Respiratory nanoparticle-based vaccines and challenges associated with animal models and translation

Gourapura J. Renukaradhya[1], **Balaji Narasimhan**[2], and **Surya K. Mallapragada**[2,*]

[1]Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691

[2]Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

Abstract

Vaccine development has had a huge impact on human health. However, there is a significant need to develop efficacious vaccines for several existing as well as emerging respiratory infectious diseases. Several challenges need to be overcome to develop efficacious vaccines with translational potential. This review focuses on two aspects to overcome some barriers – 1) the development of nanoparticle-based vaccines, and 2) the choice of suitable animal models for respiratory infectious diseases that will allow for translation. Nanoparticle-based vaccines, including subunit vaccines involving synthetic and/or natural polymeric adjuvants and carriers, as well as those based on virus-like particles offer several key advantages to help overcome the barriers to effective vaccine development. These include the ability to deliver combinations of antigens, target the vaccine formulation to specific immune cells, enable cross-protection against divergent strains, act as adjuvants or immunomodulators, allow for sustained release of antigen, enable single dose delivery, and potentially obviate the cold chain. While mouse models have provided several important insights into the mechanisms of infectious diseases, they are often a limiting step in translation of new vaccines to the clinic. An overview of different animal models involved in vaccine research for respiratory infections, with advantages and disadvantages of each model, are discussed. Taken together, advances in nanotechnology, combined with the right animal models for evaluating vaccine efficacy, has the potential to revolutionize vaccine development for respiratory infections.

Graphical abstract

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*Corresponding author: 2031 Sweeney Hall, Iowa State University, Ames, IA 50011-2230, Ph: 515-294-7407, suryakm@iastate.edu.
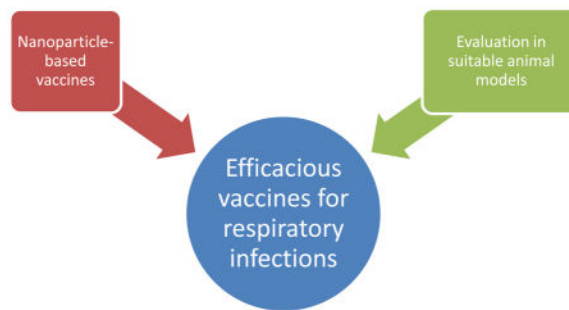
Keywords

Nanovaccines; animal models; translation; vaccine efficacy

1. Introduction

Respiratory infectious diseases remain a significant global threat to human health [1] and impose a heavy burden on our healthcare system [2]. These include diseases caused by well-known pathogens such as *Bacillus anthracis, Streptococcus pneumonia, Mycobacterium tuberculosis, Yersinia pestis*, influenza virus, and respiratory syncytial virus (RSV); as well as emerging zoonotic ones including severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), new strains of influenza, and Henipavirus. The influenza virus alone has caused more deaths in the 20th century than any other virus [3]. While vaccines have been enormously successful in combating infectious diseases [4] and are estimated to prevent over 3 million deaths worldwide annually [5], significant challenges remain on many fronts [6] as a result of which there are no effective vaccines currently available for several of the diseases [7, 8] listed above.

The current challenges facing vaccine development and translational research include identification of suitable antigen candidates, eliciting appropriate immune responses for protection, providing cross-protection against different strains of the pathogens, maintenance of the cold chain, repeated administration, route of administration and the need to identify appropriate animal models that will lead to similar responses in humans. To address potential solutions being pursued to address all these varied challenges would be outside the scope of any review, and several other review articles have focused on approaches to overcome many of these challenges [9, 10]. This review specifically focuses on two aspects: recent developments in nanoparticle-based delivery of respiratory vaccines; and choice of suitable animal models for respiratory infections to address the challenges associated with effective vaccine development.

Historically, vaccine development for influenza and many other viral infections has focused on the use of live or attenuated viruses [4], which require egg-based manufacturing systems that suffer from drawbacks of limited capacity and flexibility [3]. However, with recent developments in nanotechnology, materials science and advances in immunology, subunit vaccines have gained prominence [9]. Nanoparticles (NPs) based on synthetic polymers as well as virus-like particles (VLP) offer several advantages and ways to address many of the

challenges listed above. NPs provide the ability to deliver combinations of antigens in a single dose, target the vaccine formulation to specific cell types such as antigen presenting cells (APCs), allow encapsulation of either protein or DNA antigens or whole viruses, encapsulate domains that can elicit strong cross-neutralizing antibodies to protect against different strains [11], act as adjuvants or immunomodulators, allow for sustained antigen release, enable single dose delivery to enhance patient compliance, and potentially obviate the cold chain [12]. There are several lead nanoparticle vaccine candidates that have shown great promise in mouse and other pre-clinical models, but have failed in clinical trials [13]. Therefore the selection of appropriate animal models for vaccine development is a key requirement that can aid in the translation of promising vaccine technologies to the clinic for many of these diseases [14]. Both of these pathways for the development of more effective vaccines – nanoparticle-based vaccines and use of appropriate animal models for vaccine evaluation, are described in greater detail below. Taken together, advances in nanotechnology, combined with the right animal models for evaluating vaccine efficacy, has the potential to revolutionize vaccine development and translation for respiratory infections.

