovations in this model system have provided the opportunity to evaluate the effects of environmental conditions on the transmission of influenza viruses [51,52]. Transmission of virus through the air among guinea pigs was most efficient in a cool, dry environment but regardless of relative humidity conditions, was abolished at 30 °C [53]. A shortcoming of this model is that guinea pigs lack many clinical signs found in humans following influenza virus infection, making it difficult to assess virulence. However, because infection is not fatal, as is observed with certain strains of influenza virus in mice and ferrets, they can be useful in studying particular disease parameters and treatment strategies that may not be otherwise possible [54]. Ferrets have become the model of choice for many influenza virus research laboratories despite a general lack of available immunological reagents [48,50,55,56]. Outbred ferrets are highly susceptible to influenza virus infection without prior adaptation of the virus, exhibit clinical signs similar to those of infected humans, and display the general transmissibility phenotypes of influenza viruses reported in humans. Studies demonstrating the similarities between the distribution of influenza virus receptors and the PVA in ferret respiratory tract tissues compared to those of humans provide evidence of the suitability of ferrets for the modeling of influenza virus infections [39–41,57]. Respiratory droplet transmission models have been developed in ferrets to identify influenza viruses capable of transmitting through the air [58]. Respiratory droplets may consist of larger droplets or smaller droplet nuclei so this term refers to transmission of virus through the air in the absence of direct or indirect contact between the animals. Critical information regarding the molecular correlates of virulence and transmission of influenza viruses has been gained with the use of ferrets, providing genetic markers for the early detection of influenza viruses with the potential to cause disease and transmit readily among people.

Analysis of the composition and deposition of influenza virus in aerosols

Before influenza virus infections can be accurately modeled in laboratory animals, an understanding of the composition of the respiratory secretions passed from one host to the next is critical. Aerosols play a key role in the transmission of influenza viruses among people, and several environmental, host, and viral factors directly influence the size and number of aerosols expelled from infected individuals that are subsequently deposited in the respiratory tracts of susceptible hosts [16,58]. Studies conducted using human volunteers show that during tidal breathing, the majority of exhaled aerosols are <1 μm and reach concentrations of >10 000 particles/l with a substantial range from person to person [59,60]. Influenza virus can be detected in exhaled aerosols of this size from people but has been largely limited to detection of viral RNA, demonstrating the presence of influenza viral material in respirable aerosol particles and the need to improve methods to preserve virus viability in sampled aerosols [16,61]. Aerosol shedding profiles have been evaluated in the ferret model and, similarly, the majority of aerosols ($>53\%$) exhaled by ferrets during tidal breathing and sneezing are <1 μm [46]. Several air sampling devices have been evaluated for their ability to isolate and preserve the infectivity of influenza virus in aerosols. In a recent study, the SKC BioSampler, which uses a liquid medium for aerosol collection, maintained virus viability better than that of other filter-based samplers [62]. However, these devices do not provide size fractionation of aerosols. The National Institute for Occupational Safety and Health developed a two-stage cyclone bioaerosol sampler that separates aerosols by size (<1, 1–4, and >4 μm) and recently reported improvements in methods that enhance viable virus recovery from nebulizer-generated aerosols (15–34% recovery) [63]. When this device was used to measure influenza virus in aerosols generated by individuals during coughing, detection of viral RNA proved to be

