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Ferrets as a Mammalian Model to Study Influenza Virus-Bacteria Interactions

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Abstract

Ferrets represent an invaluable model for the study of influenza virus pathogenicity and transmissibility. Ferrets are also employed for the study of bacterial pathogens that naturally infect humans at different anatomical sites. While viral and bacterial infection studies in isolation using animal models are important for furthering our understanding of pathogen biology and developing improved therapeutics, it is also critical to extend our knowledge to pathogen coinfections in vivo, to more closely examine interkingdom dynamics that may contribute to overall disease outcomes. We discuss how ferrets have been employed to study a diverse range of both influenza viruses and bacterial species and summarize key studies that have utilized the ferret model for primary influenza virus challenge followed by secondary bacterial infection. These copathogenesis studies have provided critical insight into the dynamic interplay between these pathogens, underscoring the utility of ferrets as a model system for investigating influenza virus-bacteria interactions.

Keywords

ferret; influenza virus; bacteria; pathogenesis; transmission; coinfection

Seasonal influenza epidemics occur annually, claiming 290 000 to 650 000 lives globally to influenza-associated respiratory causes, and an estimated 99 000 to 200 000 lives to lower respiratory tract infections directly caused by influenza virus [1]. An estimated 9.2 million to 35.6 million influenza-related illnesses were recorded during 6 influenza seasons

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over 2010–2016, including 140 000 to 710 000 influenza-related hospitalizations in the United States alone [2]. The severity of a primary influenza infection, both in seasonal and pandemic outbreaks, can be exacerbated by secondary bacterial infections that present either concurrently during the viral infection or after the virus is cleared by the host immune system [3]. In support of this, a majority of autopsies performed on victims of the 1918 influenza A virus (IAV) pandemic indicated bacterial pneumonia as the cause of death [4]. The mammalian respiratory tract has a wide diversity of bacteria; the most frequently occurring species include *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* [3]. Respiratory tract infections with *S. aureus* alone were estimated to be associated with over 1 million deaths in 2019, highlighting the substantial disease burden posed by bacterial species [5]. As such, there is a need to study the interplay between influenza viruses and commensal bacteria. However, their study can be a challenge given the complexity of these interactions and the diversity of the mammalian microbiome.

In vitro studies researching viral-bacterial interactions have indicated that interactions between eukaryotic viruses and bacteria in different host environmental niches can be synergistic or antagonistic. For example, poliovirus infectivity was enhanced by exposure to bacteria or their surface polysaccharides, including lipopolysaccharide (LPS) and peptidoglycan (PGN) [6]. Furthermore, both gram-negative and gram-positive bacteria promoted reovirus infection by enhancing virion thermostability during an environmental insult through the bacterial envelope components LPS and PGN, respectively [7]. Binding of the H1N1 IAV, A/Puerto Rico/8/1934 (PR8), to gram-positive and gram-negative bacterial pathogens of the respiratory tract facilitated increased adhesion of these bacterial pathogens to human respiratory cells [8]. Collectively, these studies highlight the capacity for interkingdom synergism between viruses and bacteria. In contrast, LPS from a gastrointestinal tract bacterial isolate (*Escherichia coli* O111:B4) significantly decreased the stability of a human IAV strain in a temperature-dependent manner, by binding to and altering the morphology of the virion envelope, highlighting the potential for antagonistic virus-bacteria interactions [9].

These in vitro studies have inherent limitations, as investigations of viral pathogenicity, transmissibility, and tropism are characterized by complex interactions between multiple tissues, which can be challenging to model outside of a living host. In vivo models to study viral-bacterial interactions can be invaluable to address these limitations. Many small mammalian models have been used to study respiratory viruses including mice, ferrets, and guinea pigs [10], while bacterial pathogenicity and transmissibility have been studied in a range of species including mice, ferrets, and chinchillas [11]. Among these, ferrets represent a robust model in laboratory settings for studying many bacterial and viral pathogens, both in isolation and in coinfection scenarios. Here, we review use of the ferret in modelling IAV and respiratory bacterial infections, secondary bacterial complications following primary IAV infection, and coinfection with both pathogens, with an emphasis on studies evaluating IAV and bacterial pathogens of the respiratory tract.

