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Review Article

Low Pathogenic Avian Influenza and Coinfecting Pathogens: A Review of Experimental Infections in Avian Models

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SUMMARY. Low pathogenic avian influenza virus (LPAIV) usually causes mild disease or asymptomatic infection in poultry. LPAIV has, however, become a great threat to poultry industry due to mixed infections with other pathogens. Coinfections do frequently occur in the field but are not easily detected, and their impact on pathobiology is not clearly defined due to their complicated nature, but it is well known that there is an impact. One way to increase our knowledge of coinfections in poultry is to challenge birds in experimental and controlled conditions. While many articles report *in vivo* experiments with LPAIV in avian models, only a few have studied coinfections. Moreover, researchers tend to choose different bird types, ages, inoculation routes, and doses for their experiments, making it difficult to compare between studies. This review describes the state of the art for experimental infections with LPAIV alone or associated with coinfecting pathogens in avian models. It also discusses how best to mimic field infections in laboratory settings. In the field of avian diseases, experimental design is obviously directly linked with the research question addressed, but there is a gap between field and experimental data, and further studies are warranted to better understand how to bring laboratory settings closer to field situations.

RESUMEN. *Estudio Recapitulativo.* Influenza aviar de baja patogenicidad y patógenos co-infectantes: Revisión de las infecciones experimentales en modelos aviares.

Los virus de la influenza aviar de baja patogenicidad (con las siglas en inglés LPAIV) generalmente causan enfermedad leve o infección asintomática en las aves comerciales. Sin embargo, los virus de la influenza aviar de baja patogenicidad se han convertido en una gran amenaza para la industria avícola debido a infecciones mixtas con otros patógenos. Las co-infecciones ocurren con frecuencia en el campo pero no son detectadas fácilmente y su impacto en la patobiología de estos virus no está claramente definido debido a su naturaleza complicada, pero se reconoce que existe un impacto. Una forma de aumentar el conocimiento de las co-infecciones en las aves comerciales es desafiar a las aves en condiciones experimentales y controladas. Mientras que muchos artículos reportan experimentos *in vivo* con virus de la influenza aviar de baja patogenicidad en modelos aviares, sólo unos pocos han estudiado co-infecciones. Además, los investigadores tienden a elegir diferentes tipos de aves, edades, rutas de inoculación y dosis para sus experimentos, lo que dificulta las comparaciones entre los estudios. Esta revisión describe lo más reciente con las infecciones experimentales con virus de la influenza aviar de baja patogenicidad, solos o asociados con patógenos de co-infectantes en modelos aviares. También se analiza la mejor manera de replicar las infecciones de campo en condiciones de laboratorio. En el campo de las enfermedades aviares, el diseño experimental está obviamente relacionado directamente con la hipótesis de investigación abordada, pero existe una brecha entre los datos experimentales y de campo y se necesitan más estudios para comprender mejor cómo adecuar los parámetros de laboratorio a las situaciones de campo.

Key words: low pathogenic avian influenza viruses, coinfection, avian models

Abbreviations: AI = avian influenza; AIV = avian influenza virus; AP = *Avibacterium paragallinarum*; C = intraocular; CFU = colony-forming unit; EID₅₀ = 50% egg infectious dose; HA = hemagglutination; HPAIV = highly pathogenic avian influenza virus; IBV = infectious bronchitis virus; IC = intraocular; ID₅₀ = 50% infectious dose; IM = intramuscular; IN = intranasal; IO = intraoviduct; IT = intratracheal; IV = intravenous; LPAIV = low pathogenic avian influenza virus; MG = *Mycoplasma gallisepticum*; NA = neuraminidase; NDV = Newcastle disease virus; O = intraocular; ORT = *Ornithobacterium rhinotracheale*; PO = per orally; SA = *Staphylococcus aureus*; SPF = specific-pathogen-free; TCID = tissue culture infectious dose; TOC = tracheal organ culture

In poultry farms, infections with avian influenza viruses (AIVs) are dreaded as they are very often associated with severe economic losses. To better understand the clinical outcomes, pathogenesis, and transmission in the field, researchers have developed animal models to study infections in laboratory settings. Here we present a survey of the state of the art for experimental infections with low pathogenic avian influenza virus (LPAIV) alone or associated with coinfecting pathogens in avian models. On this basis we then discuss how to best mimic field infections in laboratory settings and highlight a gap between field and experimental data that requires further studies to fill. This review is not a meta-analysis but was performed using

PubMed and a combination of the following keywords: low pathogenic avian influenza virus, coinfection, chicken, turkey, duck, poultry, experimental infection, *in vivo*. Only studies with experimental infections of birds with a LPAIV alone or with a coinfecting pathogen were considered here.

AVIAN INFLUENZA VIRUSES: HIGH AND LOW PATHOGENICITY

Avian influenza (AI) is caused by avian influenza viruses (AIVs) that belong to the genus Influenza virus A of the family *Orthomyxoviridae*. AIV harbors a segmented genome of eight distinct single-stranded RNA molecules, which encode at least 10

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different viral proteins (50). On the basis of antigen variations of the surface glycoprotein hemagglutinin (HA) and neuraminidase (NA), there are 18 antigenically distinct HA and 11 NA subtypes of influenza A viruses (82). With the exception of the recently described influenza A (H17N10 and H18N11) viruses of bats, all other subtypes circulate in birds (84). AIVs are classified into two pathotypes in gallinaceous birds known as a highly pathogenic avian influenza virus (HPAIV) and a low pathogenic avian influenza virus (LPAIV) based on the existing World Organization for Animal Health criteria (16). For AIVs to be highly pathogenic, they must meet one of two criteria: 1) intravenous pathogenicity index > 1.2 or lethality for 75% or more of intravenous susceptible chickens (*Gallus gallus domesticus*) or (2) viruses of H5 or H7 subtypes with multiple basic amino acids at the HA cleavage site. All other AIVs are characterized as LPAIVs (78). HPAIVs are responsible for rapid and fatal systemic infection inducing mortality up to 100% in broilers, layers, and breeders, while LPAIVs lead to asymptomatic or milder infections. All HPAIVs identified up to date belong to H5 and H7 subtypes, even though all H5 or H7 viruses are not always highly pathogenic (86). Pathogenicity of HPAIVs is associated with polybasic amino acids (arginine and lysine) at their HA cleavage site motif (85), which enable them to replicate and damage a wide range of vital organs and tissues, ultimately resulting in the death of the infected birds (64). In contrast, LPAIVs are capable to replicate only in limited tissues of respiratory and digestive systems. In the field, LPAIVs can cause severe disease if the host is stressed or if other diseases are present (1,47). In cases of subclinical LPAIV infections, production losses are observed due to anorexia in meat birds and drop in egg production in breeders/layers. LPAIVs of H1N1 and H9N2 subtypes were shown to replicate in chicken and turkey reproductive tracts (57,58,63,83,89). In the present review we choose to focus on LPAIVs.

