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AI	avian influenza
AIV(s)	avian influenza virus(es)
ABSL-2	animal biosafety level 2
aMPV	Avian metapneumovirus
Ct	cycle threshold
DMEM	Dulbecco's Modified Eagle's Medium
Dpi	days post-inoculation
EID_{50}	mean egg infective dose
ELISA	enzyme-linked immunosorbent assay
HA	Hemagglutinin
HE	hematoxylin/eosin
HI	hemagglutination inhibition
HPAI	highly pathogenic avian influenza
HPAIV(s)	highly pathogenic avian influenza virus(es)
IHC	Immunohistochemistry
IBV	Infectious bronchitis virus
ILTV	Infectious laryngotracheitis virus
LPAI	low pathogenic avian influenza
LPAIV(s)	low pathogenic avian influenza virus(es)
MDCK	Madin-Darby canine kidney
M1	matrix protein
M2	membrane ion channel protein
NEPs	nuclear export proteins
NA	Neuraminidase
NP	Nucleoprotein
NS1	nonstructural protein 1
NS2	nonstructural protein 2
NDV	Newcastle disease virus
OIE	World Organization for Animal Health
PA	polymerase acidic protein
PBS	phosphate buffer saline
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
qRRT-PCR	quantitative real time RT-PCR
SHS	Swollen head syndrome
μg	Microgram
μM	Micromolar
μl	Microlitre
μm	Micrometre
mL	Milliliter
Min	Minute(s)

INTRODUCTION

1. General introduction

Respiratory disease is a multifactorial problem in poultry, with viral and bacterial respiratory pathogens often concurrently present and most probably influencing one another (Marien *et al.*, 2007). A community of mucosal dwelling microorganism colonize healthy upper respiratory tract including both commensals and potential pathogens kept under control by the host immune system. There are some evidence demonstrating that bacterial colonization can be enhanced by viral priming (Brealey *el al.*, 2015).There are two hypotheses which can explain the underlying mechanism. One hypothesis is that viral infections may lead to bacterial superinfection by damaging the respiratory tissue, characterized by loss of cilia and ciliated cells (Bakaletz, 1995; Matthijs *et al.*, 2009), decreased ciliary activity and mucociliary clearance (Wilson *et al.*, 1996), and leading to efficient bacterial attachment with damaged respiratory tissue (El Ahmer *et al.*, 1999). A second hypothesis is that the dysfunction of immune system may increase the colonization of bacterial infections after viral infection. Previous virus infection led to decreased phagocytic activity of macrophages and heterophils (Engelich *et al.*, 2002; Navarini *et al.*, 2006). Moreover, bacterial colonization may also be enhanced by severe granulotoxic effects of the innate anti-viral responses (type I interferons (IFN)) (Navarini *et al.*, 2006; Matthijs *et al.*, 2009). A significant higher number of pathogens are seen in tissues of superinfected animals than in tissues of animals infected with only one pathogen. Furthermore, inflammatory cytokines can be over produced during superinfection leading to exacerbated immune responses and damage to host tissue (Beadling & Slifka, 2004; van der Sluijs *et al.*, 2006; Speshock *et al.*, 2007).

Usually opportunistic bacterial pathogens are detected during respiratory virus infections such as *Escherichia coli* (*E. coli*). Uptil now, unidirectional view of avian influenza virus (AIV) /bacterial interactions has been studied, where viral infection proved beneficial to bacterial infection and led to bacterial superinfection at the respiratory tract. Respiratory viruses and bacteria may interact in a bidirectional way, where bacteria may also influence host

susceptibility to viral infection. Moreover, cotransmission of virus and bacteria may be possible as infectious agents of respiratory tract are transmitted through aerosol or direct contact with respiratory excretions (Brealey *et al.*, 2015). Low pathogenic Avian influenza virus (LPAIV) infection is an emerging respiratory problem, isolated from different birds from a number of countries and has been reported to have zoonotic potential (Swayne, 2008; Liu *et al.*, 2014; Umar *et al*., 2016a). LPAIV may be transmitted from aquatic birds to domestic poultry leading to economic losses (Swayne, 2008). Turkeys are an important host in influenza virus ecology because they are susceptible to infection with these viruses and are often involved in inter species transmission. Several previous studies reveal that waterfowl-origin influenza viruses can be more easily transmitted to domestic turkeys than to chickens (Abid *et al*., 2016).

An important natural route of avian influenza (AI) infection in farms is inhalation of contaminated dust. Aerosols may contribute to the transmission of AI between birds in addition to the faecal-oral route. For example, some high pathogenic avian influenza virus (HPAIV) (H5N1) can replicate in feather follicles of waterfowl, which may serve as a potential source for aerosol transmission (Yamamoto *et al.*, 2007). Spread of viruses in the air has been suspected when outbreaks of AI have occurred downwind from infected flocks or when contaminated manure has been spread on land in the proximity of poultry buildings (Lv *et al.*, 2012). Experimental studies have shown that some subtypes of AIV could be transmitted between flocks of chickens via the air (Tsukamoto *et al.*, 2007; Shi *et al.*, 2010; Yao *et al.*, 2011; Guan *et al.*, 2013), and more information on virus infection by aerosols and the corresponding host immune response is needed to improve the understanding of aerosol transmission of AIV. Viruses that travel in the air can be carried as aerosols; aerosols contain particles < 5 μm in size. Aerosols can stay in the air longer and travel farther than large droplets and hence are more likely to be responsible for airborne transmission of viruses (Nicas *et al.*, 2005). Commonly used methods of experimental infection, such as intranasal and intratracheal inoculations, bypass the deep air sac access of virus particles. In addition, the minute size of virus aerosols facilitates their reach into the lower respiratory tract and causes more severe

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disease (Tellier, 2006; Guan *et al.*, 2015). A human clinical study of influenza infection reported that the 50% infectious dose (ID50) by aerosol inhalation was approximately 100-fold less than that by inoculation with intranasal drops (Tellier, 2006). In accord with that report, studies in chickens have shown that the ID50 values of both HPAIV (H5N1) and LPAIV (H9N2) were substantially lower by aerosol inoculation than by intranasal drops (Guan *et al.*, 2013; Sergeev *et al.*, 2013). Sergeev et al. (2013) found that aerosolised HPAIV was rapidly spread to various organs via respiratory infection in chickens. LPAIV H9N2 is currently widespread in domestic poultry and occasionally transmitted to mammalian species, including humans (Alexander, 2007; Kwon *et al.*, 2008; Liu *et al.*, 2014). Respiratory tract infections in turkeys due to viruses and bacteria frequently result in considerable financial losses due to increased production losses, mortality rates and medication costs. Respiratory diseases in turkeys are triggered by several pathogens, alone or in combination with the support of other non-infectious factors. The respiratory viruses influenza virus type A, paramyxovirus types 1, 2 and 3 and avian metapneumovirus (avian pneumovirus, turkey rhinotracheitis virus) (aMPV) have been shown to induce respiratory problems. However, clinical signs following experimental inoculation with these viruses are less severe than those observed in the field (Marien *et al.*, 2007). It is generally accepted that secondary bacterial pathogens are often involved, with amongst others *E. coli.* These bacterial agents differ from the viral pathogens in that it is not always straightforward to reproduce clinical signs following experimental infection. *E. coli,* a notorious infectious pathogen in poultry, illustrates this apparent paradox.

LPAIV H6N1 has been circulating in French Poultry industry and may cause severe economic losses especially due to secondary bacterial infection by *E. coli* (Prof.Jean Luc Guerin personal communication) and *E. coli* infections often occur between production onset and slaughter. Thus, in the current field situation bacterial infections appeared to be the predisposing agents rather than AIV. Whether this virus, which belongs to the genus *Orthomyxovirus* and this Gram-negative bacterium merely act separately or in a synergistic or additive way remains to be elucidated. Only a few experimental studies have been undertaken in chickens to study

possible mutual interactions between AIV and *E. coli* (Barbour *et al.*, 2009). The lack of documentation in scientific journals is surprising since AI and *E. coli* co-infections are serious emerging issues in the poultry industry. Previous studies have shown that pathogenicity of the LPAIV was typically enhanced by secondary bacterial infections, resulting in chicken morbidity (Kishida *et al.*, 2004). Recently, one H3N8 virus, Dk/BJ/40/04, caused a fatal disease when coinoculated with *E. coli* indicating H3N8 subtype viruses can be pathogenic to chickens under field conditions (Pu *et al.*, 2012). Moreover, Barbour *et al.* (2006) and Stipkovits et al. (2012ab) reported that clinical signs were aggravated during mixed infections of AIV and *Mycoplasma* in poultry (Barbour *et al.*, 2006; Stipkovits *et al.*, 2012ab).

Poultry industry is one of the biggest industry in Pakistan and France. However, turkey industry is much bigger and developed in France than in Pakistan. Percapita meat consumption is lower in Pakistan than in France. Therefore, poultry industry in Pakistan needs big improvement to fulfill the demands of increasing human population. Comparison of poultry production for both countries is presented in Table 1 (FAOSTAT, 2014). The present study was undertaken to study the molecular epidemiology of respiratory viruses in Pakistani poultry and to develop a dual infection model for AIV and *E. coli* in turkeys using the intratracheal and aerosol inoculation routes. During our experimental work on turkeys, we performed different pilot experiments to compare aerosol and intratracheal route of inoculation. We found that aerosol route of inoculation is better than intratracheal route of inoculation in that it better mimics field conditions. Aerosol inoculation may help to shorten the gap between field and laboratory conditions. However, it is still difficult to generate 100% field conditions in laboratory settings because there are some other factors which contribute in the production of diseases e.g. dust, pollution, humidity, temperature, ammonia production, housing stress etc. Different settings were tested between infections, with clinical signs, gross lesions, histology and bacterial/viral titration as parameters for evaluating possible synergistic/additive potential between both agents.

Parameters	France	Pakistan
Total poultry population	216,587 (1000 heads)	858,700 (1000 heads)
Chickens	167,906 (1000 heads)	855,000 (1000 heads)
Turkeys	20,190 (1000 heads)	NA
Total poultry meat production	1,718 (1000 tonnes)	1,074 (1000 tonnes)
poultry meat consumption per capita	26.4 kg	7 kg

Table 1: Comparison of Pakistani and French poultry production (FAO STAT, 2014)

2. The avian respiratory system

The principal function of the respiratory system in birds is exchanging oxygen (O_2) and carbon dioxide (CO_2) between atmosphere and blood, but also temperature regulation and phonation. Because of its role in gas exchange, the respiratory system is regularly in direct interaction with the outside environment. Every day many liters of air go through the lungs, providing a constant challenge of airborne particles and microbes. In complete contrast to the tidally ventilated mammalian respiratory system, where fresh inhaled air is mixed with residual stale air in the respiratory airways, the avian lung is a flow-through system (Reese *et al.*, 2006). The respiratory tract begins at the nares, consists of passages between conchae in the head and subsequently leads inhaled gas to the larynx. The trachea extends from the larynx, and branches into two extrapulmonary primary bronchi. From each primary bronchus, four groups of secondary bronchi (medioventral, mediodorsal, lateroventral and laterodorsal) arise and from the secondary bronchi multiple parabronchi develop (Fedde, 1998) (Figure 1). Ventilation is achieved through a unique action of the air sacs as there is no diaphragm in birds. Therefore, avian lungs have a highly complex structure and are fixed in thoracic walls. Air sacs occupy every available space in the body cavity which is not occupied by other viscera. There are nine

air sacs in most of the birds: paired cervical air sacs, an unpaired clavicular air sac that is connected to each lung, paired cranial thoracic air sacs, paired caudal thoracic air sacs, and paired abdominal air sacs. The cervical, clavicular, and cranial thoracic air sacs arise from the medioventral secondary bronchi, and they are often called the cranial air sacs. The caudal thoracic and abdominal air sacs (the caudal air sacs) arise from the lateroventral and mediodorsal secondary bronchi and from the continuation of the intrapulmonary primary bronchus. The air sacs are auxiliary structures that pump air through the respiratory tract, but do not contribute to the gas exchange with the blood (Fedde, 1998; Reese *et al.*, 2006). The O_2 and CO_2 exchange only occurs in the lungs. During inspiration, active contraction of some muscles of the body wall causes an increase in the volume of the air sacs which results in pressure in the air sacs less than that in the atmosphere and gas moves through the lungs into the air sacs. The inspired air completely bypasses the cranially lying openings of the medioventral secondary bronchi, a process which is called inspiratory aerodynamic valving (Reese *et al.*, 2006). In contrast, during the inspiratory phase as well as the expiratory phase, air flows in the mediodorsal and lateroventral secondary bronchi. About one half of the inspired volume passes through the paleopulmonic parabronchi and in this way in the cranial air sacs, and the remainder passes through the much smaller neopulmonic parabronchial network to the caudal air sacs, and through the direct connection from the intrapulmonary primary bronchus to the abdominal air sacs. During expiration, reduction in coelomic volume (decrease in the volume of the air sacs) increases the pressure in the air sacs and air moves out of the air sacs. Some of the air from the caudal air sacs again traverses the neopulmonic parabronchi and most of the air enters the paleopulmonic parabronchi, travelling in the same direction as during inspiration. Air from the cranial air sacs flows through the medioventral secondary bronchi to exit the lung without contacting any parabronchial gas exchanging surfaces (Figure 2). Thus exchange of O_2 and CO_2 between air and blood occurs both during inspiration and expiration in birds and nearly all of the air that was inhaled, has passed over paleopulmonic parabronchial gas exchanging surfaces during some part of the respiratory cycle. The walls of the parabronchi

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are perforated by numerous openings that lead to the respiratory atria. Funnel-shaped infundibulae arise from the atria and open into the air capillaries. The inhaled air flows through the parabronchial lumen and then into the exchange tissue through the atria, the infundibulae, and the network of air capillaries. A complex network of blood capillaries closely surrounds air capillieries forming efficient gas exchange system in birds (Reese *et al.*, 2006). The blood-gas barrier in the avian lung is approximately 56-67% thinner than that of a mammal of the same body mass and the respiratory surface area is approximately 15% greater (Maina *et al.*, 1989). Respiratory efficiency is enhanced by large surface area and thin tissue barrier. However, these structural features make birds more susceptible towards pulmonary injury from environmental toxicants and invasion by pathogenic organisms (Reese *et al.*, 2006).
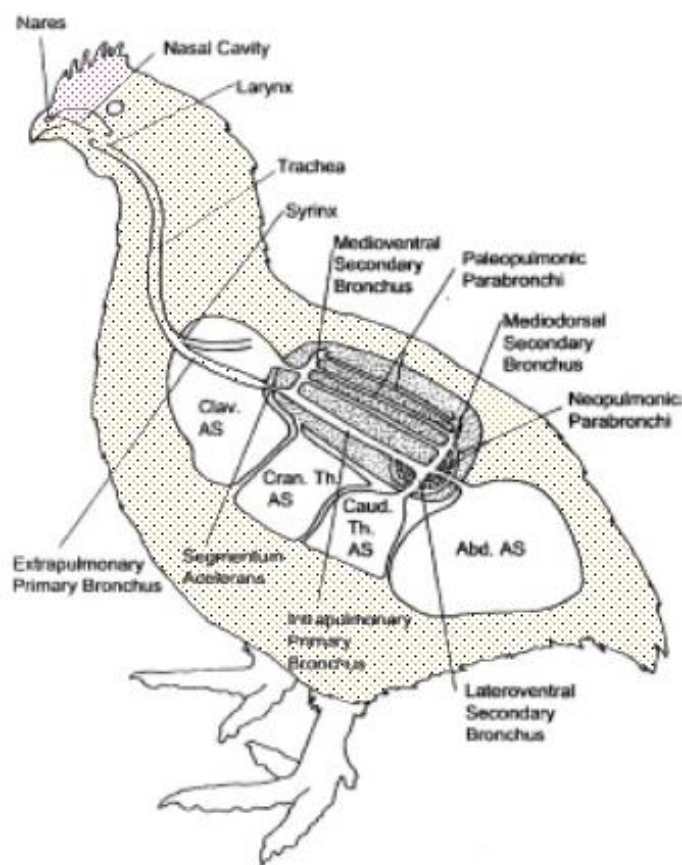
Figure 1: Respiratory system of chickens (Clav. AS= clavicular air sac; Cran. Th. AS=/cranial thoracic air sac; Caud. Th. AS=caudal thoracic air sac; Abd. AS=abdominal air sac). There is expansion of airsacs during inhalation drawing air from trachea and primary bronchi towards the the caudal air sacs and paleopulmonic parabronchi (Fedde, 1998).
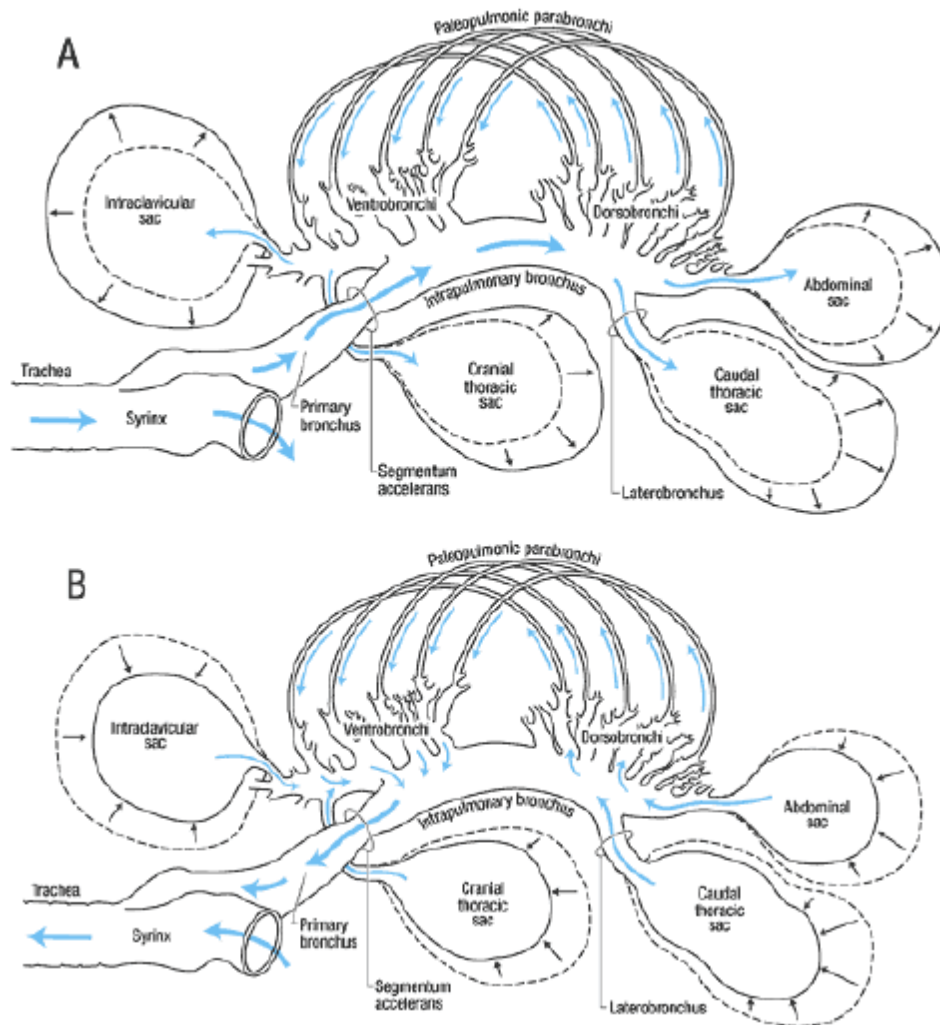
Figure 2: Mechanism of respiration in birds. Mechanism and pathway of gas flow and exchange through the pulmonary system during inspiration and expiration in right paleopulmonic lung and air sacs of chicken. **A:** Inspiration. **B:** Expiration (Source: http://www.people.eku.edu/ritchisong/birdrespiration.html)

2. Defense mechanisms and immune responses in birds

The divergence of mammals and birds from a common reptilian ancestor occurred 200 million years ago. Despite this evolutionary time period, the fundamental principles of both the innate and adaptive immune systems of mammals and birds are the same. The availability of the chicken genome has helped improve our understanding of the avian immune system. The

respiratory system harbours the most extensive and thinnest surface across which the body is exposed to the external environment. Due to this characteristic, a vast array of proteins and pathogens are challenging this system on a daily basis. To cope with these pathogens, birds have well-developed defence mechanisms.

3.1. Innate resistance

The first non-specific arm of the avian immune system is known as the innate System. As in mammals, the most well characterised family of non-specific pattern recognition receptors (PRRs) are membrane-bound Toll-like receptors (TLRs) expressed by various cell types including epithelial cells and sentinel cells such as antigen presenting cells (APCs), dendritic cells and macrophages. In the chicken, TLRs are also expressed by heterophils, a polymorphonuclear leukocyte and homologue of mammalian neutrophils. TLRs detect structurally conserved microbial specific motifs. Thirteen TLRs have been described in the chicken; 11 are also present in mammals while two are chicken-specific (TLR-15 and TLR-21) (Temperley *et al.*, 2008). Despite differences in TLR families, similar microbial motifs are recognised by both TLR repertoires. The initial line of defense for the airway is the nasal and tracheal epithelium, which prevents pathogens from entering the body. Multiple mucous glands within the pseudostratified ciliated columnar epithelium produce mucus which forms a layer on top of the cilia of the epithelial surface. Particulate material that is caught in the mucus gets transported by the movement of the cilia in an oral direction, where it is swallowed and digested or excreted by coughing and sneezing (Sharma, 2003; Koch *et al.*, 2009). Furthermore, mucus contains antibacterial enzymes which impede the attempts of pathogens to colonize.

Phagocytic cells that include heterophils and macrophages, and natural killer (NK) cells are important components of innate immunity. Monocytes-macrophages, cells belonging to the mononuclear phagocytic system, are considered to be the first line of immunological defense. These cells originate from the bone marrow and subsequently enter the blood circulation. Upon migration to various tissues, monocytes mature and differentiate into tissue macrophages (Dietert *et al.*, 1991; Qureshi *et al.*, 2000). Macrophages then get involved in innate and

acquired immunity (Qureshi *et al.*, 2000). Since the respiratory surface is in proportion much larger than that of mammals and the tissue barrier is much thinner (Maina, 1989; Maina *et al.*, 1989), one can expect that, as stated above, the avian respiratory tract is relatively more easily attacked by pathogens than the mammalian one (Nganpiep & Maina, 2002). One would hence assume that for a similar defense competence, more residing avian respiratory phagocytes (ARP = macrophages and polymorphonuclear leukocytes such as heterophils) would arise on the surface of avian lungs. Paradoxically, the normal, steady-state avian respiratory system has very low numbers of residing ARP in comparison to the mammalian system, and as a consequence birds must rely heavily on the influx of ARP into the site of infection for non-specific defense against bacteria and other pathogens (Ficken *et al.*, 1986; Qureshi *et al.*, 1994, 2000; Klika *et al.*, 1996; Toth, 2000). Interestingly, ARPs the were present atria and the infundibulae and were never found on the surface of the air capillaries which represent the functional equivalent to the mammalian alveoli (Nganpiep & Maina, 2002; Reese *et al.*, 2006). Thus, macrophages seem to be located at strategic check points where fresh air is distributed into the gas exchange areas and where particles can be trapped and removed (Marien *et al.*, 2007). The paucity and even lack of ARPs in birds has been used to explain a purported high susceptibility of poultry to respiratory diseases. Nganpiep & Maina (2002), however, showed that a composite defense armament has additionally developed in the avian respiratory system. A highly lytic upper airway epithelium endowed with lysosomes (apparently lacking in mammals), generally robust ARPs, and efficient translocation of subepithelial macrophages onto the respiratory surface, play a role in the protection of the respiratory system (Nganpiep & Maina, 2002). In the air sacs, being thin walled and lacking an elaborate ciliated epithelium, particle clearance is largely accomplished by phagocytic cells albeit significantly lower than in the lungs (Nganpiep & Maina, 2002; Reese *et al.*, 2006).

3.2. Adaptive immunity

The second arm of the chicken immune system is the adaptive one involved in both cellular and humoral (antibody) responses, as well as the production of memory cells. The avian

antigenic repertoire is more compact than that of the mammalian system; one reason for this is that birds only possess 2 of the Major Histocompatability complex class (MHC), compared to the 6 of mammals (Kaufman, 2000). When pathogens cannot be withheld by physical barriers nor controlled by innate immune defense mechanisms, adaptive immunity (specific immune response) is required to specifically focus defense mechanisms on that particular antigen resulting not only in the elimination of the pathogen but also in protecting in case of a repeat encounter with the same pathogen (memory). Adaptive immunity is mediated by a variety of cells, of which T lymphocytes, B lymphocytes, and macrophages are the most important.

In poultry as in mammals, adaptive immunity is critically dependent on regulation by T lymphocytes (T cells), the coordinators of the immune response. Maturation of the T cells takes place in the thymus, a feature shared with mammalian species (Arstila *et al.*, 1994). Before T cells can initiate and participate in an adaptive immune response to a pathogen, the antigen has to be presented by host cells in the context of their MHC molecules, i.e., as an antigenic peptide bound to the MHC molecule. The MHC molecules come in two forms: the MHC class I is expressed by essentially all nucleated cells, whereas the MHC class II is expressed mainly by cells of the immune system, the so-called antigen presenting cells (APC) such as macrophages, dendritic cells and B lymphocytes (B cells). These APC also deliver other signals equally important to the T cell activation, the so-called second or costimulatory signals (Arstila *et al.*, 1994). Activation of T cells results in proliferation of the activated T cells and their differentiation into subpopulations of diverse effector cells, helper T cells (CD4+), suppressor T cells, and cytotoxic T cells (CD8+), or memory cells. Effector functions of T helper cells primarily involve production of cytokines (soluble molecules secreted to the extracellular space), and expression of membrane-bound cell-surface molecules, all affecting other cells of the immune system. The cytotoxic T cells, in contrast, are mostly killers that are specialized in the elimination of intracellular antigens. The latter include those that have entered cells *via* the endocytic pathway (exogenous antigens; e.g., phagocytosed bacteria) or were produced within the cell such as viral proteins and proteins resulting from neoplastic transformation of the cell

(endogenous antigen) (Erf, 2004). Another lineage of T cells exists (γδ T cells), but their physiological significance remains largely a matter of speculation (Marien *et al.*, 2007).

Besides the T lymphocytes, other cells important to the cellular immune response include macrophages, dendritic cells, NK cells, and effector cells of antibody dependent cellular toxicity (Sharma, 2003). NK cells can also be regarded effector cells of specific cell-mediated immunity as they greatly benefit from T helper mediated activity (Erf, 2004). Unlike mammals, birds have a special organ, the bursa of Fabricius, where the development of B lymphocytes (B cells) from their immature precursors takes place. For humoral immunity, B cells differentiate into plasma cells that secrete antigen-specific antibodies. Antibodies can prevent disease caused by pathogens and provide protection, but they are primarily effective in preventing entry of pathogens through mucosal surfaces (e.g., secretory IgA) and in eliminating extracellular antigens (Koch *et al.*, 2009). Most organisms stimulate both cell-mediated immunity and humoral immunity, although the type of immunity most critical for defense may vary with the organism (Vandaveer *et al.*, 2001; Sharma, 2003; Erf, 2004).

3. Respiratory diseases and associated pathogens in turkeys and chickens

Respiratory diseases are continuing to cause heavy economic losses in the poultry industry due to high production losses, mortality and medication costs (Van Empel & Hafez, 1999). Respiratory disease in poultry is a multifactorial problem, with viral and bacterial respiratory pathogens often concurrently present and most probably influencing one another (Marien *et al.*, 2007). In addition to these infectious organisms, non-infectious factors, such as climatic conditions (e.g. inadequate ventilation, high ammonia levels, too high or too low temperature) can also help in diseases progress. The severity of clinical signs, duration of the disease and mortality are extremely variable and are influenced by many factors such as a virulence and

pathogenicity of the infectious agent as well as by many environmental factors. In many cases, respiratory disease observed in a flock may be a component of a multisystemic disease or it may be the predominant disease with lesser involvement of other organ systems. Respiratory tract infections increase the overall cost of production in terms of the provision of services of qualified veterinary personnel and the cost of medication for possible treatment. It is therefore important to reduce if not eliminate, respiratory infections among poultry flocks to the barest minimum to have good production and maximize profit of the producer. Various pathogens including a variety of viruses, bacteria, and fungi may initiate respiratory diseases in poultry. Environmental factors may augment these pathogens to produce the clinically observed signs and lesions. Poultry respiratory diseases are known to be caused by many pathogens (Table 2) including Newcastle disease virus (NDV), AIV, Infectious Bronchitis Virus (IBV), aMPV, *Mycoplasma gallisepticum* (*M. gallisepticum*), *Mycoplasma synoviae* (*M. synoviae*) *Mycoplasma meleagridis* (*M. meleagridis*), *Mycoplasma iowae* (*M. iowae*), *Ornithobacterium rhinotracheale* (*O. rhinotracheale*), *Pasteurella multocida* and *Avibacterium paragallinarum*, *Bordetella avium, Chlamydophila psittaci* and *E. coli* with associated significant economic losses to the industry (Van de Zande *et al.*, 2001).