MODELING HUMAN IAV INFECTION IN FERRETS

Ferrets have been employed extensively for the study of influenza virus infection since the 1930s. Lung physiology and to-pography of the upper respiratory tract in ferrets closely resembles that of humans, as does the distribution of sialic acid receptors throughout the upper and lower respiratory tract of ferrets [10]. For these reasons, IAVs isolated from both human and zoonotic sources, as well as influenza B viruses isolated from humans, can infect ferrets without prior host adaptation, providing a low-cost small mammalian model to study pathogenesis and disease progression, transmission, and host immune responses to a wide range of influenza viruses [12]. Clinical symptoms of influenza infection in humans (which include sneezing, nasal discharge, fever, lethargy, anorexia, and weight loss) are closely recapitulated in ferrets; a similar scope of symptomology is not present in other small animal models such as mice and guinea pigs [10], further illustrating the utility of the ferret model for IAV research applications.

While both seasonal and zoonotic IAV can lead to productive infection in ferrets postinoculation (typically following a high dose of virus administrated intranasally in a liquid inoculum), the severity of disease can vary widely depending on the virus examined. Seasonal IAVs are typically restricted to replication in respiratory tract tissues (primarily the nasal passages), with spread to the lungs and selected extrapulmonary tissues (such as the intestinal tract) possible depending on the strain used to infect (Figure 1). In contrast, selected zoonotic strains, such as highly pathogenic avian influenza viruses of the A(H5N1) subtype, can cause robust infection in both the upper and lower respiratory tract, and result in extrapulmonary spread to multiple organs, including the brain [13]. While seasonal IAV is generally associated with a transient, mild infection in ferrets following standard intranasal inoculation, van den Brand et al showed that ferrets could be used to model moderate to severe pneumonia caused by a range of seasonal IAV when inoculated by the intratracheal route [14]. This group also showed that A(H1N1) viruses from the 2009 pandemic caused more severe pneumonia in ferrets than A(H1N1) influenza viruses circulating pre-2009 [15], illustrating the potential for strain-specific variance in viral pathogenesis in this species.

Beyond modeling viral pathogenesis, ferrets have become invaluable for studies of IAV transmission; data from ferret experiments inform risk assessment models generated by the Centers for Disease Control and Prevention Influenza Risk Assessment Tool (CDC IRAT) and the World Health Organization Tool for Influenza Pandemic Risk Assessment (WHO TIPRA) [16, 17]. Virus transmissibility is routinely assessed in laboratory settings using 2 models: a direct contact transmission (DCT) model, and a respiratory droplet transmission (RDT) model. In the DCT model, the virus-infected animal is housed in the same cage with a naive ferret. Cohousing infected and uninfected ferrets causes sharing of bedding, food, and water, and breathing the same air, representing the most permissive mode of transmission because it promotes transmission through any part of the shared space between ferrets. In the RDT model, inoculated and naive ferrets are separated by a partition that prevents direct contact but allows exchange of air between animals. This model represents the most restrictive evaluation of virus transmission, as many viruses that demonstrate the capacity for transmission when ferrets are placed in direct contact are not transmitted by the respiratory droplet or aerosol route [18]. Seasonal IAVs generally transmit efficiently in both

settings. Zoonotic strains can vary widely; viruses with enhanced binding to α−2,6 sialic acids are more prone to transmission in DCT models, with selected strains transmitting with varying degrees in RDT models.