2. Nanoparticle-based vaccine delivery systems

Nanoscale materials have unique physicochemical properties in terms of their size, surface area, chemical composition and structure [15]. Unique properties of nano or microparticles (100–1000 nm size) have been widely utilized in drug and vaccine delivery [16–18]. Encapsulation of vaccine antigens (Ags) in biodegradable polymer-based NPs, or VLP-based strategies have proved to be powerful vaccine delivery systems [19, 20], especially to mucosal sites. In addition, targeting particulate vaccines to mucosal M cells helps in rapid internalization, processing and presentation of Ags by APCs to naïve T cells [21, 22]. Soluble Ags might be poorly immunogenic, but when entrapped in NPs, elicit strong immune responses as the NPs can act as adjuvants [23, 24]. NP size and surface characteristics can control their opsonization and clearance kinetics [25]. For optimal uptake of particulate Ags by APCs, the preferred size of NPs should be around 500 nm [26]. NP-based vaccines of approximately 500 nm size co-administered with toll-like receptor (TLR) ligands have been shown to induce long-lasting antigen-specific T cell response and production of high-affinity neutralizing antibodies [27]. Particles of up to 5 μm in size have been shown to protect encapsulated antigens and drugs from enzymatic or ionic degradation *in vivo* [17, 28]. Immunization using NP (200–600 nm)-based vaccines was found to enhance IFN-γ production and provided long-lasting antigen-specific humoral and cell-mediated immune responses [27, 29–31].

On the basis of physiological parameters such as hepatic filtration, tissue extravasation, tissue diffusion, and kidney excretion, the optimal size of NPs used *in vivo* varies. In addition to surface characteristics, the size of NPs plays a critical role in the bio-distribution of NPs [32]. NPs of 5–250 nm size range are found to be beneficial for drug delivery systems because of their ability to overcome multiple biological barriers and releasing a therapeutic load in the optimal dosage range [32]. However, when NPs of 50–500 nm size were delivered *in vivo,* high levels of agglomeration of the larger sized NPs was found in the liver [33]. Blood clearance of the smaller sized NPs is twice faster than larger formulations [32]. Orally delivered NPs of 20–40 nm are taken up readily by intestinal epithelial cells,

while NPs larger than 100 nm are taken up mainly by specialized follicular epithelial cells called M cells [34], which in turn deliver them to underlying APCs to initiate immune response [35]. In contrast, studies in rats using larger size polystyrene and poly(lactic acid) NPs (100–500 nm) showed uptake of particles exclusively by M cells [36–38].

2.1. Nanoparticle-based vaccine delivery to mucosal sites

Delivery of the vaccines to mucosal sites is ideal for eliciting appropriate immune responses to combat respiratory infections [39] because it mimics the entrance pathway of many of the pathogens [40]. Approximately 80% of the body's total immune cells are present at mucosal surfaces and mucosa-associated lymphoid tissues (MALT) [41]. The MALT is strategically located to orchestrate local immune functions against infections. Key immune cells in MALT involved in initiation of mucosal immunity are epithelial 'M' (Microfold, Membranous or Microvilli) cells and 'professional' APCs such as dendritic cells (DCs) and macrophages (Mφs) [42]. M cells are dedicated to sampling, capture and transcytosis of microorganisms and particulate Ags to underlying APCs in the MALT, and are thus considered as the principal targets of the mucosal vaccine delivery system [21].

However, a variety of factors limit the mucosal delivery of vaccines, and these include mucociliary clearance, presence of deteriorating enzymes, pH extremes, low permeation, and metabolic degradation. To overcome these limitations, mucoadhesive polymeric NPs can be designed to pass through the mucus barrier and thus are promising mucosal delivery vehicles for vaccines [21]. Particulate Ags administered directly to mucosal sites have an inherent affinity for mucosal M cells and APCs, and are phagocytosed passively by APCs [43]. Particulate Ags delivered through an intranasal route have been found to be sampled readily by M cells of the nasal associated lymphoid tissues (NALT) and are delivered to underlying APCs in the respiratory tract [44]. M cells are strategically located in the epithelium of the small and large intestines, tonsils and adenoids, and airways, involved in sampling of particulate antigens [45]. But presence of M cells is still not clear in the mucosal sites of the reproductive tract and the deep airways. However, cells with typical features of M cells have been reported in the nasal passage epithelium of mice, suggesting the NALT-independent mode of antigen sampling in the respiratory tract is also possible [46]. NPs protect entrapped protein Ags from protease-mediated degradation at mucosal surfaces, thus aiding in preserving intact Ags for long periods of time at mucosal surfaces, and facilitating extended availability for sampling by M cells and APCs [47].