INFLUENZA A VIRUSES AND EVOLUTION

Because AI RNA-polymerase does not possess a proofreading function, faulty nucleotides are integrated during replication with high mutation rates of 10^{-3} to 10^{-4} substitutions/site/year. Besides mutations, viruses with segmented genomes change genetically through genetic reassortment (antigenic shift) by exchange of one or more segments between two related viruses that infect a host cell at the same time (4,6). Genetic variability is of vital importance for the survival of AIVs, and it is ensured through mutations (antigenic drift) and reassortment (antigenic shift) during the replication cycle of the virus (26). Infections with multiple strains of AIV are well documented in ducks and lead to a high viral genetic diversity. In contrast, mixed infections in poultry are relatively rare and generally seen in areas where several endemic strains circulate (1,79). In nature, the high prevalence of mixed infections in chickens and ducks can then lead to genome reassortment and result in antigenic shift (27,59). It has been confirmed that genetic reassortment in LPAIVs has led to novel phenotypes and increased virulence (37,40). Previous studies have shown that seven genes of the H5N1 virus isolated in the Hong Kong outbreak in 1997 have high sequence similarity to LPAIV virus H6N1 (69). Similarly, in Pakistan extensive cocirculation of H9N2 viruses with other AIVs, including highly pathogenic H5N1 and H7N3 subtypes, coupled with extensive vaccination

has generated a novel variant H9N2 with possibly increased epizootic and zoonotic potentials (51). The novel H9N2 viruses (A/chicken/Pakistan/UDL-01/08-like virus) currently circulating in Pakistan have acquired PB2, PB1, PA, and NS gene segments from HPAI H7N3 viruses with the other genes originating from G1-like lineage H9N2 viruses (40). Some LPAIVs, such as H9N2, can indeed break species barriers and provide genes to other influenza virus, which could present a risk for severe human infection (31).

LPAIVS AND COINFECTING PATHOGENS IN FIELD CONDITIONS

Over the last 15 years, influenza viruses of the H9N2 subtype have been isolated from outbreaks in poultry in various countries such as Germany, Italy, Ireland, Saudi Arabia, Iran, Egypt, Israel, Pakistan, China, Hong Kong, South Africa, and the United States (1,2,7,16). Laboratory examination of specific-pathogen-free (SPF) chicken showed that H9N2 avian influenza virus causes little disease, but in the last decade Asian and Middle Eastern countries have faced frequent outbreaks of H9N2 infection with high mortality (8,31). It was reported that outbreaks of H9N2 influenza viruses in Iranian broiler chicken farms caused a 20–65% mortality rate, and the most prominent lesions in affected dead birds were respiratory airway hyperemia and severe exudation, which lead to tubular cast formation in the tracheal bifurcation, extending to the lower bronchi (52,53,66). However, it is also documented that LPAIVs, such as the H9N2 subtype in domestic poultry, manifest mild clinical signs and respiratory diseases with low mortality, not exceeding 5% (79). Disease effects of AI may be far more devastating in the presence of other organisms or other forms of stress (1). It is proposed that concurrent infections may play a key role in exacerbating mortality in chicken infected with mild AIVs. Coinfection with other respiratory pathogens may complicate the respiratory disease syndrome during outbreaks of non-highly pathogenic avian influenza viruses and cause severe disease and high mortality. Strains of infectious bronchitis viruses (IBVs) were isolated from several broiler flocks during the course of the H9N2 outbreak in Iran (55). Previous studies demonstrate that H9N2 virus infection contributes to respiratory distress and is involved in diseases caused by other respiratory pathogens in the poultry industry (36,79). Mixed infections of influenza virus with other respiratory pathogens have been found to be responsible for high mortality and resulted in great economic losses (1,8,29,49). Mixed infections with Newcastle disease virus (NDV) and LPAIVs have been reported in waterfowl, and competition between viruses during isolation suggests an underestimation of coinfections in the laboratory (24).

Other respiratory copathogens, such as *Mycoplasma gallisepticum* (MG), *Escherichia coli*, and IBV, have been commonly identified in the field and could have increased the severity of clinical syndromes accompanying H9N2 AI virus infections (70). MG and *E. coli* were isolated from the field cases and may have played a role as copathogens to AI virus in the clinical disease syndrome. Fibrinonecrotic casts in the tracheal bifurcation were reported in turkeys during the 1999 outbreak of H7N1 AI in Italy in association with secondary bacterial pathogens such as *E. coli*, *Riemerella anatipestifer*, and *Pasteurella multocida* (17). MG in combination with other respiratory pathogens, including LPAIVs, can cause severe airsacculitis (9,10,53). This suggests a common pathogenic mechanism

with multiple lineages of AIVs causing extensive damage to respiratory airways, followed by additional damage by secondary pathogens. In severe cases, death was perceived as the result of occlusion of the airway and resulting asphyxiation. The individual role of H9N2 AI virus and copathogens needs to be determined in future studies.

LPAIVs IN EXPERIMENTAL STUDIES

The pathogenicity and transmission of many LPAIV strains have been investigated in experimental conditions in avian models. SPF chickens are the most frequently used as a standard well-characterized and relatively homogeneous host to carry out LPAIV experimental infections. Table 1 summarizes the main experimental infections of avian species with LPAIVs. Considering that different research teams used various animal models (in term of species and age), LPAIV strains, routes of inoculation, and doses, Table 1 illustrates the difficulty one may have to compare data generated from different studies.

Avian models of 3–4 wk of age are commonly used for experimental studies, likely because of their easy handling and low feed cost at this age. Moreover, the intranasal route (IN) for the inoculation of respiratory pathogens in avian models is the most common followed by intratracheal (IT), per orally (PO), intraocular (O), intramuscular (IM), intravenous (IV), intraoviduct (IO), and intracloacal (C). The aerosol route is the most common route for the spread of respiratory pathogens, which is why many researchers choose IN or IT routes to simulate respiratory transmission of LPAIV and complicating pathogens. Inoculation routes (IN, IC, and O) were compared in a study where hens were inoculated with 10^6 EID₅₀ of A/chicken/CA/1255/02(H6N2) or A/chicken/NJ/12220/97(H9N2). Hens inoculated IN with H6N2 virus presented mild clinical signs and shed higher virus titers in the higher respiratory tract (as measured in oropharyngeal swabs) than their H6N2-inoculated counterparts. Birds were less susceptible to H9N2, which was detected only in oropharyngeal swabs and only when inoculated IN. Clinical signs and lesions were also less pronounced when the pathogen was inoculated through oral and cloacal routes (57). In this latter study the IN route of inoculation was hence the most efficient. But it may be virus strain-specific and linked with preferential binding of these H6N2 and H9N2 viruses to the respiratory rather than digestive tract of the chickens.