Disease	Aetiology	Main Clinical signs /lesions	Prevention /control
Avain influenza (AI)	Avian influenza virus (AIV)	Mild to severe respiratory signs depend on virus subtype	Vaccine available Good biosecurity measures
NewCastle disease (ND)	Newcastle disease virus (NDV)	Variable: mild to severe respiratory clinical signs and lesions	Vaccine available Good biosecurity measures
Infection bronchitis (IB)	Infection bronchitis virus (IBV)	Tracheitis, airsacculitis, pneumonia, nephritis	Vaccine available Good biosecurity measures
Infectious laryngotracheitis (ILT)	Infectious laryngotracheitis (ILTV)	Teacheitis	Vaccine available Good biosecurity measures
Swollen head syndrome/ turkey rhinotracheitis	Avian metapneumovirus (aMPV)	Swollen head, tracheitis, airsaculitis,pneumonia	Vaccine available Good biosecurity measures
Mycoplasmosis	*Mycoplasma gallisepticum*	Chronic tracheitis; chronic	Mycoplasma free progeny.

		airsacculitis	Vaccination Possible
Mycoplasmosis	*Mycoplasma synoviae*	Moderate tracheitis and airsacculitis. Arthritis	Mycoplasma free progeny
Infectious Coryza	*Avibacterium paragallinarum*	Conjunctivitis, sinusitis, airsacculitis	Vaccination possible
Colibacillosis	*E. coli*, often associated with other respiratory pathogens, e.g. IBV, NDV, mycoplasma	Fibrinous pericarditis, airsacculitis, tracheitis	Vaccine available against some *E. coli* serotypes. Reduce dust in shed
Pasteurellosis (Fowl cholera)	*Pasteurella multocida*	in chronic form e.g. conjunctivitis, tracheitis; in acute form septicaemia	Vaccination possible Good biosecurity measures
Ornithobacteriosis	*Ornithobacterium rhinotracheal*	Tracheitis, airsacculitis	Vaccination possible Good biosecurity measures

Table 2: Respiratory pathogens and related diseases in poultry

These respiratory pathogens are of major importance because they can cause disease independently, in alliance with each other or in association with other bacterial and viral agents (Ali & Reynolds, 2000; Yashpal *et al.*, 2004). Viral agents are mostly being attributed a triggering role, since the clinical signs following experimental inoculation with these viruses are less severe than those observed in the field. Viral infections generally cause rather acute respiratory problems from which birds usually can recover fairly easily. The problems, however, become more critical when bacterial pathogens are involved. With these bacterial agents, it is not always straightforward to reproduce clinical signs following experimental infection. This has led to a still contemporary discussion point whether the different bacterial agents are primary or rather secondary pathogens (Marien *et al.*, 2007). In the present thesis, the experimental research focuses on the co-infection in turkeys with AIV, and *E. coli*. Hence, the most important literature data on these agents will be discussed below.

4. Avian influenza

Avian influenza (AI) was first identified as a distinct disease entity of poultry in 1878, in Italy. It was called "fowl plague" and was defined as a highly lethal, systemic disease of chickens. From the 1870s into the early 1900s, fowl plague spread from Northern Italy into the rest of Europe, and by the 1930s it was endemic in parts of Europe and Africa (Schafer, 1955). Likewise, in the United States the disease was reported in 1924-1925 and 1929 (Stubbs, 1948). Into the mid-twentieth century, fowl plague had been diagnosed in North Africa, South America, North America, and most of Europe (Swayne & Halvorson, 2008). The agent responsible for the human influenza initially isolated from pigs in 1931 and later from humans in 1933 (W. Smith *et al.*, 1933; Shimizu, 1997). Prior to that, one of the most devastating influenza pandemics in human history, the "Spanish Flu" (H1N1 subtype), hit the population in 1918, causing thousands of deaths (Taubenberger *et al.*, 2000). Even if Centanni & Savonuzzi (1901) had already demonstrated the existence of a filter-passing agent, the viral etiology of fowl plague was unknown until 1955, when the disease was determined to be caused by influenza A virus (Schafer, 1955).

In gallinaceous birds (i.e., chickens and turkeys), AI viruses are classified as being highly pathogenic AI (HPAI) or low pathogenic AI (LPAI) viruses. Although molecular criteria have been established by the World Organization for Animal Health (OIE) for the identification of the HPAI virus based on the protein sequence of the HA proteolytic cleavage site, *in vivo* testing used to be the gold standard. An AI virus isolate is classified as being HPAI if it kills at least 75% of susceptible 4- to 6-week-old chickens within 10 days postinoculation by the intravenous route. Some isolates will cause 100% mortality by 36–48 hours postinoculation. All other isolates are considered to be LPAI viruses. Biologically, the difference between HPAI and LPAI is that HPAI is a systemic infection and LPAI remains localized to the respiratory and intestinal tracts. For unknown reasons, all HPAI viruses have been either H5 or H7 HA subtypes (David L. Suarez & Swayne, 2008). Nevertheless, mild clinical forms of AI, characterized by respiratory disease and drops in egg production, were first recognized in 1949 in chickens and,

subsequently, in several domestic poultry species (Easterday & Tumova, 1972). Therefore, since 1971, H5 and H7 viruses have been isolated and characterized not only as HPAIVs but also as LPAIVs (Smithies *et al.*, 1969). Although wild birds were already suspected to participate in fowl plague transmission, it was not until 1961 that the first proof of AIV infection in wild birds arose, in an outbreak in South Africa affecting common terns (Sterna hirundo) with high mortality (Becker, 1966). Since then, and particularly during the recent past decades, numerous surveys have been conducted in migratory waterfowl, confirming asymptomatic infection by AIV of healthy wild aquatic birds, especially in the orders *Anseriformes* and *Charadriiformes* (Hinshaw & Webster, 1982).

Type A influenza strains are classified by the serological subtypes of the primary viral surface proteins, the hemagglutinin (HA) and neuraminidase (NA). The HA has 16 subtypes (H1–H16) and contains neutralizing epitopes. Antibodies against the NA are not neutralizing, and there are nine neuraminidase or "N" subtypes. The "H" and N subtypes seem to be able to assort into any combination, and many of the 144 possible combinations have been found in natural reservoir species, although some combinations are more common than others. All 16 subtypes have been found in ducks, gulls, or shorebirds, the natural reservoir host species of the virus and two of HA and NA (HA17–HA18 and NA10–NA11) have been isolated from bats (Tong *et al.*, 2012, 2013). However, in these species certain subtypes are more common than others; for example, H3, H4, and H6 are most common in ducks in North America and although there is no clear association between host range or host restriction based on HA subtype, some subtypes are more common in some species than others, i.e., H1 and H3 in swine, H3 in horses, and H5 and H7 in chickens (David L. Suarez & Swayne, 2008).

It was not until 1997 that AI became considered a disease not only of birds, when the occurrence of fatal disease in poultry and humans in Hong Kong was associated with the HPAIV H5N1 strain (Claas *et al.*, 1998). This episode increased the international interest in HPAIV among the veterinary and public health community, because it was the first indication that AIV (H5N1) could potentially be the precursor to a human pandemic viruses (Sims &

Brown, 2008). Indeed, over the next decade, HPAIV (H5N1) in poultry spread across three different continents with unprecedented socioeconomic consequences. These concerns were amplified because of the reassortment possibility with a human influenza A virus, which could create a new virus capable to produce the next human influenza pandemic (David L. Suarez & Swayne, 2008).

4.1. Etiology and Classification

Influenza viruses belong to the *Orthomyxoviridae* family (orthos, Greek for "straight"; myxa, Greek for "mucus") and are classified into five different genera: influenza A, influenza B, influenza C, Thogotovirus, and Isavirus (Cheung & Poon, 2007). The most serious types that cause dangerous outbreaks with high morbidity and mortality are influenza A viruses because they mutate more rapidly and have a wider range of hosts (Khanna *et al.*, 2008). Influenza A viruses infect animals, including birds, pigs, horses, whales, seals, and also humans (Ito & Kawaoka, 2000; Reperant *et al.*, 2009). Type B and C are generally found in humans, in addition to some mammals like seals, with less severity than influenza A. The main differences between the three main types of influenza viruses (A, B and C) are outlined in table 3. Wild aquatic birds of the order of *Anseriformes* (ducks, geese and swans) and *Charadriformes* (gulls, terns, surfbird and sandpiper) are considered to be the natural reservoir of all types of influenza A viruses. In these hosts, viral replication occurs mainly in the gastrointestinal tract, and to a lesser extent in the respiratory tract. The infected birds generally have no apparent signs of illness, with some exceptions after infection with highly pathogenic avian influenza viruses (Munster *et al.*, 2007).

Features	Influenza A virus	Influenza B virus	Influenza C virus
Number of gene segments	8	8	7
Surface glycoproteins	Haemaglutinin and neuraminidase (HA and NA)	HA and NA	HEF (Haemagglutination esterase Fusion)
Host range	Wide range of hosts (humans, pigs, horses, whales, seals and birds)	Humans and seals	Mainly humans (also found in swine)

Table 3: Comparison of major properties of influenza viruses (Cheung & Poon, 2007).

4.2. Morphology and molecular organization

Influenza viruses are roughly spherical with a size of around 100 nm or filamentous in shape, often in excess 300 nm in length (Bouvier & Palese, 2008). Morphological structure is known to be affected by several viral proteins (HA, NA) and matrix proteins (M1 and M2), in addition to the nature of the host cells (Cheung & Poon, 2007). Influenza viruses are enveloped with surface glycoprotein spikes and a segmented RNA genome of negative sense (complementary to mRNA). RNA of influenza A virus is organized into 8 segments, in total around 13600 nucleotides long (Webster *et al.*, 1992). These are the polymerase basic (PB1 and PB2), the polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural (NS) genes (Samji, 2009). Influenza A viral gene segments are known to encode at least ten proteins which are the RNA polymerase complex proteins (PA, PB1, and PB2), surface glycoproteins (HA, and NA), nucleoprotein (NP), matrix proteins (M1 and M2), and nonstructural proteins (NS1, NS2) (Samji, 2009; Wang & Taubenberger, 2010). In addition, PB1–F2 and a new viral protein (N40) which is translated from segment 2 have been recently identified in some influenza A virus isolates (Wise *et al.*, 2012). Moreover, two more proteins, PA-X and M42 which are translated from segment 3 and 7, respectively, have been recently found (Jagger *et al.*, 2012; Wise *et al.*, 2012) (Figure 3).
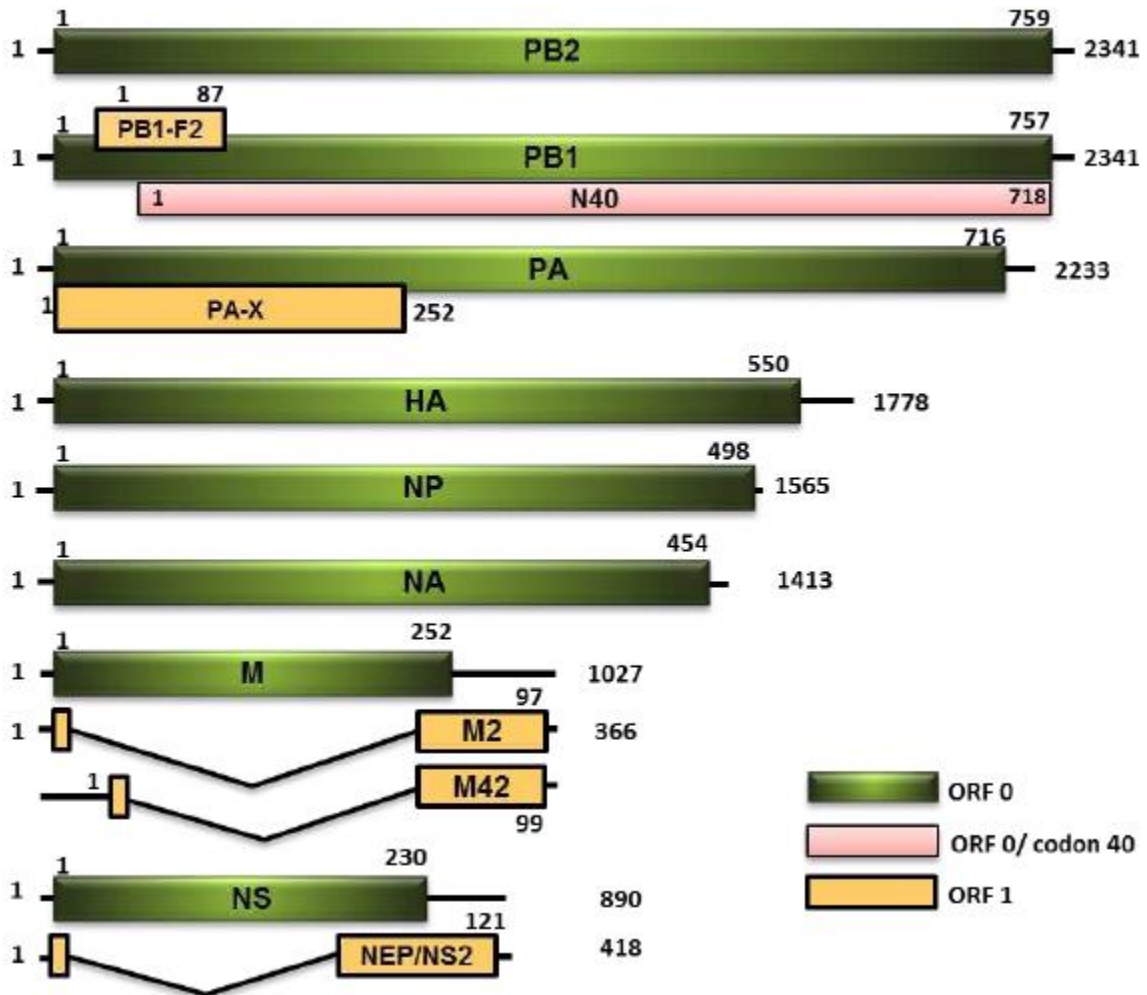
Figure 3: Genomic structure of influenza A virus

RNA segments (in nucleotides) shown in positive sense and their encoded proteins (in amino acids). The lines at the 5´ and 3´ termini represent the coding regions. The PB1 segment encodes three proteins, two of them (PB1 and N40) translated from ORF 0, and PB1–F2 protein translated from ORF 1. The M2, M42 and NEP/NS2 proteins are encoded by spliced mRNAs (the introns are indicated by the V–shaped lines) (Jagger *et al.*, 2012; Wise *et al.*, 2012).

Each viral RNA segment is surrounded by nucleoprotein (NP) forming ribonucleoprotein (RNP) and encapsidated by one copy of trimeric polymerase (PB1–PB2–PA complex) which is essential for viral replication (Digard *et al.*, 1999). The structural organization of viral ribonucleoprotein can be seen in figure 4.
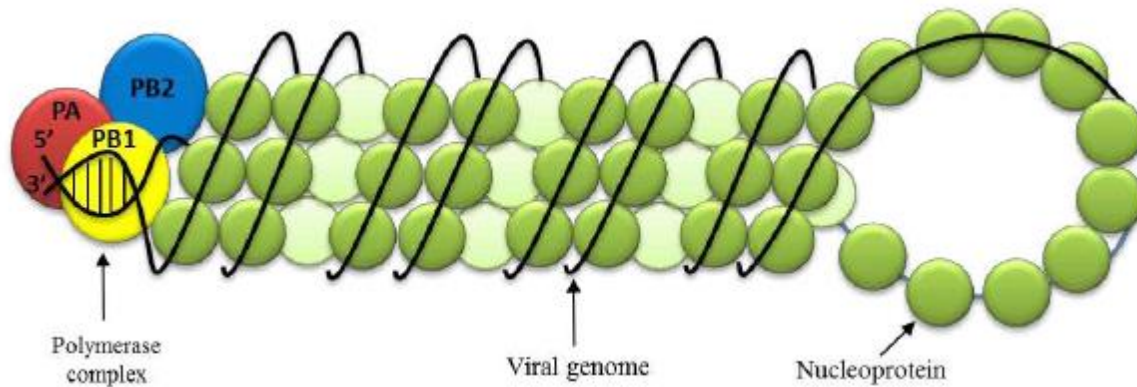
Figure 4: Structure of influenza virus ribonucleoprotein (vRNP).

Green spheres represent NP monomers, and the black line shows the associated single–stranded vRNA molecule. Influenza RNP folds into a double–helical hairpin structure. A short duplex formed between the 5´ and the 3´ ends provides the binding site for the heterotrimeric RNA–dependent RNA polymerase (Portela & Digard, 2002).

Four virus proteins (PB2, PB1, PA, and NP) are responsible for virus transcription and replication of the viral genome in the nuclei of infected cells. PB1–F2 protein plays a role in pro–apoptotic activity, while N40 protein, which is encoded by the same gene (PB1), interacts with the polymerase complex in the cellular environment but does not contribute to transcription function (Wise *et al.*, 2012). PA-X protein has been shown to modulate host response and viral virulence (Jagger *et al.*, 2012). Haemagglutinin (HA or H) plays a role in virus attachment to the host cell and subsequent fusion with cell membranes, while neuraminidase (NA or N) supports the release of viruses from the host cell surface by hydrolyzing sialic acid from glycoproteins which helps to release the progeny virus particles from host cells (McCauley & Mahy, 1983). Non–structural protein 1 (NS1) has a major role in inhibition of host immune response *via* limitation of interferon (IFN) production (Hale *et al.*, 2008). NS2 (also called nuclear export protein or NEP) plays a role in the export of RNPs from the nucleus to the cytoplasm during viral replication, in addition, it also regulates virus transcription and replication processes (Robb *et al.*, 2009). Matrix protein 1 (M1), the major structural protein, is the dominant protein in determining virus morphology and also plays an

important role in virus assembly and budding (Rossman & Lamb, 2011). Matrix protein 2 (M2) is the ion channel that regulates the pH, and is responsible for virus uncoating, the step that follows virus entry into the host cell (Holsinger *et al.*, 1994). In addition, this protein also plays an important role in membrane scission in the last stage of virus life cycle (Roberts *et al.*, 2013). Matrix protein 42 (M42) can functionally replace M2 and support efficient replication in null M2 influenza viruses (Wise *et al.*, 2012). Table 4 summarizes the length of each viral segment and the function of protein(s) encoded by each segment.

Segment	Segment size (nt)	Encoded proteins	Protein length (a.a)	Protein roles
1	2341	PB2	759	Polymerase subunit; plays a role in RNA replication by mRNA cap recognition
2	2341	PB1	757	Polymerase subunit; RNA elongation during replication
		PB1–F2	87	Pro–apoptotic activity
		N40	718	Polymerase complex interaction
3	2233	PA	716	Polymerase subunit, endonuclease activity
		PA-X	252	modulates the host response and viral virulence
4	1778	HA	550	Major surface antigen, receptor binding and fusion activities, main target for neutralizing antibodies
5	1565	NP	498	RNA binding protein, nuclear import regulation
6	1413	NA	454	Minor surface glycoprotein for neutralizing antibodies; sialic acid activity, cleavage of progeny virions from host cell receptors and virus release
7	1027	M1	252	Major component of virion, RNA nuclear export regulation, viral assembly and budding
		M2	97	Ion channel for controlling pH during virus uncoating and HA synthesis (viral release)
		M42	99	functionally replace M2 in M2-null viruses
8	890	NS1	230	Interferon antagonist protein, regulation of hostgene expression
		NEP	121	Control export of RNP from nucleus

Table 4: Influenza A virus gene segments, their proteins and functions. Typical gene and protein sizes are shown, though strain variation occurs (Bouvier & Palese, 2008).

The viral envelope is made of a lipid bilayer which is derived from the host cell's plasma membrane. Three surface viral antigens are embedded in the lipid bilayer: the HA spike, which has a rod like–shape, represents approximately 80% of the total surface proteins; the NA spike, which is almost mushroom–shaped, represents 17%; with minor components of M2 represented by few molecules (only 16 to 20 molecule per virion) (Schroeder *et al.*, 2005; Nayak *et al.*, 2009). Underneath the lipid bilayer, the M1 protein forms a layer that separates the viral segments from the virus membrane. Inside the virion, 8 segments of different length are associated with the nucleocapsid protein (NP) and three large proteins (PB1, PB2, and PA). NEP is also associated with the virus but in low amounts (Cheung & Poon, 2007). Figure 5 illustrates the typical structure of influenza A virus.
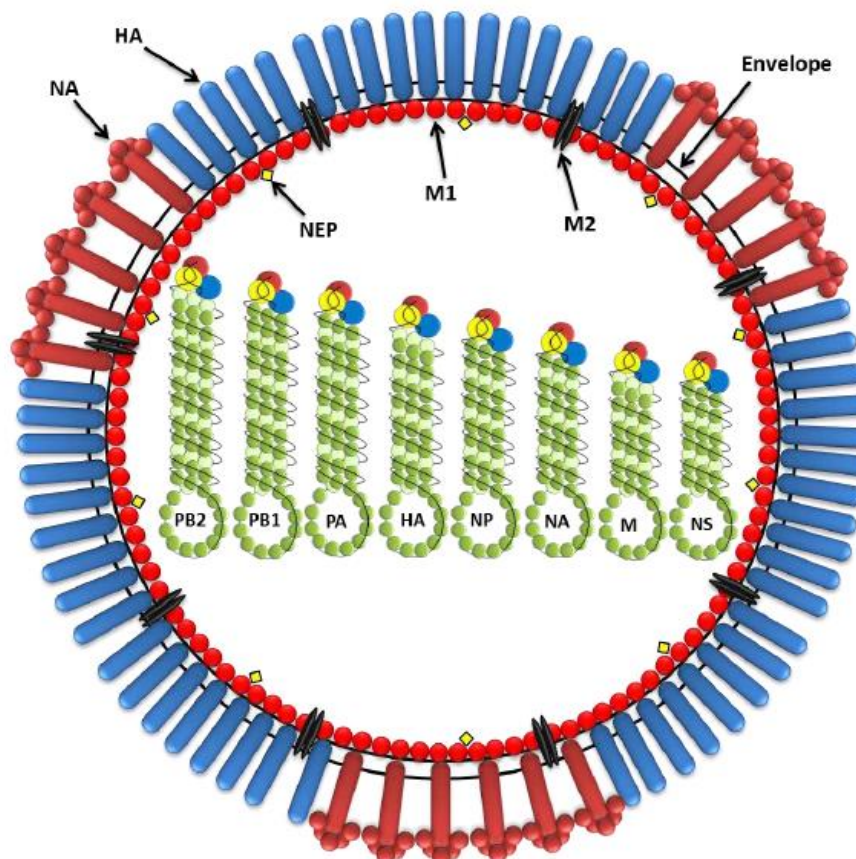
Figure 5: Schematic diagram of an influenza A virus particle.

The RNA is segmented and each segment encodes one or more proteins. The segments are not identical in length (ranging from 2341 to 890 nucleotides). The longest segment encodes PB2 protein and the shortest encodes NS protein. The RNA segments are coated with nucleoprotein forming ribonucleoprotein (RNP), and a small amount of transcriptase (polymerase complex) represented by PB1, PB2, and PA is also associated with it. The haemagglutinin (HA), neuraminidase (NA), and M2 proteins are inserted into the host–derived lipid envelope. The matrix (M1) protein underlies the lipid envelope. A nuclear export protein (NEP) is also associated with the virus (Al-Mubarak, 2014).

4.3. Replication of influenza A viruses

The first step of viral replication is virus attachment to its host cell. The host specificity of each type of influenza virus is mainly determined by differences in the host cell receptors (Naeve *et al.*, 1984). There are two main types of host cell receptors with which influenza viruses have the affinity to bind. The linkage between neuraminic acid and the sugar (galactose) determines whether influenza virus binds to avian or mammalian cells (Figure 6). Avian influenza viruses preferentially bind to the neuraminic acid α 2,3 galactose receptors while mammalian influenza viruses bind to neuraminic acid α 2,6 galactose (Auewarakul *et al.*, 2007; Pillai & Lee, 2010)
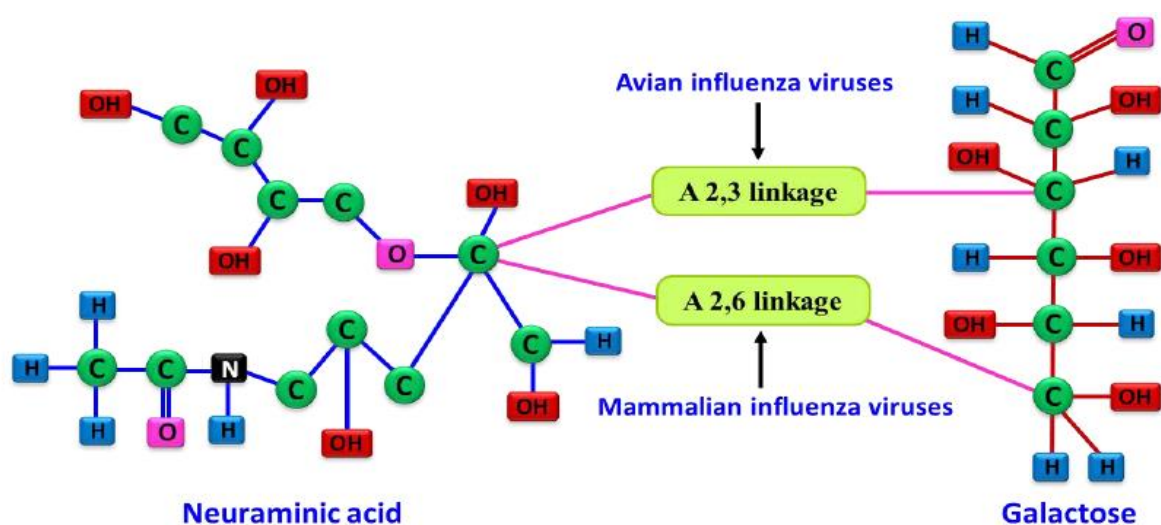
Figure 6: Overview of receptor predilections of avian and mammalian influenza viruses (Nelli *et al.*, 2010).

Once a host cell is infected with influenza virus, the HA glycoprotein is produced as a precursor, HA0, which is cleaved into two subunits (HA1 and HA2) by host serine proteases before virus particles become infectious (Klenk *et al.*, 1975). The HA1 portion contains the antigenic sites (the receptor binding domain), while the HA2 portion mediates fusion of the virus envelope and cell membranes (Steinhauer, 1999). Virulent and avirulent avian influenza A viruses can be differentiated by the sequence of a few basic amino acids at the point where the HA0 is cleaved (cleavage site); the so–called cleavage sequence (Zambon, 1999). The virus enters the host cell via receptor (clathrin) mediated endocytosis at the inside face of the plasma membrane forming an endosome (Rust *et al.*, 2004). The endosome has a low pH of around 5 to 6, which induces a conformational change in HA0, displaying the HA2 fusion peptide. This fusion peptide inserts itself into the endosomal membrane and mediates the fusion of the viral envelope with the endosomal membrane. This mechanism is not only important for inducing the conformation change in HA0, but also opens up the M2 ion channel during fusion of viral and endosomal membranes, allowing the virion interior to become acidic which releases the vRNP from M1. This permits the vRNP to enter the host cell's cytoplasm (Pinto & Lamb, 2006).