Mucus membrane is a single layer of epithelial cells in the mucosa protecting the body from entry of extraneous substances. Mucus is a viscoelastic gel layer that protects the mucosa, and it is composed of crosslinked and entangled mucin fibers secreted by goblet cells and submucosal glands [26–28]. The bulk viscosity of healthy human mucus is typically 1,000–10,000 times higher than the viscosity of water. Mucosal delivery of vaccines is a challenge due to adverse physiological conditions at the mucosa such as mucociliary clearance, pH extremes, deteriorating enzymes, low permeation, and metabolic enzymatic degradation [48]. Therefore, mucoadhesive biodegradable polymer based NPs provide numerous advantages in delivery of vaccines and drugs [49]. They have desired chemistry to attach to mucus, inhibit the action of proteolytic enzymes, and modulate epithelial permeability once

they reach the mucosa [50]. Thus, such polymer derived NPs are highly useful in mucosal delivery of vaccines, especially in the respiratory tract [51].

NPs of 40–120 nm size engineered to display strong adhesive interactions with mucus and cell membranes are taken up by intestinal epithelial cells and facilitate the transport of conjugated substances into the lamina propria [52]. Similarly, mucus-penetrating NPs are also used in drug and gene delivery, as they avoid rapid mucus clearance mechanisms and provide targeted or sustained drug delivery for localized therapies in mucosal tissues. Neutrally charged hydrophobic NPs of approximately 200 nm size undergo rapid transport in thick sputum than anionic particles, suggesting that the surface charge of NPs is an important parameter in governing the rate of transport of NPs in mucus [53]. Diffusion of PLGA NPs coated with anionic DNA via the cationic surfactant dimethyl dioctadecyl ammonium bromide was found to imporve NPs transport rate by 10-fold in pig gastric mucus compared to slightly smaller, hydrophobic polystyrene particles [53, 54]. All these data suggest that charge, size, and chemistry of the particles play a critical role in mucosal delivery systems, and both mucoadhesive and mucus-penetrating NPs facilitate the delivery of cargo to mucosal tissues. Thus, appropriate selection of the polymer to synthesize nanoparticle delivery systems is critical to deliver drug or vaccine to mucosal tissues.

2.2 Synthetic and natural polymers for NP-based vaccine delivery

The use of next generation biomaterials to develop polymeric nanovaccine delivery vehicles offers several advantages over more conventional vaccine adjuvants (Alum, MPLA, MF59) including thermal stability, reduced reactogenicity, shelf-life stability of the payload, and ability to induce mucosal immunity with both antibody- and cell-mediated responses [55] [56–61]. By tailoring polymer chemistry and degradation kinetics, antigen release kinetics can be controlled and it is anticipated that the optimal nanovaccine will more closely mimic the immune response induced by the natural infection [12]. In this context, rapidly degrading (i.e., hydrophilic) nanoparticles may release antigen quickly and reduce antigen availability, impacting the ability of such nanovaccine formulations to induce sustained antibody responses [57]. In addition, the danger signals presented to the immune system by such formulations are weak as evidenced by waning profiles of pro-inflammatory cytokines [62]. Thus, hydrophobic and pathogen-mimicking nanovaccine formulations that enhance antigen availability and lead to the production of long-lived plasma cells more effectively prime the immune response and lead to long-lived protection [57]. Polymeric adjuvants can be further tailored to incorporate immunomodulatory properties [12]. A wide variety of biodegradable and biocompatible natural and synthetic polymers have proven to be useful for vaccine delivery and are approved by the US Food and Drug Administration (FDA) and European Medicines Agency [18, 28]. They include natural polymers like albumin, alginate, chitosan, collagen, cyclodextrin and gelatin; and synthetic polymers like polyesters, polylactides, polyacrylates, polylactones, polysulfones, polyanhydrides and poly(lactic-co-glycolic acid) (PLGA) [17, 63]. These polymers also act as adjuvants, but additional potent adjuvants in the vaccine formulations are usually necessary to boost the vaccine efficacy [64, 65]. Biodegradable NPs made of chitosan, PLGA, polyanhydrides, and liposomes have been in use to deliver candidate vaccines to mucosal sites [66].