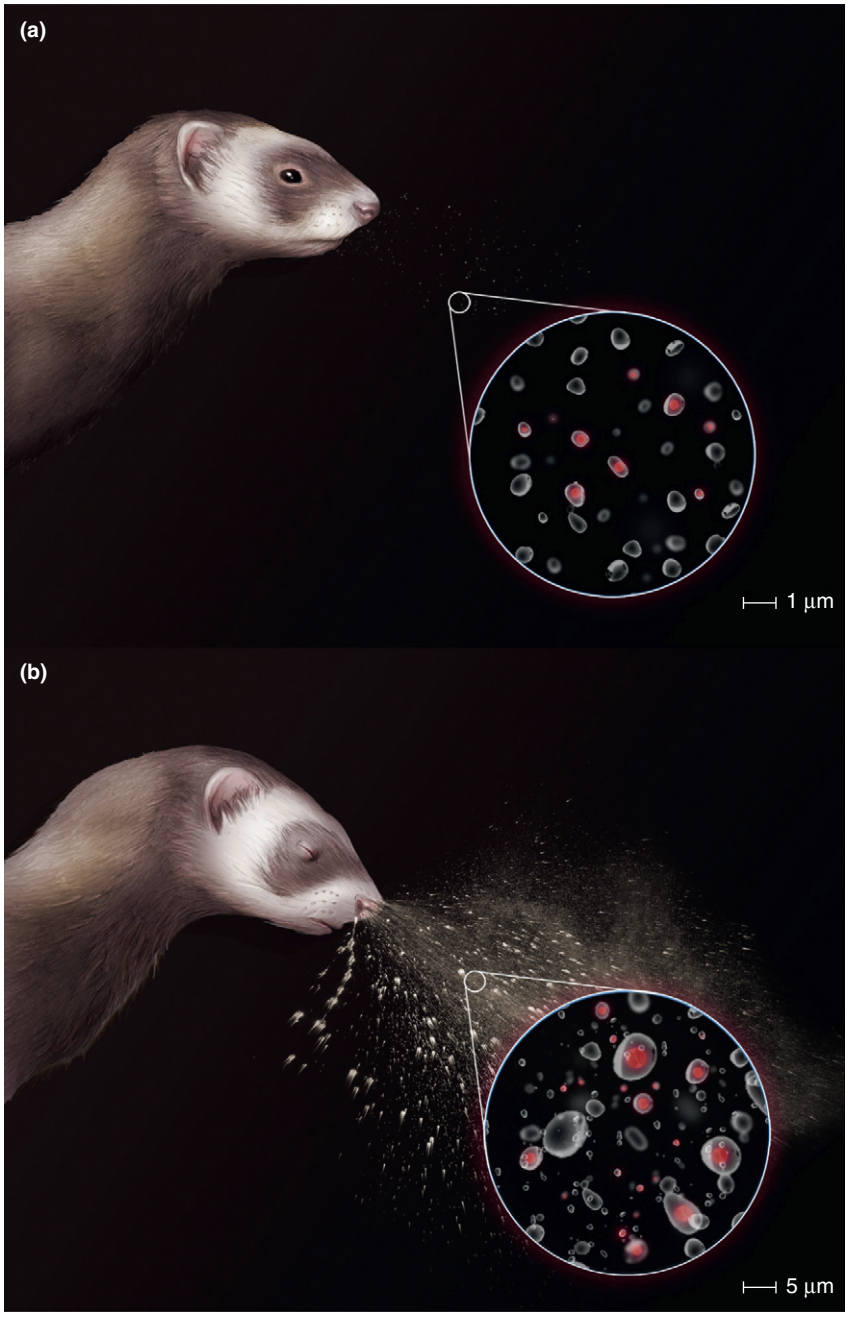

Figure 1. Cartoon representation of aerosols shed from influenza virus-infected ferrets during **(a)** normal breathing and **(b)** sneezing. The majority of aerosols are <1 μm, but infectious virus (shown in red) is detectable in small and large particles.

much more reliable than recovery of viable virus; however, the enhanced detection method for viable virus was not used during this study [61]. When this sampling device was used during ferret transmission studies, detection of influenza viral RNA in respirable aerosols exhaled from ferrets allowed the identification of viral genetic factors associated with transmission of the 2009 H1N1 virus through the air [64]. Using another sampling device, a cascade impactor, and modified procedures, viable virus was detected in respirable aerosol particles collected during tidal breathing and sneezing with recovery rates estimated at 19–31% (Figure 1; [46]). Although there was variability among ferrets, infectious virus shedding was detected at ~2 plaque forming units (pfu)/l during normal breathing. The devices and updated procedures described in these studies represent innovations in bioaerosol sampling that enhance our ability to understand the composition of aerosols shed from people and animal models during infection and how influenza viruses transmit through the air.

Deposition of aerosol particles in the human respiratory tract has been extensively studied and occurs via several mechanisms that can be affected by factors such as aerosol size, concentration and composition, breathing rate, and tidal volume [65]. Generally, particles <0.5 and >5 μm deposit in the URT passages, particles <0.5 and 5–10 μm deposit in the tracheobronchial region, and aerosols <5 μm

are capable of reaching the bronchioles and alveoli. Animal models of aerosol deposition have been useful for inhalation toxicology, physiology, and drug delivery studies [66]. Despite apparent anatomical differences compared to humans, valuable information can be extrapolated from large animal models like NHPs but also from rodent species and ferrets [67,68]. Advances in molecular imaging technology [69] have facilitated the visualization of deposition patterns of influenza virus after inoculation procedures in ferrets [46] as well as bacterial pneumonia related to influenza virus infection in mice [70]. Using these techniques to characterize aerosols exhaled by and deposited in the respiratory tracts of laboratory animals has been instrumental in the development of more relevant methods of inoculation.