Transcription and replication occur inside the nucleus. Because of the negative sense of the viral genome, the viral RNA is copied into positive sense mRNA by the polymerase complex to act as a template for the production of the viral RNAs. The polymerase complex responsible for viral transcription and replication is formed by PB1, PB2, and PA. The viral RNA transcription is catalyzed by the RNA dependent RNA polymerase. The resultant positive sense viral mRNA is exported to the cytoplasm through nuclear pores to start viral translation by ribosomes. Positive sense viral mRNA also serves as a template to produce the negative sense RNA that is packaged into new virions (Swayne, 2008). Polymerase basic (PB1 & PB2), nonstructural (NS1 & NS2), NP, PA, and M1 proteins are synthesized in the host cell cytoplasm then transported to the nucleus to participate in matrix and nonstructural splicing, transcription and replication. Surface glycoproteins (HA and NA) are synthesized by ribosomes and then enter the endoplasmic reticulum (ER), where they are glycosylated, and then folded in the Golgi

apparatus. These proteins are incorporated in the cell membrane and assembled with vRNP complex (Sidorenko & Reichl, 2004). Progeny RNPs are released to the cytoplasm and packaged into new virus particles. New virions get enveloped with the plasma membrane with integrated virus proteins through budding (Palese & Shaw, 2007). Progeny virions are released from the cell surface using NA, which cleaves the sialic acid residues from the cell surface (Roberts *et al.*, 2013). The stages of influenza virus replication start from attachment of the virus onto host cells and end with the release of the progeny particles (Figure 7).
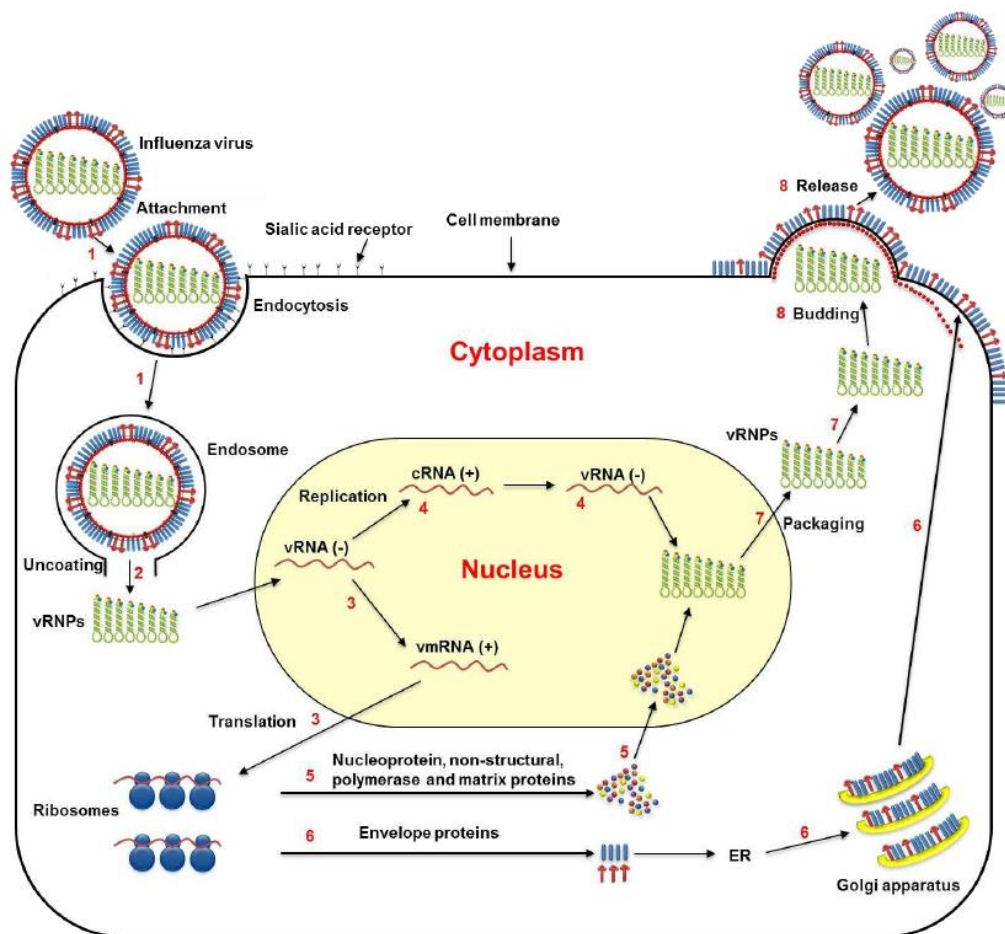
Figure 7: Life cycle of influenza viruses.

Stages involved in the replication process are:**1**. Attachment to host receptor and entry to host cell via endocytosis.**2**. Virus uncoating and releasing RNPs to the cytoplasm.**3**. Transcription and translation of viral RNA. **4**. Replication of viral RNA.**5**. Production of nucleoprotein, non-structural, matrix, polymerase acidic, and polymerase basic proteins.**6**. Production of envelope proteins (surface glycoproteins HA and NA, and M2) and their transportation to cell

membrane.**7**. Viral RNPs packaging and assembly.**8**. Virion budding and release from the cell membrane (Al-Mubarak, 2014).

4.4. Pathogenicity of influenza A viruses

According to the pathogenicity and severity of the disease in chickens, avian influenza A viruses can be classified into two pathotype groups: HPAIV and LPAIV. The mortality rates of the poultry flocks infected with HPAIV may reach 100%, while infection with LPAIV cause only milder and primarily respiratory disease (Capua & Marangon, 2000). In HPAIV, the region that encodes the cleavage site of the surface glycoprotein (HA) molecule contains multiple basic amino acids (arginine and lysine) which allows cleavage of the HA molecule by cellular endogenous proteases widely distributed throughout the cells of the body (Wood *et al.*, 1993). This molecular structure is important in determining the virulence of these strains because it allows the virus to replicate in a considerably broader tissue range, causing widespread damage in tissues and death of the bird, with a mortality rate approaching 100% (Kim *et al.*, 2009; Adams & Sandrock, 2010). The most pathogenic subtypes of avian influenza are restricted to subtypes H5 and H7. On the other hand, LPAIV have only one basic amino acid (arginine) in the cleavage site of the HA molecule. This limits the site for the viral cleavage by trypsin–like host proteases, and as a consequence, the replication process occurs in limited tissues and organs, particularly in respiratory and digestive tracts, causing only mild disease (Alexander, 2000). LPAIV which cause asymptomatic or low pathogenic infection may mutate and convert to HPAIV through an adaptation process after infection of poultry (Mundt *et al.*, 2009).

4.5. Influenza A viruses evolution

During influenza viral replication, genetic variations occur frequently. This is due to the structure of the viral RNA (segmented) and the low fidelity of the RNA dependent RNA polymerase which generates replication errors during virus life cycle (Zambon, 1999; Zambon, 2001). Consequently, influenza A viruses can undergo recurrent antigenic changes. The resultant change in structure allows the virus to evade neutralizing antibody, the main

mechanism of protective immunity against influenza infection. Such changes may lead to the creation of a new virus strain distinctive from those previously circulating viruses (Zambon, 1999).

Antigenic shift is a result of reassortment and it occurs when two or more different influenza A viruses subtypes infect a single cell simultaneously. Because influenza A viruses are segmented, it is possible to produce new viruses with a variety of segment combinations by the acquisition of entirely new gene segments. The newly assembled progeny virions may have mixed genes from the two parent viruses (Holmes *et al.*, 2005; Nelson *et al.*, 2008). This may result in the emergence of new subtypes which may be more pathogenic than the original parent viruses and may be associated with pandemics (Neumann *et al.*, 2009; Van-Tam & Sellwood, 2010). Pigs are thought to play an important role in influenza virus ecology because of their ability to become infected with different types of influenza A viruses (avian and human viruses), and thus they act as an intermediate host, or mixing vessel (Figure 8). The new reassortant strain may cause a pandemic or panzootic because the hosts (humans or birds) have little or no immunity against it (Van-Tam & Sellwood, 2010). Such a scenario happened recently in April 2009 with the H1N1 pandemics caused by swine origin quadruple reassortant virus with of avian, swine and human origins (Michaelis *et al.*, 2009).
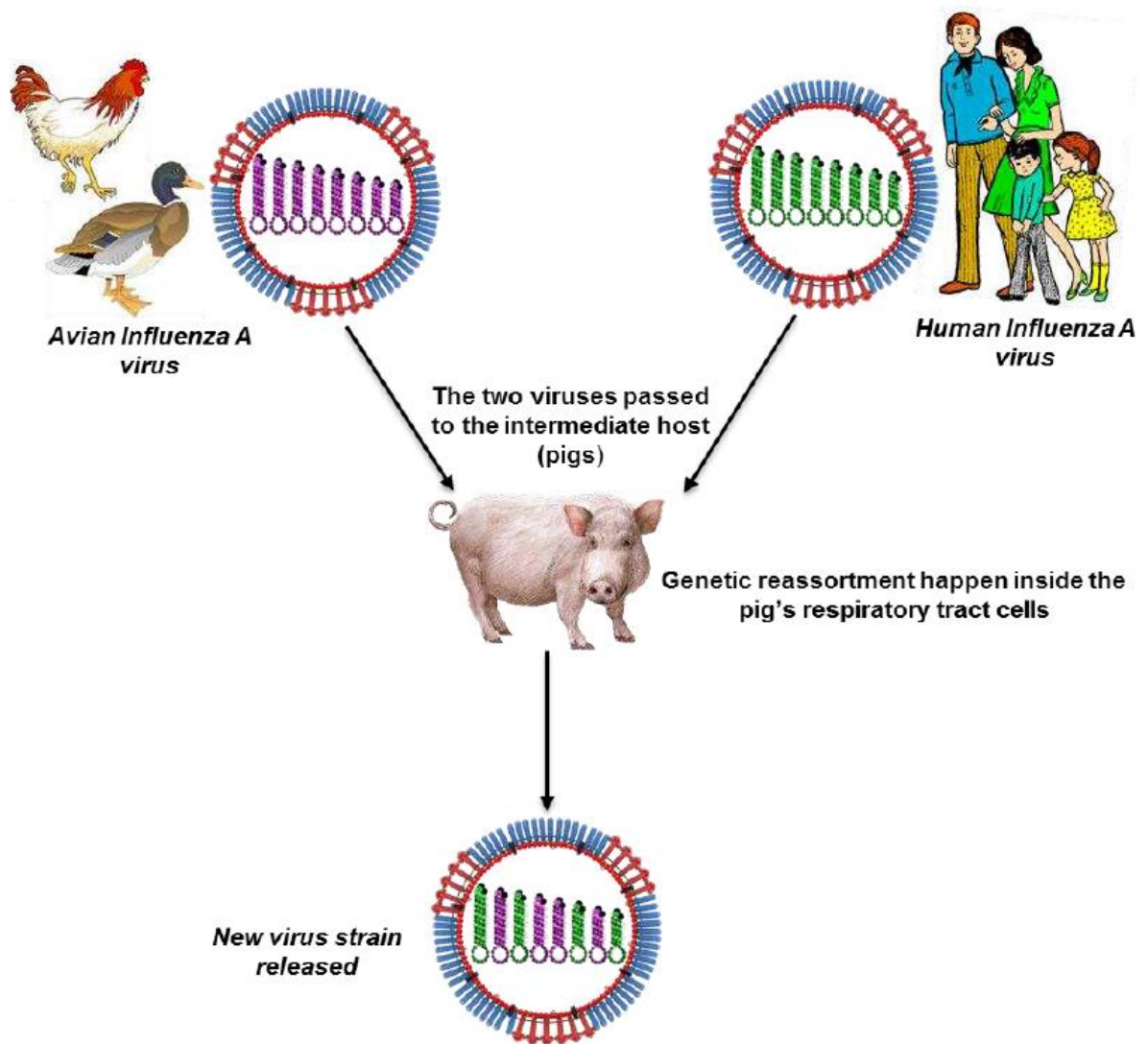
Figure 8: Schematic diagram of the antigenic shift process.

Genetic change in influenza A virus also occurs by 'antigenic drift'. This is due to the accumulation of point mutations over time, which results from a lack of proofreading mechanism in the RNA polymerase, leading to incorrect ribonucleotide insertions during replication (Zambon, 1999; Adams & Sandrock, 2010). Such changes occur progressively over a period of time accompanied by a gradual change in surface glycoproteins (HA and/ or NA). The accumulation of basic amino acids in the HA gene product may result in the transition of low pathogenic viruses to high pathogenic forms (Adams & Sandrock, 2010). Antigenic drift gives rise to immune-escape variants and can decrease a vaccine's efficacy (Figure 9). There are circumstantial evidences indicating that viruses may have drifted to escape vaccine induced

immunity in poultry (Connie Leung *et al.*, 2013). As a result of this, influenza vaccines must be updated each year with changes in the circulating influenza viruses to achieve the best match with the circulating strain possible
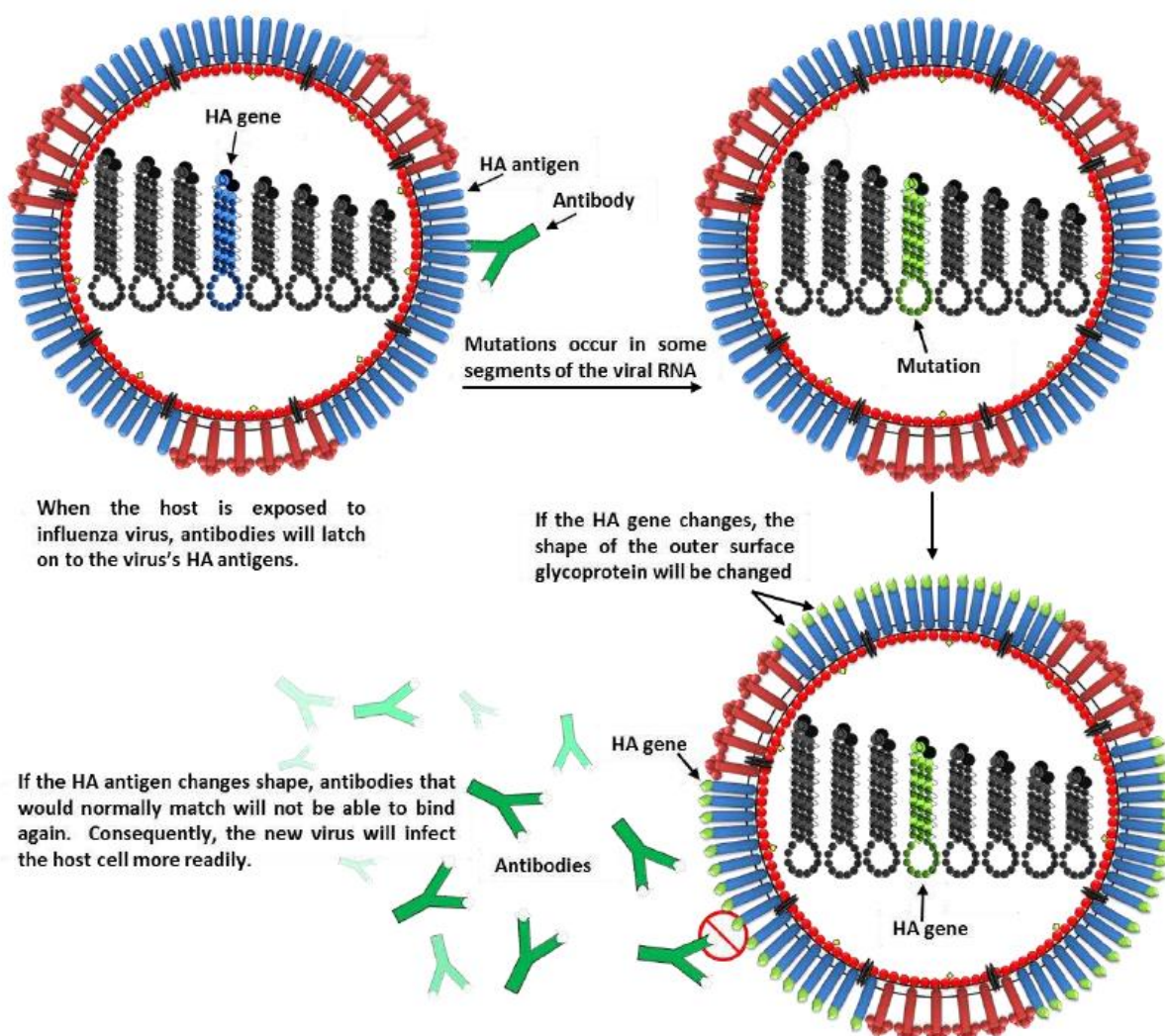
Figure 9: Schematic diagram of antigenic drift process (Al-Mubarak, 2014)

This occurs when the genes encoding viral surface antigens undergo progressive mutation which leads to antigenic changes in the protein. Such changes allow the newly formed viruses to infect the host because of the absence of the specific antibodies against the altered surface antigen.

4.6. Mode of transmission of influenza A viruses

All influenza A subtypes can be transmitted in two main ways: inhalation of contaminated aerosols and by direct contact. Many studies have shown that inhalation of aerosol

and infectious respiratory droplets play an essential role in the spread of the disease (Tellier, 2006, 2009). Transmission by contact may occur directly from the infected persons or animals or indirectly by touching contaminated tissues and surfaces (Collier & Oxford, 1993; Tellier, 2006; Van-Tam & Sellwood, 2010). Persons who are in contact with infected birds may be infected with the highly pathogenic strains (Khanna *et al.*, 2008). Such transmission could happen in wet markets where live birds are sold, leading to direct close contact with infected poultry, *via* feather plucking and preparation of poultry for consumption, as well as poultry slaughtering facilities, commercial poultry farms, and eating of raw or poorly cooked animal parts (Tambyah & Leung, 2006). Transmission between birds usually occurs by the faeco–oral route which is the predominant mean of spread in wild bird reservoirs. The stability of avian influenza viruses in water may enable transmission of the virus to other birds such as shore birds and also to aquatic mammals such as seals and whales (Stallknecht *et al.*, 1990). Mallard ducks are of great interest because they are widely distributed and can travel large distances carrying the viruses from one region to another (Achenbach & Bowen, 2011). Transmission also occurs through inhalation of respiratory secretions contaminated with influenza virus particles (Zambon, 1999).

4.7. Clinical signs and pathology of influenza A viruses in birds

The incubation period of influenza A in birds extends from one to seven days and is followed by the appearance of clinical signs (Swayne, 2008). Clinical signs displayed by birds that are infected with avian influenza viruses can differ considerably. Factors influencing the course of the disease include: 1.) Strain (Low/High Pathogenicity AI but also subtype) 2.) Host family and subfamily (for example turkey vs. chicken) 3.) Gender 4.) Age 5.) Presence of secondary pathogens (respiratory or digestive tract pathogens) 6.) Management conditions 7.) Route of inoculation. The main clinical signs which appear in poultry infected with HPAIV include decreased food and water consumption, sudden drop in egg production, rales, sinusitis, ruffled feathers, excessive lacrimation, respiratory signs, cyanosis of the head and skin (purplish–blue coloring), edema of the face and head, diarrhea and nervous system disorders,

including loss of the ability to walk and stand. The birds can be markedly depressed and sudden death of large number of poultry is common (Peiris *et al.*, 2007; Neumann *et al.*, 2009). Other signs include sneezing, coughing, blood tinged oral and nasal discharges, loss of egg pigmentation and shell less eggs (Swayne, 2008). Usually HPAIV cause significant mortality in chickens but are typically benign in ducks and geese with some exceptions of highly pathogenic avian H5N1 which may cause dark green diarrhea, anorexia and sometimes neurological signs (Neumann *et al.*, 2009). Infection of poultry with LPAIV is usually subclinical (asymptomatic), however, it may cause decreased egg production and mild respiratory signs (Swayne, 2008).

With low pathogenic virus infections in chickens, the major areas affected with pathological lesions are the respiratory and urogenital systems. These pathological lesions include pulmonary congestion, air saculitis, pneumonia, congestion of the ovary, and hemorrhagic ovarian follicles. During the latter stages of outbreaks, gross or histologic lesions which are identified within the urinary system include visceral urate deposition, nephritis, renal tubule necrosis, and swollen kidneys (Swayne, 2008). In contrast, during infection with high pathogenic viruses in chickens, the pathological lesions are more prominent in comparison with low pathogenic viruses. The lesions may also involve the intestine, liver, spleen, and the brain. The major lesions are congestion and neuronal degeneration in brain tissues and severe congestion, edema and hemorrhage in lung tissues. The main pathological findings in the liver, spleen, and kidneys are hyperemia, cell degeneration and necrosis (Vascellari *et al.*, 2007).

4.8. Diagnosis of influenza A viruses

Because pathological lesions of AI are not definitive, several tests have been developed for an accurate diagnose when AIV infection is suspected and for surveillance programs. Such tests are based either on the detection of the virus or the detection of the host immune response. Besides, a series of techniques are used to characterize the virus and study the pathogenesis of

the disease (Spackman *et al.*, 2008). Many diagnostic techniques for influenza virus infection have been used and are classified into direct and indirect techniques. The direct methods include direct detection of viral particles, rapid antigen detection such as immunofluorescence techniques, and Enzyme Linked Immunosorbent Assay (ELISA) (Stallknecht *et al.*, 2008; OIE, 2012). The indirect methods involve conventional and rapid cell culture, eggs or animal inoculation for growing and also typing of the viruses. Hen's eggs are usually used for such propagation. Virus isolation is the reference standard method to diagnose AI in both poultry (Spackman *et al.*, 2008) and wild birds (Stallknecht *et al*., 2008). Influenza viruses can be isolated by using cell culture techniques whereby a specimen is inoculated in a live culture system and the virus is then detected after a given period of incubation. Madin Darby Canine Kidney (MDCK) cells are frequently used to detect viral replication by observing the cytopathic effects (CPE) on infected cells. However, it is not utilized for routine diagnosis because of being expensive and time-consuming (Spackman *et al.*, 2008). Further investigations of influenza virus have been done by nucleic acid testing (RT– PCR), and serological diagnostic tests (complement fixation (CF), haemagglutination inhibition (HI), and neutralization tests). All of these diagnostic tests have different sensitivity rates with some advantages and disadvantages (Spackman *et al.*, 2008). In recent years, molecular methods have become an essential tool for the diagnosis and surveillance of AIV (Rose *et al.*, 2006; Spackman *et al.*, 2008; Stallknecht *et al.*, 2008). RT–PCR is generally more sensitive and specific and is not time consuming. It provides accurate detection, and facilitates the typing and subtyping of influenza viruses (Spackman *et al.*, 2008). In addition, multiplex PCR can be used to detect the infection by including a universal primer set in one amplification reaction, to determine the presence of more than one genome segment in the same reaction (Swayne, 2008). Furthermore, quantitative RT–PCR (qRT–PCR) is considered the more sensitive and accurate method for influenza A virus detection and quantitation. This test is usually used for the detection of viral M gene, the most conserved gene for all influenza A virus subtypes (Swayne & Halvorson, 2008). Serological tests, particularly haemagglutination inhibition and complement fixation are not only used for

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detection of infection, but also to determine the host's response to influenza vaccination. Viral subtype testing through Hemagglutination (HI) and neuraminidase (NI) inhibition assays is generally deferred to national or international AIV reference laboratories. The HI can be used as a confirmatory test for the presence of subtype-specific AIV in hemagglutinating egg fluids, to further characterize AIV isolates by identifying the HA subtype or to identify subtype-specific antibodies to AIV in serum, plasma or egg yolk (Thayer & Beard, 1998; Pedersen, 2008; Kida *et al.*, 1980). HI results have also proven inconsistent because: 1) antibodies are often not detectable against killed intact virus (Kida *et al.*, 1980); 2) nonspecific inhibitors may be present in serum samples; and 3) serum may cause nonspecific agglutination of chicken erythrocytes. Some of these problems can be solved using different methods such as pretreatment of the serum with chicken erythrocytes (Swayne & Halvorson, 2008).

4.9. Control and prevention of influenza A viruses

Since identification of influenza A virus as the cause of fowl plague or high pathogenicity avian influenza (HPAI) in 1955, 30 epizootics of HPAI in birds have occurred around the world. Traditional control strategies for HPAI have relied upon four basic categories of components: 1) education (including behavioral change communications), 2) biosecurity (including modifications to the ways in which poultry are reared and sold, movement management, and cleaning and disinfection), 3) diagnostics and surveillance, and 4) elimination of infected poultry, usually through culling or depopulation (Swayne, 2008). In 1995, a fifth category was added, decreasing host susceptibility, with the field implementation of vaccines and vaccination in Mexican and Pakistani control programs. In the future, increasing host resistance through genetics may replace or at least augment vaccination as a means to decrease host susceptibility (Swayne, 2012). The decision to eradicate by a stamping-out program, or to use other control measures that may include vaccination, is often a difficult decision that must consider multiple factors. This may include how many premises have infected birds, how widely distributed are the affected farms, the strength of the veterinary infrastructure, the impact

on trade, the availability of appropriate vaccines, the availability of indemnity, and so on. The principals of stamping out are clear: Diagnose when a disease is introduced into a country or region, identify farms with infected animals, and then destroy the infected animals and those animals that are likely to have been exposed to the infected animals as quickly as possible before they have a chance to spread the infection to naïve animals. If the index case and close contacts are identified quickly the rapid destruction of the flock or herd can be the most costeffective approach for control. For countries with a good veterinary infrastructure and an efficient veterinary diagnostic laboratory system, the use of stamping out has been shown to be an effective method for control of disease outbreaks (Suarez *et al.*, 2002; Swayne & Suarez, 2005 ; Stallknecht & Brown, 2008). Surveillance is the second critical component for reducing potential domestic animal and public health impacts. Effective surveillance supports efficient disease control through early detection of the disease, definition of risk factors, better assessment of vaccination programs, improved understanding of genetic evolution of the virus, and clearer elucidation of the epidemiology of the disease. Therefore, surveillance activities should focus on specific geographical sampling sites and periods, and targeted to pre defined species of high risk of exposure (Rose *et al.*, 20006; Olsen *et al.*, 2006).

4.9.1 Vaccination

One of the primary alternatives to stamping-out programs is to vaccinate and try to protect uninfected animals. Effective vaccination programs can lead to a robust immune response that can reduce clinical disease symptoms, reduce the amount of virus that birds excrete if infected, and increase the resistance of the bird to being infected. All these factors can help break the transmission chain that can end an epidemic (Swayne & Kapczynski, 2008). However, vaccination if improperly applied, either by ineffective application, insufficient coverage, or through poor antigenic matching of vaccines to field strains may contribute to the persistence of infection and disease in the region (Swayne & Halvorson, 2008; Swayne & Kapczynski, 2008; Swayne *et al.*, 2011). One argument against the use of vaccination is that vaccinated animals could not be easily differentiated from naturally infected animals. Most

importing countries have viewed the presence of antibody as evidence of prior or active infection with the disease agent and will actively block the importation of live poultry and poultry products. This negative effect on trade is a major concern for exporting countries. The two major poultry transboundary viruses that affect trade are AIV and NDV. For AI, any HPAI or, alternatively, LPAI of the H5 andH7 subtype are reportable to the World Organisation for Animal Health (WHO, 2014) and typically result in trade sanctions on the reporting country. There are five general types of avian influenza vaccine which include inactivated, DNA vaccines, live attenuated virus, recombinant vectors expressing foreign genes and subunit vaccines. All have specific advantages and disadvantages but only inactivated and recombinant type AI vaccines have received licensure for commercial use (Swayne *et al.*, 2011).

Inactivated whole AIV vaccines

Inactivated viral vaccines are essentially inert antigens that induce CD4+ T cell and humoral responses. The overwhelming majority of AI vaccines produced and sold for use in poultry are oil emulsion, inactivated whole AI virus vaccines that are administered either subcutaneously or intramuscularly (Koch *et al.*, 2009). Conventional inactivated vaccines aimed at H5, H7 and H9 subtypes are now commercially available and have been licensed for use in a number of countries (Naeem & Siddique, 2006; Swayne *et al.*, 2011). These vaccines have the advantage of being very safe as, although most of the proteins that induce the protective immune response are present, the organism cannot replicate and therefore establish a persistent infection or revert to a virulent form. With reverse genetics, vaccine strains have been incorporated the HA and NA of recent field AIVs and remaining six gene segments from a high growth influenza A vaccine virus. This type of technology allows to convert HPAIV into LPAIV vaccines by mutating the HA cleavage site. Parenteral administration is required for these vaccines, which can be a limitation from the pragmatic viewpoint. In addition, the immune response tends to be weaker so multiple doses, as well as adjuvantation, is required to induce sufficient protection. As discussed earlier, LPAIV (H6 and H9) are becoming serious threats to

poultry and have become endemic in most countries. Different killed vaccines (single or combined) are being used to overcome LPAIV (H9N2) in different countries e.g. Poulshot®, CEVAC® FLU-KEM, CEVAC®, Intervac-H9, Jova Zeit 7, GALLIMUNE™ Flu H9, NOBILIS® Influenza H9N2 and many more (http://www.cfsph.iastate.edu/Vaccines/disease_list.php?disease=avian-influenza&).

