Advances in viral respiratory infections: new experimental models

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The wide array of models available for the study of respiratory viral infections is extremely valuable for the development of novel therapeutic and prophylactic options against these highly prevalent diseases. In addition, through these models we have gathered considerable insight into the cellular and molecular mechanisms involved in the pathogenesis of these infections and the inflammatory and immune responses they elicit in the host. This article reviews new promising models introduced recently in this field.

Introduction

Viral respiratory infections remain an important cause of morbidity and mortality worldwide. Paramyxoviruses are the most common respiratory pathogens in children. In particular, the respiratory syncytial virus (RSV) infects nearly all children within the first two years of life [1], resulting in more than 120,000 hospitalizations annually in the United States. Because immunity after the first infection is not complete, RSV is also a common cause of respiratory morbidity in older children and adults and mortality in the elderly. In addition to the acute care costs, a common sequela of early-life RSV infection is the development of recurrent wheezing and asthma [2]. A new paramyxovirus genetically related to RSV, the human metapneumovirus (hMPV), has been discovered recently and characterized as a frequent cause of lower respiratory tract infections [3]. Other common viral respiratory pathogens include influenza viruses, rhino-

viruses, and adenoviruses. Given the considerable public health burden, it is important to develop relevant disease models using *in vitro*, *in vivo* and *in silico* techniques to better understand the acute and chronic effects of viral infection on the respiratory tract.

In vitro models

In vitro assays using mammalian cells have long been used as models for the diagnosis, prevention, and therapy of respiratory viral infections. However, the actual prevalence of respiratory viruses is probably underestimated because of the use of diagnostic techniques that not only lack sensitivity, but are also time-consuming. Often the time necessary to obtain the results of a viral culture exceeds two weeks, at which point most therapeutic options against respiratory viruses are virtually useless.

Polymerase chain reaction (PCR)

This technique has improved dramatically our ability to diagnose respiratory viral infections. Real time PCR analysis is now used to detect influenza A and B, RSV and the severe

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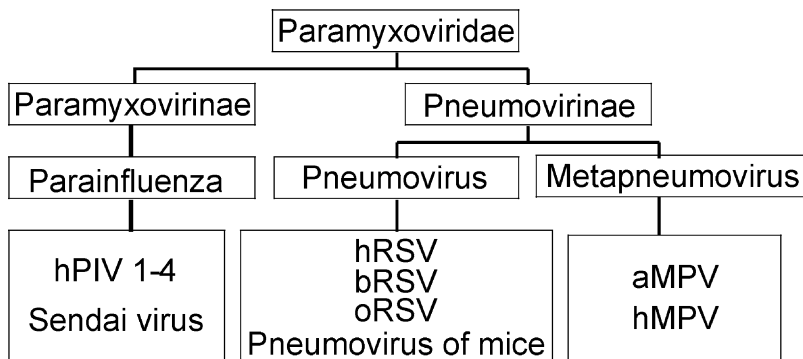
Figure 1. *Paramyxoviridae*. A new paramyxovirus closely related to RSV, the human metapneumovirus (hMPV), has been discovered recently and characterized as a frequent cause of infant bronchiolitis.

acute respiratory syndrome-associated coronaviruses (SARS-CoV) in humans [4–6]. In lung transplant recipients, PCR has been used to rapidly detect respiratory viruses with a sensitivity up to 84%, which is significantly better than any other available method [5]. In addition to studying viruses during the acute infection, PCR has also been used to ascertain viral persistence in peripheral blood mononuclear cells during the convalescent phase [7].

Rapid reverse-transcription PCR has been introduced recently for the detection of rhinovirus in human subjects with excellent results. Steininger and colleagues [8] using nested RT-PCR were able to diagnose rhinovirus infection within 48 h, versus the two-week delay necessary for viral culture. Similar protocols have been employed by other researchers to study enteroviruses [9]. Na *et al.* [10] have developed multiplex PCR to detect and type 6 serotypes of respiratory adenovirus, and a similar assay has been developed for influenza and RSV [4].