As discussed above, primary infection with IAV is often studied using serologically naive ferrets, but this model is also employed to study subsequent reinfection with homologous and heterologous IAV strains. As in humans, repeated infection with seasonal IAV in ferrets can result in cross-protective immunity, which significantly limits infection with antigenically distinct viruses. In support of this, Laurie et al showed that prior infection with both antigenically related and unrelated viruses provided protection from subsequent infection or modified the infection kinetics of the challenge virus, with multiple prior infections associated with both reductions in viral replication and onward transmissibility of the challenge virus [19]. Importantly, this study showed that coinfection was a consistent outcome in ferrets when exposure to both influenza viruses occurred within 3 days. Coinfections with IAV could give rise to novel reassortants, so understanding their true frequency and likelihood of occurrence is necessary for public health reasons [20]. Ferrets previously exposed to seasonal influenza H3N2 virus exhibited stronger immune protection and transmission was blocked following subsequent challenge with homologous or heterologous virus [21]. Collectively, these studies support that the ferret is well suited to study both primary influenza virus infection and transmission, in addition to more dynamic coinfection/repeated exposure events.

MODELING HUMAN RESPIRATORY BACTERIAL INFECTION IN FERRETS

The natural susceptibility of ferrets to human pathogens extends beyond their use as a valuable model to study viruses, to also encompass a range of bacterial pathogens [22]. As in humans, numerous bacterial pathogens are present in ferrets depending on the anatomical site examined (Figure 1). Common respiratory bacterial pathogens of ferrets include species of *Streptococcus* and *Pseudomonas, Klebsiella pneumonia*, *E. coli*, and *Bordetella bronchiseptica*. Common gastrointestinal bacterial pathogens include species of *Salmonella*, *Clostridium perfringes* type A*, Campylobacter jejuni*, and *Lawsonia intracellularis* [23]. While the majority of these bacterial pathogens are common to both humans and ferrets, there are bacterial species specific to ferrets, such as *Helicobacter mustelae* [24]. *H. mustelae* infection occurs at birth, and unless eradicated, will remain a lifelong infection, making it a necessary consideration of coinfections in the gastrointestinal tract when employing this species [23]. Discovery of a novel *Mycoplasma* species in ferrets further underscores the susceptibility of ferrets to a diverse range of bacterial pathogens beyond what have been previously identified in humans [25]. As with all biomedical models, appropriate biosecurity and husbandry practices are necessary to prevent unintentional infection or disturbance of host microbial homeostasis in ferrets that may confound research or pose a risk to the health of laboratory personnel.

Similar to influenza viruses, numerous respiratory and gastric bacterial pathogens exhibit parallel clinical outcomes between ferrets and humans [24, 26]. As such, ferrets have been employed as an in vivo model to study a range of bacterial pathogens of public health importance, or to recapitulate bacterial involvement present in different clinical diseases

of humans. These studies include establishment of ferret models to study pathogenicity and transmissibility of *Mycobacterium bovis*, *M. tuberculosis*, and *M. avium* [27–29]. *H. mustelae* has been used to model human infection of *Helicobacter pylori* [24, 30, 31] as, similar to human infection with *H. pylori*, ferrets infected with *H. mustelae* developed ulcer formation whereas naive ferrets did not [24]. Ferret models of chronic obstructive pulmonary disease (COPD) presented with overt bacterial infection around the airways, similar to humans [32]. Furthermore, ferrets with cigarette smoke-induced COPD inoculated with nontypeable *H. influenzae* presented with significant morphological and functional declines in lung function in comparison to control ferrets that were exposed to uncontaminated air [33], modeling clinical symptoms and pathophysiologies associated with COPD in humans. While challenge studies with live bacterial pathogens in this species are fewer in number compared with respiratory viral pathogens, these studies nonetheless support the need and utility for ferrets in modeling bacterial pathogens associated with human infection.