Live LPAIV vaccines

A live attenuated vaccine is a live virus that has lost its virulence while maintaining its ability to replicate. Live attenuated vaccines induce humoral and cellular immunity and provide superior and longer lasting protection compared with inactivated vaccines. In addition, these vaccines have a relatively low manufacturing cost as they do not require adjuvants in the formulation. However, these vaccines are not recommended in field conditions because: 1) they may produce important economically losses associated with clinical signs of AI; 2) they can easily spread among birds and farms; 3) they can potentially revert to a HPAIV; and 4) they can reassort with field AIVs (Swayne & Kapczynski, 2008).

Live vectored vaccines

Recombinant vaccines for avian influenza viruses have been produced by inserting the gene coding for the influenza virus HA protein into a live virus vector and using this recombinant virus to vaccinate against influenza. These live vaccines are ideal as they replicate, presenting the foreign antigen to the immune system in the context of an intracellular infection, with the expectation of stimulating humoral and cellular immunity. Many vector supports have been studied, but the most frequently reported systems have been the recombinant fowl poxvirus (rFPV) and the recombinant Newcastle disease virus (rNDV) with H5 or H7 AI HA gene inserts (Swayne *et al.*, 2000). These vaccines replicate in the host providing similar immune protection than a live vaccine. Disadvantages include a limited host range and vaccine failure if the birds to be vaccinated already possess immunity to the vector (Swayne *et al.*, 2011).

DNA vaccines

DNA vaccination involves the introduction of DNA expression vectors encoding immunogenic proteins into cells, thereby inducing a CD8+ cytotoxic T cell response. Using this method, plasmid DNA-based vaccines that express the AI HA gene can provide protective immunity towards influenza. Following vaccine application and uptake by host cells, the HA gene is transcribed into RNA and transported to the cytoplasm for protein translation. The endogenously expressed protein antigen is processed intracellularly via MHC class I proteins, stimulating cytotoxic T cells, or by MHC class II molecules for the stimulation of humoral immunity (Swayne & Kapczynski, 2008). DNA vaccines are safe as the production of plasmids does not require handling of infective agents and, because immunity is only directed towards the plasmid encoded antigen, it is easy to differentiate infected from vaccinated animals. However, although DNA vaccines do induce a humoral and cellular response, they have a relatively low efficacy and a large amount of DNA is required to produce a strong response (Lee & Suarez, 2005; Ullah *et al.*,2013).

HA subunit vaccines

These vaccines are based on portions of influenza proteins (HA) that are chemically synthesized and formulated into a vaccine to stimulate a protective immune response in the host. The main disadvantage is that the peptide alone stimulates a very weak immune response. However, this can be improved using adjuvants or another method of delivering the peptide to the immune cells (Bertran *et al.*, 2013). In France, similarly to other developed countries (Swayne *et al.*, 2011), preventive or routine vaccination of domestic poultry against AI is prohibited, although emergency vaccination programs previously approved by the European Commission (EC) could be used (Swayne & Kapczynski, 2008). Preventive vaccination programs with vaccines licensed for chickens (mainly inactivated) might be useful when targeted to high value or high risk non-poultry populations, such as zoo birds, hunting or endangered species (Swayne *et al.*, 2011). In this light, the EC approved preventive vaccination programs against H5N1 HPAIV for birds kept in zoos in 17 Member States to avoid stamping out measures for captive wild bird species (Pittman *et al.*, 2007). Additionally to the EU

program, vaccination of zoo or captive held non poultry birds has been conducted in several other countries (Swayne *et al.*, 2011).

5. *E. coli* infection in poultry

E. coli is a Gram-negative, non-spore forming, rod-shaped bacterium, of the family *Enterobacteriaceae*. Most strains are motile and have petrichous flagella (Barnes *et al.*, 2003). *E. coli* is present in the normal microbiota of the intestinal tract, other host mucosal surfaces and in the bird's environment, only a certain number of these strains possessing specific virulence attributes, designated as avian pathogenic *E. coli* (APEC), are able to cause disease (Dho-Moulin & Fairbrother, 1999; Vandekerchove, 2004). *E.coli* are usually opportunistic pathogens and can cause severe disease in the presence of immunosuppressive agents (Umar *et al*.,2014; Rehman *et al*.,2016) Since serotyping for the somatic antigen (O-serotyping) is still the most frequently used typing method for diagnostic purposes, the O-type is often used for APEC description. O1, O2 and O78 are reported as the main serotypes in poultry (Barnes *et al.*, 2003; Vandekerchove, 2004). Many other serotypes (O8,O15,O18,O35 etc) can also cause colibacillosis in poultry and have been found less frequently (Barnes *et al.*, 2003). Colibacillosis refers to any localized or systemic infection (e.g. septicemia, peritonitis, cellulitis, salpingitis, osteomyelitis, synovitis, omphalitis, airsacculitis, and coligranuloma) caused entirely or partly by APEC, and is the most frequently reported disease in surveys of poultry diseases or condemnations at slaughter, hence responsible for severe economic losses (Dho-Moulin & Fairbrother, 1999; Barnes *et al.*, 2003). Most, if not all avian species, are susceptible, although clinical disease is reported most often in chickens, turkeys and ducks. Susceptibility and severity of infection are greatest in young birds (Barnes *et al.*, 2003; Rodriguez-Siek *et al.*, 2005). Horizontal infection with *E. coli* usually occurs through contact with other birds, or through faeces, contaminated water and feed. Natural respiratory tract infection of poultry by APEC is thought to occur via the inhalation of faeces contaminated dust (Dho-Moulin & Fairbrother, 1999). Carlson & Whenham (1968) have demonstrated that the risk of

colibacillosis increases with the level of environmental contamination. Dust in poultry houses may contain 10^5-10^6 colony forming units (cfu) of *E. coli*/g. These bacteria may persist for long periods, particularly under dry conditions (Harry, 1964; Barnes *et al.*, 2003). Vertical infection results from the transmission of *E. coli* from breeders, via contaminated shells during hatching, or *in ovo*, as a result of salpingitis. The virulence mechanisms of avian pathogenic *E. coli* have not been clearly characterized yet. A number of potential virulence factors have been identified in APEC strains isolated from diseased birds, but their role in causing disease is not completely understood (Barnes *et al.*, 2003). Besides bacterial virulence factors, probably also host resistance is a great determinant of colibacillosis occurrence (Barnes *et al.*, 2003). In fact, colibacillosis is usually considered to be a secondary disease, following a primary infection with respiratory pathogens and/or unfavorable environmental conditions (Barnes *et al.*, 2003; Vandekerchove, 2004). Different known virulence factors of APEC have been summarized in Table 5.

Function	Name	Reference
Adhesins	Type I fimbriae	La Ragione *et al.*(2000)
	Stg fimbriae	Lymberopoulos *et al.* (2006)
	P fimbriae	Kariyawasam and Nolan (2009)
	Autotransporter adhesion AatA	Li *et al.* (2010)
	Curli	La Ragione *et al,* (2000)
	Temperature sensitive hemagglutinin Tsh	Dozois *et al.* (2000)
	Yqi	Antao *et al.* (2009)
	E. coli common pilus (ECP)	Stacy *et al.* (2014)
Iron acquisition	Aerobactin	Gao *et al.* (2015a)
	Salmochelin	Caza *et al.* (2008)
	System Sit	Sabri *et al.* (2008)
	Heme utilization/transport protein ChuA	Gao *et al.* (2012)
Antiphagocytic activity/serum resistance	K1 capsular polysaccharide	Mellata *et al.* (2003a)
	Increased serum survival (Iss)	Nolan *et al.* (2003)
	Degenerate type III secretion system 2 (ETT2sepsis)	Ideses *et al.* (2005)
	O78 LPS	Mellata *et al.* (2010)

Metabolism	Phosphate transport system (pts)	Lamarche *et al.* (2005)
	Nitrite transporter (NirC)	de Paiva *et al.* (2015)
	Sugar metabolism (Aec35-37)	Chouikha *et al.* (2006)
Two-component regulatory systems	RstA/RstB	Gao *et al.* (2015b)
	PhoB/PhoR	Bertrand *et al.* (2010)
	BarA/UvrY	Herren *et al.* (2006)
Miscellaneous	SsrA/SmpB	Mu *et al.* (2013)
	IbeA and IbeB	Flechard *et al.* (2012)
	Type VI secretion systems	Ma *et al.* (2014)
	Transcriptional regulator (YjjQ)	Li *et al.* (2008)
	Vacuolating autotransporter toxin (Vat)	Parreira and Gyles (2003)
	Flagella (FliC)	Dziva *et al.* (2013)
	Group 4 capsule	Dziva *et al.* (2013)

Table 5. Validated virulence factors of APEC (adapted from Guabiraba *et al.,* 2015)

5.1. **Host immune response to *E. coli* infections in poultry**

Avian colibacillosis generally starts as a respiratory infection that evolves to generalised infection resulting in fibrinopurulent lesions of internal organs. Uptil now, the mechanisms of APEC virulence are not well defined. Colonisation of the trachea and air sacs is considered the first step of a systemic infection by APEC, followed by the colonization of the liver and the pericardium, with subsequent bacteraemia (Guabiraba & Schouler, 2015). There have been fast advances in chicken immunology after the study of full chicken genom (Wong *et al*., 2004). New technologies has allowed to understand chicken immune responses in a better way against important poultry pathogens such as *E. coli* (Lowenthal *et al*., 2013). Host immunity responds to invading pathogens through different receptors such as the Toll-like receptors (TLRs), which distinguish different classes of pathogen-associated molecular patterns (PAMPs) (Roy & Mocarski, 2007). Immune cells are activated by the invasion of microbes which leads to activation of intracellular signaling pathways related to production of pro- and/or anti-inflammatory cytokines and microbial killing mechanism (Kogut *et al*., 2015). Bacterial motifs are recognized with the help of four TLR in chicken including TLR-2, which senses

peptidoglycan; TLR-4, which recognises lipopolysaccharide (LPS); TLR-5, which binds flagellin; and TLR-21, which recognises unmethylated *CpG* DNA commonly found in bacteria (Keestra *et al*., 2010). TLRs expressed by epithelial and resident phagocytic cells probably recognise APEC in lungs leading to stimulation of phagocytic cells and the production of inflammatory cytokines. So far, the dynamics of TLR expression in the pneumonic form of colibacillosis in birds have not been studied. Therefore, mechanisms of APEC and TLR interactions in bird's respiratory tract remain largely unknown (Guabiraba & Schouler, 2015). Macrophages and heterophils are efficiently activated by immune responses within the respiratory tract of birds to APEC infection (Figure.10). However, there is very limited knowledge on the interaction and adhesion mechanisms of APEC to these cell populations. These phagocytic cells are likely to play a key role on the initial host defence not only to APEC but also to other pathogens that might predispose the avian lung to a more efficient bacterial adherence and invasion. Blood monocytes and tissue resident macrophages certainly play different roles in recognition and initiation of immune responses to APEC invasion. However, there is no study integrating phenotyping and activation status of circulating monocytes and resident macrophages in the course of APEC infection in chickens (Guabiraba & Schouler, 2015).

Heterophils, the most abundant granulocyte in most avian species, play an crucial role in the avian immune response to pathogens by using a range of cytotoxic and prostimulatory molecules such as cytokines, chemokines and lipid mediators (Kogut *et al*., 2001). Heterophils play a vital role for the recruitment and activation of other innate immune cells and mediate the inflammatory responses (Kogut *et al*., 2003). Heterophils are likely to be the first leukocyte population to be recruited from the bloodstream to the infected site by chemokines within few hours after the establishment of infection forming purulent discharge during pulmonary infection of APEC (Horn *et al*., 2012). Although, heterophils help in bacterial clearance but they can also lead to tissue damage and complicate disease outcomes if inflammation persist for longer time (Figure 10). Natural killer (NK) cells have been functionally and

morphologically defined in chickens (Jansen *et al.*, 2010), and recent progress has been made on the role of diverse types of NK cell receptors (Straub *et al.*, 2013). Peripheral blood leukocytes (PBL) of chicken infected with an APECO1 strain showed upregulation of genes related to NK cell mediated toxicity however, the phenotype and function of this cell population largely remains unexplored *in vivo* upon APEC infections (Sandford *et al.*, 2012). So far, the mechanism of leukocyte recruitment into chicken's lungs upon APEC infection is under debate. Although heterophils and macrophages are likely to be the most important cell populations to respond to invading bacteria in the lungs, one cannot exclude the role of NK cells and other innate lymphoid cells in early or later moments of the host immune response to infection, which can be better studied with new cell surface markers and flow cytometry strategies that have arisen in the last years (Sperling *et al.*, 2015). Our current knowledge on the inflammatory response to respiratory colibacillosis in chicken's lungs and air sacs and the dynamics of cell populations involved in recognition and elimination of APEC is summarised in Figure 10.

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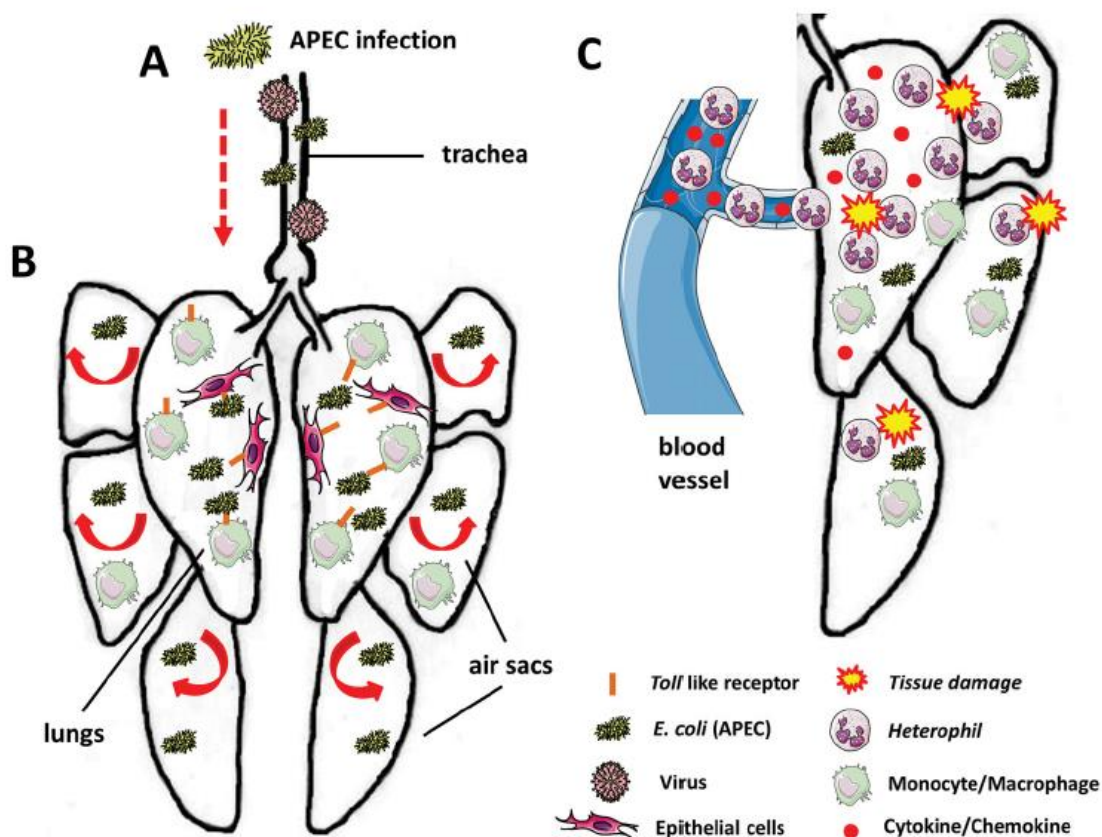
Figure 10: Schematics showing chicken's inflammatory response to APEC in the respiratory tract. (**A**) Upon inhalation of contaminated aerosol particles, APEC might interact with and infect epithelial cells already in the trachea. A previous viral infection, such as IBV, may damage the respiratorymucosa and facilitate APEC colonization. (**B**) Resident cells such as epithelial cells and macrophages in lungs and air sacs will be the first line of defense through recognition of bacterial PAMPs by TLRs or bacteria phagocytosis. (**C**) These responses will result in proinflammatory cytokines and chemokines production that will activate and/or recruit other leukocytes to the infected site. Inflammation will also contribute to tissue damage and impairment of lung function if bacterial colonization persists. Heterophils are likely to be the first leukocyte population to be recruited from the bloodstream to the infected site by chemokines within few hours after the establishment of infection. Later in the inflammatory response to APEC, monocytes arrive in the second wave of leukocyte immigration into the lungs not only to optimize bacterial clearance but also to contribute to phagocytosis of dying heterophils and thus contribute to tissue repair. The kinetics of these cellular events and the mediators of inflammation that participate in the immigration and activation of heterophils or monocytes/macrophages in APEC-infected chicken lungs remain largely unexplored (Guabiraba & Schouler, 2015)

5.2. Clinical signs and lesions caused by *E. coli*

One of the most common forms of colibacillosis begins as a respiratory tract infection and, if unattended, this infection may evolve into a bacteraemia and a generalised infection which manifests as a polyserositis (Pourbakhsh *et al.*, 1997; Dho-Moulin & Fairbrother, 1999; Barnes *et al.*, 2003). Barnes et al. (2003) and Dho-Moulin & Fairbrother (1999) described in details other localized and systemic colibacillosis-associated disease syndromes. Respiratory-origin colisepticemia affects both chickens and turkeys and is the most common type of colisepticemia (Barnes *et al.*, 2003). Lesions are prominent in respiratory tissues (trachea, lungs, and air sacs), pericardial sac and peritoneal cavities and are typical of the subacute

polyserositis stage of colibacillosis. Infected air sacs are thickened and often have caseous exudates on the respiratory surface. Pneumonia is more common in turkeys than chickens.

5.3. Diagnosis of *E. coli*

The diagnosis of colibacillosis is first suggested by the clinical picture and by the presence of typical macroscopic lesions such as airsacculitis, sometimes associated with pericarditis and perihepatitis. Diagnosis needs to be confirmed by the isolation of pathogenic *E. coli* from the heart blood and affected tissues, like liver, spleen, pericardium or bone marrow, on selective media like McConkey, eosin methylene blue or drigalki agar. Care must be taken to avoid faecal contamination of samples. Further identification of the isolated colonies is based on biochemical reactions (Dho-Moulin & Fairbrother, 1999). The diagnosis is strengthened if the isolated culture belongs to a known pathogenic serogroup. Different ELISAs have been developed for the detection of antibodies, although they have limited value because they can only detect homologous APEC types (Leitner *et al.*, 1990; Bell *et al.*, 2002). Virulance associated factors can not be used to identify APEC because these factors have been detected in both fecal isolates and in strains isolated from colibacillosis lesions. Currently, molecular based techniques such as PCR improves both the speed of detection and the level of sensitivity and has been increasingly used to identify several APEC strains from food and clinical samples.

5.4. Control of *E. coli*

Colibacillosis is mainly treated with antimicrobials. *E. coli* may be sensitive to many drugs such as ampicillin, chloramphenicol, chlortetracycline, enrofloxacin, neomycin, nitrofurans, gentamicin, nalidixic acid, oxytetracycline, polymyxin B, spectinomycin, streptomycin and sulphonamides (Barnes *et al.*, 2003). *E. coli* isolates from poultry are frequently resistant to one or more drugs, since they have been largely used in the poultry industry over a long period (e.g. tetracyclines) (Barnes *et al.*, 2003; Vandekerchove, 2004). It is not only important to analyse the isolates for their antimicrobial resistance patterns, one must also take care that the animals

receive a sufficiently high dose of the antimicrobial and moreover, ingest it especially when they are diseased, to obtain the necessary therapeutic effect. It is impossible to stop all the factors that cause colibacillosis. Autogenous killed *E.coli* vaccines have been tried but they provide protection against homologous challages only. But with Poulvac® *E. coli*, the only commercially available modified-live *E. coli* vaccine, it is possible to protect against both the disease and productivity loss. Despite the potential for developing an efficacious vaccine to combat this economically important poultry disease, several obstacles hinder such efforts. Those obstacles include the cost, vaccine delivery method and timing of vaccination. Measures should be taken to prevent introduction of pathogens that promote infections with APEC (Barnes *et al.*, 2003;Azeem *et al*., 2017). The housing climate (humidity, ventilation, dust and ammonia) and the stocking density must be kept optimal (Dho-Moulin & Fairbrother, 1999; Vandekerchove, 2004). Vaccines are not being used on a large scale in poultry due to great diversity among APEC strains (Dho-Moulin & Fairbrother, 1999; Vandekerchove, 2004).

6. Other viral respiratory pathogens

Respiratory diseases in domestic poultry species are caused by various viruses which complicate disease prognosis in the presence of other opportunistic bacteria such as *E. coli.* Below is a brief introduction to common poultry viral pathogens other than AIV.

6.1. Newcastle disease virus (NDV)

Newcastle disease (ND) is an economically important and highly infectious disease of both wild and captive birds (Saif *et al.*, 2008) caused by Newcastle disease viruses (NDV) synonymous with avian paramyxovirus- 1 (APMV-1). NDV is a member of the *Avulavirus* genus in the *Paramyxoviridae* family. Encompassing a diverse group of single-stranded, negative sense, non-segmented, enveloped RNA viruses of approximately 15.2kb; NDV has a broad host range and is known to infect over 200 bird species (Alexander, 2001). NDV was first reported in 1926, in Java, Indonesia and Newcastle-on-Tyne, England, and since then

various genotypes have been responsible for different ND panzootics. NDV is distributed worldwide and its continual presence in multiple avian species presents a constant threat to all poultry industries and other activities that involve the raising or keeping of birds (Anonymous, 2011). Based on its virulence in chickens, the NDV is classified into three main pathotypes: velogenic, mesogenic, and lentogenic (Alexander *et al.*, 2012). The velogenic strains are more virulent than the mesogenic strains, but both are classified as being virulent. The lentogenic strains, on the other hand, are considered avirulant. The presence of virulent strains (velogenic and mesogenic strains) in poultry requires monitoring and control measures even in countries where they are endemic because the existence of the virus severely impacts commercial productivity and the international trade in poultry and poultry products (Alexander *et al.*, 2012). The NDV is tentatively diagnosed based on the clinical outcome, postmortem lesions particularly pin-point hemorrhages in proventriculs and hemorrhages in cecal tonsils, mortality pattern, vaccine history. However, geometric mean titer (Haemaaglutination inhibition test), and virus isolation are being considered the gold standard method in identifying ND outbreaks and subsequent biological and molecular characterization of the genotype involved. The vaccines currently being used to prevent ND are not able to control the disease and sporadic outbreaks are reported every year even disease remains endemic throughout the year in developing countries. Therefore, the introduction of efficient vaccination strategies is needed in order to rise to the occasion (Saif *et al.*, 2008).

6.2. Avian metapneumovirus (aMPV)

Avian Metapneumovirus (aMPV) infections are a huge economical issue for the poultry industry worldwide, which has been associated with upper respiratory tract infections and reductions in egg production in various avian species. The aMPV causes turkey rhinotracheitis (TRT) and is associated with swollen head syndrome (SHS) in chickens, which is usually accompanied by secondary infections that increase mortality. It was first reported in 1978 in South Africa and since then, it has been reported in most regions of the world. aMPV,

previously called avian pneumovirus (APV) or turkey rhinotracheitis virus (TRTV), is a member of the *Paramyxoviridae* family, *Pneumovirinae* subfamily, within the new genus *Metapneumovirus* (Lamb *et al.*, 2000; Gough & Jones, 2008). It was classified into four subgroups called: A, B, C and D. TRT and SHS are characterized by tracheal rales, sneezing, swollen sinuses, swollen head, and nasal and ocular discharge. The aMPV can also lead to transient drop in egg production and/or an increase in egg abnormalities in both turkeys and hens. Chickens may have antibodies, without having clinical signs. Transmission requires direct contact among birds. Its spread over long distances is uncertain, but wild birds are postulated as probable chain links (Umar *et al.*,2016b). Clinical signs are not pathognomonic for a diagnosis of aMPV. A diagnosis may be made by serology, PCR or virus isolation. The diagnosis of this disease poses a challenge due to difference in clinical features and genome. Avian metapneumovirus can be very difficult to isolate and the success rate might be low. To confirm the identity of the aMPV electron microscopy or immunochemical methods can be used. There are three immunochemical methods that are used for detecting aMPV in turkeys; immunofluorescence (IF), immunoperoxidase (IP) and immunogold staining. For detecting aMPV in chickens IF and IP are being used. The value of these tests under field conditions has not been fully evaluated, nor have any scientific studies been undertaken to compare the sensitivity and specificity of IF and IP. Good biosecurity and immune interventions are effective and necessary aspects of the control program. Live vaccines predominantly control avian aMPV infection in poultry flocks, but vaccine virus can be found for extended periods after application that may lead to reversion of vaccine virus (Cook & Cavanagh, 2002; Shin *et al.*, 2002; Gough & Jones, 2008; Catelli *et al.*, 2010; Cecchinato *et al.*, 2010; Cecchinato *et al.*, 2014).

6.3. Infectious bronchitis virus (IBV)

Infectious bronchitis is a highly contagious upper-respiratory tract disease of chickens and causes a severe economic burden on the poultry industry worldwide (Fraga *et al.*, 2013). The causative agent, infectious bronchitis virus (IBV), has been found in many types of birds.

IBV belongs to genus *Gammacoronavirus* of family *Coronaviridae* within order Nidovirales (Jackwood, 2012). This round, enveloped virus usually found in pleomorphic shape has size of approximately 120 nm in diameter (Cavanagh, 2007). The positive sense RNA genome of this virus is 27.6 kb in size and encompasses 3′ and 5′ untranslated regions (UTRs) with a poly (A) tail. Clinical signs of the disease in chickens include watery eyes, mucus in the nares and trachea, gasping, coughing and tracheal rales. The disease leads to a decrease in egg production and egg quality, and some strains of the virus can cause an interstitial nephritis. The virus replicates in epithelial cells causing lesions in the nasal turbinates, trachea, kidney, gonads, oviduct, lungs and air sacs. Lesions can also be found in the gastrointestinal tract, bursa of Fabricius and caecal tonsils (Cook *et al.*, 2012; Jackwood, 2012). The viral distribution is virtually global. It was first described in the 1930s in the USA and more than 50 variants or serotypes have been documented globally. IBV causes serious problems to poultry health when coinfected with other pathogens e.g. *E. coli*, influenza viruses. It has been demonstrated that infectious bronchitis live vaccine, exacerbates the manifestation of experimental H9N2 AIV infection in broiler chicken (Haghighat-Jahromi *et al.*, 2008; Seifi *et al.*, 2012). Mutation and recombination processes are involved in the genetic and phenotypic variations of IBV, leading to the emergence of new variant strains, and give rise to virus population diversity to be modelled by the host, particularly by the immune system. The consequence is the continuous emergence of new variants with regard to pathotypes, serotypes, and protectotypes (Umar *et al.*, 2016c). The viral genes encoding the spike, replicase and nucleocapsid proteins can be considered the main genomic regions, which indicate the evolution processes of IBV.Conventional and more advanced methods have been used for the diagnosis of IBV infection. In the past, serological assays such as virus neutralization (VN) and haemagglutination inhibition (HI) were used widely for detecting and serotyping IBV strains. These tests also have been used to measure flock protection following vaccination. Serotype-specific antibodies usually are detected using HI, even though the HI test is less reliable. On the other hand, ELISA assays are more sensitive and easily applied for field use and in

monitoring antibody response following vaccination or exposure. However, emergence of different IBV serotypes that do not cross-react with commonly available antisera generally made serological tests less applicable and nonconclusive in classifying new or emerging IBV isolates. Virus isolation has been the gold standard for the diagnosis of IBV. The stringent technique requirements and factors, such as the time required for several passages of virus in egg or cell culture, limit the use of virus isolation as a diagnostic method of choice for IBV infection. In view of their increased sensitivity and reduced reporting time, molecular methods such as PCR, have nearly replaced conventional serology and virus cultivation methods of IBV diagnosis (Bande *et al.*, 2016). Despite the use of vaccination, new IBV variants are evolving and circulating in the field and should be considered as initial candidates for vaccine development. The new generation vaccines developed against locally prevailing IBV strains may be more helpful and avoid the reversion of virulence in live vaccine viruses. Understanding the mechanisms underlying the evolution of IBV has basic relevance and, without doubt, is essential to appropriately control and prevention of the disease.