Another important implication is that PCR is sensitive enough to detect respiratory viruses in stored biological materials. Recently, multiplex PCR has been used to detect respiratory viruses in archival nasal swab specimens, thus allowing for retrospective diagnostic and epidemiological work [9]. PCR techniques have also been instrumental for the diagnosis and classification of new pathogens, as with the recent addition of hMPV [11] to the *Paramyxoviridae* family (Fig. 1).

DNA microarrays

One of the most recent advances in this field is the introduction of microarray technology, based on the use of arrays of DNA probes immobilized on solid supports for the simultaneous identification of multiple target sequences. Microarrays can be used not only for the diagnosis of viral infections, but also for molecular typing and for the investigation of virus–host interactions [12]. Generic, multiplex or random PCR amplification of viral target sequences in clinical specimens increases substantially the versatility of this methodology [13].

The early diagnosis of a respiratory viral pathogen is of particularly crucial importance in clinical practice, especially because the already limited therapeutic options become even less effective as the infection progresses. A comprehensive and unbiased analysis of viral prevalence in a given biological setting is facilitated by 70 mer oligonucleotide DNA microarrays capable of detecting hundreds of viruses simultaneously. Viral serotypes can be distinguished by the unique hybridization pattern generated by each virus. Furthermore, combining random PCR amplification with microarray analysis multiple viruses can be detected in human respiratory specimens without the use of sequence-specific probes. This expands the spectrum of detectable viruses in a single assay, whereas simultaneously providing the capability to discriminate among viral subtypes.

Recently, microarray techniques were used as a diagnostic tool to track the evolution of SARS-CoV. Using high-throughput, high-density re-sequencing arrays, Wong *et al.* [14] were able to define SARS-CoV mutations associated with different clinical patterns. This study illustrates the development and validation of an oligonucleotide re-sequencing array for the entire 30-kb SARS-CoV genome which is both rapid and cost effective. Through the study of SARS-CoV mutation patterns, tracing index cases and contacts in human populations is made much easier. Thus, this DNA microarray methodology is ideal for global epidemiologic monitoring of SARS-CoV and other small-genome pathogens, and has also been used as a complementary technique for typing and sub-typing influenza viruses, which could lead to more effective tracking of flu epidemics [15].

DNA microarrays have also been used recently to monitor host responses induced by respiratory viruses in humans. For example, using microarrays Zhang *et al.* [16] profiled the kinetics and patterns of chemokine expression in

RSV-infected lower airway epithelial cells. In particular, this study explores the temporal progression in the epithelial expression of chemokines and other factors responsible for the recruitment and activation of inflammatory cells to RSV-infected lower airways.

In vivo models

Animal models have provided considerable insight into the pathophysiology of viral respiratory infection, the mechanisms of virus–host interactions, and the development of candidate vaccines. In vivo models are also used to simulate the chronic sequelae of respiratory viral infection in humans. The most commonly used animal models for respiratory virus research are rodents, particularly mice (Mus musculus), rats (Rattus norvegicus), and guinea pigs (Cavia). The advantages involved in using rodents include lower purchase costs, wide availability, and smaller/cheaper barrier housing facilities compared to larger animals. The use of non-human primates has typically been reserved for respiratory virus vaccine research.

Inflammation

Mouse models have been developed for many viruses and have led to better understanding of the immuno-pathogenic processes activated by these infections. In addition, mouse models have contributed to our understanding of virus replication in the airways [17] and of the role of cytokines and chemokines [18]. A major advantage of mouse models has been the wide availability of reagents and specific antibodies for the study of immuno-inflammatory and signal transduction pathways. More importantly, targeted deletion or over-expression of specific genes, which is typically conducted in mice, has been instrumental in understanding the molecular basis of virus–host interactions. By contrast, the small size of litters limits the use of this model for studies of early-life infections, especially when physiologic measurements are involved. These studies are usually conducted in larger rodents, like rats and guinea pigs.