Beyond traditional challenge studies, ferrets have been employed to examine a range of host-pathogen interactions with bacteria. Inoculation of ferrets with radiolabeled staphylococci (*S. aureus, S. epidermidis*, and *S. saprophyticus*) supported a role for respiratory mucins in efficient adherence of respiratory bacterial pathogens to the nasal mucosa [34]. Ferrets can serve as an effective in vivo model to investigate the role of virulence determinants of bacterial pathogens, such as the role of *S. aureus* alpha toxin in modulating disease severity in mammals [35]. Similar to the influenza virus challenge models described above, these ferret challenge models can be employed to assess the efficacy of vaccine and therapeutic agents against bacterial populations [27, 35]. Collectively, these findings support the ferret model as beneficial to human research with bacterial pathogens of both the respiratory and gastrointestinal tract, and the need to consider perturbations of these populations when examining viral pathogens, such as influenza viruses, which can replicate at both sites (Figure 1).

MODELING BACTERIAL COMPLICATIONS AND TRANSMISSION FOLLOWING A PRIMARY INFLUENZA VIRUS INFECTION IN FERRETS

While relatively few, key studies conducted in ferrets to model interkingdom interactions between influenza viruses and bacteria have provided valuable insight into influenza-bacteria coinfection dynamics and how those interactions modulate severity of disease. These studies have largely investigated the association between a primary influenza virus infection and a subsequent bacterial infection in the ferret model by first inoculating sex-and age-matched ferrets with influenza virus, followed by a bacterial challenge 2 to 5 days after viral challenge (Table 1). Typically, ferret nasal wash specimens are collected prior to and serially during viral and subsequent bacterial challenge, with viral and bacterial titers obtained from these specimens as a measure of pathogenicity. Donor and contact ferret nasal washes are obtained to quantify viral and bacterial titers, to assess pathogen transmissibility between hosts. Clinical correlates of disease are assessed in both donor and contact animals by monitoring temperature and weight loss in ferrets daily.

Research encompassing viral-bacterial interactions within a living host are inherently complex, due to the presence of multiple infectious agents at play coupled with the host immune system. Studies of this nature performed to date typically employ a high dose (10^5–10^6 infectious units) of influenza virus for primary challenge, and a high concentration (10^5–10^7 colony forming units) of bacteria for secondary challenge, although the volumes of each may vary between studies (Table 1). As the majority of studies conducted employ only a single viral and bacterial strain, identifying the contribution of strain-specific differences between studies can be challenging. Most studies are conducted with young (<1 year old) ferrets, but selected studies have employed juvenile or neonate animals [36, 37]. When assessed, viral titers are typically reflective of infectious virus loads in specimens examined. However, bacterial loads are variably reported in these studies, based on culture, molecular detection, bioluminescent signals, or a mixture of these approaches (see references in Table 1). While less frequently examined, variability between groups in experimental conditions to assess transmissibility, and methodology employed to assess immune responses and histopathological alterations postinfection, further complicates direct extrapolation of findings between different studies. Despite this experimental- and protocol-based variation, identification of trends across independently performed studies are still possible, as discussed below.

Most of these copathogenesis studies have shown an increase in severity of clinical symptoms and immunological parameters, including higher white blood cell counts and histopathologic abnormalities in the upper respiratory tract of ferrets that received both influenza virus and bacterial challenge, compared to those that received influenza virus or bacterial challenge only [37, 38] (Table 1). During the acute phase of infection, higher bacterial titers were detected in nasal wash specimens obtained from ferrets that received both influenza virus and secondary bacterial challenge across multiple studies, with bacterial colonization in the nasopharynx [37–39]. Interestingly, reduced viral titers have been observed in nasal washes of ferrets with a secondary pneumococcal infection relative to challenge with influenza virus alone [40]. Furthermore, a majority of animals exposed to both influenza virus and bacterial challenges developed upper respiratory tract complications, including sinusitis or otitis media. Importantly, these interactions can be strain specific, with A(H3N2) viruses associated with greatest respiratory tract complications, followed by A(H1N1) and influenza B viruses, suggesting that viral strain-specific differences can regulate the development of secondary bacterial complications in mammalian hosts [37]. Primary influenza infection also augmented the incidence and severity of secondary bacterial disease in a pneumococcal strain-specific manner. For example, secondary bacterial complications had a higher association with an invasive pneumococcal strain, BHN97, following A(H3N2) influenza challenge than with BHN54, a carrier isolate of pneumococcus [41]. Taken together, these studies emphasize the importance of how differences in both viral and bacterial strains can impact disease outcome in vivo.