Innovative methods of influenza virus animal inoculations

Inoculation of laboratory animals with influenza viruses can be accomplished using a variety of techniques depending on the question being addressed, but by far the method used most frequently is administration of liquid virus suspensions directly to the respiratory tract, most notably by intranasal instillation to the nares of the animal. This method has become standard practice among influenza virus laboratories, using a range of volumes depending on the animal model and the region of the respiratory tract being targeted [49,55]. A reduced volume may be used to avoid delivering a substantial portion of the inoculum to the LRT. However, a caveat of intranasal inoculation is that a portion of the inoculum may be swallowed and diverted away from the respiratory tract, because some animals may swallow during the inoculation procedure [46]. Alternatively, intratracheal inoculation may be used to bypass the URT and gastrointestinal tract and has allowed researchers to observe differences in disease parameters that may be associated with the route of inoculation [71,72]. Studies using ocular inoculations have provided evidence that replication-independent spread of virus from the eye to the respiratory tract can occur, likely via the lacrimal and nasolacrimal ducts but that ocular tissues can also support influenza virus replication [35,73,74]. Studies like these demonstrate that the method of inoculation is an important component of experimental designs. There are important advantages to establishing a method of inoculation that closely mimics a natural route of infection, and the administration of a liquid inoculum does not represent how influenza virus infections occur in nature.

Many studies presenting either epidemiologic or experimentally derived data have concluded that droplets and aerosols are involved in influenza virus transmission in humans and in animal models [16,58]. Aerosol exposure of animal models such as mice, guinea pigs, and ferrets to influenza virus has been described. Studies in mice showed that, compared to intranasal inoculations, aerosol delivery of H3N2 influenza virus caused increased morbidity and mortality [75] and that aerosol delivery of antivirals and live-attenuated influenza vaccine virus is an effective method of disease prevention in this animal model [76,77]. Guinea pigs inoculated with aerosolized seasonal influenza virus reportedly were as contagious as those inoculated intranasally [52]. Ferrets inoculated with aerosolized HPAI H5N1 virus exhibit earlier onset and a broader spectrum of disease compared to those intranasally inoculated [46,78], whereas ferrets inoculated with aerosolized seasonal influenza virus exhibited illness that was indistinguishable from naturally acquired infections. Specifically, when ferrets were infected by exposure to other infected ferrets, similar virus shedding kinetics and disease characteristics were noted compared to low-dose aerosol inoculated ferrets [46]. Moreover, the 50% ferret infectious dose of a seasonal influenza virus delivered via aerosols was 1.9 pfu [46] or 4 focus forming-units [79], similar to the 50% human infectious dose of aerosol-delivered influenza virus (0.6–3 50% tissue culture infectious doses [80]), providing additional support to the relevance of the aerosol method of inoculation and the ferret as a model of influenza virus infection. Although these studies require specialized instrumentation (described within noted references), they demonstrate the applicability of aerosol inoculations to influenza virus research and how bioaerosol exposure systems can be individualized to meet specific experimental needs. Validation of bioaerosol systems should be conducted prior to animal exposures to ensure the consistent and reliable delivery of presented doses to animals, and consideration should be given to the bioaerosol shedding profiles of infected hosts under the environmental conditions being modeled. These innovations in inoculum delivery methods have provided improvements to animal modeling of influenza virus infection, pathogenesis, and transmission by more closely recreating natural, airborne infections in a laboratory setting.

Limitations and future directions

Over the past 50 years, techniques in experimental research have been developed that facilitate improvements in our understanding of how respiratory viruses and, in particular, influenza viruses cause disease and spread through the human population. These advances would not have been possible without the ability to model respiratory virus infections in the laboratory, but despite these advances many limitations still exist. Regardless of how closely respiratory infections are recreated in animal models, they will never provide an exact representation of infections in humans. Complicated and often unpredictable behavioral and environmental influences govern the interactions of people and the environmental conditions in which respiratory viruses spread, and all of these scenarios cannot be accommodated in a controlled laboratory setting. Continued evaluation of environmental parameters affecting respiratory pathogens and, when feasible, results from ethically conducted human studies will provide continued improvements in animal models and in the procedures used to recreate respiratory infections in the laboratory. The application of aerobiological techniques to animal models of influenza virus infections by delivery of aerosolized inocula and characterization of aerosol shedding is an important step to this end. Manipulation of the size and composition of aerosolized inocula delivered to animal models offer opportunities to better understand the mechanisms involved in the various modes of transmission. Performing these experiments under well-controlled

conditions that accurately reflect both indoor and outdoor environments of temperate and tropical regions will provide a more relevant understanding of the behavior of exhaled respiratory secretions and the stability of respiratory pathogens transmitted in them. As researchers continue to make innovative strides in infectious disease experimental research, biosafety and biosecurity will undoubtedly be at the forefront of discussion. Careful consideration of the potential dual use applications and the risk versus benefit to public health via information sharing should be undertaken by the scientific and public health community. Nevertheless, studies aimed at improving our knowledge of the pathogenesis and transmission of respiratory viruses will facilitate advances in current treatment and prevention strategies and aid in the development of new antivirals and vaccines with broad coverage that lessen the disease burden on the human population.

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