Caution should be exercised when choosing a rodent model for experimental respiratory infections, as there is significant variation in how different species and strains respond to pathogens. For example, Brown Norway (BN) rats exposed to respiratory viruses mount a predominant TH2-type allergic inflammatory response followed by a chronic asthma-like syndrome [19], which is in stark contrast to the response of Fischer 344 (F344) rats to the same pathogens. We developed a model of RSV bronchiolitis in F344 rat [20], which is unique in that this strain mounts a strong immune response and clear RSV from the lungs, much like the self-limiting disease process that occurs in normal, immunocompetent children. By contrast, guinea pigs [21] and mice [22] are unable to clear this virus even after several months, developing practically life-long infec-

tions that are highly unusual in humans. Thus, our model is particularly useful to study the long-term physiological abnormalities after a mild and transient lower respiratory tract infection with RSV.

In addition, the inoculation of F344 weanling rats at two weeks of age allows for evaluation of the long-term effects of early RSV infection [23]. In this model, important developmental changes in the distribution of neurogenic inflammatory responses across the respiratory tract were observed as the rats aged. Stronger inflammatory responses developing in the lower respiratory tract early in life might explain why RSV bronchiolitis tends to occur in infants, whereas this infection tends to be restricted to the upper respiratory tract infection in older children and adults. Furthermore, remarkable age-related differences were found in mast cell distribution [24], leukotriene-dependent inflammation [24], and in the expression of neurotrophic factors and receptors [25] in RSV-infected lungs.

Prophylaxis

In vivo models have been used to test vaccines and antibodies against respiratory viruses. An excellent example is palivizumab (Fig. 2), a humanized monoclonal antibody against the RSV fusion protein, which prevents spreading of the infection to the lower respiratory tract and is currently used worldwide for the passive prophylaxis of RSV disease in high-risk infants. The neutralizing activity of this antibody was titrated in cotton rats [26], and its protective effects against RSV-induced acute airway inflammation [27], chronic hyperreactivity [28], and apnea [29] were shown in F344 rats (Fig. 3). The same models are currently being used to test new generation, more potent anti-RSV antibodies [30]. Also, monoclonal antibodies against SARS-CoV nucleocapsid protein were prepared recently by immunizing mice [31].

Animal models have been used to experiment new strategies to generate and deliver vaccines to humans. A recent example is the development of a recombinant multi-epitope peptide comprising repeats of influenza virus hemagglutinin-neutralizing epitopes expressed in Escherichia coli. Administration of this peptide to mice and rabbits (Oryctolagus cuniculus) induced a high level of specific antibodies, suggesting that recombinant multi-epitope peptides can provide a new way to develop effective vaccines against influenza virus [32].

A new technique called epidermal powder immunization (EPI), which utilizes the dermal route for the delivery of vaccines, has recently been tested using in vivo models. Chen et al. [33] have inoculated mice and non-human primates with influenza vaccine using EPI and have demonstrated that this technique evokes significantly higher serum hemagglutination inhibition titers than the traditional intramuscular route, especially when combined with an adjuvant. The epidermal route for vaccine delivery has also been evaluated for RSV vaccination using two G protein-derived molecules
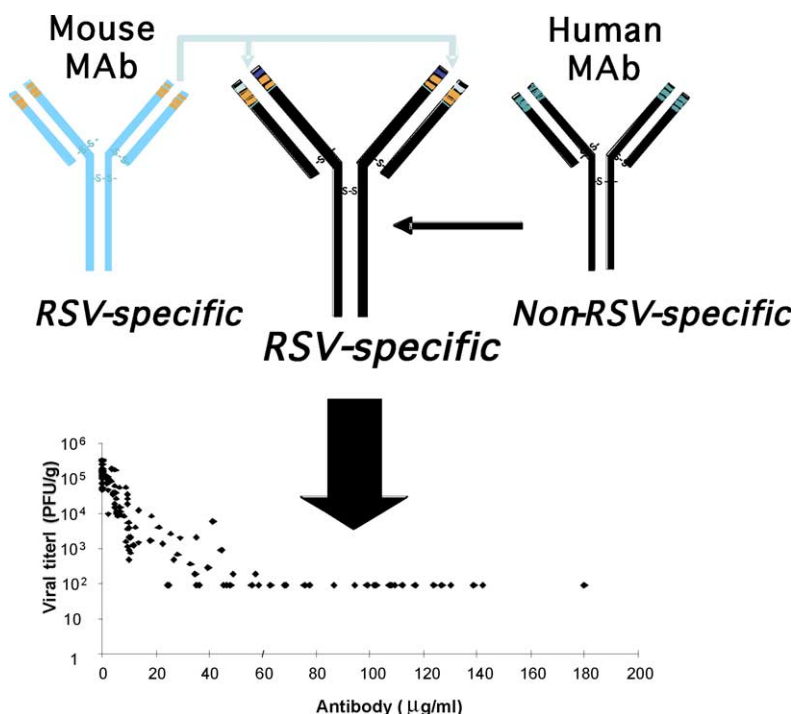
Figure 2. RSV Prophylaxis. Construction of anti-RSV MAbs by transfer of complementarity determining regions (CDR) from mouse antibody to a human frame (top) and determination of their neutralizing activity *in vivo* in a cotton rat model of RSV infection (bottom).