PLGA is a widely used polymer in drug and vaccine delivery [48, 49, 67, 68], and it is non-toxic even at very high doses [67]. Until now, greater than 60% of patents filed on the development of PLGA NP vaccines involve delivery through the intranasal route [48]. NPs entrapping a killed influenza virus vaccine along with an adjuvant was administered intranasally to mice, rabbits, and pigs and found to elicit a protective immune response. In pigs, the intranasal route conferred better immunity compared to the intramuscular route of vaccination [69]. A single dose of intranasally delivered PLGA NP-entrapped *Schistosoma mansoni* Ags in mice elicited protective neutralizing antibody response detected in both the lungs and blood [70]. PLGA NP-entrapped bovine parainfluenza type 3 virus inoculated intranasally once elicited enhanced antibody response in mice [71]. A hallmark of PLGA NP-mediated vaccine delivery is its ability to induce enhanced and balanced Th1 and Th2 immune responses, essential for complete clearance of intracellular pathogens [72–74]. Therefore, to reinforce the efficiency of PLGA NPs mucosal vaccines, it is required to target the vaccine to mucosal M cells and DCs with the help of M cell targeting molecules, such as *Ulex Europaeus* Agglutinin-I (UEA), specific immunoglobulins, TLR ligands, etc. [44, 75, 76]. Intranasal vaccination of mice with PLGA NPs entrapping HIV peptides and UEA elicited enhanced and prolonged antibody and T cell response at both mucosal and systemic sites compared to vaccination by other routes [77]. UEA entrapped in PLGA NPs targets particles to M cells resulting in a 2–4 fold increase in specific antibody titers [77]. Surface anchored UEA on PLGA NPs entrapped with hepatitis B virus enhanced the vaccine targeting to M cells *in vitro.* And in orally vaccinated mice, significantly augmented SIgA and Th1 cytokines production was observed compared to NPs without M cell targeting agents [78]. In mice, PLGA NPs entrapped with hepatitis B, rotavirus, influenza, or parainfluenza viruses generated protective immune response when delivered to mucosal sites [69, 71, 79, 80].

PLGA NPs also mediate activation, maturation, and antigen presentation by APCs [81]. Since NPs facilitate sustained release of vaccine Ags, they mediate induction of robust B and T cell responses [82]. NP-based vaccines are capable of eliciting cell-mediated and humoral response in the lungs of mice [56–61]. PLGA NPs can provide slow release of antigens and can elicit robust effector and memory immune responses [83]. A recent study has shown that the duration of available vaccine Ags to effector T-cells (also to a lesser extent memory T cells) can control the magnitude of CD4 and CD8 T-cell responses, which require sustained antigenic stimulation for their maximal expansion [84]. PLGA NPs have been shown to promote cross-presentation of vaccine Ags, as the phagosome-disruptive properties of PLGA NPs facilitate delivery of Ags to the cytosol for MHC class I loading and CD8$^+$ T cell activation [85–90]; which in turn helps in the generation of memory T cell response and efficient clearance of invading pathogens [83]. The interaction of NPs with pathogen recognition receptors on APCs (especially B cells) can lead to affinity maturation and production of highly avid antibodies [91].

Polyanhydride-based nanoparticle vaccine delivery systems have been designed against several respiratory diseases, including influenza, pneumonia, anthrax, and pneumonic plague [92–105]. Polyanhydrides are biodegradable materials suitable for intranasal delivery in the form of micro or nanoparticles [106], and can enable sustained release kinetics of

encapsulated antigen, resulting in long-lived, high avidity antibody titers induced with otherwise suboptimal doses of antigen [97, 103, 107]. Polyanhydride nanoparticles have been shown to be a versatile vaccine adjuvant/delivery platform that can enhance the immune response to recombinant proteins [97, 103, 107]. Amphiphilic polyanhydride nanoparticles have been demonstrated to preserve the structure and antigenicity of recombinant proteins upon release [98, 108–112]. In addition to amplifying humoral immunity, polyanhydride nanoparticle-based vaccines (i.e., nanovaccines) activate APCs [105, 113, 114], have been shown to be immunomodulatory [97], and are capable of promoting cell-mediated immunity by expansion of antigen-specific memory CD8$^+$ T cells [115].

In addition to PLGA, and polyanhydrides, other polymeric systems, liposomes, nano-emulsions [8] and micellar delivery systems [116–118] have shown promise as NP-based vaccine delivery systems. Chitosan, a cationic mucoadhesive polymer, has shown efficacy in NP-vaccines for diphtheria and other infections [119, 120]. Chitosan NPs have shown an immune potentiating ability that is mediated by innate immune cells, in addition to enhanced production of IL-6 and IFN-γ [121]. Phagocytosed NPs induce activation and maturation of mouse APCs due to their inherent adjuvant properties [88]. In an earlier study, rapid uptake of chitosan NPs by APCs derived from mice followed by gradual release of Ags, and increased expression of co-stimulatory molecules and activation of DCs and antigen presentation by MHC class I and II molecules was observed [122, 123]. Phagocytosis of polystyrene latex microspheres by Mφs activate the signal transduction events in innate immune cells [124]. Other polysaccharides such as dextran derivatives [125], alginate [126] and starch [127] micro and nanoparticles have shown good macrophage uptake, high antibody titers, mucosal and systemic immune responses, and protection against challenge.