As far as inoculation dose is concerned, the most common dose used in experimental studies is 10^6 EID₅₀ for LPAIVs, and this dose usually induces clinical signs and lesions. The latter are indeed dose dependent. Thus, quail and turkeys infected oro-nasally with increasing doses (10^3 – 10^6 EID₅₀/0.1 ml) of LPAIV (A/quail/Hong Kong/G1/97) showed more disease and lesions when inoculated with higher virus doses. Quail were more susceptible than turkeys; they were readily infected with lower challenge doses (13).

Ex vivo tracheal organ culture (TOC) models have been proposed as possible alternative to experimental LPAIV infections of the respiratory tract to address some specific questions: TOC may be of interest to compare the host susceptibility to different LPAIV strains by monitoring induction of ciliostasis, necrosis of the epithelium, or viral replication (61). But tissue cultures show obvious limitations since they cannot address accurately the contribution of the immune response.

LPAIVs AND COINFECTING PATHOGENS IN EXPERIMENTAL STUDIES

It is well appreciated that upper respiratory tract viral infections in poultry are often complicated by more serious bacterial diseases. While influenza virus is most commonly thought of in this context, other respiratory viruses, including NDV, IBV, and infectious laryngotracheitis virus, may also predispose to secondary infections. Several different bacteria have also been implicated, including MG, *E. coli*, *Avibacterium paragallinarum*, *Ornithobacterium rhinotracheale* (ORT), and *Staphylococcus aureus* (SA) (35,36,77). It is thought that certain pairings of organisms better complement each other than other potential pairings. Coinfections of poultry present a complicated clinical picture confusing the identification and diagnosis, and unfortunately little is known on the interactions between coinfecting pathogens (19). Tables 2 and 3 summarize the literature on experimental infections of birds with LPAIVs and bacteria, and LPAIVs and avian viruses, respectively. Coinfection of poultry with more than one bacterial and/or viral agent is common and often results in increased clinical signs when compared to single-agent infections (56,73,74). Conversely, infection of a host with one virus may affect infection by a second virus, a phenomenon explained by the occurrence of viral interference; cells infected by a virus may not permit multiplication of a second virus (22). In addition, viral interference may be detrimental to detecting viruses in coinfecting flocks since lower or undetectable virus titers might fail to give a complete diagnosis (24). Coinfection of LPAIV A/chicken/Iran/SH-110/99 (H9N2) with infectious bronchitis live vaccine led to increased clinical signs and mortality rates as well as longer virus shedding in chickens (36).

Significantly higher antibody titers against AIV was observed during coinfection with IBV, which may indicate that IBV could promote the propagation of H9N2 AIV (A/chicken/Iran/SH110/99(H9N2)) or stimulate the immune response (67). IBV and LPAIV coinfections have not been thoroughly studied, but live attenuated IBV vaccine has been shown to interact with LPAIV H9N2 infection, leading to a more severe disease outcome than with LPAIV alone (35,36). Coinfection of LPAIV A/mallard/MN/199106/99(H3N8) and NDV (mallard/US(MN)/AI06-978/2006) in ducks resulted in a higher number of cloacal swabs detected positive for LPAIV and a lower number of cloacal swabs detected positive for NDV (25). Coinfection of chickens and turkeys with NDV and LPAIV (A/turkey/VA/SEP/67/2002(H7N2) affected the replication dynamics of these viruses but did not alter clinical signs (19). ORT infection could lead to a higher mortality and economic losses in presence of H9N2 AIV in chicken (56). Coinfection of H9N2 influenza virus with SA or *A. paragallinarum* enhances the replication of the virus in chickens, resulting in exacerbation of the H9N2 virus infection (42). Bacterial and viral infections cause huge economic losses in the form of morbidity and mortality of birds. Poor diagnosis, antibiotics, and vaccination cost for the prevention or treatment of viral and bacterial infections (and emergence of antibiotics-resistant bacteria) are major issues for poultry industry. The medical community has expressed concern that antibiotic use in food animals may promote the development of antibiotic-resistant strains of bacteria that could impact human health too (32). Antibiotic resistance is also an economic burden on the health care system. This global trend toward restriction of antibiotics use in poultry farms may lead to an increase of bacterial coinfections and

Table 1. Summary of LPAIV infections in different avian models in experimental conditions.^A

Influenza virus strain	Animals/age	Infection dose (f/bird)	Infection route	Main findings	References
A/cx/Pakistan/UDL-01/08(H9N2)	3-wk-old chicken (broiler and white leghorn), common quail, red jungle fowl (hen pullet)	10 ^{6.6} EID ₅₀	IN	<ul style="list-style-type: none"> • Efficient virus replication in all species except for crows • Efficient direct contact transmission within a given species for all species except for crows • Efficient broiler to sparrow transmission • Higher virus shedding in quail than in other species 	(39)
A/cx/Shaanxi/01/2011	Sparrow, crow (age not known) 30-wk-old SPF white leghorn layers	10 ⁶ EID ₅₀	IN, IC, or IO	<ul style="list-style-type: none"> • Clinical signs of depression and respiratory distress • Efficient virus replication in magnum and uterus • Severe gross lesions in uterus and magnum, gradual decrease in egg production with deteriorated egg shell quality 	(63)
A/cx/Shaanxi/11/2012	30-wk-old SPF white leghorn layers	O, IN		<ul style="list-style-type: none"> • Efficient virus replication in magnum and uterus • Significant tissue damage and apoptosis in oviductal parts 	(83)
A/cx/CA/1255/02(H6N2) and A/cx/NJ/12220/97(H9N2)	46-wk-old SPF chicken (layer)	10 ⁶ EID ₅₀	IN IC O	<ul style="list-style-type: none"> • Decreases expression of mRNA expression of IFN-α • Efficient H6N2 virus replication but poor H9N2 virus replication • Efficient transmission of H6N2 but not of H9N2 virus; both viruses can also infect chickens through other routes besides the IN route 	(57)
A/cx/United Arab Emirates/99 (H9N2)	3-wk-old chicken (broiler)	10 ^{8.8} EID ₅₀	O IN IT	<ul style="list-style-type: none"> • Efficient virus replication in lungs • Little gross pathology • Necrosis of parabronchial epithelium, oedema, and fibrin exudation in airways and air capillaries (severe pneumonia) 	(21)
A/cx/Pakistan/UDL-01/08(H9N2)	3-wk-old chicken (broiler)	4 HA units	IV	<ul style="list-style-type: none"> • Mild clinical signs • Gross and microscopic lesions mainly on lungs and kidneys 	(75)
A/cx/Iran/SH-110/99(H9N2)	3-wk-old chicken (broiler)	10 ⁶ EID ₅₀	IT	<ul style="list-style-type: none"> • IHC detection of viral antigen in kidney and lungs • Virus affinity for respiratory and urinary systems • Clinical signs: depression, puffing, face edema, conjunctivitis, ruffled feathers (2–4 dpi) 	(34)
A/cx/Lebanon/1/04(H9N2)	3-wk-old chicken	7 TCID ₅₀	IT	<ul style="list-style-type: none"> • Gross lesions in respiratory tract and kidneys • No mortality • Low morbidity (<50%) 	(68)
A/cx/Chile/176822/02(H7N3)	4-wk-old chicken	10 ⁶ EID ₅₀	IN IV	<ul style="list-style-type: none"> • Increased pathogenicity after virus passaging (higher morbidity, higher gross, and microscopic lesions) • After IV inoculation: mortality, nephrosis, interstitial nephritis, and oedema • After IN inoculation: no morbidity or mortality • No gross pathologic and histologic lesions irrespective of inoculation route except the caecal tonsils in IN inoculated birds 	(41)
A/cx/California/1772/02(H6N2)	6–8-wk-old chicken	10 ⁷ EID ₅₀	IN	<ul style="list-style-type: none"> • Efficient virus replication in all inoculated and contact birds • Efficient transmission • Higher respiratory than digestive virus shedding • No clinical signs but mild oedema and congestion of kidney and lungs at necropsy 	(87)