Beyond studies of pathogenicity, ferrets have been used to model bacterial transmission following primary influenza virus infection, typically employing the gram-positive bacterium *S. pneumoniae* (pneumococcus) following experimental intranasal challenge of donor ferrets with human influenza strains. These studies have reported higher bacterial

titers in nasal wash specimens obtained from ferrets with a preceding influenza infection than ferrets with bacterial challenge alone. Prior influenza infection in donor ferrets increased pneumococcal transmission to previously naive contact ferrets in both DCT and RDT models relative to donors who were not previously infected with influenza virus; bacterial transmission was further augmented in both transmission settings when contact animals had a prior influenza virus infection [39], supporting that antecedent influenza challenge amplified secondary pneumococcal transmission in the ferret model. Similar to studies assessing viral-bacterial pathogenicity, bacterial strain-specific differences contributed to changes in secondary pneumococcal infection and transmission in ferrets after a primary influenza challenge [39]. Prior work by Rowe et al identified that key bacterial factors that play a role in secondary pneumococcal transmission in ferrets following a primary influenza virus infection included genes involved in metabolism and transcriptional regulators [41]. Taken together, these studies support that primary influenza virus infection can often predispose the mammalian respiratory tract to secondary bacterial coinfection, with complex and manifold mechanisms by which influenza promotes these secondary infections.

MOVING FORWARD: NOVEL AND NEEDED APPROACHES TO STUDY INFLUENZA VIRUS-BACTERIA INTERACTIONS IN FERRETS

Due to the versatility of the ferret model, this species has been used to model in vivo interactions between influenza viruses and *S. pneumoniae* beyond the experimental designs presented above. For example, reduction of nasopharyngeal bacteria using a topical antibiotic application on donor ferret nostrils abolished transmission of an IAV to contact ferrets [42]. Thus, blocking influenza virus transmission by antibiotic-mediated reduction of host bacterial flora could serve as a novel approach to control influenza infection that remains to be investigated. In agreement with the dynamic role both viruses and bacteria contribute in the context of transmission of both pathogens, aerosols expelled from coinfected ferrets were recently shown to contain both live IAV and *S. pneumoniae* [43]. Further illustrating the importance of microbiome homeostasis to prevent influenza-bacteria coinfections, primary influenza virus infection in ferrets resulted in microbial dysbiosis in the ferret upper respiratory tract during the acute phase of infection; microbial populations were restored to preinfection levels following ferret convalescence [44]. There is a need to better understand these multifactorial aspects of influenza-bacterial coinfections in the respiratory tract, to aid in the development of novel approaches for disease prevention and effective therapeutics.

As valuable as the studies discussed here have been in furthering our understanding of the complexity of viral-bacterial interactions in the context of both pathogenicity and transmissibility, particularly for the study of influenza virus as well as bacterial infections in isolation, and to a lesser extent for studying influenza-bacterial copathogenesis, they concurrently shed light on areas warranting closer examination. Of note, the majority of these studies have been performed using seasonal or well-adapted human IAVs only; as evidenced by the current coronavirus disease 2019 (COVID-19) pandemic, zoonotic viruses are capable of crossing species barriers and causing severe and transmissible