2.3 Virus-like particles for vaccine delivery

VLP are formed from assemblies of viral proteins that can effectively crosslink B cell receptors, exhibiting immunostimulatory properties of viruses, without the potential for infection [128]. VLPs are structurally diverse and functionally versatile and can trigger both arms of the immune response and are taken up by APCs. The licensed human papilloma virus vaccine is based on VLP technology [129]. Different antigens can be displayed on the surface of VLPs. VLPs can be commercially produced in various host systems such as *E. coli*, yeast, insect cell cultures and CHO cells, enabling facile production of efficient vaccines for diseases such as influenza in several expression systems [3]. Several VLP-based vaccine candidates are currently in clinical trials (Fig. 1) [129]. VLPs delivered intranasally have been shown to produce high lung mucosal antibody titers without additional adjuvants [130]. However, prediction of functional epitopes and the ability to present native 3D structures of the epitopes on the carriers are challenges that need to be overcome. Enhanced molecular modeling and computational design efforts to help overcome these challenges, and biomolecular engineering advances to present large antigens on VLPs are urgently needed to reduce vaccine development time and high vaccine costs associated with VLP vaccine production [129].

3. Animal models for human respiratory disease studies and vaccine development

Identifying suitable large animal surrogates for understanding the efficacy of novel candidate vaccines as well as insights on human disease pathogenesis, is likely to have high impact for translational research to improve human health, as the predictive validity of preclinical animal models has been disappointing [13]. In some disease areas, greater than 90% of promising new treatments failed in clinical trials [131] because the targeted pathogenic process can be expressed differently in the animal model as opposed to humans. In general, there are fundamental immunobiological differences between young inbred mice and human patients [132]. Inbred mice also lack genetic diversity and the exposure to environmental pathogens. Large animals models, such as non-human primates (NHPs) and large farm animals can potentially provide better predictive validity [13]. Basic biological research using murine models has brought enormous knowledge following the development of technologies for production of monoclonal antibodies, and transgenic and gene knockouts. However, many recent studies have questioned the utility of mouse models for understanding certain diseases, vaccines, and drug screening efforts [133–136]. For example, mice are resistant to the development of classic TB disease, minimizing their utility for evaluation of TB vaccines [137]. There are substantial differences in pathogenesis in RSV mouse models versus in human patients [138].

The route of administration (intranasal, subcutaneous, etc.) has an impact on the choice of the animal model and the efficacy of the vaccine. The mucosal immune inductive sites in the respiratory tract are concentrated in aggregates of MALT adjacent to mucosal surfaces of the nasal cavity, nasopharynx, larynx, trachea and bronchus; these are called NALT, lymphoid tissues of the Waldeyer's ring in the nasopharynx (tonsils), larynx and trachea associated lymphoid tissues (LALT and TALT), and bronchus-associated lymphoid tissues (BALT) [41, 139]. Unlike in rodents, in farm animals (such as pigs, cattle, sheep, and horses), tonsils and LALT are well developed, and LALT is on the *epiglottis* in the *vestibulum laryngis* and the *plica aryepiglottica* [140] present in the form of lymphatic nodules [139].

3.1. Non-human primate models

NHPs are excellent preclinical models for vaccine development and translation because of their evolutionary proximity to humans, similarities in host defense components as well as in clinical and pathological presentation of disease, and their large size allow for frequent blood sampling [141]. There is an expanding genome sequence data for various NHPs, leading to the greater use of omics-approaches to profile NHP responses to viral infections and vaccinations [142].

Asian macaques are one of the most frequently used NHPs in infectious disease research. NHPs and the macaques species in particular, are naturally susceptible to TB infection and display all the typical features of human TB, including identical histological structure of TB granulomas [143]. NHPs such as chimpanzees have also been used, but suffer from ethical constraints [144]. Unfortunately, all of the existing animal models for TB other than NHPs suffer from specific drawbacks and fail to mimic the human disease perfectly [137].

Macaque models have also aided research related to H5N1 and H1N1 pandemic influenza disease models [142] and are emerging as viable animal models for MERS-CoV [145].

African green monkeys (AGM) also provide a good model for influenza studies [146, 147] as the distribution of sialic acid receptors is similar to that in humans, and live attenuated pandemic influenza virus vaccines were found to replicate similarly in these monkeys as in humans [147]. AGMs also provide a more uniformly lethal model of henipavirus infections compared to the more variable response seen in squirrel monkeys [148] and are considered the gold standard for testing the efficacy of vaccines against henipaviruses. They have also shown utility as models for evaluating countermeasures against RSV infections [149].