Table 1. Continued.

Influenza virus strain	Animals/age	Infection dose (/bird)	Infection route	Main findings	References
A/mallard/Delaware/415/05 (H6N2), A/shorebird-environment/Delaware/251/05(H3N6), A/mallard/Maryland/1159/06 (H5N1), A/mallard/Delaware/418/05(H7N3)	2-wk-old chicken (broiler) and turkey	10 ⁶ EID ₅₀	IT IC	<ul style="list-style-type: none"> No clinical disease, gross lesions, or mortality with any tested virus in chickens or turkeys Replication of A/mallard/Maryland/1159/2006(H5N1) in the upper respiratory tract of turkeys Replication of A/mallard/Delaware/418/2005(H7N3) in the upper respiratory and intestinal tracts of turkeys and to a lesser extent of chickens Replication of A/shorebird-environment/Delaware/251/2005(H3N6) in the upper respiratory and intestinal tracts of chickens 	(44)
A/Chile/3536/2009 (H1N1)	53-wk-old laying turkeys	10 ^{5.3} TCID ₅₀	IN, IC, IU	<ul style="list-style-type: none"> No clinical sign, lesion, virus shedding, and drop in egg production was shown by turkeys inoculated through IN with pH1N1 Turkeys inoculated IU presented with mild diarrhea, drop egg production, lesions in reproductive tract, virus shedding in oropharyngeal, and cloacal swabs Turkeys inoculated by the IC route showed diarrhea, no lesions in reproductive tract, no effect on egg production, and virus shedding in cloacal swabs only 	(58)
A/qu/Hong Kong/G1/97(H9N2)	4-wk-old turkey and Japanese quail	10 ³ to 10 ⁶ EID ₅₀	PO, IN	<ul style="list-style-type: none"> Quail more susceptible than turkeys to H9N2 but with less severe clinical signs (quail can act as a silent reservoir for H9N2) 	(13)
A/cx/Hong Kong/G9/97(H9N2), A/qu/Hong Kong/G1/97(H9N2), A/qu/Hong Kong/A17/99(H9N2), A/Hong Kong/1073/99(H9N2), A/pigeon/HK/FY6/99(H9N2)	Adult quail, pigeon, and chicken	10 ³ EID ₅₀ (quail) 10 ^{6.5} EID ₅₀ (chicken) 10 ⁶ EID ₅₀ (pigeon)	PO, IN, O	<ul style="list-style-type: none"> Efficient direct contact transmission in quail and turkeys No clinical signs in quail or chicken Higher virus shedding in respiratory tract than intestinal tract of quail Efficient transmission of A/Hong Kong/1073/99(H9N2) to contact quail Poor/no replication of all viruses in pigeon; no contact transmission in pigeons 	(33)
A/anas platyrhynchos/Spain/1877/09(H7N2)	8-wk-old European quail	10 ⁶ EID ₅₀	IN	<ul style="list-style-type: none"> No clinical signs or pathology in inoculated and contact quail 	(11)
A/cx/Iran/772/98(H9N2)	23-wk-old chukar partridges (<i>A. chukar</i>)	10 ⁷ EID ₅₀	O IN	<ul style="list-style-type: none"> Efficient replication and transmission Clinical signs: coughing, sneezing, depression, and anorexia 	(54)
A/Anas crecca/Spain/1460/08 (H7N9)	9-wk-old red-legged partridge (<i>Alectoris rufa</i>)	10 ⁶ EID ₅₀	IN	<ul style="list-style-type: none"> Good virus replication and wide tissue tropism No clinical signs or histopathological lesions 	(12)
A/cx/California/1772/02(H6N2)	6–8-wk-old chicken 8–12-wk-old Pekin ducks and quail (7-wk-old)	10 ⁷ EID ₅₀	IN	<ul style="list-style-type: none"> Only short-term viral shedding and seroconversion Efficient virus aerosol transmission from chickens to chickens and quail; poor aerosol transmission from chickens to ducks 	(88)

Table 1. Continued.