infections in humans [3]. Understanding how these interkingdom viral-bacterial interactions are modulated at host-range barriers represents an area needing further study. Influenza B viruses have been associated with secondary bacterial infection but have also been understudied in this context [3]. Furthermore, these studies have also been generally limited to evaluation of respiratory tissues or specimens only. Considering the capacity for influenza viruses to spread and replicate in extrapulmonary tissues, it is prudent to evaluate coinfection studies at nonrespiratory sites; the ferret is uniquely suited to conduct these investigations [45]. Additionally, ferret models have been established to emulate a range of health states to assess the contribution of comorbidities to disease severity [22]. Assessment of bacterial populations in these models has only been infrequently performed [46] and could provide a wealth of information to better understand how influenza-bacterial coinfections are modulated in the context of immuno-suppression and/or other backgrounds. As in vivo studies investigating viral-bacterial interactions can possess additional complexity beyond viral or bacterial challenges in isolation, adherence to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines when reporting results from these studies will ensure that future studies in this area includes all necessary methodological criteria for interpretability and reproducibility by others in the field [47].

The sequenced genome of the ferret makes this animal model advantageous for large-scale functional genomic analyses [48]. Technological advances to study global changes in the ferret host following influenza virus infection have more recently included multiomics-based approaches. These include assessment of the host lipidome, metabolome, and proteome in the ferret respiratory tract after infection. Combining multiomics data with virologic and histopathologic findings has enabled linking of host responses to severity of pandemic influenza virus infection [49]. Furthermore, the availability of next-generation sequencing techniques has been instrumental in deciphering the influenza infectome of ferrets at different stages of the infection [50]. Collectively, omics technologies together with molecular mechanistic approaches could be vital for improved design and development of influenza therapeutics that could combine both antiviral as well as antibacterial drug targets to counter disease exacerbation and deaths due to influenza virus infections and/or bacterial complications arising from these infections.

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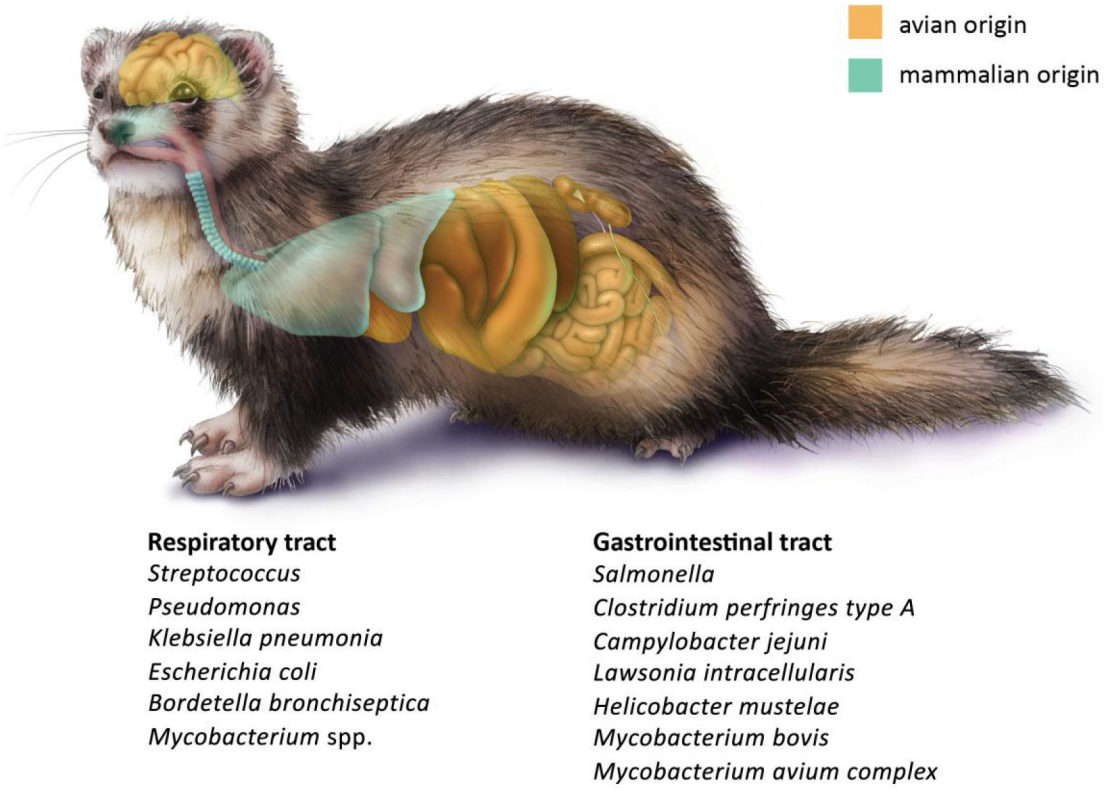