RSV has been found to cause severe respiratory failure, especially in infants. There is no vaccine available for RSV, partly because of the difficulties associated with the lack of a suitable animal model to study the pathophysiology of the disease and evaluate vaccines [150]. Infant baboon models of RSV have shown clinical and pathological changes similar to those observed in human infant patients and offer a promising model for the disease [138]. In contrast, chimpanzee (and other monkey models) experience only mild infections and do not experience the lower respiratory tract infections seen in human infant RSV [138]. Since RSV vaccines need to elicit mucosal immunity at both upper and lower respiratory tracts to effectively prevent RSV infection, the baboon model provides advantages over the chimpanzee model for RSV [151]. Baboons have also been shown to be excellent animal models for pertussis, with 100% inoculated baboons developing clinical pertussis, compared to only 25% of rhesus macaques [152].

SARS-CoV studies have been limited in NHP models, with a few studies involving African green monkeys, marmosets, cynomolgus and rhesus macaques. Variability in the results points to a need for more studies with large sample sizes to draw meaningful conclusions [153]. Cynomolgus macaques are however preferred for Ebola studies since the immunologic and physiologic responses are thought to be similar to those in humans [154], and they are also good models for anthrax infections [155]. Conflicting results have been reported with respect to the efficacy of pneumonic plague vaccines based on the F1-V fusion protein when evaluated in cynomolgus macaques and African green monkeys [156, 157]. While protection was demonstrated in the macaques, there was considerably less success in the African green monkey, once again underlining some of the complexities associated with using NHPs as preclinical animal models for vaccine efficacy against respiratory infections.

However, the cost, availability, need for specialized personnel and facilities, and ethical concerns prevent large-scale use of NHP models for vaccine development studies [13] and limit their use. The U.S. federal government recently announced that captive chimpanzees will also be protected under the Endangered Species Act. Moreover, because species such as the rhesus macaques are genetically diverse and outbred, sufficient statistical power requires the use of larger animals and greater numbers of replicates [142].

3.2 Small animal models

Due to commonalities in the course of clinical infection with TB in guinea pigs and humans, they are commonly used for vaccine evaluation. However, there is a limited resource of

immunological reagents for quantitative evaluation of the immune responses in this model [137] compared to mice. Guinea pigs were also one of the earliest models to be used for henipavirus infections, but variability in results led to reduced interest in this model [158]. Cotton rats are commonly used models for RSV and other respiratory infections [159] such as metapneumovirus infections [160]. RSV replication occurs in lower airways and the virus is present in both the upper and lower respiratory tracts, leading to pneumonia [161]. Golden Syrian hamsters are good animal models for SARS-CoV because their respiratory tracts enable virus replication and can elicit good neutralizing antibody response [153]. These hamsters have also been used for henipaviruses to study transmission and pathogenesis [162]. Rabbits are relatively resistant to *Mycobacterium tuberculosis*, but are susceptible to *M. bovis*, producing pulmonary cavities like humans [137]. They are also a potential model for MERS-CoV infections [163] and for anthrax [164, 165]. In many of these cases, however, limited immunological reagents and techniques available compared to mouse models, which limit studies of immunological pathways.

Ferrets are one of the earliest and the most widely used model for influenza [166] because they are susceptible to a wide range of human isolates without prior adaptation [167]. They have similar influenza receptor distributions to that of humans in the respiratory system and exhibit upper respiratory infection patterns and clinical symptoms similar to that in humans [168]. Moreover, ferrets are the only mammalian model suitable for studies of both pathogenesis and transmission [169] of influenza viruses [167], as they can transmit human viruses to naïve animals by direct contact or respiratory droplets [170, 171]. In addition, histochemical studies in ferrets correlate well with differences in pathology between seasonal [172] and pandemic viruses [173] seen in humans leading to similarities in the different disease outcomes seen in both species [174]. An aged ferret model has also been developed to investigate influenza morbidity in the elderly population [175]. Ferrets have also been shown to be good models for pathogenesis of other respiratory viruses such as paramyxoviruses such as henipaviruses [166] as they develop the full spectrum of diseases seen in humans. However, unlike in hamsters, no correlation between challenge dose and clinical outcome was seen in ferrets with henipavirus infections [176]. In the case of coronaviruses, there was variability in the infection and presentation of disease symptoms for SARS-CoV in a ferret model [177, 178]. Ferrets and other small animal models were found not to be susceptible to MERS-COV and do not enable replication of the virus, restricting the efficacy evaluation of MERS vaccines in small animal models [153, 179]. The sequencing of the ferret genome opens new avenues, but the greatest disadvantage of ferret models remains the limitation of species-specific reagents.

Fruit bats are natural reservoirs for several viruses such as henipaviruses, Ebola, etc., and the viruses usually cause asymptomatic infections in the hosts. However, viruses, after crossing the species barrier turn virulent. They have been used in the generation of neutralizing antibodies and the mechanisms by which viral replication in bats is controlled are being investigated [158]. Cats are also susceptible to henipavirus infections and become clinically ill, and can be used to assess the potential of vaccines [158].