7. Other respiratory bacterial pathogens

Bacterial pathogens are playing a crucial role in causing respiratory diseases in domestic poultry. Bacterial pathogens may play a primary or secondary role in diseases. Usually, primary viral or environmental damage to respiratory tissue attract bacteria to colonize the respiratory system. IBV infections predispose birds to *E. coli* superinfection is an example of secondary bacterial invasion. In other cases, bacterial act as a primary factor and initiate the disease process in birds. Infectious coryza and fowl cholera infections in chicken are examples of primary bacterial respiratory diseases in poultry. Below is a brief introduction to common poultry bacterial pathogens other than *E. coli*.

7.1. *Ornithobacterium rhinotracheale*

Ornithobacterium rhinotracheale (*O. rhinotracheale*) infection, also known as ornithobacteriosis, is a contagious disease of avian species, primarily turkeys and chickens. *O. rhinotracheale* is a gram-negative staining rod, belonging to the family *Flavobacteriaceae* in the phylum Bacteroidetes. It has been variously referred to as Pasteurella-like, Kingella-like, Taxon 28, or pleomorphic gram- negative rod until the present name *O.rhinotracheale* was suggested in 1994 (van Empel & Hafez, 1999; Chin *et al.*, 2008). In chickens and turkeys, *O.rhinotracheale* causes a contagious disease characterized by respiratory distress, decreased growth, and mortality. Besides drops in egg production, decrease of egg shell quality, and reduced rate of hatchability, decreased weight gains, increased mortality, and high condemnation rates due to purulent airsacculitis cause considerable economic losses. Other respiratory lesions, such as catarrhal tracheitis (Gavrilović *et al.*, 2010) and bilateral exudative pneumonia (Gornatti Churria *et al.*, 2012), have also been found in chickens affected by ornithobacteriosis. The severity of clinical signs, duration of the disease, and mortality are extremely variable and are influenced by virulence and the pathogenicity of the causative isolate as well as by many environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia level, concurrent diseases, and secondary infections (Chin & Charlton, 2008; Chin *et al.*, 2008). Moreover, *O. rhinotracheale* infection can induce higher economic losses and mortality if H9N2 AIV is also present (Pan *et al.*, 2012). *O. rhinotracheale* spreads horizontally by direct and indirect contact through aerosol or drinking water (Chin *et al.*, 2008). Besides its isolation from ovaries, oviduct, hatching eggs, infertile eggs, dead embryos, and dead-in-shell chickens and turkeys (van Empel & Hafez, 1999; Chin *et al.*, 2008), there are circumstantial evidences of vertical transmission in birds affected by ornithobacteriosis (van Empel *et al.*, 1997). Diagnosis consists of case history, clinical investigations, and postmortem examination, as well as isolation or detection of ORT DNA by PCR (Gornatti Churria *et al.*, 2012). Isolation and identication of the causative agent are essential for differential diagnosis as clinical signs and postmortem lesions of *O. rhinotracheale* infections are similar to other bacterial and virus infections. Currently 18

different ORT serotypes designated A to R have been identified. Serotyping can be done with reference antisera by agar gel precipitation test (AGP) or enzyme linked immunosorbent assay (Chin & Charlton, 2008). Currently AGP is the method of choice for serotyping. Serological tests are useful for flock monitoring and/or the diagnosis of *O. rhinotracheale* infection (Chin *et al.*, 2008). ELISA can be a useful diagnostic tool as it can detect presence of antibodies against *O. rhinotracheale* in 1-day-old birds and in egg-yolks, as well as in birds with clinical signs. PCR assays have shown to be useful for identication and genotyping purposes (Gornatti Churria *et al.*, 2012). The best strategy for the control or prevention of *O. rhinotracheale* infection is probably vaccination, because most worldwide *O. rhinotracheale* isolates have acquired resistance against the antibiotics regularly used in poultry (Schuijffel *et al.*, 2006). In spite of the availability of autogenous vaccines, economic losses related to *O.rhinotracheale* infections in the poultry industry are estimated in hundreds of millions of dollars annually in the United States. Bacterins, live vaccines, and subunit recombinant vaccines have been developed and reported, with variable results for the control of experimental and natural infections associated with *O.rhinotracheale* (Gornatti Churria *et al.*, 2012). Moreover, antibiotic susceptibility profiles should be used for the selection of antibiotic for the treatment.

7.2. *Avibacterium paragallinarum*

Infectious coryza is an upper respiratory disease of chickens caused by infection with *Avibacterium paragallinarum* (*A. paragallinarum*) previously known as *Haemophilus paragallinarum* (Blackall *et al.*, 2005). The bacteria exhibit pleomorphic rod or coco-bacilli morphology with a tendency to form filament like arrangement with short chains. Ordinarily, the size of bacterium ranges from 1 to 3 μm in length and from 0.4 to 0.8 μm in width. In most of the cases, the organisms are grown under micro aerobic or anaerobic conditions with increased levels of CO_2 (5-10%). The organism causes severe upper repiratory disease characterized by swollen infraorbital sinuses, nasal discharge, and depression leading to very significant drop in egg production in layer chicken. At initial stage of infection, the clinical

signs are sero-mucus discharge(s) mainly from nostrils and eyes, lately transforming into caseous flake like exudative deposits in the subcutaneous tissue, around the para- nostrils area as well as eyes, consequently resulting in sticky closures of eyes of birds (Chukiatsiri *et al.*, 2010).

Besides, primarily involving upper respiratory tract, the infection also transcends down to trachea, air sacs as well as causes pneumonia in lungs in very extreme cases. In older and egg laying chicken, the organism predominantly affects the reproductive organs *viz*; ovary and salpinx and are responsible for poor egg quality and decreased egg production. In meat type birds, due to inanition, the feed conversion efficiency drastically declines, that mainly results in poor flesh development and extreme culling at tender age. The infection does not spread readily from farm to farm without movement of contaminated people or fomites from farm to farm because *A. paragallinarum* does not survive well outside of its host. An infected flock may potentially shed the organism for the life of the flock. For this reason, *A. paragallinarum* infection may persist indefinitely on farms having multiple-age chickens, particularly multiple-age layer or breeder farms (Priya *et al.*, 2012). In yester years, various ancillary tests viz; isolation of bacterium along with biochemical characterization used to be set procedures to confirm the presence of organism but with delayed intimation. The improvement in molecular diagnostics has largely curtailed the awaiting time for disease reporting in matter of hours to begin suitable therapeutic regimen in shortest possible time point. Apart from this, the cogent identification of the provincial sero/immuno vars to conclusive, comprehensive region specific vaccine development has immensely appreciated these molecular diagnostic approaches for its boon. PCR, a molecular tool has now become largely indispensable to its identification with having advantages closely accurate to conventional techniques i.e. culture, with much rapidity that avoids concept of false negativity. Biosecurity and vaccination are usually adopted to prevent infectious coryza outbreaks at farms. Good biosecurity procedures should prevent exposure of chickens to *A. paragallinarum* infection; however, vaccination must be used to minimize the impact of the disease especially on those farms where *A. paragallinarum* is

endemic. Inactivated whole-cell bacterins vaccies are available commercially containing one or several isolates. Broilers are seldom vaccinated against infectious coryza; however, replacement layers and breeders are routinely vaccinated in many countries (Deshmukh *et al.*, 2015).

7.3. *Pasterulla multocida*

Pasteurella multocida, the causative agent of fowl cholera, is of major economic importance worldwide (Glisson *et al.*, 2003; Singh *et al.*, 2014).The other subspecies of Pasteurella, for example, *Pasteurella multocida* subspecies septica, and pasteurella-like bacteria from the *Pasteurellaceae* family (e.g., *Avibacterium* spp., *Galibacterium* spp.), can cause similar clinical signs. *Pasteurella multocida* is a gram-negative, nonmotile, and facultatively anaerobic bacterium. *Pasteurella multocida* displays wide serologic and genetic diversity. *Pasteurella multocida* is classified into the serogroups A, B, D, E, or F based on their capsular composition and 16 somatic (lipopolysaccharide) serotypes. When present as an acute disease, high morbidity and mortality can occur. In this acute form, clinical signs are typically only seen shortly before death. Typical clinical signs seen in the acute form of the disease are fever, ruffled feathers, mucus discharge from the mouth, diarrhea, and increased breathing rate (Glisson *et al.*, 2008). The disease also presents as a chronic form, in which localized infection of joints and sinuses can follow the acute form, or on occasion, be the only form of the disease present in a flock. In the chronic form, the typical signs include swollen wattles, eyes, sinuses, leg or wing joints, or foot pads; twisted necks (torticollis); and respiratory gurgles (rales). In the chronic form, birds that recover become carriers of the disease, can remain infected for life, and become a reservoir of infection for further outbreaks (Glisson *et al.*, 2003). The disease has been recognized as a putative in the emerging free range layers (Singh *et al.*, 2013) and backyard poultry (Christensen *et al.*, 1998) in the developed world and in village chickens in the developing world (Muhairwa *et al.*, 2001). But, no reports of fowl cholera outbreaks in free range, organic broiler flocks in the developed world appear to have been reported. The recurrence of fowl cholera outbreaks on properties has been reported in turkeys (both shedded

and free range) and free range layers and ducks (Singh *et al.*, 2013). The explanation for these recurring outbreaks could be carrier chickens or environmental persistence of *Pasteurella multocida*. An alternative explanation could be a common source reintroducing the strain at intermittent periods (Singh *et al.*, 2013). Since all types of birds are susceptible to fowl cholera, wild birds are a potential source of reintroduction of strains. It has been shown that sparrows and pigeons can become infected with *Pasteurella multocida* from infected chickens and once infected can transmit it to susceptible chicken. However, it seems only the isolates of *Pasteurella multocida* from pigs and cats that have been shown to be consistently pathogenic for fowl. Cats have consistently been identified as a potential source of introduction of *Pasteurella multocida* into flocks. However, definite evidence linking cats with fowl cholera outbreaks does not exist (Glisson *et al.*, 2003, 2008). The diagnosis of fowl cholera is based on the occurrence of typical signs and lesions and on detection of bacteria by using microscopy techniques in tissues, such as liver, heart, lung, and blood. However, in many cases, confirmation depends on the isolation and identification of the causative *Pasteurella multocida* bacterial species. Conventional methods of identification, such as capsular serotyping, have been largely replaced by DNA-based methods (PCR) that are sensitive and specific for a rapid diagnosis. Confinement is probably the most effective way to prevent introduction of *Pasteurella multocida*. However, extensive management systems dominate in many parts of the world, and under such circumstances vaccination is recommended as a preventive measure. Unfortunately, the development of safe and efficient live vaccines still poses problems. As a result, control remains dependent on bacterins which exhibit significant disadvantages compared to live vaccines (Ahmed *et al.*, 2014).

7.4. *Mycoplasma*

Avian mycoplasmosis caused by *Mycoplasma* species was primarily described in turkeys in 1926 and in chickens in 1936 (Nascimento *et al.*, 2005; Kleven, 2008). There are more than 120 named *Mycoplasma* species and more than 20 species are known to infect avian hosts (Nascimento *et al.*, 2005; Purswell *et al.*, 2011). Of these, *M. gallisepticum* and *M.*

synoviae are the major pathogens, and *M. meleagridis*, and *M. iowae* are of importance in turkeys (Sprygin *et al.*, 2011). *M. gallisepticum* infection is usually designated as chronic respiratory disease of chickens and infectious sinusitis in turkeys. Recently, it has been reported that *Mycoplasma gallisepticum* modifies the pathogenesis of influenza A virus in the avian tracheal epithelium (Sid *et al.*, 2016). It is characterised by respiratory rales, coughing, nasal discharges, and frequently by sinusitis in turkeys. *M. synoviae* infection is usually known as infectious synovitis, an acute-to-chronic infectious disease for chickens and turkeys involving primarily the synovial membranes of joints and tendons sheaths. However, during recent years, *M. synoviae* has less frequently been associated with synovitis but more frequently associated with airsacculitis in chickens and sometimes in turkeys (Khalifa *et al.*, 2013). Transmission may be transovarian or lateral via respiratory aerosols and direct contact. Infection occurs via the respiratory tract and usually affects 100% of the birds. Following infection birds become persistently infected with *M. synoviae* and remain carriers for life. As a result of the expansion of poultry production and the concentration of large, multiage production complexes within a restricted geographic area, it is becoming more and more difficult to maintain flocks that are free of *M. synoviae*. Both diseases are economically important, egg transmitted and hatchery disseminated. They lead to tremendous economic losses in poultry production as a result of decreased hatchability and egg production, reduced quality of day-old chicks, reduced growth rate, increased costs of eradication procedures which involve site cleaning and depopulation, and increased costs of medication and vaccination (Ferguson-Noel & Williams, 2015). For many years, diagnosis of avian mycoplasmosis was based on serological assays to detect antibody production and/or on isolation and identification of the organism. Serological tests include serum plate agglutination test (SPA), Haemaglutination inhibition test (HI) and Enzyme linked immunosorbant assay (ELISA) for *M. gallisepticum*, *M. synoviae*, or *M. meleagridis* (Ferguson-Noel *et al.*, 2012; Khalifa *et al.*, 2013). Difficulties encountered with the use of serologic tests for *Mycoplasma* have been described extensively. Serologic testing is related to imperfections in the interpretation of results (Kleven, 2008). The problems arise primarily from

the multiplicity of serotype strains isolated and their coexistence in the same isolate. Cultivation techniques are laborious, slow, and expensive and require sterile conditions. Problems experienced with culture include overgrowth by faster-growing *Mycoplasma* species or other organisms, or no growth in subculture. Culture can take 3-4 weeks, and even then, the result can be negative or be hampered by mixed infections (Ferguson-Noel *et al.*, 2012). For these reasons, use of rapid and sensitive detection methods, like PCR, can be advantageous because it provides a better sensitivity and specificity facilitating the detection of pathogens in clinical samples collected from asymptomatic animals, or those who are under treatment with antibiotics (Evans *et al.*, 2005; Peebles *et al.*, 2014,Umar *et al.*,2017b).

Control of avian mycoplasma infections is, in theory, quite simple and straightforward, especially because the pathogenic avian mycoplasmas are egg transmitted and lack a cell wall, rendering them susceptible to environmental influences. One begins with mycoplasma-free replacement stock that is placed on a single-age farm with all-in, all-out type management, good biosecurity, and a consistent monitoring programme (Kleven, 2008). Multi-age commercial egg complexes are mostly positive for *M. gallisepticum* and *M. synoviae*, and in some parts of the world, both infections are widespread in commercial broiler production. *M. gallisepticum* vaccines are used with increasing frequency in areas where control is not feasible such as large poultry populations in small geographic areas and multiple-age farms that never depopulate. Vaccine has been used extensively; it is reported to be very immunogenic and mildly virulent in chickens and is effective in displacing virulent (field) strains from poultry operations. Control of poultry mycoplasmas consists of three general aspects: prevention, medication, or vaccination (Kleven, 2008).

8. Multicausal respiratory disease

Respiratory diseases are a matter of great concern for turkey producers as these are causing huge economic losses to turkey industry. According to an estimation, nearly all turkey farms flocks face respiratory issues leading to expensive antibiotic treatment, loss of production

and carcass condemnation at slaughter (Hall *et al.*, 1975; Anderson *et al.*, 1978). Several respiratory pathogens namely: influenza virus type A subtypes H6 and H9, avian paramyxovirus 1, 2 and 3, avian pneumovirus (APV), *E. coli*, *O.rhinotracheale*, Mycoplasma species and *Chlamydophila psittaci* have been associated with respiratory disease in turkeys in Europ (Van Beek *et al.*,1994.; Macpherson *et al.*, 1983; Vanrompay *et al.*, 1993, 1997; Van de Zande *et al.*, 2001; Vandemaele *et al.*, 2002; Kishida *et al.*, 2004; Corrand *et al.*, 2012; Bertran *et al.*, 2013; Costa-Hurtado *et al.*, 2014). Although much is known about the single agents responsible for respiratory diseases in poultry, however, coinfection involving different pathogens largy remains unexplored. Complicated infections involving multiple etiologies with viruses, mycoplasmas and other bacteria, immunosuppressive agents, and unfavourable environmental conditions are more commonly observed than single infections under commercial conditions. AIV has been found to be involved in multicausal respiratory infections where interaction with Mycoplasma was thought to be responsible for high mortality (Bano *et al.*, 2003; Roussan *et al.*, 2008; Stipkovits *et al*.,2012ab, Egyed, *et al.*, 2012; Sid *et al.*, 2015). Multiple respiratory infections may be related to poor hygiene or prophylactic measures. Other studies have shown that influenza virus infection together with bronchitis live vaccine may lead to an extension of the shedding period of H9N2 virus and to an increase in the severity of clinical signs (Haghighat-Jahromi *et al*., 2008). It was previously speculated that multi-infection may have been responsible for high mortalities in poultry flocks (Stipkovits *et al*., 2012ab; Egyed, *et al.*, 2012). Experimental co-infection of *M. gallisepticum* with aMPV or avian influenza increased clinical signs and reduced weight gain (Naylor *et al.*, 1992; Stipkovits *et al*., 2012ab; Glavits, *et al.*, 2012).

In turkeys, only a few studies have been performed to elucidate the effects of combined action of viruses and other micro-organisms. The virus being regarded as having a very important role in turkeys is aMPV, and as a consequence, most studies have included aMPV as triggering agent. Cook et al. (1991) demonstrated that *B. avium* and *Pasteurella*-like organisms were able to colonize after an aMPV infection. Infection was somewhat more severe (slightly more severe

clinical symptoms and thickened air sacs) when bacteria were included in the inoculum, but no poults in any of the experiments appeared sick and no mortality was recorded. In 1992, Naylor et al. demonstrated that an infection with aMPV accelerated the colonization of the lower tract by *M. gallisepticum* and that simultaneous infection resulted in respiratory disease of greater morbidity than following infection with either agent alone. However, for the affected birds, the severity of disease in the mixed infection group was not greater than that in the *M. gallisepticum* group. In an experiment to investigate the possible pathogenicity of *M.imitans* for turkeys, Ganapathy et al. (1998) showed that in 1-day-old turkey poults, the presence of aMPV enhanced the ability of *M.imitans* to invade and colonize. *M. imitans* was only isolated from the upper respiratory tract in single infection, but was recovered also from lung and air sacs in the presence of the virus. After dual infection, they saw a significant increase in clinical signs and lesions, although these still remained relatively mild. On the contrary, dual infection of turkey poults with aMPV and *M. synoviae* did not result in detectable synergism, i.e. no increase in severity of clinical disease, nor gross and microscopic lesions due to aMPV (Khehra *et al.*, 1999). Several *O. rhinotracheale* strains, isolated from turkey, chicken or partridge, were used for aerosol challenge of turkeys of various ages (Van Empel *et al.*, 1996). In turkeys, infection was aggravated by the prior administration of aMPV or NDV. In these studies, no airsacculitis nor pneumonia were seen in the absence of virus. Van de Zande et al. (2001) reported that aMPV/*E. coli* dual infection in turkey poults results in respiratory disease with a higher morbidity, higher incidence of lesions, and higher isolation of *E. coli* from inoculated poults compared with groups given single infections. Clinical symptoms such as depression and anorexia were only seen with dually infected birds and correlated well with the high incidence of gross lesions such as pneumonia, airsacculitis, perihepatitis and pericarditis. Mortality, however, as often seen in the field in aMPV/*E. coli* infected birds, was not encountered in their trial. In 13-wk-old turkeys, dual infection with aMPV and *E. coli* resulted in more severe clinical signs compared with single infection (Van de Zande *et al.*, 2001). In a study with a US aMPV isolate (Colorado strain), a dual infection in turkeys with either a turkey Newcastle

disease virus isolate or broiler *E. coli* isolate resulted in increased morbidity rates and gross lesions compared with single infection, and more synergism was observed with the viral Newcastle infection than with *E. coli* (Turpin *et al.*, 2002). Most poults receiving aMPV/*E. coli* exhibited mild clinical signs (mild depression) early during infection, but swelling of sinuses, as frequently reported in the field, was not observed. In the aMPV/NDV infected birds more severe symptoms were found, ranging from decreased food consumption and in most of the birds nasal exudates with infraorbital, periocular and submandibular swelling. Very recently, Jirjis et al. (2004) used an aMPV subgroup C strain present in the US, to experimentally inoculate turkey poults together with different bacterial species. They found that infection was more severe (increase in severity or incidence of clinical scores, nasal discharge, swollen sinuses, microscopic inflammatory changes in both upper and lower respiratory tract, and gross lesions in air sacs and lungs) in the turkey poults inoculated with aMPV when *B.avium* was administered either alone or in combination with *E. coli* and *O.rhinotracheale*. They concluded that *B.avium* had an additive effect on aMPV infection in turkeys, but this effect was not seen with aMPV in combination with *E. coli* or *O. rhinotracheale*. Loock et al. (2006) examined the pathogenicity of an aMPV superinfection in *C.psittaci* predisposed turkeys. The aMPV infection during the acute phase of a *C.psittaci* infection aggravated the severity of clinical signs, macroscopic lesions, pharyngeal AMPV excretion and histological tracheae lesions. Some of the single aMPV and single *C.psittaci* infected turkeys excreted nasal exudates with or without swollen sinus, whereas a higher percentage of dually infected turkeys showed similar and more long-lasting symptoms. In contrast, no clear interaction could be established after AMPV infection in latently *C.psittaci* infected SPF turkeys.

In some studies, other viruses were used in turkeys in order to try to reproduce severe clinical respiratory disease. Back et al. (1997) infected SPF turkeys with *O.rhinotracheale* and with *O.rhinotracheale* in combination with Newcastle disease vaccine virus, but were not able to reproduce neither clinical signs nor mortality. Charlton et al. (1993) experimented with dual *P.anatipestifer* and NDV infection via different inoculation routes in turkeys, but were unable

to reproduce clinical symptoms. They were only able to demonstrate some differences in histopathology. Sivanandan et al. (1991) evaluated the effect of an apathogenic avian influenza virus (AIV) subtype (H5N2) on the ability of the respiratory tract of turkeys to clear bacterial infections and suggested that AIV infection contributed to increased numbers and decreased clearance of *P.multocida*. Clinical symptoms were not mentioned. Experiments in turkeys have also been done with two bacterial strains, e.g. De Rosa et al. (1997) found that *B. avium* may enhance pathogenicity of *O.rhinotracheale*, although no convincing results were reported, and Droual & Chin (1997) were not able to find a synergistic effect between *O.rhinotracheale* and *E. coli* after intra air sac inoculation. Ficken et al. (1986) found that the clearance of *E. coli* from the air sacs was little affected after infection with *B.avium*. Van Alstine & Arp (1987) found in an infection experiment designed to study the effects of *B.avium* infection on the pulmonary clearance of *E. coli* in turkeys, that *B.avium* had no effect on the numbers *E. coli* in the lungs, but was associated with increased numbers of *E. coli* in tracheae. Severe airsacculitis was found more often in *B.avium* pre-infected turkeys.

When considering the different results obtained from the various challenge studies, it can be noted that it is generally problematic to reproduce respiratory disease similar as seen in the field. For instance, mortality is frequently seen in natural outbreaks of respiratory disease, especially when *E. coli* is involved. None of the above mentioned studies was able to reproduce this phenomenon. Furthermore, it is very difficult to really compare the different experimental studies, since a lot of different variables have to be taken into account. For instance, the virulence and pathogenicity characteristics from the different challenge isolates, the different inoculation routes applied, the varying intervals between the different microbiological inoculations, the age, strain and health status of the inoculated hosts may influence the clinical outcome of an experimental inoculation (Umar *et al.,* 2017a).
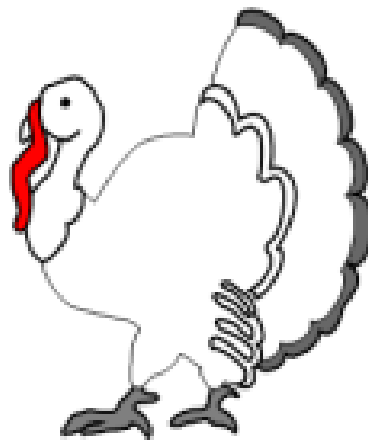
OBJECTIVES OF THE STUDY

Respiratory pathogens and diseases are a matter of great concern for poultry farmers all over the world including in Pakistan. Respiratory disease causes important financial losses in the turkey industry worldwide due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter and these are usually associated with coinfections which are not easy to detect in field condition due to complex clinical picture. A variety of respiratory pathogens, both bacterial and viral, and adverse environmental conditions are the main factors contributing to the development of this disease. The pathogenesis of infections with these respiratory disease agents and their mutual interactions, are far from fully unraveled. This is partly responsible for the fact that therapeutic measurements against different bacterial infections are virtually entirely based on the administration of antimicrobials. In the literature, it is frequently mentioned that although different antimicrobial products are available, the clinical effects are very variable in the field especially during coinfections thus leading to excessive use of antibiotic in poultry especially in turkey farming. So far, coinfecting pathogens interactions upon coinfection largly remains unexplored or only poorly investigated. This is to a great extent rooted in the fact that often no suitable infection models are available. To extend our knowledge on coinfections and control of respiratory infections in turkeys, the present thesis was initiated. Single and combined infections with AIV and *E. coli*, important respiratory pathogens, were studied.

Bacterial and viral infection causing huge economic losses to economy in the form of morbidity and mortality of birds, poor diagnosis and excess medicine, vaccination costs. Antibiotic resistance and vaccination cost for the prevention or treatment of viral and bacterial infections are two 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 (Grahem *et al.,* 2007). Antibiotic resistance is also an economic burden on the healthcare system. On the other hand, limiting the use of antibiotics in poultry farms may lead to an increase of bacterial infections, and also of co-infections, confirming the need of a better understanding of these complex interactions *in vivo*. LPAIV

H6N1 has been circulting in France but their interactions with co-circulating pathogens are still rela tively unknown (Corrand *et al.*, 2012). To the best of our knowledge no data are available that describe the co infection of LPAIV (H6N1) with certain respiratory pathogens such as *E. coli*. Antimicrobial drugs are used extensively to fight against co-infection in turkeys resulting in antimicrobial resistance and economic losses. Growth-promoting antibiotics have been banned from animal feed in the European Union (feed additives regulation1831/2003/EC). One of the negative consequences associated with the prohibition of antibiotic growth promoters in commercial poultry production is the increase in secondary bacterial infections, such as colibacillosis. Personal experiences showed that some turkey flocks which had been suffering from *E. coli* infection showed extraordinary high mortality due to H6N1 infection. Recently, some researchers have reported on coinfections in field in French poultry industry (Corrand *et al.*, 2012a, 2012b, Croville *et al.*, 2017 in preparation) while there is no data available on the coinfections in poultry in Pakistan. The impact of co-infections is not very well known so far especially in turkeys. One way to increase our knowledge on coinfections is to perform experimental studies in poultry models. Co-infection studies is one approach in defining possible synergistic or additive effects of different organism on each other. For the present PhD thesis work, we intended to link the coinfections observed in Pakistani poultry farms with coinfection studied in experimental conditions and experimental studies were set out to examine the possible pathogenic interplay between *E. coli* and LPAIV H6N1, and as such contributing to the unravelling of the respiratory disease complex in turkeys. Our main objective was to develop experimental infection models using these agents and subsequently use these models to study the role of *E. coli* on the pathogenesis of LPAIV H6N1 or *vice versa*. We hope that our coinfection study will help toward better diagnosis and ultimately reduce the use of antimicrobials in turkey industry. Random treatments (vaccines or antimicrobials) are applied to fight against these infections in Pakistan without knowing the actual cause of disease. Epidemiological work in Pakistan will help to screen and characterize respiratory pathogens involved in respiratory diseases thus leading to efficient implementation of control measures

against respiratory diseases. We used non specific pathogen free (SPF) commercial turkeys in our experiements because they better mimics field conditions than SPF turkeys. Moreover, turkeys have longer life span than chicken and in their longer life span they encounter different respiratory infections/coinfections. During coinfections in turkeys, different antibiotics are used to fight against these infections in field. Better knowledge on these coinfections may help to reduce the use of antibiotics in field conditions.

More specific goals of the study were:

- To develop a coinfection model in poultry

- To study the impact of these coinfection *in vivo*

- To screen and chacterize avian respiratory viruses in Pakistani poultry

- To assess the burden of co-infections in poultry in Pakistan

- To link the coinfections observed in Pakistani poultry farms with coinfection studied in experimental conditions.