Influenza virus strain	Animals/age	Infection dose (/bird)	Infection route	Main findings	References
A/ry/WI/66(H9N2), A/ty/MN/38391/95(H9N2), A/shorebird/DE/9/96(H9N2), A/cx/Beijing/1/94(H9N2), A/qu/Hong Kong/G1/97(H9N2)	3–12-wk-old SPF white leghorn chicken and 5-wk-old Pekin white duck	10 ⁷ EID ₅₀ (chickens) 10 ⁶ EID ₅₀ (ducks)	IV, PO, IT, or IN	<ul style="list-style-type: none"> • Efficient replication of all viruses in chickens and ducks • No clinical signs except with A/chicken/Beijing/1/94(H9N2) • Up to 80% mortality with A/chicken/Beijing/1/94(H9N2) in chicken • Increased susceptibility to A/chicken/Beijing/1/94(H9N2) with age • Differential tracheal/cloacal shedding (virus-specific) 	(31)
A/cx/Korea/323/96(H9N2), A/cx/Korea/006/96(H9N2), A/cx/Hong Kong/G9/97(H9N2), A/cx/Hong Kong/G23/97(H9N2), /dk/Hong Kong/Y439/97 (H9N2), and A/dk/Hong Kong/Y280/97(H9N2) inoculated in chickens;					
A/cx/Hong Kong/G9/97(H9N2), A/qu/Hong Kong/G1/97(H9N2), and A/dk/Hong Kong/Y439/97(H9N2) inoculated in ducks	4-wk-old Japanese quail (<i>Coturnix coturnix</i>) Chicken (white leghorn), mallard, and Pekin ducks	5 × 10 ⁶ EID ₅₀	IT, PO, IN	<ul style="list-style-type: none"> • Efficient replication of duck viruses in quail and ducks but not in chickens • Higher replication in the respiratory than intestinal tract of quail • Direct and aerosol transmission of A/duck/HK/702/79(H9N2) and A/quail/Hong Kong/A28945/88(H9N2) in quail; direct transmission only of A/quail/Hong Kong/A28945/88(H9N2) in chickens • Good replication of all viruses • Higher vial shedding in turkeys than ducks or chickens • Higher pathogenicity in turkeys (severe clinical signs and mortality) • Only 3 isolates in ducks and 6 isolates in chickens induced mild clinical signs without mortality 	(60)
A/dk/HK/702/79(H9N2), A/dk/HK/448/78(H9N2), A/dk/HK/366/78(H9N2), A/dk/HK/149/77(H9N2), A/dk/HK/86/76(H9N2), A/dk/HK/147/77 (H9N6), and A/qu/Hong Kong/A28945/88(H9N2)	4-wk-old chicken 2-wk-old duck and turkey	10 ⁶ EID ₅₀	IT	<ul style="list-style-type: none"> • No detectable clinical signs with either virus • Replication of both viruses in lungs and air sacs until 3 dpi (locally extensive interstitial, exudative, and proliferative pneumonia) • Replication of H2N3, but not H13N6 virus, in intestinal mucosa and cloacal bursa (no lesion) • Higher shedding of H2N3 virus in cloacal than pharyngeal swabs 	(72)
A/cx/MD/MinhMa/04(H7N2), A/mallard/OH/421/87(H7N8), A/pintail/MN/423/99(H7N3), A/ ruddy turnstone/DE/1538/00 (H7N9), A/cx/NJ/15086-3/94 (H7N3), A/ty/NY/4450-4/94 (H7N2), A/cx/NY/3112-1/95 (H7N2), A/cx/NY/12273-11/99 (H7N3), A/cx/NY/30749-3/00 (H7N2), A/ guinea hen/MA/148081-11/02 (H7N2), A/cx/PA/9801289/98(H7N2), A/ty/V/A/SEP-67/02(H7N2)	22-wk-old mallard duck	10 ⁸ EID ₅₀	PO IT	<ul style="list-style-type: none"> • No detectable clinical signs with either virus • Replication of both viruses in lungs and air sacs until 3 dpi (locally extensive interstitial, exudative, and proliferative pneumonia) • Replication of H2N3, but not H13N6 virus, in intestinal mucosa and cloacal bursa (no lesion) • Higher shedding of H2N3 virus in cloacal than pharyngeal swabs 	(20)
A/mallard/New Brunswick/1/06 (H2N3), A/gull/Ontario/680-6/01 (H13N6)					

Table 1. Continued.

Influenza virus strain	Animals/age	Infection dose (/bird)	Infection route	Main findings	References
A/mallard/MN/355779/00(H5N2) A/mallard/MN/182761/98(H7N3) A/mallard/MN/199106/99(H3N8)	10–16-wk-old duck (mallards, red heads, wood ducks, laughing gulls)	10 ⁶ EID ₅₀	IN	<ul style="list-style-type: none"> No morbidity, mortality, gross and histopathological lesions in all type of ducks Efficient replication of all virus strains H7N3 virus shedding in respiratory tract only Cloacal shedding of H3N8 and H5N2 in mallards but oropharyngeal shedding in other ducks and gulls Efficient virus replication No clinical signs but high virus shedding 	(18)
A/mallard/Minnesota/199106/99 (H3N8)	4-wk-old duck	10 ^{6.5} EID ₅₀	IN	<ul style="list-style-type: none"> No morbidity, mortality, gross and histopathological lesions in all type of ducks Efficient replication of all virus strains H7N3 virus shedding in respiratory tract only Cloacal shedding of H3N8 and H5N2 in mallards but oropharyngeal shedding in other ducks and gulls Efficient virus replication No clinical signs but high virus shedding 	(15)

^Acx: chicken, dk: duck, dpi: days post inoculation, EID: egg infectious dose, HA: hemagglutination, IC: intracloacal, IHC: immunohistochemistry, IN: intranasal, IO: intraoviduct, IP: intraperitoneal, IT: intratracheal, IU: intrauterine, IV: intravenous, LPAIV: low pathogenic avian influenza virus, O: intraocular, PO: per orally, qu: quail, SPF: specific-pathogen-free, TCID: tissue culture infectious dose, ty: turkey.

stresses the need for better understanding of these complex interactions *in vivo* to define innovative approaches of control.

As for LPAIV infections alone, the most common avian models for the study of viral and bacterial coinfections are chickens. Ducks, turkeys, quail, and partridges have also been used to address different research questions. One possible reason for the most widely use of chicken as an avian model may be their easy handling, availability, low price, and early maturity as compared with other avian models. In addition to this, chickens are also the most economically important (largest industry) type of poultry worldwide.

The classical doses used for bacteria inoculation (MG, *E. coli*) as described in literature are 10⁶ to 10⁹ colony-forming units (CFUs). Clinical signs and lesions severity have been shown to increase in a dose-dependent manner. Bacterial coinfections lead to more severe lesions when the bacterial were inoculated IN or via an aerosol route. In an experimental coinfection study with LPAIV H3N8 (A/mallard/Hungary/19616/07) and MG inoculated through aerosol spray, the authors showed that LPAI H3N8 virus alone did not cause any clinical signs, but MG infection caused clinical signs, reduction of body weight gain, and colonization of the inner organs. These parameters were more severe in the birds coinfecting with MG and LPAIV H3N8 than in the group challenged with MG alone. Coinfection with LPAIV H3N8 thus enhanced the pathogenesis of MG significantly (73,74). Barbour *et al.* coinfecting chickens IT or intrathoracically with H9N2 LPAIV and *E. coli*. High and acute mortality was observed with the intrathoracic inoculation route for *E. coli* (9,10).

LPAIV coinfection with other viruses promotes replication of LPAIV, leading to increase the severity of clinical signs, mortality rate, and gross lesions (35,67). However, these viruses can interfere during viral replication phase as reported in previous studies. França and colleagues documented higher shedding of LPAIV in cloacal swabs when mallards were coinfecting with LPAIV and NDV on the same day. Conversely, marked reduction of NDV in cloacal swabs was observed during the study. At the same time, reduced LPAIV and NDV was observed in oropharyngeal swabs. However, coinfection with LPAIV and NDV did not affect replication of LPAIV (H3N8) in intestine and bursa of Fabricius. Viral interference during replication can be a possible reason for the decrease of NDV shedding in cloacal swabs, and LPAIV may have reduced or inhibited NDV replication (25). Similarly, Costa-Hurtado and colleagues reported that coinfection of chicken and turkeys with LPAIV (H7N2) and NDV can affect replication dynamics of these viruses but did not affect clinical signs. This virus replication pattern was dependent on timings of inoculation and bird species, suggesting that coinfection with two different viruses can result in temporary cell receptor binding competition (19). During this study, chickens and turkeys were infected with an NDV vaccine strain (LaSota) and a H7N2 LPAIV (A/turkey/VA/ SEP-67/2002) simultaneously or sequentially 3 days apart. No clinical signs were observed in chickens coinfecting with NDV and LPAIV, while all turkeys showed mild clinical signs during coinfection. The replication dynamics of these viruses was, however, affected by the coinfection: lower virus titers and fewer birds with virus replication were recorded, especially when LPAIV was followed by NDV. These results suggest that infection with a heterologous virus may result in temporary competition for cell receptors or competent cells for replication, most likely interferon-mediated, which decreases with time (19).