3.3 Large farm animal models

Although rodents and primates are taxonomically closer than pigs, the divergence of all three occurred over only half a million years ago, such that taxonomic relationships are largely irrelevant [180]. Calibrated comparisons show that the rodent genome has changed faster since that divergence, resulting in greater identity between pig and human than human and mouse [181, 182]. The availability of the swine genome sequence (assembly 10.2) [183] and new transgenic and gene knockout capabilities [184–188] have together increased the use of pigs in biomedical research. The pig is a better suited biomedical model than rodents in many respects, due not only to its size but also to its anatomic, physiologic, and genomic similarities to humans [189, 190]. In addition, like humans, the pig is monogastric, omnivorous, and an outbred species, and it shares a comparable immune system. For all these reasons, the pig is considered as an important large animal model species for human health research including obesity, reproductive, cardiovascular and nutritional disorders, infectious diseases, and vaccine research [191–194].

In particular, the pig is considered as an ideal animal model for studies related to respiratory infections because, its lung has marked similarities to that of humans in terms of the tracheobronchial-tree structure, airway morphology, abundance of airway submucosal glands, and in production of cytokines and chemokines [195–197]. The electrophysiological properties of the airway epithelium and submucosal glands of the pig resembles that of humans [198–200]. Therefore, the pig has been used for studies on chronic bronchitis and cystic fibrosis [201, 202]. Unlike rodents, the pig is a natural host for wide range of influenza viruses, because its airway epithelial cells contain receptors preferred by both avian and mammalian influenza viruses [203–205]. In addition, cytokines profiles in influenza infected pigs and humans are highly comparable [206, 207]. Pigs also offer advantages for cutaneous delivery of vaccines compared to mice because of larger surface areas, possibility of repeated sampling and similarities between human and pig skins, compared to those of mice [208]. Therefore, pigs represent an excellent, but underutilized animal models for respiratory infections such as influenza [209].

The pig is an ideal model for NP-based vaccine studies. NP-based delivery vehicles for vaccines (i.e., nanovaccines) and drugs have been extensively evaluated in mouse models. However, there are several limitations in translating novel rodent findings to improve human health [210]. Therefore, the pig may serve as a useful large animal model for nanovaccine research. Rapid uptake of PLGA NPs entrapped UV-killed porcine reproductive respiratory syndrome virus (PRRSV) (NanoPRRS) by porcine Mφs and DCs, translocation of viral Ags into their endosomal compartment, and increased expression of the activation marker CD80/86 was observed [211]. Studies have demonstrated immune potentiating activity of NPs in mice, pigs, and macaques; but immune correlates were not evaluated in detail in vaccinated and virus challenged animals [212]. In a pre-challenge study, NanoPRRS vaccine was administered intranasally to pigs and observed significantly increased frequency of CD8+T cells, Th/memory cells, with increase in the secretion of innate (IFN-α), proinflammatory (IL-6), and Th1 (IFN-γ) cytokines [211].

In pigs, a single dose of NanoPRRS vaccine administered intranasally and challenged with a virulent heterologous virus, showed reduced clinical PRRS symptoms with decreased gross

and microscopic lung lesions and reduction in viremia (but not viral load in the lungs). Immunologically, enhanced virus-specific IgA in the lungs, increased virus neutralization (VN) titers (3 $\log_2$) and high levels of IFN-γ production were detected [211, 213]. In a similarly vaccinated and homologous virus challenged pigs, viremia was cleared early with augmented antibody and cytokine responses [214]. To further improve the efficacy of NanoPRRS, the vaccine was co-administered with a potent adjuvant (*M. tuberculosis* whole cell lysate, *M. tb* WCL) that we identified earlier [205, 215], and observed complete clearance of detectable infectious challenged heterologous PRRSV (genetically 15% different) from the lungs [73, 74]. Immunologically, increased VN titers (4 log2) and IFN-γ^+ lymphocytes were observed [74, 213]. In yet another study, coexpressed GP3 and GP5 of type 1 PRRSV coated on chitosan NPs was administered twice intramuscularly with Quil A adjuvant and challenged with a homologous virus, results suggested one log reduction in the viral RNA load in the blood and lungs. Immunologically, increased IFN-γ production was associated with amplified CD4$^+$ and CD8$^+$ T cell frequencies and lymphocyte proliferation with VN titers of 4 $\log_2$ [216]. This study confirmed the advantages of NP-mediated delivery of viral vaccine to pigs.