Ethics statement

State of the art laboratory material and machines were at our disposal and, unless otherwise indicated, all laboratory procedures were carried out in accordance with the Manual on Animal Influenza Diagnosis and Surveillance of the World Health Organization. Experimentations were conducted in accordance with European and French legislations on Laboratory Animal Care and Use (French Decree 2001-464 and European Directive CEE86/609) and animal protocols approved by the Ethics Committee "Sciences et santé animale", committee number 115.

Low pathogenic avian influenza and co-infecting pathogens: a review of experimental infections in avian models

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Short title: co-infections in avian models

Abstract

Low pathogenic avian influenza virus (LPAIV) usually causes mild disease or asymptomatic infection in poultry. LPAIV have however become a great threat to poultry industry due to mixed infections with other pathogens. Co-infections do frequently occur in the field but are not easily detected, and their impact on pathobiology is not clearly defined due to the complicated nature but it is a well-known fact that there is an impact. One way to increase our knowledge on co-infections in poultry is to challenge birds in experimental and controlled conditions. While many articles report *in vivo* experiment with LPAIV in avian models, only few studied co-infections. 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 a state of the art on experimental infections with LPAIV alone or associated with co-infecting pathogens in avian models. Second, it aims at discussing how to best mimic field infections in laboratory settings. In the field of avian diseases, experimental design to use 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 the laboratory settings closer to the field situations.

Abbreviations: AI: avian influenza; AIV: avian influenza virus; CFU: colony forming unit; EID50:50% egg infectious dose; HA : hemagglutination; HPAIV: Highly pathogenic avian influenza virus; AP: *Avibacterium paragallinarum;* IT: intratracheal; IN: intranasal; IV:intravenous; IC: intracloacal; IBV: Infectious bronchitis virus; LPAIV: Low pathogenic avian influenza virus; MG: *Mycoplasma gallisepticum*; NDV: Newcastle disease virus; ORT: *Ornithobacterium rhinotracheal*; O: ocular; PO: per orally; SPF: specific pathogen free; SA: *Staphylococcus aureus*; TOC: tracheal organ culture

In poultry farms, infections with avian influenza viruses 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 state of the art on experimental infections with low pathogenic avian influenza virus alone or associated with co-infecting pathogens in avian models. On this first basis we then discussed 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, co-infection, chicken, turkey, duck, poultry, experimental infection, in vivo. Only studies with experimental infections of birds with LPAIV alone or with a co-infecting 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 8 distinct single-stranded RNA molecules, which encode at least 10 different viral proteins (50). On the basis of antigen variations of the surface glycoprotein hemagglutinin (HA) and neuraminidase (NA), there are eighteen antigenically distinct HA and eleven 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). AIV 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 (OIE) criteria (17). For AIV to be highly pathogenic, they must meet one of two criteria: 1) intravenous pathogenecity index (IVPI) > 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 AIV are characterized as LPAIV (78). HPAIV are responsible for rapid and fatal systemic infection inducing mortality up to 100% in broilers, layers and breeders while LPAIV lead to

asymptomatic or milder infections. All HPAIV 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 HPAIV 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 death of the infected birds (64). In contrast, LPAIV are capable to replicate only in limited tissues of respiratory and digestive systems. In the field, LPAIV can cause severe disease if the host is stressed or if other diseases are present (1,47). In cases of sub-clinical LPAIV infections, production losses are observed due to anorexia in meat birds and drop in egg production in breeders/layers. LPAIV of H1N1 and H9N2 subtypes were indeed shown to replicate in chicken and turkey reproductive tracts (57, 58, 63,83,89). In the present review we chose to focus on LPAIV.

Influenza A viruses and evolution

Because avian influenza RNA-polymerase does not possess a proof-reading-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 which infect a host cell at the same time (6). Genetic variability is of vital importance for the survival of avian influenza viruses 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 there 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 LPAIV has led to novel phenotypes and increased virulence (37,39). 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 co-circulation of H9N2

viruses with other avian influenza viruses, 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 has acquired PB2, PB1, PA and NS gene segments from HPAI H7N3 viruses with the other genes originating from G1-like lineage H9N2 viruses (39). Some LPAIV, 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).

LPAIV and co-infecting 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 (1,2,7,17). 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 (54, 66). However, it is also documented that LPAIV, 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. Co-infection 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 (IBV) 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 (36,77). 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 LPAIV have been reported in waterfowl, and competition between viruses during isolation suggests an underestimation of co-infections in the laboratory (24).

Other respiratory co-pathogens, such as *M. gallisepticum* (MG), *E. 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). *M. gallisepticum* and *E. coli* were isolated from the field cases and may have played a role as co-pathogens to AI virus in the clinical disease syndrome. Fibrino-necrotic 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* (16). *M. gallisepticum* in combination with other respiratory pathogens, including LPAIV, can cause severe airsacculitis (9,10,54). This suggests a common pathogenic mechanism with multiple lineages of AIV 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 co-pathogens needs to be determined in future studies.

LPAIV in experimental studies

The pathogenicity and transmission of many LPAIV strains have been investigated in experimental conditions in avian models. Specific pathogen free (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 LPAIV. 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 weeks of age are commonly used for experimental studies, likely because of their easy handling and low feed cost at this age. Moreover, intranasal route (IN) for the inoculation of respiratory pathogens in avian models is the most common followed by intra-tracheal (IT), oral (PO), intraocular (O), intramuscular (IM), intravenous (IV), intra-oviduct (IO) and intra-cloacal (C). 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 EID_{50} 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 (58). 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 EID_{50} for LPAIV 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 EID_{50}/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 ciliostatis, 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.

LPAIV and co-infecting 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 laryngotrachitis virus (ILTV) may also predispose to secondary infections. Several different bacteria have also been implicated, including *M. gallisepticum, E. coli, Avibacterium paragallinarum, Ornithobacterium rhinotracheal* (ORT) and, *Staphylococcus aureus* (35,36,77). It is thought that certain pairings of organisms better complement each other than other potential pairings. Co-infections of poultry present a complicated clinical picture confusing the identification and diagnosis and unfortunately little is known on the interactions between co-infecting pathogens (19). Tables 2 and 3 summarize the literature on experimental infections of birds with LPAIV and bacteria, and LPAIV and avian viruses, respectively. Co-infection 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 co-infected flocks since lower or undetectable virus titers might fail to give a complete diagnosis (24). Co-infection of LPAIV A/chicken/Iran/SH-110/99 (H9N2) virus 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 co-infection 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). Else IBV and LPAIV co-infections 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). Co-infection 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). Co-infection 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). Co-infection of H9N2 influenza virus with *S. aureus* 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 healthcare system. On the other hand, limiting the use of antibiotics in poultry farms may lead to an increase of bacterial infections, and also of co-infections, confirming the need of a better understanding of these complex interactions *in vivo*.

As for LPAIV infections alone, the most common avian models for the study of viral and bacterial co-infections are chickens. Ducks, turkeys, quail, and partridges have also been used to address different research questions. One possible reason for most widely use of chicken as 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 (*Mycoplasma, E. coli*) as described in literature are 10^6 to 10^9 colonies forming units (CFU). Clinical signs and lesions severity have been shown to increase in a dose dependent manner. Bacterial co-infections lead to more severe lesions when the bacterial were inoculated IN or via an aerosol route. In an experimental co-

infection study with LPAIV H3N8 (A/mallard/Hungary/19616/07) and *M. gallisepticum* inoculated through aerosol spray, the authors showed that LPAI H3N8 virus alone did not cause any clinical signs but *M. gallisepticum* infection caused clinical signs, reduction of body weight gain and colonization of the inner organs. These parameters were more severe in the birds co-infected with *M. gallisepticum* and LPAIV H3N8 than in the group challenged with *M. gallisepticum* alone. Co-infection with LPAIV H3N8 thus enhanced the pathogenesis of *M. gallisepticum* significantly (73,74). Barbour et al co-infected chickens IT or intra-thoracic with H9N2 LPAIV and *E. coli*. High and acute mortality was observed with the intra-thoracic inoculation route for *E. coli* (9,10).

LPAIV co-infection 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 co-infected 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, Co-infection 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 on NDV shedding in cloacal swabs and LPAIV may have reduced or inhibited NDV replication (25). Similarly, Costa-Hurtado and colleagues reported that co-infection 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 co-infection with two different viruses can result in temporary cell receptor binding competition (19). During this study, chickens and turkeys were infected with NDV vaccine strain (LaSota) and a H7N2 LPAIV (A/turkey/VA/ SEP-67/2002) simultaneously or sequentially three days apart. No clinical signs were observed in chickens co-infected with NDV and LPAIV while all turkeys showed mild clinical signs during co-infection. The replication dynamics of these viruses was

however affected by the co-infection: 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).

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 (79). 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 human (H1N1). It was reported that increase in the proportion and number of rhinovirus can decrease of influenza virus diagnoses in human, suggesting that rhinoviruses may compete with influenza virus for receptor binding and replication (4). Bacterial and viral co-infections usually show synergistic effects and exacerbate clinical signs and lesions. No competition between pathogens is observed then.

Timing of co-infections

In an attempt to put together the available methods reported in the literature for co-infections in experimental conditions, and mainly to see which time line may best fit field co-infections situations, we drew time arrows summarizing LPAIV and co-infecting bacteria experiments (Figure 1) as well as LPAIV and co-infecting viruses experiments (Figure 2). The studies in experimental conditions conducted so far reported 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 panels of the Figures, respectively). The 3 days interval chosen between subsequent infections was pretty consistent throughout literature (9,19,42,35,73,74). 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 *Staphylococcus aureus* infection followed by LPAIV H9N2 inoculation (42). The use of two different bacteria made a systematic comparison of the timing of co-infection difficult. Pan et al co-infected 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 co-infections have been more systematically studied (19,25). While minimal effect of the LPAIV and NDV co-infection was observed on a clinical point of view, altered shedding pattern was detected both in mallards and chickens, irrespective of the timing of the co-infection (19,25). When LPAIV and IBV live vaccine were both administered to chickens, the co-infection lead to more severe clinical signs and longer LPAIV shedding, again irrespective of the timing of co-infection (35). In the laboratory setting, the timing of co-infections does therefore not seem to play an essential role on pathogenesis. While common knowledge in virology associates co-infections with a virus coming first followed by a bacteria, the timing in the field may actually rather be opposite with commensal bacteria that are there first and may become a problem when a virus super-infects the birds (14). *Manheimia haemolytica*, *Gallibacterium anatis*,

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 presence of a co-infecting pathogen or in difficult environmental conditions (3, 5, 48,71).

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 (38,81,65,30). 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*., (44) 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 one rethinks the choice of bird type for LPAIV pathogenesis studies. To mimic the field situation commercial birds seem of course 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 in order to better understand disease outcome in the field thought should be given into 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: birds type, single or co-infections, timing, dose of infection(s), environmental and management

variables play a critical but poorly controlled role in the disease outcome in farms. Environment pollution can also play a very important role in disease outcomes in field conditions. 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 intra-nasally or intra-tracheally. In a clinical study, authors compared the 50% infectious dose (ID50) by aerosol and intranasal 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 intra-nasally or tracheally. 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, the review of literature suggests that to study co-infections with LPAIV and a complicating pathogen in experimental conditions, using young (3-4 weeks old) birds, 10^6 EID_{50} 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 really assess the cost-benefit of using commercial birds and to determine the adequate timing of the co-infection in relation to the field situations. Environmental factors (temperature, relative humidity, ammonia level, etc) should also be taken into account as they

definitely play a role in the field but have so far not been much looked at in experimental settings.

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Figures legends.

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

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

Table 1: Summary of LPAIV infections in different avian models in experimental conditions

Influenza virus strain	Animals/age	Infection dose (/bird)	Infection route	Main findings	References
A/cx/Pakistan/UDL-01/08(H9N2)	3 week-old chicken (broiler and white leghorn), common quail, red jungle fowl (hen pullet) sparrow, crow (age not known)	$10^{6.6}$ EID$_{50}$	IN	- 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	(40)
A/cx/shaanxi/01/2011	30-week-old SPF White Leghorn layers	10^{6} EID50	IN, IC, or IO	-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-week old SPF White Leghorn layers		O, IN	Efficient virus replication in magnum and uterus Significant tissue damage and appotosis in oviductal parts	(83)

				-Decreases expression of mRNA expression of IFN-α	
A/cx/CA/1255/02(H6N2) and A/cx/NJ/12220/97(H9N2)	46 weeks-old SPF chicken (layer)	10^6 EID$_{50}$	IN IC O	- 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	(58)
A/cx/United Arab Emirates/99 (H9N2)	3 week-old chicken (broiler)	$10^{8.8}$ EID$_{50}$	O IN IT	- 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 week-old chicken (broiler)	4 HA Units	IV	- mild clinical signs - gross and microscopic lesions mainly on lungs and kidneys - IHC detection of viral antigen in kidney and lungs - virus affinity for respiratory and urinary systems	(75)
A/cx/Iran/SH-110/99(H9N2)	3 week-old chicken (broiler)	10^6 EID$_{50}$	IT	- clinical signs: depression, puffing, face edema, conjunctivitis, ruffled feathers (2-4 dpi) - gross lesions in respiratory tract and kidneys	(34)

A/cx/Lebanon/1/04(H9N2)	3 week-old chicken	7 $TCID_{50}$	IT	- no mortality - low morbidity (<50%) - increased pathogenicity after virus passaging (higher morbidity, higher gross and microscopic lesions)	(68)
A/cx/Chile/176822/02(H7N3)	4 week-old chicken	10^6 EID_{50}	IN IV	- 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 ceacal tonsils in IN inoculated birds	(41)
A/cx/California/1772/02(H6N2)	6-8 week-old chicken	10^7 EID_{50}	IN	- 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)
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 week-old chicken (broiler) and turkey	10^6 EID_{50}	IT IC	- 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	(45)

				- 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	
A/Chile/3536/2009 (H1N1)	53 week old laying turkeys	$10^{5.3}$ TCID50	IN, IC, IU	-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	(57)
A/qu/Hong Kong/G1/97(H9N2)	4 week-old turkey and Japanese quail	10^3 to 10^6 EID$_{50}$	PO IN	- quail more susceptible than turkeys to H9N2 but with less sever clinical signs (quail can act as a silent reservoir for H9N2) - efficient direct contact transmission in quail and turkeys	(13)

Virus	Host	Dose	Route	Findings	Ref
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	- 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 week-old European quail	10^6 EID$_{50}$	IN	- no clinical signs or pathology in inoculated and contact quail - efficient replication and transmission	(12)
A/cx/Iran/772/98(H9N2)	23 week-old chukar partridges (*A.chukar*)	10^7 EID$_{50}$	O IN	- clinical signs: coughing, sneezing, depression and anorexia - good virus replication and wide tissue tropism	(52)
A/*Anas crecca*/Spain/1460/08 (H7N9)	9 week-old red-legged partridge *(Alectoris rufa)*	10^6 EID$_{50}$	IN	- no clinical signs or histopathological lesions - only short-term viral shedding and seroconversion	(11)
A/cx/California/1772/02(H6N2)	6-8 week-old chicken, 8-12 week-old pekin ducks	10^7 EID$_{50}$	IN	- efficient virus aerosol transmission from chickens to chickens and quail; poor aerosol transmission from chickens to ducks	(88)

Virus	Host	Dose	Route	Findings	Ref
	and quail (7 week old)				
A/ty/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) 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), A/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	3-12 week-old SPF white leghorn chicken and 5 week-old Pekin white duck	10^7 EID$_{50}$ (chickens) 10^6 EID$_{50}$ (ducks)	IV, PO, IT, or IN	- 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/dk/HK/702/79(H9N2), A/dk/HK/448/78(H9N2), A/dk/HK/366/78(H9N2), A/dk/HK/149/77(H9N2),	4 week-old Japanese quail (*Coturnix coturnix*),	5×10^6 EID$_{50}$	IT, PO, IN	- efficient replication of duck viruses in quail and ducks but not in chickens - higher replication in the respiratory than intestinal tract of quail	(60)

A/dk/HK/86/76(H9N2), A/dk/HK/147/77 (H9N6), and A/qu/Hong Kong/A28945/88 (H9N2)	chicken (white leghorn), mallard and Pekin ducks			- 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	
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/VA/SEP-67/02(H7N2)	4 week-old chicken 2 week-old duck and turkey	10^6 EID$_{50}$	IT	- 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	(72)
A/mallard/New Brunswick/1/06 (H2N3), A/gull/Ontario/680-6/01 (H13N6)	22 week-old mallard duck	10^8 EID$_{50}$	PO IT	- 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)	(20)

				- 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	
A/mallard/MN/355779/00(H5N2) A/mallard/MN/182761/98(H7N3) A/mallard/MN/199106/99(H3N8)	10-16 week-old duck (mallards, red heads, wood ducks, laughing gulls)	10^6 EID$_{50}$	IN	- 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	(18)
A/mallard/Minnesota/199106/99 (H3N8)	4 weeks-old duck	$10^{6.5}$ EID$_{50}$	IN	- efficient virus replication - no clinical signs but high virus shedding	(15)

LPAIV: low pathogenic avian influenza virus, cx: chicken, dk: duck, qu: quail, ty: turkey, EID: egg infectious dose, TCID: tissue culture infectious dose, IHC: immunohistochemistry, SPF: specific pathogen free, HA: hemagglutination, IC: intra cloacal, IN: intranasal, IO: intra-oviduct, IP: intraperitoneal, IT: intra-tracheal, IV: intravenous, O: intraocular, PO: per orally.

Table 2. Summary of LPAIV and bacteria co-infections in different avian models in experimental conditions

Virus and bacteria strains	Animal/age	Infection dose	Infection route	Main findings	References
A/mallard/Hungary/19616/07(H3N8), *M. gallisepticum* (strain 1226)	2 week-old chicken (broiler)	10^5 EID$_{50}$ (H3N8) 2.1 $\times 10^7$ CFU (MG)	Spray (aerosol)	- 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 co-infected birds than in *MG* only infected birds - reduced anti-mycoplasma antibody titers in co-infected versus *MG* alone infected chickens	(73,74)
A/cx/Iran/m.1/10(H9N2), R87-7/1387 (JF810491,ORT)	3 week-old chicken (white leg horn layer)	10^6 EID$_{50}$ (H9N2), 10^{10} CFU (ORT)	IT	- more pronounced clinical signs, gross lesions and mortality in co-infected birds than in birds challenged with ORT alone - significantly higher HI titers against H9N2 virus in co-infected than H9N2 infected birds	(6)
A/cx/Shandong/2011(H9N2), ORT/chicken/Shandong/2011	3 week-old chicken (broiler)	10^2 EID$_{50}$ (H9N2), 10 LD$_{50}$ (ORT)	IP and IN	- general sickness in all co-infected groups (simultaneous infection or ORT followed by H9N2) - more severe clinical signs of respiratory disease, anorexia, and mortality observed in	(56)

				co-infected birds (simultaneous infection or ORT followed by H9N2)	
				- no mortality but typical pneumonia with fibrinous airsacculitis, pericarditis, peritonitis and scattered areas of haemorrhage in the lungs in birds inoculated with H9N2 virus followed by ORT or H9N2 virus alone	
A/cx/Lebanon1/04(H9N2), *E. coli* (BVL strain)	3 week-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 CFU (*E. coli)*	IT + intra thoracic	- enhanced clinical signs and gross lesions with increasing *E. coli* doses, but more severe clinical signs and gross lesions in co-infected birds than single infections	(9)
A/dk/Beijing/40/04(H3N8), A/dk/Beijing/61/05(H3N8), *E. coli* O78	5 week-old chicken (white leghorn layer)	10^6 EID$_{50}$ (H3N8) 2.53×10^8 (*E. coli)*	IN IO IC O	- no obvious signs of disease in chickens inoculated with virus or bacteria alone - depression, diarrhoea and mortality after inoculation with A/dk/Beijing/40/04(H3N8) and *E. coli*	(62)

			- slight depression but no mortality after inoculation with A/dk/Beijing/61/05(H3N8) and *E. coli*	
A/cx/aq-Y-55/01(H9N2), A/cx/aq-Y-135/01(H9N2), A/cx/Beijing/2/97(H9N2), A/tern/South Africa/61(H5N3), *Staphylococcus aureus* (Hyogo strain) and *Avibacterium paragallinarum* (HK-1 strain)	4 week-old chicken (white leghorn layer)	10^7 EID$_{50}$ IN (influenza virus) 2.2×10^5 CFU (SA) 3×10^3 CFU (AP)	- exacerbation of clinical signs and gross lesions for co-infected birds with H9N2 virus and *SA* or *HP* compared with single infected birds - higher H9N2 viruses in co-infected than H9N2 only infected birds	(42)

LPAIV: low pathogenic avian influenza virus, cx: chicken, dk: duck, qu: quail, ty: turkey, *E. coli= Escherichia coli,* MG: *Mycoplasma gallisepticum*, ORT: *Ornithobacterium rhinotracheal,* SA: *Staphylococcus aureus*, AP: *Avibacterium paragallinarum,* EID: egg infectious dose, CFU: colony forming unit, HA: hemagglutination, IN: intranasal, IP: intraperitoneal, IT: intra-tracheal, O: intraocular IC: cloacal, IO: intra-oviduct
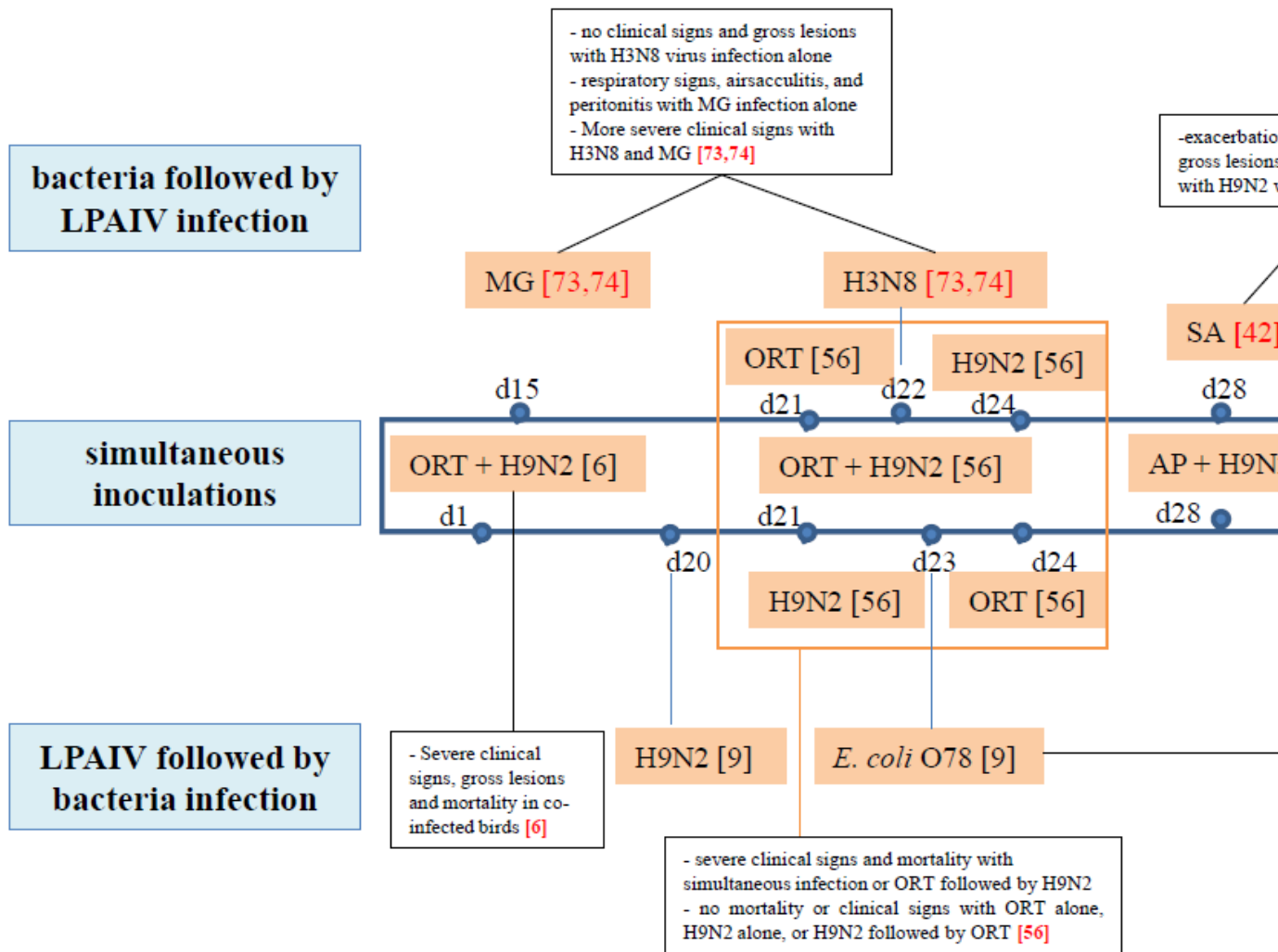
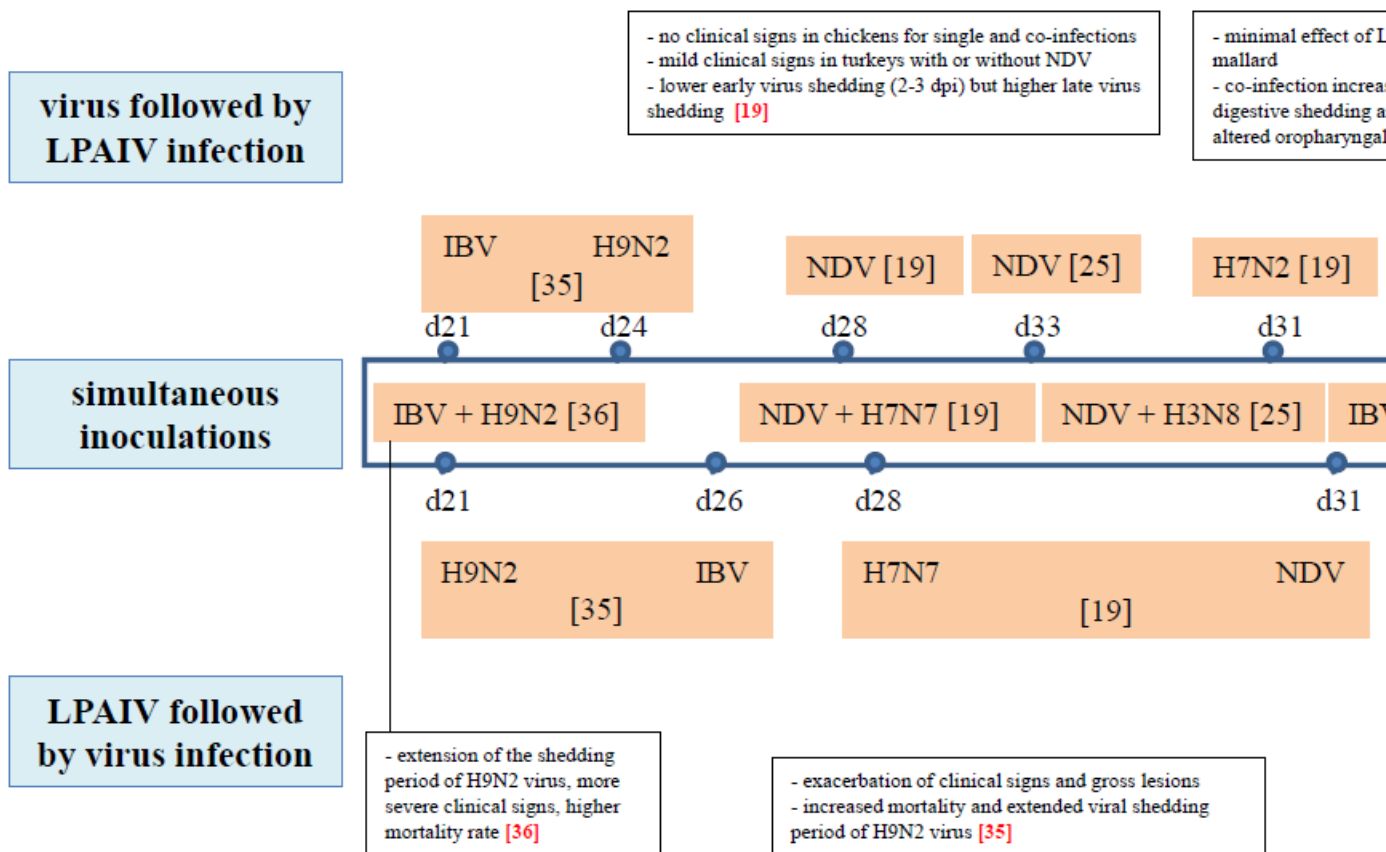**Table 3. Summary of LPAIV and avian virus co-infections in different avian models in experimental conditions**

Viral strains	Animal/age	Infection dose	Infection route	Main findings
A/ty/VA/SEP/67/02(H7N2) APMV1/cx/US(NJ)/LaSota/46 (NDV)	3-4 week-old chicken (white leghorn layer) and turkey	10^7 EI D$_{50}$	O IN	- no clinical signs and chickens and turkeys, single or co-infection - delay in virus shedd chickens
A/cx/Iran/SH-110/99(H9N2), IBV live vaccine (freeze-dried live attenuated vaccine, Mass type, H120 strain)	3 week-old chicken (broiler)	10^6 EID$_{50}$ (H9N2), 10^3 EID$_{50}$ (IBV)	IN + spray	- longer shedding peri more severe clinical s mortality in co-infecte
A/mallard/MN/199106/99(H3N8) mallard/US(MN)/AI06-978/06 (NDV)	5 week-old mallard	10^6 EID$_{50}$	IN	- efficient AIV and N - higher digestive she infected birds
A/cx/Iran/SH110/99(H9N2) IBV vaccine strain IB 4/91	3 week-old chicken (broiler)	0.2 ml homogeni-zed sample	IN	- exacerbated clinical and mortality in co-in - higher antibody titer co-infected birds

LPAIV: low pathogenic avian influenza virus, NDV: Newcastle disease virus, IBV: infectious

bronchitis virus, cx: chicken, ty: turkey, EID: egg infectious dose, IN: intranasal, O:

intraocular

bacteria followed by LPAIV infection

simultaneous inoculations

LPAIV followed by bacteria infection

- no clinical signs and gross lesions with H3N8 virus infection alone
- respiratory signs, airsacculitis, and peritonitis with MG infection alone
- More severe clinical signs with H3N8 and MG [73,74]

-exacerbatio...
gross lesions...
with H9N2 v...