Table 2. Summary of LPAIV and bacteria coinfections in different avian models in experimental conditions.^A

Virus and bacteria strains	Animal/age	Infection dose	Infection route	Main findings	References
A/mallard/Hungary/19616/07(H3N8), <i>M. gallisepticum</i> (strain 1226)	2-wk-old chicken (broiler)	10 ⁵ EID ₅₀ (H3N8) 2.1 × 10 ⁷ CFUs (MG)	Spray (aerosol)	<ul style="list-style-type: none"> No clinical signs with H3N8 alone but clinical signs and reduction of body weight with MG alone More pronounced clinical signs and reduction in weight in coinfecting birds than in MG only infected birds Reduced antimycoplasma antibody titers in coinfecting versus MG alone infected chickens 	(73,74)
A/cx/Iran/m.1/10(H9N2), R87-7/1387 (JF810491,ORT)	3-wk-old chicken (white leghorn layer)	10 ⁶ EID ₅₀ (H9N2), 10 ¹⁰ CFUs (ORT)	IT	<ul style="list-style-type: none"> More pronounced clinical signs, gross lesions, and mortality in coinfecting birds than in birds challenged with ORT alone Significantly higher HI titers against H9N2 virus in coinfecting than H9N2 infected birds 	(6)
A/cx/Shandong/2011(H9N2), ORT/chicken/Shandong/2011	3-wk-old chicken (broiler)	10 ² EID ₅₀ (H9N2), 10 LD ₅₀ (ORT)	IP and IN	<ul style="list-style-type: none"> General sickness in all coinfecting groups (simultaneous infection or ORT followed by H9N2) More severe clinical signs of respiratory disease, anorexia, and mortality observed in coinfecting birds (simultaneous infection or ORT followed by H9N2) No mortality but typical pneumonia with fibrinous airsacculitis, pericarditis, peritonitis, and scattered areas of hemorrhage in the lungs in birds inoculated with H9N2 virus followed by ORT or H9N2 virus alone 	(56)
A/cx/Lebanon/1/04(H9N2), <i>E. coli</i> (BVL strain)	3-wk-old chicken (broiler)	2 HA units (H9N2) 1.7 × 10 ⁹ 1.7 × 10 ⁶ 1.7 × 10 ⁵ 1.7 × 10 ⁴ 1.7 × 10 ³ CFUs (<i>E. coli</i>)	IT + intrathoracic	<ul style="list-style-type: none"> Enhanced clinical signs and gross lesions with increasing <i>E. coli</i> doses, but more severe clinical signs and gross lesions in coinfecting birds than single infections 	(10)
A/dk/Beijing/40/04(H3N8), A/dk/Beijing/61/05(H3N8), <i>E. coli</i> O78	5-wk-old chicken (white leghorn layer)	10 ⁶ EID ₅₀ (H3N8) 2.53 × 10 ⁸ (<i>E. coli</i>)	IN IO IC O	<ul style="list-style-type: none"> No obvious signs of disease in chickens inoculated with virus or bacteria alone Depression, diarrhea, and mortality after inoculation with A/dk/Beijing/40/04(H3N8) and <i>E. coli</i> Slight depression but no mortality after inoculation with A/dk/Beijing/61/05(H3N8) and <i>E. coli</i> 	(62)
A/cx/aaq-Y-55/01(H9N2), A/cx/aaq-Y-135/01(H9N2), A/cx/Beijing/2/97(H9N2), A/tern/South Africa/61(H5N3), <i>Staphylococcus aureus</i> (Hyogo strain) and <i>Avibacterium paragallinarum</i> (HK-1 strain)	4-wk-old chicken (white leghorn layer)	10 ⁷ EID ₅₀ (influenza virus) 2.2 × 10 ⁵ CFUs (SA) 3 × 10 ³ CFUs (AP)	IN	<ul style="list-style-type: none"> Exacerbation of clinical signs and gross lesions for coinfecting birds with H9N2 virus and SA or HP compared with single infected birds Higher H9N2 viruses in coinfecting than H9N2 only infected birds 	(42)

^AAP: *Avibacterium paragallinarum*, CFU: colony-forming unit, cx: chicken, dk: duck, EID: egg infectious dose, HA: hemagglutination, IC: cloacal, IN: intranasal, IO: intraoviduct, IP: intraperitoneal, IT: intratracheal, LPAIV: low pathogenic avian influenza virus, MG: *Mycoplasma gallisepticum*, O: intraocular, ORT: *Ornithobacterium rhinotracheale*, qu: quail, SA: *Staphylococcus aureus*, ty: turkey.

Table 3. Summary of LPAIV and avian virus coinfections in different avian models in experimental conditions.^A

Viral strains	Animal/age	Infection dose	Infection route	Main findings	References
A/ry/VA/SEP/67/02(H7N2) APMV1/cx/US(NJ)/LaSota/46 (NDV)	3–4-wk-old chicken (white leghorn layer) and turkey	10 ⁷ EI D ₅₀	O, IN	<ul style="list-style-type: none"> No clinical signs and mild clinical signs in chickens and turkeys, respectively, after single or coinfection Delay in virus shedding peak in coinfecting chickens Longer shedding period of H9N2 virus, more severe clinical signs, and higher mortality in coinfecting birds 	(19) (35,36)
A/cx/Iran/SH-110/99(H9N2), IBV live vaccine (freeze-dried live attenuated vaccine, Mass type, H120 strain)	3-wk-old chicken (broiler)	10 ⁶ EID ₅₀ (H9N2), 10 ³ EID ₅₀ (IBV)	IN + spray	<ul style="list-style-type: none"> Efficient AIV and NDV replication Higher digestive shedding of AIV for coinfecting birds Exacerbated clinical signs, gross lesions, and mortality in coinfecting birds Higher antibody titers against H9N2 virus in coinfecting birds 	(25) (67)
A/mallard/MN/199106/99(H3N8) Mallard/US(MN)/A106-978/06 (NDV) A/cx/Iran/SH110/99(H9N2) IBV vaccine strain IB 4/91	5-wk-old mallard 3-wk-old chicken (broiler)	10 ⁶ EID ₅₀ 0.2 ml homogenized sample	IN IN		

^Acx: chicken, EID: egg infectious dose, IBV: infectious bronchitis virus, IN: intranasal, LPAIV: low pathogenic avian influenza virus, NDV: Newcastle disease virus, O: intraocular, ty: turkey.