attached to a bacterial enterotoxin adjuvant, demonstrating adequate protection against RSV in mice [34].

Another novel prophylactic approach against RSV utilizes chitosan-DNA nanospheres containing a cocktail of plasmid DNAs encoding most RSV antigens, which is suitable for intranasal delivery. Mice treated with these nanospheres have a significant reduction of viral titers and viral antigen load after acute RSV infection [35]. These new prophylactic strategies can increase significantly the availability of vaccines in underdeveloped areas where refrigeration is an issue, as is the availability of sterile syringes and needles. Furthermore, methods derived from *in vivo* models are probable to be crucial in the future for rapid mass immunization against known and newly emerged airborne biological agents.

In silico models

During the past two decades, new advancements have added layers of complexity to the research in biotechnologies and drug discovery. Many newly developed techniques are computational in nature, taking their particular disciplines *in silico*. Collaborations between mathematical and computational biologists and scientists participating in the World Health Organization (WHO) global influenza surveillance network have resulted in a number of mathematical and computational advances, such as increasing the resolution of antigenic surveillance and new methods for genetic analysis of the virus. WHO has used these *in silico* techniques to increase the ability to extract information from influenza surveillance and to increase the quantitative data available for the selection of candidate vaccine strains [36].

In silico models have also been used to generate rapid diagnostic tests for respiratory viruses. Using a hexon-based fluorogenic PCR assay, a rapid and type-specific diagnostic system for adenovirus type 4 has been developed allowing for early diagnosis in high-risk patients [37]. *In silico* models have also been generated to help construct vaccines against respiratory viruses and to study the SARS-CoV spike glycoprotein. Using these models, synthetic peptides have been generated for six peptide sequences corresponding to the surface regions of SARS-CoV, which were subsequently used to elicit-specific antibodies against the virus. This synthetic peptide-based approach might provide insight into the development of a SARS-CoV vaccine [38].