Intranasal delivery of biodegradable polymeric NPs encapsulating viral vaccines has shown huge promise in eliciting enhanced breadth of cross-protective immunity in rodent models and in pigs [73, 74, 211]. PRRSV induced VN activity is weak and delayed [217, 218], but in NanoPRRS vaccinated pigs VN titers were detectable quite early and steadily increased [83]. Inactivated vaccines generally induce Th2 responses [219], but NP-based vaccines drive either balanced Th1-Th2 or Th1-biased responses [77]. In NanoPRRS administered pigs vaccinated intranasally, balanced Th1-Th2 responses were observed, indicated by increased IgG1 and IgG2 production and enhanced IFN-γ and IL-4 production [73, 74]. Recently, PLGA NPs entrapping five conserved IAV peptides delivered intranasally as a mist to pigs enhanced the breadth of the T cell response, with reduction in detectable challegned infective lung virus load, but it did not boost the VN titers (Hiremat and Renukaradhya 2015, manuscript submitted). To improve the efficacy of inactivated swine influenza virus vaccine, in an ongoing study PLGA NPs were entrapped with an inactivated H1N2 (δ1-lineage) virus and delivered as a mist intranasally to pigs, and our results indicated the complete clearance of challenged infective heterologous zoonotic H1N1 (γ-lineage) virus from the lungs of 80% of vaccinated animals (Dhakal and Renukaradhya 2015, unpublished data). All these studies have suggested the induction of enhanced cross-reactive immune responses by the NPdelivery system in pigs.

While pigs can be effective animal models for respiratory vaccines, other large farm animals such as cattle and lambs have also been explored for specific infectious diseases such as RSV. Cattle are natural hosts for bovine RSV [220], which does not infect humans, but F glycoproteins in bovine RSV and human RSV have 80% amino acid sequence identity along with some cross-reactive neutralizing epitopes [14]. The spectrum of diseases caused in calves overlaps with human RSV disease, making it a viable model [161]. Since RSV mainly affects infants and young children worldwide, similarities in developmental, structural, physiological and immunological features between newborn lamb lungs and human infants make it a good model for assessment of potential RSV vaccines [221]. In

rodents, the alveolar development occurs postnatally, while in lambs and humans, it occurs prenatally, allowing for a better model for preterm human infants. In addition, the ovine lung is susceptible to secondary bacterial infections that commonly occur with RSV infections. The ethical issues associated with use of NHPs can also be circumvented with the use of lamb models for RSV.

A summary of various animal models that have been used for different respiratory infections is provided in Table 1.

4. Conclusions and Future Prospects

There is an urgent need to develop alternative strategies to use of viral vaccines. Nanoparticle-based subunit vaccines and VLPs provide promising alternative approaches that are gaining significant momentum. The move away from viral vectors can potentially accelerate the production of vaccines, enable single dose delivery, immunomodulation, cross-protection against various strains of the pathogen and enable targeting to specific cell types. Various biodegradable polymers, both natural and synthetic, are being explored for encapsulation of the antigens for vaccine development. VLPs can provide the advantages of viruses without many of the associated challenges such as infections. However, the choice of suitable animal models for testing the efficacy of these new vaccines is very important aspect to enable successful translation of these experimental vaccines to the clinic. While mice models are easier to implement, and relatively inexpensive, they might not provide clinically relevant answers in many cases. The discussion presented herein reveals that there is no universal animal model for respiratory infections. Depending on the questions to be answered, the route of administration of the vaccine and the scale of the study, animal models should be carefully chosen to provide reliable testing of vaccine countermeasures, which can then enable successful translation to the clinic [222].

Acknowledgments

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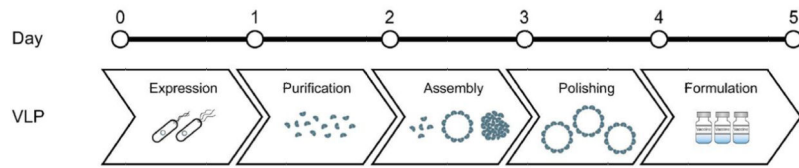
Fig. 1.
Microbial platform for VLP-based vaccines (Adapted from [129] with permission from John Wiley and Sons).

Table 1

Various animal models used in different respiratory infectious disease research to evaluate countermeasures

Animal Model	Respiratory Infectious Disease
Macaques	Influenza [142], TB [143], MERS-CoV [145], SARS-CoV [153], Ebola [154], Anthrax [155], pneumonic plague [156]
African green monkeys	Influenza [146,147], henipavirus [148], RSV [149], SARS-CoV [153], pneumonic plague [157]
Baboons	RSV [138, 151], pertussis [152]
Guinea pigs	TB [137], henipavirus [158]
Cotton rats	RSV [159, 161], metapneumovirus [160]
Golden Syrian Hamsters	SARS-CoV [153], henipaviruses [162]
Rabbits	MERS-CoV [163], anthrax [164,165]
Cats	Henipaviruses [158]
Ferrets	Influenza pathogenesis and transmission [166–175], henipaviruses [166]
Pigs	Influenza [203–209]
Cattle	RSV [220,161]
Lambs	RSV [221]