Viral interference is a phenomenon in which a cell infected by a virus does not allow replication of a second homologous or heterologous superinfectant virus (22). Viral interference can result from different mechanisms, including competing by attachment interference therefore reducing or blocking of receptor sites for the superinfecting virus, competing intracellularly for replication host machinery, and virus-induced interferon interference. NDV and LPAIV replicate in cells where there are trypsin-like enzymes such as in the upper respiratory and intestinal epithelia and might compete for the same target cells or replicate in adjacent cells (77). Both NDV and AIV bind to sialic acid-linked glycoconjugates on host cells and may also compete for host cell machinery during viral replication (25,76). In addition, previous replication in the same site of another virus may affect replication by activating antiviral immune responses. Although the LaSota NDV strain is known to be a weak interferon inducer as part of their low virulent phenotype profile, local interferon production might still be able to interfere with LPAIV replication (23). In fact, previous studies in embryonated eggs showed that LaSota NDV could suppress growth of a H9N2 LPAIV, provided NDV was inoculated prior to LPAIV (28). Influenza virus-induced interferons may be the other possible reason for the inhibited replication of NDV (43). Viral interference has also been suggested in other studies with influenza virus in humans (H1N1). It was reported that increase in the proportion and number of rhinovirus can decrease influenza virus diagnoses in human, suggesting that rhinoviruses may compete with influenza virus for receptor binding and replication (4). Bacterial and viral coinfections usually show synergistic effects and exacerbate clinical signs and lesions. No competition between pathogens is observed then.

TIMING OF COINFECTIONS

In an attempt to put together the available methods reported in the literature for coinfections in experimental conditions, and mainly to see which time line may best fit field coinfections situations, we drew time arrows summarizing LPAIV and coinfecting bacteria experiments (Fig. 1) as well as LPAIV and coinfecting virus experiments (Fig. 2). The studies in experimental conditions conducted so far report either simultaneous infections (central parts of the figures, in the arrows), or subsequent infections usually 3 days apart (bacteria or virus followed by LPAIV, LPAIV followed by bacteria or virus on the top or bottom panel of the figures, respectively). The 3-day interval chosen between subsequent infections was pretty consistent throughout literature (10,19, 35,42,73,74). Just five studies compared in parallel simultaneous and subsequent infections with similar experimental conditions. Kishada and colleagues first studied LPAIV H9N2 and *Avibacterium paragallinarum* with simultaneous inoculation and SA infection followed by LPAIV H9N2 inoculation (42). The use of two different bacteria made a systematic comparison of the timing of coinfection difficult. Pan *et al.* coinfecting chickens with LPAIV H9N2 and ORT and showed that when ORT was inoculated before or at the same time as LPAIV H9N2, the disease outcome was more severe (56). LPAIV (H7N2 in chickens or H3N8 in mallard) and NDV experimental coinfections have been more systematically studied (19,25). While a minimal effect of the LPAIV and NDV coinfection was observed on a clinical point of view, an altered shedding pattern was detected in both mallards

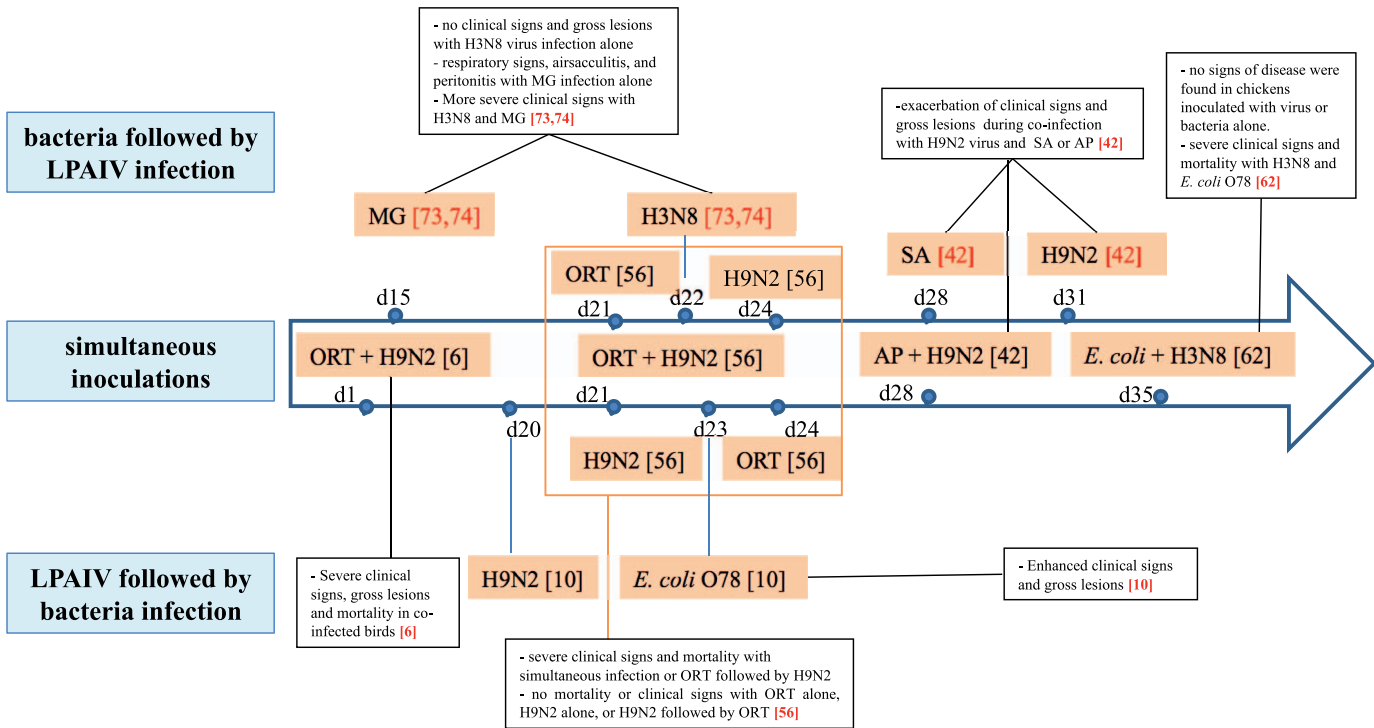


Fig. 1. Time arrow of LPAIV and bacteria coinfections in experimental conditions. AP: *Avibacterium paragallinarum*, d: day, LPAIV: low pathogenic avian influenza virus, MG: *Mycoplasma gallisepticum*, ORT: *Ornithobacterium rhinotracheale*, SA: *Staphylococcus aureus*.

and chickens, irrespective of the timing of the coinfection (19,25). When LPAIV and IBV live vaccines were both administered to chickens, the coinfection led to more severe clinical signs and longer LPAIV shedding, again irrespective of the timing of coinfection (35). In the laboratory setting, the timing of coinfections therefore does not seem to play an essential role in pathogenesis. While common knowledge in virology associates coinfections with a virus coming first followed by a bacterium, the

timing in the field may actually be opposite with commensal bacteria that are there first and may become a problem when a virus superinfects the birds (14). *Manheimia haemolytica*, *Gallibacterium anatis*, and *Pseudomonas aeruginosa* have indeed been identified as commensal bacteria of the upper respiratory tract of poultry, while they may also be found in sick birds either in the presence of a coinfecting pathogen or in difficult environmental conditions (3,5,48,71).