In silico techniques have also been used for molecular epidemiological analysis of respiratory viruses and to monitor genetic diversity in community-based studies. Researchers have designed a multiplex nested PCR assay for the analysis of clinical specimens to subtype phylogenetically the nucleotide sequences of RSV surface G glycoproteins [39]. Using these techniques, the same researchers were able to examine retrospectively biological samples gathered over a four-year period, through which they determined the emergence of two new subgroups of the virus [40]. In another study, phylogenetic analysis of rhinovirus isolates collected during successive epidemic seasons showed striking diversity of the virus strains circulating in a given community [41].
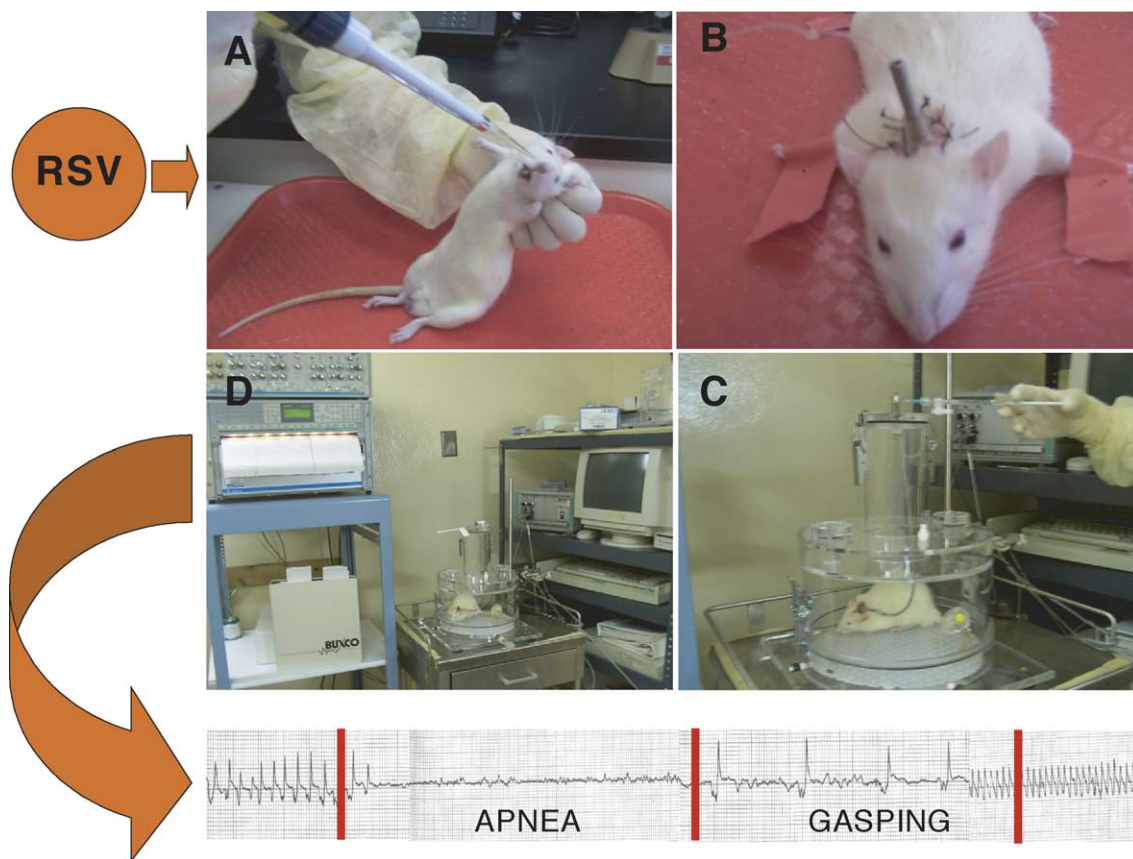
Figure 3. Rat model of RSV-induced apnea. Clockwise: (A) virus inoculation in pathogen-free Fischer 344 (F344) rat; (B) jugular venous catheter placement for drug infusion; (C) pharmacological nerve stimulation with capsaicin in an anaesthetized rat placed in whole body plethysmograph and tethered to a swivel mechanism holding the intravascular catheter; (D) plethysmographic measurements of pulmonary function and ventilatory parameters. A representative printout showing apnea evoked by sensorineural stimulation in an RSV-infected rat is shown below.

Conclusions

Viral respiratory infections remain an important cause of morbidity and mortality globally. Through the use of *in vitro* and *in vivo* disease models, a better understanding has been gained with regards to the pathophysiology of their acute and chronic clinical expression. *In vitro* techniques pose significant advantages to *in vivo* models, particularly because of their wide availability and relative ease of setup. *In vitro* models also provide for rapid detection and analysis of cytopathic effects, and because of most of these models have been characterized thoroughly they tend to be reproducible. Novel *in vitro* techniques, such as DNA microarrays, allow for the simultaneous screening of multiple viral species and strains. Unfortunately, many of these new technologies are not yet widely available to clinicians and also require experienced personnel to process the samples (Table 1).