MG [73,74]

H3N8 [73,74]

SA [42]

ORT [56]

H9N2 [56]

d15

d21

d22

d24

d28

ORT + H9N2 [6]

ORT + H9N2 [56]

AP + H9N...

d1

d21

d28

d20

d23

d24

H9N2 [56]

ORT [56]

H9N2 [9]

E. coli O78 [9]

- Severe clinical signs, gross lesions and mortality in co-infected birds [6]

- severe clinical signs and mortality with simultaneous infection or ORT followed by H9N2
- no mortality or clinical signs with ORT alone, H9N2 alone, or H9N2 followed by ORT [56]

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virus followed by LPAIV infection

- minimal effect of L
mallard
- co-infection increas
digestive shedding a
altered oropharyngal

IBV	H9N2 [35]		NDV [19]	NDV [25]		H7N2 [19]
d21	d24		d28	d33		d31

simultaneous inoculations

IBV + H9N2 [36]		NDV + H7N7 [19]	NDV + H3N8 [25]	IB
d21	d26	d28		d31

H9N2	IBV [35]		H7N7	NDV [19]

LPAIV followed by virus infection

- extension of the shedding period of H9N2 virus, more severe clinical signs, higher mortality rate [36]

- exacerbation of clinical signs and gross lesions
- increased mortality and extended viral shedding period of H9N2 virus [35]

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PRESENTATION OF EXPERIMENTAL WORK

Co-infection of turkeys with *E. coli* (O78) and H6N1 avian influenza virus

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Short title: Bacterial and virus coinfection in turkeys

Abstract

Respiratory diseases are responsible for major economic losses in poultry farms. While in most cases a single pathogen is not alone responsible for the clinical outcome, the impact of co-infections is not well known, especially in turkeys. The purpose of this study was to assess the possible synergism between *E.coli* (O78) and low pathogenic avian influenza virus (LPAIV, H6N1), in the turkey model. Four-week-old commercial turkeys were inoculated with either LPAIV subtype H6N1, *E.coli* serotype O78 or both agents simultaneously or 3 days apart. We have established an experimental infection model of turkey poults with LPAIV using aerosolization that better mimics field infections. The birds were observed clinically and swabbed on a daily basis. They were killed at 4 and 14 days post single or dual inoculation and examined for gross lesions at necropsy. Samples of the trachea, lungs, air sacs were taken for histological and immunohistochemical analyses. Combined LPAIV/*E.coli* infections resulted in more severe clinical signs, were associated with higher mortality and respiratory organ lesions, in comparison with the groups given single infections ($p<0.05$). The time interval or the sequence between H6N1 and *E. coli* inoculation (none or 3 days) did not have a significant effect on the outcome of the dual infection and disease although slightly higher ($p>0.05$) respiratory signs were observed in turkeys of the *E.coli* followed by H6N1 inoculated group. Microscopic lesions and immunohistochemical staining supported clinical and macroscopic findings. Efficient virus and bacteria replication was observed in all inoculated groups. These findings all endorse our conclusions that *E. coli* and H6N1 exercise an additive or synergistic pathogenic effect in the reproduction of respiratory disease if given simultaneously or three days apart.

Introduction

Respiratory diseases are continuing to cause heavy economic losses in the poultry industry with increased mortality rates, drops in egg production, reduction of egg shell quality, decreased hatchability, increased condemnation rates at slaughter and increased medication costs (Van Empel & Hafez, 1999). They may be induced by various viral and bacterial agents, either alone or in combination. Respiratory tract infections increase the overall cost of production in terms of the provision of services of qualified veterinary personnel and the cost of medication for possible treatment (Anderson *et al.*, 1978; Marien *et al.*, 2005). It is therefore important to reduce if not eliminate, respiratory infections among poultry flocks to the barest minimum to have good production and maximize profit of the producer. Various pathogens may initiate respiratory disease in poultry, including a variety of viruses, bacteria, and fungi. Poultry respiratory diseases are known to be caused by many pathogens including Newcastle disease virus (NDV), Avian influenza virus (AIV), Infectious bronchitis virus (IBV), avian metapneumovirus (aMPV), *Mycoplasma gallisepticum* (*M. gallisepticum*), *Mycoplasma synoviae* (*M. synoviae*), *Mycoplasma meleagridis* (*M. meleagridis*), *Mycoplasma iowae* (*M. iowae*), *Ornithobacterium rhinotracheale* (*O. rhinotracheale*), *Pasteurella multocida*, *Avibacterium paragallinarum*, *Bordetella avium, Chlamydophila psittaci* and *E. coli* with associated significant economic losses to the industry (Van de Zande *et al.*, 2001; Sid *et al.*, 2015). In chickens, respiratory disease caused by aMPV (Majó *et al.*, 1997) or IBV (Smith *et al.*, 1985; Cook *et al.*, 1986; Nakamura *et al.*, 1992) was more severe in the presence of *E. coli*. In turkeys, a few studies have been performed to elucidate the effects of the combined action of aMPV and other microorganisms (Cook *et al.*, 1991; Naylor *et al.*, 1992; Ganapathy *et al.*, 1998).

Influenza infections in poultry are important because they impact animal health and agricultural trade, and control costs are very high. Low pathogenic avian influenza virus (LPAIV) infections are becoming major threat to poultry industry and limited protection is often provided by the inactivated vaccines (improper cold chain, dose given, etc are often involved). Effective culling

strategy is not widely implemented in developing countries during outbreaks of LPAIV so no effective strategy exists for controlling LPAIV infections. Co-infection of LPAIV with various other respiratory pathogens led to increasing severity of clinical signs and mortality rates as well as the virus shedding period in chickens (Kishida *et al.*, 2004; Haghighat-Jahromi *et al.*, 2008; Barbour *et al.*, 2009; Pan *et al.*, 2012; Seifi *et al.*, 2012; Stipkovits, Egyed, *et al.*, 2012; Stipkovits, Glavits, *et al.*, 2012; Azizpour *et al.*, 2013). An important natural route of LPAIV infection in farms is inhalation of contaminated dust. 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 intra-nasal or intra-tracheal route (Nicas *et al.*, 2005; Guan *et al.*, 2013, 2015). The aerosol route of inoculation better mimics field infection and use of aerosols for *in vivo* infections may help reduce the gap between the laboratory and the field conditions. LPAIV subtype H6N1 has been isolated from commercial turkeys in France (Corrand *et al.*, 2012). However, the pathogenicity of the H6N1 isolates, alone or in combination with *E. coli*, had so far not been studied in turkeys in experimental conditions. *E. coli* infection is widely spread in poultry flocks (reviewed in Guabiraba & Schouler, 2015). Therefore, we hypothesized that LPAIV H6N1 could be easily transmitted to poultry flocks that are infected with *E. coli*. In the field, LPAIV H6N1 and *E. coli* have shown exacerbation of clinical signs, mortality and severe production loss (Dr. Jean Luc Guérin personal communication). Whether LPAIV H6N1 and *E. coli*, merely act separately or in a synergistic or additive way remains to be elucidated. The aim of the present investigation was to develop a co-infection experimental model and to study the impact of co-infections of *E. coli* (O78) on LPAIV H6N1 infections under experimental conditions in turkeys.

Materials and Methods

Turkeys

Mixed sex turkey poults (non-specific pathogen free) were purchased from a local company (GFA de Pierpont, Castelnau de Montmiral, France) originating from *M. synoviae* and *M. gallisepticum* free parent stock. The birds were housed in animal biosafety level 2 (ABSL-2) facilities. The turkeys were housed in high-efficiency particulate air (HEPA) isolators (Allen town, NJ, USA) with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^{3}h^{-1}. The isolators were equipped with nipple drinkers. The birds received 16 hours of light per day, and had free access to food and water. The birds were fed with standard feed without antibacterial agents. Strict measures were adopted to avoid cross contamination and management procedures for all groups were identical. Before inoculation with *E. coli* (O78), oropharyngeal swabs were taken from all birds in all groups for bacteriological examination, and were negative for *E. coli* (O78). All birds tested serologically negative for influenza before the start of the experiment. Cotton swabs (MWE, medical wire, UK) were used to collect oropharyngeal swabs and placed in viral transport media as recommended (WHO, 2014). Experimentations were conducted in accordance with European and French legislations on Laboratory Animal Care and Use (French Decree 2001-464 and European Directive CEE86/609) and animal protocols approved by the Ethics Committee "Sciences et santé animale", committee number 115.

Bacteria and viruses

E. coli strain (O78K80, X7122) was obtained from Professor Eric Oswald (Purpan Hospital Toulouse), originally isolated from chicken. A stock culture of *E. coli* strain was stored in 40% glycerol broth at - 80°C. *E. coli* stock culture was prepared by inoculating Mckonky's agar with a loopful of reference *E. coli* strain culture and incubating at 37°C for 24 h. To prepare *E. coli* cultures for infecting birds by aerosol, 250 ml of DMEM was inoculated with colonies from Mckonky's agar plate and incubated in an orbital shaker at 37°C for 22 to 24 h. A neutralizing medium (Hepes 25mM) was added just 1 hr prior to inoculation. The estimated colony count

was confirmed by plating 0.1 ml of 10^5 dilution of the final culture onto separate Mckonky agar plates.

LPAIV H6N1 (A/turkey/France/09010-1/2009) was isolated from commercial turkeys in France (Corrand *et al.*, 2012). Viruses used in this study were propagated in fertile SPF hen's eggs (PFIE, INRA, Nouzilly, France). Viral titers were measured with standard plaque assays [plaque-forming units (PFU)/mL] as described previously (Matrosovich *et al.*, 2006).

Animal experiment

Turkeys were individually identified and assigned into different groups. Turkeys were housed in different isolators and each isolator served as an experimental treatment group. Bacteria and virus were administered as aerosols at a dose of 10^9 colony forming units and 10^6 plaque forming units per isolator diluted in PBS, respectively. For the aerosol inoculations, a compressor nebuliser CompAir Pro NE C29 E (OMRON, Japan) was used to aerosolize the pathogens for 20 min. Turkey groups were as follows: (negative control) non infected group, (*E. coli* alone) Bacteria inoculated group, (H6N1 alone) Virus inoculated group, (*E. coli*+H6N1) group inoculated bacteria and virus simultaneously, (*E. coli*/H6N1) group inoculated with bacteria and followed by virus inoculation three days later, (H6N1/*E. coli*) group inoculated with virus and followed by *E. coli* inoculation three days later. The experimental setup is summarized on Fig.1. Experimentations were conducted in two batches for animal facilities space constraints.

Clinical examination and sampling

Throughout the experiment the birds were monitored at least once a day to evaluate the clinical signs they may display. We paid attention to any kind of pathology but especially to disorders of the respiratory system (head swelling, nasal discharge, sneezing, tracheal rales, coughing, and difficult breathing). A scoring system was used to evaluate the severity of clinical signs. Each clinical sign was scored by the following scale: 0, no sign; 1, mild or slight; 2, moderate; 3, severe. The mean clinical score was based on the sum of clinical scores for each sign divided

by the number of birds in each group at each observation time as previously described (Jirjis *et al.*, 2004).

Macroscopic and microscopic lesions examination

Birds were euthanized using an intracephalic injection of' pentobarbital sodium (Anpro Pharmaceutical, Arcadia, CA) at 4 and 14 days post-infection (dpi). Necropsy was performed immediately after the birds were euthanized. The presence of pathologic lesions was examined. The lesions of the trachea were scored as follows: 0, no redness of mucosal membrane and no mucus; 1, reddish mucosal membrane, congestion, and small amount of mucus; 2, intensive redness, congestion of mucosal membrane, and significant amount of mucus. The lesions of the lungs were scored as follows: 0, no lesions; 1, mild congestion and no fibrinous exudate; 2, moderate congestion and small amount of fibrinous exudate; 3, severe congestion and significant amount of fibrinous exudate. Lesions of air sacs were scored grossly for severity. Briefly, all air sacs were scored together, all on a scale of 0 to 3: 0, no lesions; l, cloudy air sac walls; 2, thickened air sac walls and small amounts of serofibrinous exudates; and 3, thickened air sac walls and meaty in consistency, with large accumulation of fibrinous exudates. The sum of scores in one group was used for statistical comparison of the severity of the lesions between the groups. Trachea, lungs and air sacs samples were taken from each necropsied bird and fixed in 10% formalin for the determination of microscopic lesions. After fixation, tissues were routinely processed in paraffin blocks, sectioned at 3 µm and stained with haematoxylin and eosin for microscopic examination. Lesions were assessed histologically and graded as follwos: (-) no lesion, (+) light, (++) moderate or (+++) marked lesions as described previously (Corrand *et al.*, 2012).

Immunostaining was performed on paraffin-embedded sections of trachea with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody (Argene, 11-030, pronase 0,05% retrieval solution, 10 min at 37 °C: antibody dilution 1/50, incubation overnight, at 4°C). The immunohistochemical staining was revealed with a biotinylated polyclonal goat anti-mouse Immuno-globulin conjugated with horseradish peroxidase (HRP) (Dako, LSAB2 system-HRP,

K0675) and the diaminobenzidine chromagen of the HRP (Thermo Scientific, TA-125-HDX). Negative controls comprised sections incubated either without specific primary antibody or with another monoclonal antibody of the same isotype (IgG2). Histopathological analyses were carried out by two veterinary pathologists certified by the European College of Veterinary Pathologists.

Determination of Virus Shedding

Buccal (B) swabs were collected daily from 1 to 7 days post virus inoculation to assess virus shedding in 1 mL of PBS containing 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Invitrogen) and kept at -80°C until further used. RNA was extracted using the QIAamp viral RNA (Qiagen, Valencia, CA) and Macherey-Nagel viral RNA isolation kits (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturers. The virus titer of each sample was determined by using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) targeting the influenza virus M gene (M52C 5'CTTCTAACCGAGGTCGAAAG 3' and M253R 5'AGGGCATTTTGGACAAAKCGTCTA 3') using QuantiTect® SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany). A 250 bp fragment of M gene was amplified (Fouchier *et al.,* 2000). The quantitative RT-PCR reactions were performed on a *Light Cycler*®480, Real-Time PCR system (Roche diagnostics, Germany) with the following program: 50°C for 30 min, 95°C for 15 min followed by 40 cycles of 94°C for 10s and 60°C for 40s. M gene copy number was calculated using a standard curve as described previous (Ducatez *et al.*, 2016). The virus titer was represented by the mean ±SEM (Standard error of the mean) of the virus titer per mL of sample ($\log_{10}$ cDNA copies/ml).

Determination of bacteria shedding

Bucal swabs were collected in PBS 1 to 7 days post-infection for the determination of bacterial shedding. Swabs were vortexed and tenfold serial dilutions were inoculated in duplicate Meckonkys agar. After 24-48h of incubation at 37°C, viable counts were performed. Bacterial titres were expressed as $\log_{10}$ colonies forming units (CFU)/ml PBS solution.

Serology

For detection of AIV antibodies, blood samples were taken at day 0 and day 14 post virus challenge for haemaglutination inhibition (HI) assays. The serological response to the influenza virus was assayed with the same virus batch as for challenge with the haemagglutination inhibition test using 8 haemagglutinating units. Sera were treated with receptor destroying enzyme (RDE) to remove non specific inhibitors as described previously (WHO, 2014). 1% chicken red blood cells suspension (v/v in PBS) was used. Serum titers of 1:10 or lower were considered negative for antibodies against AIV.

Statistical Analysis

Data was expressed as means ±SEM (standard error of the mean) and analyzed using the GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). One way analysis of variance (ANOVA) including Bonferroni correction was performed to assess putative differences in virus shedding in oropharyngeal swabs, and in clinical signs and lesions at different time points in different groups. Differences were considered statistically significant when *p < 0.05*.

Results

Clinical signs

Birds in the negative control group remained healthy and active throughout the experiment period and did not show any clinical sign. The turkey poults remained active and became alert upon clapping and tapping the walls of shed. Mild clinical signs (ruffled feathers, hunched posture and depression) were shown by the birds affected with colibacillosis. Infection with AIV H6N1 alone caused clinical disease with various respiratory signs including swollen infraorbital sinuses, snicking, open mouth breathing and frothy discharge in the mouth cavity. There was gradual loss of condition and dullness. The H6N1 and *E. coli* (O78) dual infection resulted in a higher morbidity and mortality than single infections with either agent (Fig. 2 and 3). Survival rates were 52%, 58% and 78% in the H6N1/*E.coli*, *E.coli*+H6N1 and *E.coli*/H6N1 groups respectively (Fig.3). The clinical symptoms in themselves were similar in nature to those caused by H6N1 alone, but they were more severe and persisted markedly longer. From 4 dpi

onwards, turkeys in the *E.coli*/H6N1 group showed slightly higher respiratory clinical scores in comparison with *E.coli*+H6N1 and H6N1/*E.coli* infected birds. However, general sickness and head swelling was more pronounced in turkeys of the H6N1/*E.coli* group than in turkeys of the *E.coli*/H6N1 and *E.coli*+H6N1 groups. The clinical signs were more severe in co-infected turkeys indicating a synergistic or additive effect between the pathogens.

Macroscopic and Microscopic lesions

No bird in the negative control group demonstrated body cavity lesions and all organs were normal in size, shape and consistency. Tracheas were lined with pseudostratified ciliated columnar respiratory epithelium with few goblet cells and varying numbers of mucoid glands. The lamina propria was one to three cells wide and no infiltrates were present. A few single granulocytes per section were present. Plasma cells were present in low numbers varying from 5 to 30 plasma cells per section. The capillary area of parabronchi, infundibula and atria appeared open and aerated. The atrial septa consisted of a thin stroma lined with flat epithelium. In the capillary area predominantly erythrocytes, air capillary epithelium and endothelium were recognized; a few mononuclear cells were also present, mostly fibrocytes and macrophages. The wall of the cranial thoracic air sacs consisted of a thin layer of stroma covered with serosa of one cell thickness. On the respiratory side areas covered with columnar ciliated epithelium of the respiratory tract alternated with areas covered with flat epithelium. No oedema, infiltrates and follicles were present (supplementary Fig.1 and 2).

Macroscopically, the pericardium, epicardium, and hepatic peritoneal sac were markedly thickened with caseous exudates in *E. coli* infected turkeys. Scoring of macroscopic lesions is summarized in Fig.4. Mild hyperemia or congestion of lungs was observed. The air sac, peritoneum, mesentery, and gastrointestinal serosa had similar changes. In severely affected turkeys, there were adhesions between the pericardium and epicardium. Adhesions between the hepatic peritoneal sacs and the hepatic capsule were seen occasionally but those separated easily. The liver and spleen were moderately enlarged. The hock joint of one bird was swollen, and filled with yellowish exudates. At day 14 post infection, macroscopic lesions were milder

in severity than 4 dpi. In the trachea, slight hyperplasia of the epithelial cells and glandular cells with heterophilic and lymphocytic infiltrations in the lamina propria were noted. Epithelial cells of the air sac were hyperplastic, degenerate, and necrotic-resulting in erosion with fibrinous exudates. In the subepithelial tissue of the air sac, there was infiltration of heterophils, lymphocytes, and plasma cells with macrophages and fibroblasts. Loss of epithelial cells with fibrinous exudate and infiltration of heterophils, lymphocytes and plasma cells with macrophages and fibroblasts in subepithelial tissue. Lungs hardly showed any lesion except mild congestion and few localized granulomatous foci. Moreover, minimal or no lesions were observed at 14 dpi.

On the other hand, in turkeys inoculated with influenza virus, lesions were confined to the lung and air sacs: diffuse and opacification (mild to moderate) of air sacs was reported in birds 4dpi and only a few birds showed slight airsacculitis at 14 dpi. Microscopic lesions consisted of proteinaceous fluid, fibrin, heterophils, macrophages and sloughed epithelial cells within parabronchi, atria, and infundibula. The main specific lesion was a focal to diffuse fibrino-necrotic exudate covering the trachea, primary bronchi and intra-lung airways. These lesions were observed in all turkeys but with different severity levels. Spleen enlargement and congestion was also observed. The main histopathological lesions were observed in the trachea, bronchi, lung and air sacs (Table 1). These lesions consisted of a marked loss of cilia, hyperplasia of tracheal epithelium and infiltration of monocytes. A moderate exudative fibrinoleucocytic airsacculitis was also present. Atrial and infundibular epithelial cells were swollen, cuboidal in appearance and hyperplastic. There was an intense infiltration of the atrial and infundibular interstitium, surrounding air capillary beds, and the lamina propria of adjacent secondary bronchi with lymphocytes, macrophages, heterophils and plasma cells with many blood vessels cuffed with lymphocytes. By 14 dpi, the epithelium had returned to normal and small well-developed lymphoid nodules were present in bronchial lamina propria and the interstitium of the atria and infundibula. Similar gross and histopathological lesions were

observed for co-infected turkeys, however, the intensity of lesions were more severe in turkeys of these groups.

In negative control turkeys, influenza nucleoprotein was not detected in air sacs, trachea or lungs. In influenza virus-inoculated poults at 4 dpi, influenza A nucleoprotein was occasionally to commonly detected within areas of pneumonia in most poults. The majority of positive cells were hypertrophied atrial and infundibular epithelial cells or mononuclear cells which morphologically resembled macrophages. In trachea and bronchi, a few respiratory epithelial cells had staining for nucleoprotein. In the air sacs, a few ciliated columnar and non-ciliated hypertrophied (cuboidal) epithelial cells had staining for nucleoprotein. On 6 dpi, nucleoprotein was detected in a similar number of poults and had a similar tissue distribution as seen on 4 dpi. However, the total number of positive cells and intensity of staining was decreased. At 14 dpi, influenza nucleoprotein was not detected in air sacs, tracheas or lungs. Results for immunohistochemical detection of influenza A nucleoprotein are reported in supplementary Table 1.

Determination of virus shedding

Efficient virus replication was observed in all virus inoculated groups. However, virus shedding pattern in the buccal swabs of influenza virus inoculated turkeys did not significantly differ between groups ($p > 0.05$) except in turkeys in H6N1/*E.coli* group who showed significantly higher virus shedding than other group turkeys ($p < 0.05$) at day 6 post-inoculation (Fig. 5A & 5B).

Determination of bacterial Shedding

E. coli (O78) was not recovered from bucal swabs from any of the birds in the negative control group. The results of *E. coli* (O78) titrations of the bucal swabs of the remaining groups are shown in Figure 5C. Mean titres ($\log_{10}$ CFU/ml) for each experimental group are depicted. Bacteria shedding was slightly higher in co-infected groups however this difference was non-significant ($p > 0.05$).

Serology

Serum samples collected pre-inoculation were all negative for avian influenza antibodies (HI titers <10). Moreover, serum samples collected at 14 dpi showed seroconversion against H6N1 in all virus infected birds (320 ≤ HI titers ≤ 1280).

Discussion

Respiratory diseases are matter of great concern worldwide and these are often associated with confections (Watanabe *et al.*, 1977; Sakuma *et al.*, 1981; Yashpal *et al.*, 2004) resulting in poor animal welfare, economic losses, and increased antibiotics consumption. It is well appreciated that upper respiratory tract viral infections in poultry are often complicated by more serious bacterial diseases. It is thought that certain pairings of organisms better complement each other than other potential pairings. Co-infections of poultry present a complicated clinical picture confusing the identification and diagnosis and unfortunately little is known on the interactions between co-infecting pathogens (Costa-Hurtado *et al.*, 2014). Influenza virus is commonly thought of in this context along with other respiratory viruses and bacteria.

Natural infections with AIV are more severe than experimental infections, suggesting that secondary agents or other factors play prominent role in the clinical disease process. Natural AIV/bacterial problems are expected to occur at the same time and have been reported in poultry (Pan *et al.*, 2012; Pu *et al.*, 2012), but the effects of such combinations on the health status of poultry is not well known. In the field, co-infections of AIV do occur with other respiratory pathogens but are not easily detected, and the impact of co-infections on pathobiology is unknown. The mixed AIV infections may provide increased virulence, posing a substantial risk to poultry and public health. The coinfection of *E. coli* and influenza viruses in poultry has been observed in field conditions. During the last decade, the outbreaks of H9N2 influenza virus with severe clinical signs, high mortality (20-65%) and low production (up to 75%) have been reported in commercial poultry farms (Nili & Asasi, 2002; Bano *et al.*, 2003; Nili & Asasi, 2003; Swayne, 2008). Similarly, an outbreak of H9N2 influenza virus infection in chickens in Hong Kong (A/chicken/Hong Kong/739/94) was associated with coughing and respiratory distress in 75% of the birds, and with 10% mortality. Treatment with antibiotics

reduced the mortality rate, suggesting that bacteria may play a role in the exhibition of the clinical signs (Kishida *et al.*, 2004). However, H9N2 viruses in domestic poultry cause mild clinical signs and respiratory diseases with low mortality (less than 5%). It is proposed that concurrent infection may play a key role in exacerbating mortality in chicken infected with H9N2 influenza virus (Haghighat-Jahromi *et al.*, 2008; Pan *et al.*, 2012; Seifi *et al.*, 2012). It was speculated that severe clinical signs linked to AIV H9N2 infections in the field were, probably due to *E. coli* involvement (Bano *et al.*, 2003). Similarly, these lesions were commonly reported in turkeys during the 1999 outbreak of H7N1 AIV in Italy in association with secondary bacterial pathogens such as *E. coli*, *Riemerella anatipestifer* and *Pasteurella multocida* (Capua & Marangon, 2000).

In the present study, we tried to come as close as possible to field conditions in our experiments by choosing commercial turkeys and using aerosol route of inoculation. However, it is still difficult to reproduce field conditions in laboratory settings because many other factors contribute in the production of diseases such as dust, pollution, humidity, temperature, ammonia production, housing stress etc.

Clinical signs and lesions were significantly more severe and persisted longer in co-infected turkeys than in birds infected with a single pathogen (p<0.05). Respiratory distress was slightly higher in turkeys inoculated with *E.coli* first and followed by H6N1 while co-infected turkeys showed more head swelling when H6N1 was inoculated first followed by inoculation of *E.coli*. However, this difference was found non significant (p>0.05). Similarly, mortality was significantly higher in co-infected groups. AIV H6N1 has a unique ability to form purulent exudate in the lower part of the respiratory tract which latter becomes thick and blocks respiratory passage leading to respiratory distress and ultimately death. There was no significant impact of *E.coli* and H6N1 inoculation sequence in co-infected groups on disease outcome. All inoculated turkeys in co-infected groups showed more or less similar clinical picture. Histopathological analysis supported clinical and gross findings. However, virus and bacteria shedding did not differ much between birds groups. Two hypotheses may explain this pattern.