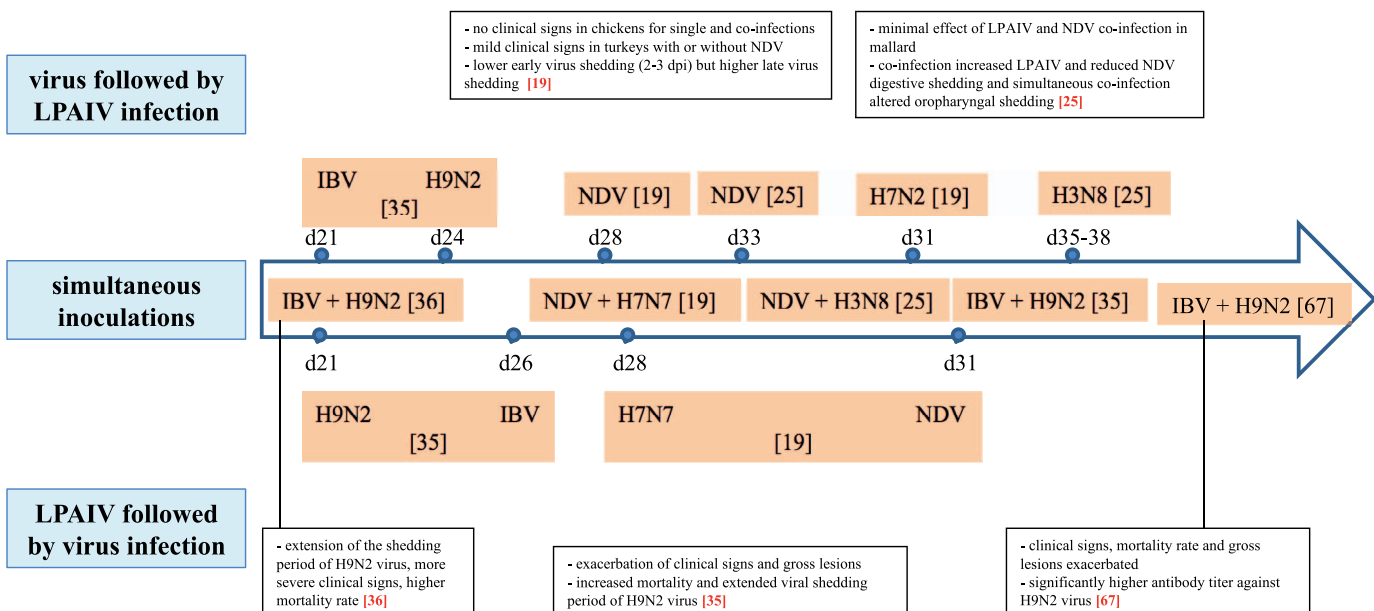


Fig. 2. Time arrow of LPAIV and avian virus coinfections in experimental conditions. d: day, IBV: infectious bronchitis virus, LPAIV: low pathogenic avian influenza virus, NDV: Newcastle disease virus.

SPF VERSUS COMMERCIAL BIRDS FOR EXPERIMENTAL INFECTIONS

In chickens, to avoid interference with other pathogens and in an attempt to “standardize” experiments, researchers usually inoculate SPF birds. The absence (or very limited supply) of SPF turkeys, quail, and other poultry species makes it of course difficult: commercial birds are then used in experimental conditions. The SPF versus commercial bird (broiler, layer, or breeder) comparison for LPAIV infections (with or without complicating pathogens) has, however, never been properly investigated. The immune responses of two types of chickens have been compared to some extent in vaccine studies that aimed at understanding differences in vaccine protection in the laboratory and in the field (30,38,65,81). These studies show a better antibody response of SPF chickens than commercial birds, likely due to the differences among genetic lines of chickens in antibody development.

To our knowledge a single study compared SPF and commercial birds for LPAIV pathogenicity. Ladman *et al.* (45) indeed observed respiratory signs, airsacculitis, and microscopic lesions in the trachea and lung of broilers infected with LPAIV H7N2 but very rarely in SPF layers. Systemic (serum) antibodies were also detected earlier in broilers than in SPF layers. The authors suggested that one rethink the choice of bird type for LPAIV pathogenesis studies. To mimic the field situation, commercial birds of course seem more appropriate also in experimental settings, but care should be taken to control the health status of the birds. Vaccination history should also be respected to be closer to the farm situation.

To study LPAIV pathogenesis to better understand disease outcome in the field, thought should be given to the design of experimental settings. Depending upon the research question asked, different protocols may be selected. In addition to the variables we have just reviewed, bird type, single infections or coinfections, timing, dose of infection(s), and environmental and management variables play a critical but poorly controlled role in the disease outcome in farms. Contaminated dust is a known source of respiratory pathogens, which is very difficult to reproduce in laboratory settings. One way to address the spread of respiratory pathogens with dust in experimental conditions is to inoculate birds by aerosol rather than IN or IT. In a clinical study, authors compared the 50% infectious dose (ID₅₀) by aerosol and IN inhalations and showed a 100-fold lower ID₅₀ in the first case (80). Access of virus particles to the deep air sacs is actually bypassed when birds are infected IN or IT. The use of aerosols for *in vivo* infections may help reduce the gap between the laboratory and the field. Temperature and humidity can now also be regulated (and brought closer to the farm conditions) in poultry isolators with relevant technicity. All these improvements in experimental conditions will contribute to bringing laboratory settings closer to the field situation, but one should stay aware of the remaining gap between the two configurations and not overinterpret experimental results.

CONCLUSION

Taken together, this review of literature suggests that to study coinfections with LPAIV and a complicating pathogen in experimental conditions, using young (3–4 wk-old) birds, 10⁶ EID₅₀ of virus, and 10^{6–9} CFU of bacteria per bird, IN, would most likely lead to clinical observations and would allow for better

comparison of the findings with previously published studies. While using SPF birds (for chickens) makes comparison with published data easier, commercial birds with a carefully checked health status better reflect the field situation and may be even more susceptible to infections than their SPF counterparts. Further studies are warranted to truly assess the cost benefit of using commercial birds and to determine the adequate timing of the coinfection in relation to the field situations. Environmental factors (temperature, relative humidity, ammonia level, etc.) should also be taken into account because they definitely play a role in the field but have so far not been much looked at in experimental settings.

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