Despite the broader availability and lower cost of *in vitro* analysis, *in vivo* models remain essential for the investigation of many respiratory viral infections. The chief advantage of these models is that they approximate closely the complexity of inflammatory pathways, cell–cell communications, and pathogen–host interactions at work in humans, often produc-

cing disease states that mimic the ones seen in clinical practice. However, as discussed above, careful consideration must be given to the species and also the strain chosen, as there is significant variation in disease patterns that makes it difficult comparing *in vivo* models for a given respiratory virus. Nevertheless, *in vivo* models have been and still remain instrumental in exploring the acute and chronic pathophysiology of respiratory viral infections, as well as in the development of vaccines and anti-viral antibodies and therapeutics.

Advances in biotechnology have brought forth *in silico* techniques, which allow for rapid and accurate processing of bioinformatics data and have already been used successfully for monitoring respiratory virus outbreaks. However, these models are underutilized at present and require further refinement to increase acceptance and use by the scientific community. Through combinations of the disease models presented in this article, the comprehensive investigation of complex aspects of known and emerging respiratory viral infections evolving from pathophysiology and diagnosis, to genomics and epidemiology, to prevention and therapy is probable to experience rapid and dramatic progress in the future.

Table 1. Comparison summary table

	In vitro models	*In* vivo models	*In* silico models
Pros	Reduced complexity of the system High sensitivity Rapid detection Lower cost	Complexity similar to human systems Intact immuno-inflammatory pathways Pathophysiological responses to viral infection can be determined	Increased resolution of antigenic surveillance Rapid genetic analysis Rapid multiplex detection
Cons	Morphological/functional differences between cultured cells/tissues and their *in vivo* counterpart Absence of neuro-endocrine influences Absence of immuno-inflammatory networks	Time consuming Higher costs Requires specialized housing facilities and trained personnel Ethical implications and considerations Species-/strain-specific responses to respiratory viruses	Expensive Require specially trained personnel and facilities
Best use of model	Diagnosis and typing Mechanisms of viral attachment, entry, signal transduction Anti-viral therapeutic design	Mechanisms of virus–host interactions Chronic sequelae Vaccine testing Antibody testing	Analysis of candidate vaccine strains Molecular epidemiology Monitoring viral genetic diversity in a target community
References	[7,11,14]	[29–31]	[35,38]

Links

- Stanford University Microarray Database: http://genome-www.stanford.edu/
- National Institute of Health (NIH) National Institute of Allergy and Infectious Diseases (NIAID) Pathogen Genomics Center: http://www.niaid.nih.gov/dmid/genomes/
- NIH/NIAID Vaccine Research Center: http://www.niaid.nih.gov/vrc/
- NIH Rat Genomics and Genetics Center: http://www.nih.gov/science/models/rat/resources/index.html
- NIH Model Organisms for Biomedical Research: http://www.nih.gov/science/models/
- National Library of Medicine: http://www.nlm.nih.gov/medlineplus/viralinfections.html
- WHO Severe Acute Respiratory Syndrome (SARS): http://www.who.int/csr/sars/en/
- Centers for Disease Control Respiratory and Enteric Viruses Branch: http://www.cdc.gov/ncidod/dvrd/revb/index.htm
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Related articles

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Outstanding issues

- Development of *in vivo* models reproducing genetic and environmental factors that predispose to severe infections and/or chronic sequelae.
- Use of disease models to develop safe and effective anti-viral therapies and vaccine strategies.
- Advancement of DNA microarray technology for global epidemiological monitoring.
- Refinement of high-throughput analysis for the rapid detection and typing of respiratory viruses in clinical settings.
- Utilization of gene "knockout" models to further characterize the molecular basis of immuno-inflammatory host responses against respiratory viruses.

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