Figure 1.

Common bacterial pathogens at different anatomical sites of ferrets that support influenza virus replication. Anatomical sites of ferrets that support replication of human and zoonotic influenza viruses include the respiratory tract and the gastrointestinal tract. Mammalian-origin strains are typically restricted to replication in the respiratory tract (shaded in green). Selected avian-origin strains are capable of extrapulmonary spread (shaded in orange). Commonly detected bacterial pathogens potentially present during influenza virus replication at either respiratory or gastrointestinal sites are listed.

Table 1.

Studies Reporting Secondary Bacterial Infection Following a Primary Influenza Virus Challenge in Ferrets

Study	Primary Influenza Challenge[a]	Secondary Bacterial Challenge[b]	Increased Severity Of Clinical Symptoms[c]	Increased Viral Titers[d]	Increased Viral Transmission[e]	Increased Bacterial Titers[d]	Increased Bacterial Transmission[e]	Increased Immune Responses[f]	Increased Histopathological Alterations[f]
Husseini et al 1983 [36]	H1N1, H3N2	Mixed infection	Yes	Yes	NA	Yes	NA	Yes	Yes[g]
Peltola et al 2004 [38]	H3N2	*S. pneumoniae*	Yes	Yes	NA	Yes	NA	Yes	No
Peltola et al 2006 [37]	H1N1, H3N2 (2 strains), B	*S. pneumoniae*	Yes	No	NA	Yes	NA	Yes	Yes[g]
McCullers et al 2010 [39]	H3N2	*S. pneumoniae* (2 serotypes)	Yes	No	NA	Yes[g]	Yes	NA	NA
Rowe et al 2019 [41]	H3N2	*S. pneumoniae*	NA	NA	NA	Yes	NA	NA	NA
Rowe et a I 2020 [42][h]	pdmH1N1	*S. pneumoniae*	NA	NA	NA	NA	NA	NA	NA
Mueller Brown et al 2022 [40]	pdmH1N1	*S. pneumoniae* (2 serotypes)	Yes	No	No	Yes	NA	NA	NA
French et al 2023 [43][h]	pdmH1N1	*S. pneumoniae*	NA	NA	NA	NA	NA	NA	NA

Abbreviation: NA, not assessed.

[a] All studies listed challenged naive ferrets with human influenza A and B viruses; pdm denotes H1N1 viruses derived from the 2009 pandemic.

[b] Secondary bacterial challenge was performed with *Streptococcus pneumoniae*, with the exception of Husseini et al [36], which reports influenza challenge followed by a naturally acquired bacterial infection in the absence of subsequent challenge.

[c] Clinical symptoms as defined by weight loss and temperature increase relative to preinfection baseline.

[d] Titers measured from nasal wash specimens.

[e] Transmission in a respiratory droplet model.

[f] Immune responses and histopathological alterations as defined by increased inflammatory cell counts and the presence of inflammatory cell infiltrates and bacteria in lung tissues.

[g] Strain-specific differences in viral and/or bacterial pathogenicity were noted.

[h] Ferrets in these studies were challenged with bacteria following primary influenza virus challenge in the absence of a control group.