First, the aerosol route of inoculation may have deposited pathogens in the lower parts of the respiratory tract in extensive amounts (parabronchi, air sacs) and may thus have led to excessive multiplication of pathogens along with purulent exudates in the lower rather than upper part of respiratory system. Purulent exudate could not be expectorated to the buccal cavity due to blockage of air passages with mucous plugs. We determined pathogens shedding in buccal swabs that actually reflect the pathogen shedding in the upper and not lower respiratory system. Secondly, we can assume that the severity of lesions may also be attributed to the innate immune response.

Our experimental AIV H6N1 caused lesions are in agreement with field outbreak (Corrand *et al.*, 2012). Inflammation and deciliation of the trachea, degeneration of the mucous gland cells and damage to the respiratory epithelium were likewise previously demonstrated (Corrand *et al.*, 2012). We recently reviewed the literature on LPAIV and co-infecting pathogens in experimental conditions and our current results are also in agreement with the general finding of LPAIV + co-infecting bacteria studies: a more severe disease outcome (Umar *et al.*, 2016).

In conclusion, it was shown that the *E. coli* strain (O78) and H6N1 alone are able to produce mild respiratory infection through adhesion and colonization of the respiratory tract, but without each other help do not induce severe respiratory disease and mortality in suspected turkeys. The results obtained in the present study clearly indicate the occurrence of marked synergistic or additive effects between two distinct respiratory pathogens important in poultry. The established *E. coli* (O78) single and AIV/*E. coli* dual infection models can be used to further investigate the mechanism of *E. coli* colonization and the AIV/*E. coli* synergy. Further, the AIV/*E. coli* dual infection model may be used to test preventive and curative measures to combat the respiratory disease. With consideration of the significant spread of *E. coli* infections as well as LPAIV in poultry, a surveillance of LPAI infection and regular diagnosis of *E. coli* infection and anti *E. coli* treatment of flocks may help to prevent development of severe clinical disease and economic losses due to such co-infections. Continuous surveillance of AI infection

and co-infections studies in experimental poultry models is warranted to find new strategies to control their circulation in domestic and wild poultry. The timing of co-infection would also require further systematic experimental studies to understand the role of prior/post/simultaneous inoculation in disease outcome, pathogenesis and virus shedding pattern. The present study contributes to the unravelling of the multi-factorial respiratory disease complex in turkeys, illustrating that the outcome of AIV infection in acutely *E. coli* predisposed turkeys is aggravated, compared to single AIV infected turkeys. Clarifying the interaction mechanisms between the different pathogens will allow a more precise diagnosis and a better treatment, reducing not only economical complications of respiratory diseases, but also the zoonotic risk.

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Table 1: Summary of microscopic lesions and their intensity

Tissue	Elementary lesion	Lesion intensity					
		Negative control	*E.coli* **alone**	**H6N1 alone**	**E.coli+H6N1**	**H6N1/***E.coli*	*E.coli***/H6N1**
Trachea primary and secondary bronchi	Tracheitis and bronchitis with: - necrosis and exfoliation of the superficial mucosal epithelium	-	-+	+	++	++	+
	- regenerative epithelial hyperplasia	-	-	+	++	++	++
	- squamous epithelial metaplasia	-	-	+	++	+++	++
	- inflammatory cellular infiltrates in the lamina propria: ➔ Heterophils ➔ mononuclear cells: lymphocytes, macrophages	-	-+	+	++	++	++
	- accumulation of fibrinoleucocytic exudate in the lumina	-	-	+	+++	+++	++
Pulmonary parenchyma (from parabronchi to respiratory air capillaries)	Focal extensive inflammatory lesions from injured bronchi with:	-	-	+	++	++	++
	accumulation of necrotic cells and fibrinous material	-	-	+	++	++	+
	oedema	-		+	+++	++	+
	inflammatory cellular	-	-	+	++	++	++

	infiltrates in parabronchi and atria: → Heterophils → mononuclear cells: lymphocytes, macrophages						
Air sacs	Airsacculitis with: - congestion and oedema	-	+	+	++	++	++
→	- accumulation of fibrinous material or fibrinoleucocytic exudate	-	++	+	+++	++	++
	- inflammatory cellular infiltrates: → Heterophils mononuclear cells: lymphocytes, macrophages	-	+	+	++	+++	+
	- presence of bacteria	-	+	+	++	++	+

- = no lesions, + = slight or mild lesions, ++ = moderate lesions, +++= marked or severe lesions

Table 1 (supplementary): Average distribution of Nucleoprotein antigen of AIV in different respiratory tissues of infected turkeys.

Group	Tissue	4 dpi	14 dpi	Predominant cell types	Associated lesions
H6N1 alone	Trachea	++	+-	Respiratory epithelium	Subacute trachitis (focal, moderated to marked heterophils accumulation, epithelial hyperplasia
	Lungs	++	+-	Respiratory epithelium, Goblet cells	Severe, subacute bronchitis (edema, loss of epithelium, exudates, infiltration of heterophils)
E.coli+H6N1	Trachea	++	+	Respiratory epithelium, Goblet cells	Focal slight subacute trachitis
	Lungs	+++	+	Respiratory epithelium	Severe subacute bronchitis, mono nuclear cells, heterophils, edema exudates,(fibrinomucotic with edema)
E.coli/H6N1	Trachea	+++	+	Respiratory epithelium, Goblet cells	Acute trachitis with minimal intensity, loss of cilia for some cells, a few heterophils
	Lungs	+++	++	Respiratory epithelium	Marked acute bronchitis,fibrinonecrotic exudates in lumen, edema, heterophils, mononuclear cells
H6N1/*E.coli*	Trachea	++	+	Respiratory epithelium, Goblet cells	Acute trachitis with minimal intensity, loss of cilia for some cells, a few heterophils
	Lungs	++	++	Respiratory epithelium	Moderate subacute bronchitis,fibrinonecrotic exudates in lumen, edema, heterophils, mononuclear cells

- = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity. dpi, days post-inoculation

Figure legends:

Fig 1: Experimental setup of the coinfection study

Turkey groups: Negative Control: PBS inoculation only. *E.coli* alone: *E.coli* inoculation only. H6N1 alone: H6N1 virus inoculation only. *E.coli*+H6N1: Simultaneous inoculation of *E.coli* and H6N1. *E.coli/*H6N1: *E.coli* inoculation followed by H6N1 virus inoculation. H6N1/*E.coli*: H6N1 inoculation followed by *E.coli* inoculation. *Blood samples collection for serology
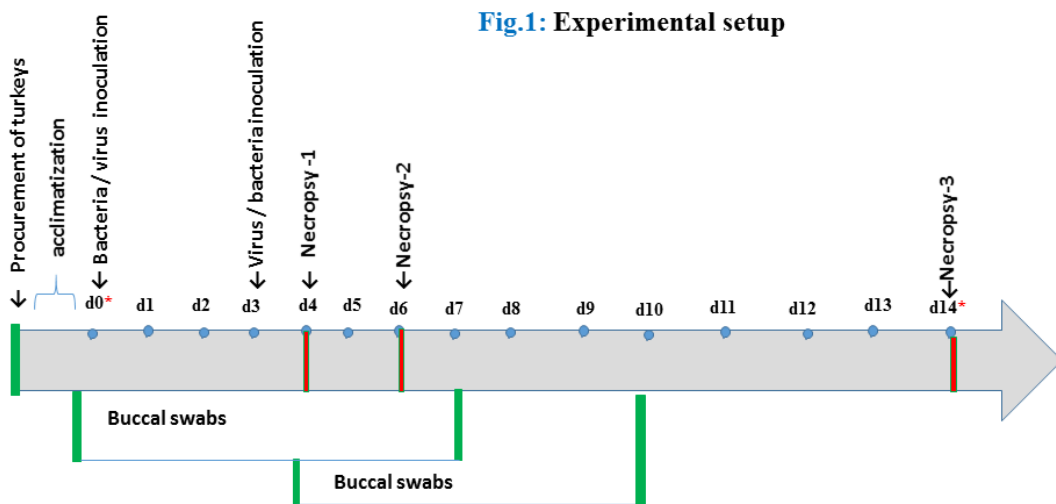

Fig.1: Experimental setup

Fig 2: Clinical scores in groups of turkeys after inoculation with avian influenza virus (H6N1) or *E. coli* (O78) or a combination of both. Each respiratory clinical sign was scored by the following scale: 0, no sign; 1, mild or slight; 2, moderate; 3, severe. Color code: black line and symbols: negative control (non infected) group; red line and symbols: *E.coli* alone group; green line and symbols: H6N1 alone group; purple line and symbols: *E.coli*+H6N1 group; blue line and symbols: *E.coli*/H6N1 group; orange line and symbols: H6N1/*E.coli* group.
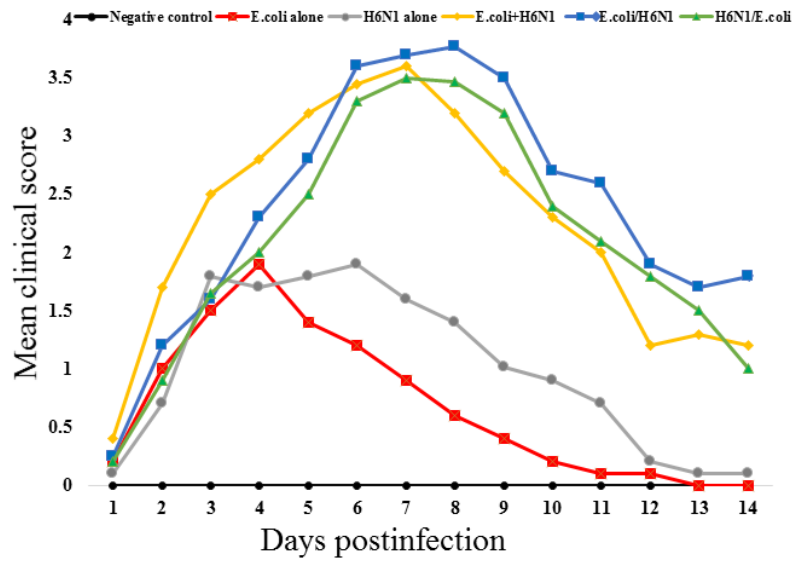
Fig 3: Mortality (%) in different groups of turkeys after inoculation with avian influenza virus (H6N1) or *E. coli* (O78) or a combination of both. Color code: black line and symbols: negative control (non infected) group; red line and symbols: *E.coli* alone group; green line and symbols: H6N1 alone group; purple line and symbols: *E.coli*+H6N1 group; blue line and symbols: *E.coli*/H6N1 group; orange line and symbols: H6N1/*E.coli* group.
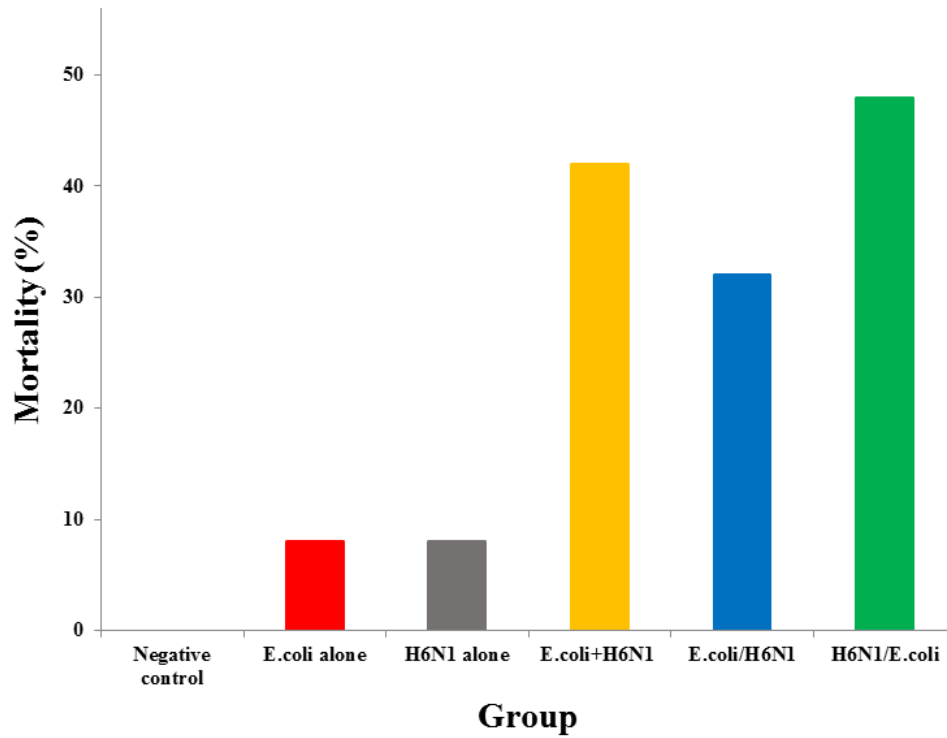
Fig 4: Scores of macroscopic lesions in trachea, lungs and airsacs of turkeys challenged with *E. coli* (O78) and AIV H6N1 singly or in combination. Color code: black line and symbols: negative control (non infected) group; red line and symbols: *E.coli* alone group; green line and symbols: H6N1 alone group; purple line and symbols: *E.coli*+H6N1 group; blue line and symbols: *E.coli*/H6N1 group; orange line and symbols: H6N1/*E.coli* group.
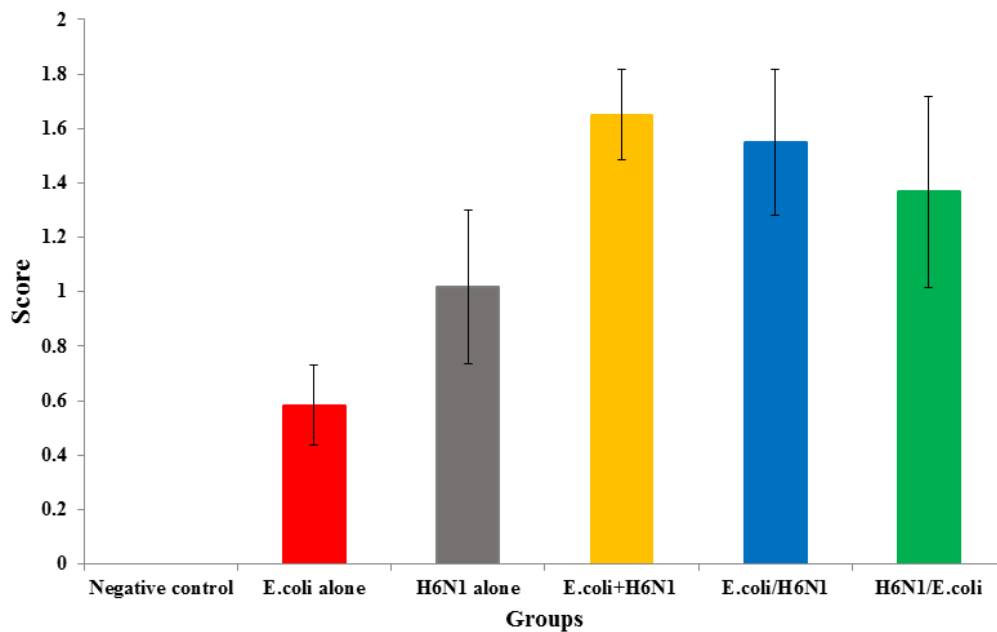
Fig.5: Virus and bacteria shedding pattern in buccal swabs in coinfection study (A) Virus shedding in co-infection study 1 showing non significant virus shedding pattern (p>0.05); (B) Virus shedding in co-infection study 2 showing non significant virus shedding pattern except in H6N1 then *E.coli* group at 6 dpi (p<0.05); (C) *E.coli* (O78) titres (log_{10} CFU/ml) in buccal swabs collected at different time points after *E.coli* (O78) inoculation. Different groups showing non significant bacteria shedding (p>0.05). Color code: black line and symbols: negative control (non infected) group; red line and symbols: *E.coli* alone group; grey line and symbols: H6N1 alone group; orange line and symbols: *E.coli*+H6N1 group; blue line and symbols: *E.coli*/H6N1 group; green line and symbols: H6N1/*E.coli* group.
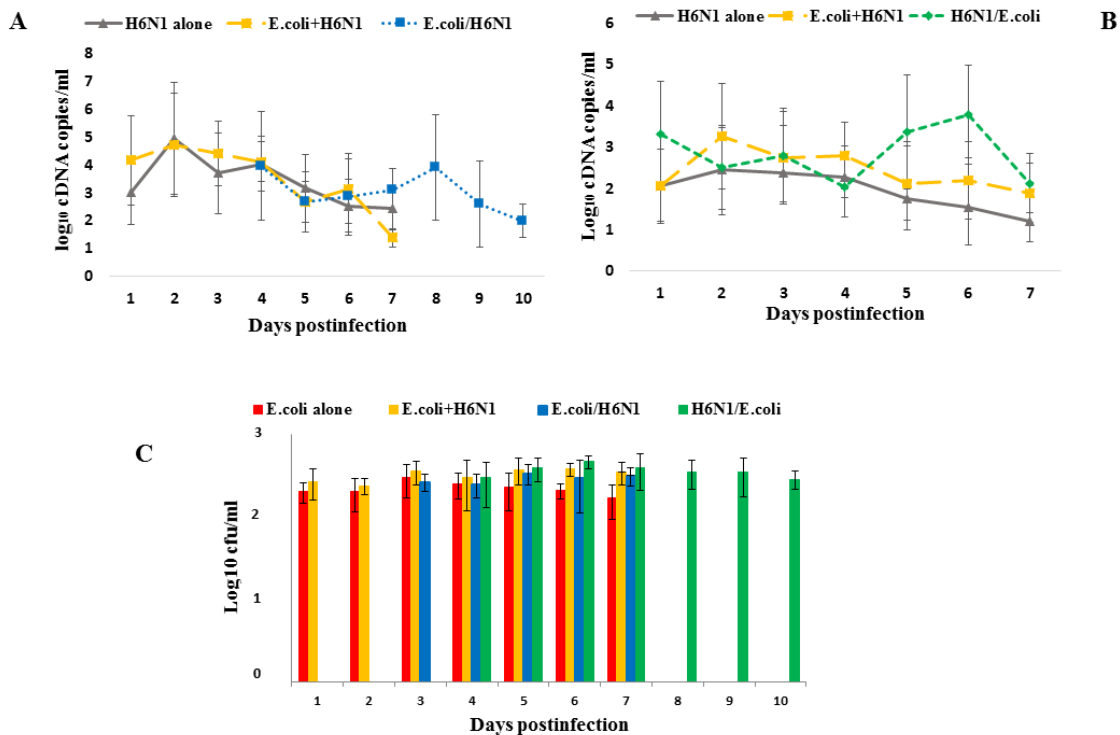

Supplementary Figure legends

Suppl. Fig. 1: Macroscopic pathologic lesions of experimentally infected turkeys. Walls of abdominal airsacs are thin, transparent and clear and there is no accumulation of exudates while airs sacs in infected turkeys are thick, cloudy and showing presence of fibrinous exudates (arrow). Similarly, lungs of negative control turkeys showing normal appearance without

exudates and pneumonia while lungs of virus infected turkeys showing edema, pneumonia and presence of thick mucus plug in bronchi (arrow).
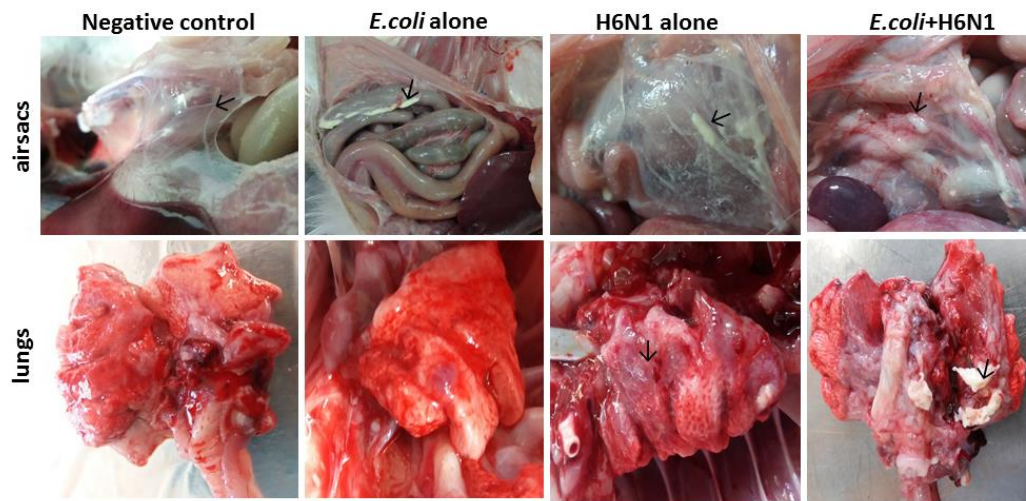
Suppl. Fig. 2: Trachea, lungs and air sacs of turkeys in single or co-infected groups at 4 dpi (Haematoxylin and eosin stain, bar = 50 µm). Normal tracheal mucosa with pseudostratified columnar epithelium bearing cilia (arrow), lamina propria of only few cells thickness and mucous glands while trachea in infected groups showing epithelial cells with irregular morphology, loss of cilia, sloughed off respiratory epithelium and in the lamina propria inflammatory cellular infiltrate of several tens of cells in thickness is present (arrow). Lung tissue sampled at 4 dpi in negative control turkeys showed normal appearance (Haematoxylin and eosin stain, bar = 50 µm) with open and aerated parabronchi, atria and infundibulum (arrow) while infected lungs showing pneumonia with heavy infiltration of inflammatory cells in the lamina propria of bronchi along with edema and presence of fibrinous exudates. Air sac sampled at 4 dpi in negative control turkeys showing normal histological structure of air sacs (Haematoxylin and eosin stain, bar = 50 µm) with columnar respiratory epithelium bearing cilia and serosa of one cell thickness while air sacs in infected groups showing thick infiltrated air sacs with edema and presence of fibrinous exudates (arrow).
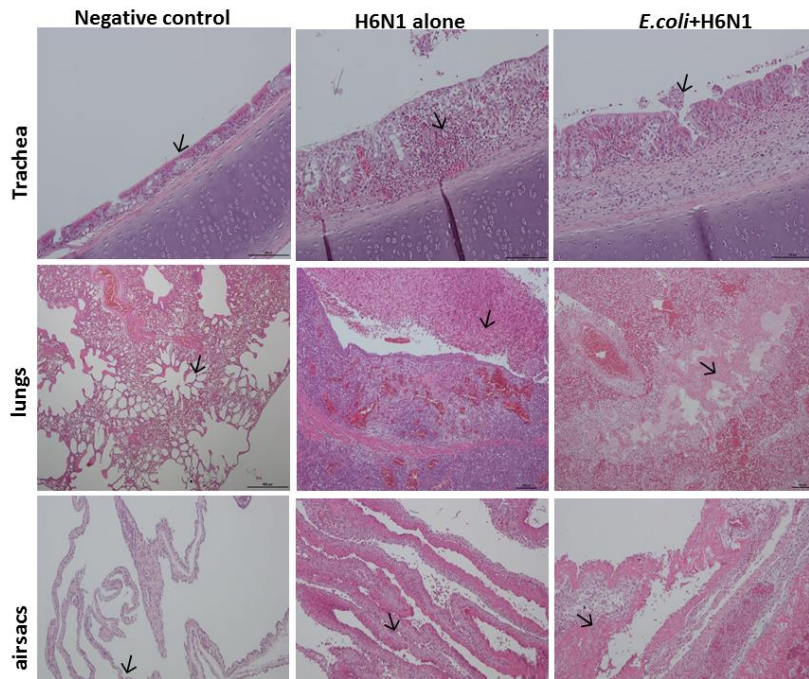
Suppl. Fig. 3: Distribution of nucleoprotein antigen in positive tissues of a turkey co-infected with *E.coli* and H6N1 simultaneously (4 dpi). (A) Positive staining in nucleus and cytoplasm of epithelial cells in trachea; (B) Positive staining in nucleus and cytoplasm of epithelial cells in lungs (IHC).
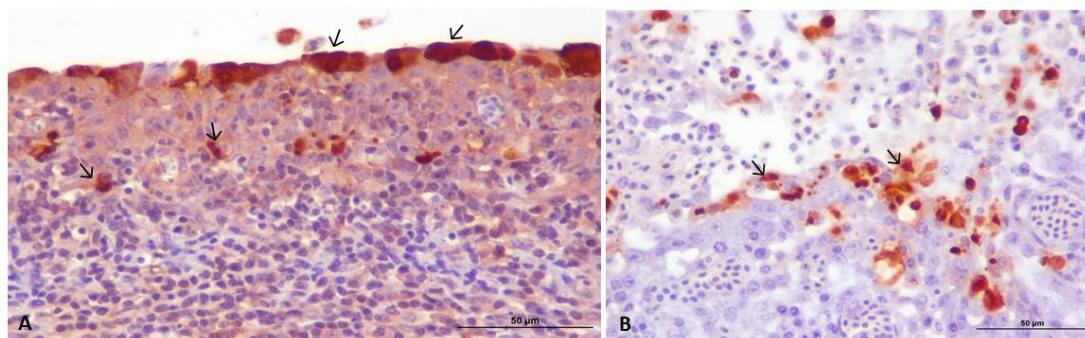
Molecular epidemiology of respiratory viruses in commercial chicken flocks in Pakistan from 2014 through 2016

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Short title: Chicken respiratory viruses in Pakistan

Abstract

Background: Viral diseases are a huge concern for poultry farmers in Pakistan. Multiple common viral respiratory diseases (CVRDs) cause huge economic loss in poultry industry. Incidence of CVRDs is not clear in many countries including Pakistan.

Results: Incidences of 5 chicken respiratory viruses: avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), avian metapneumovirus (aMPV) and infectious laryngotracheitis virus (ILTV) was assessed in Pakistanis commercial farms with respiratory problems from 2014 through 2016. While AIV and NDV were very frequently detected (in 16 to 17% of the farms), IBV and aMPV were rarer (detected in 3 to 5% of the farms) and ILTV was not detected. We characterized H9 viruses of the G1 lineage, genotype VII NDV, GI-13 IBV, and type B aMPV strains with very little genetic variability in the 2 years study period. Co-infections with AIV and NDV were common and NDV was detected despite the use of vaccines: control measures to limit the virus burden in chicken flocks are discussed.

Conclusions: Our data showed that the above mentioned respiratory viruses were the most important causes of respiratory disease in commercial poultry in Pakistan. The predominance of H9 infection indicates a need for continuous monitoring of AIV among avian species and the awareness against public health risk**.** Further studies are necessary to assess circulating strains, economic losses caused by infections and coinfections of these pathogens, and the costs and benefits of countermeasures. Furthermore, farmers need to be educated about the use of vaccines against these pathogens.

Key words: Chicken respiratory viruses, molecular epidemiology, chicken, Pakistan, avian influenza virus, infectious bronchitis virus, Newcastle disease virus, avian metapneumovirus

List of Abbreviations: aMPV=avian metapneumovirus; AIV=Avian influenza virus; CVRD=common viral respiratory diseases; DNA=deoxyribonucleic acid; dNTPs=

deoxynucleoside triphosphate; HA= Haemagglutination; IB= infectious bronchitis;

IBV=infectious bronchitis virus; ILTV= infectious laryngotracheitis virus; ND= NewCastle

disease; NDV= NewCastle disease virus; OP= oropharyngeal; PBS=phosphate buffer saline;

PCR=Polymerase Chain Reaction; RNA=ribonucleic acid; SHS=swollen head syndrome

Background

Commercial poultry in Pakistan was established in 1963 representing one of the largest agro based segment of Pakistan economy having an investment of more than 7 billion US dollars. It contributes about 1.3 percent to national GDP (Gross Domestic Product), and its share in agriculture and livestock is 6.1% and 10.8%, respectively. Furthermore, about 28% of total meat produced in the country is poultry [1]. According to a survey from the Pakistan Poultry Association, there are over 25,000 commercial poultry farms of layer, broiler and breeder birds in the country with 722.39 million broilers, 39.46 million layers and 10.19 million breeders, producing 1,220 million kg of chicken meat and about 10,000 million eggs annually [2]. In the past, small scale regional studies on surveillance of poultry diseases have been conducted in relation to seasons and region, indicating the huge economic impact of viral diseases [3–5]. Consistent outbreaks of viral diseases in the field with huge morbidity and mortality have been reported recently [6–8]. Pakistan poultry industry is growing continuously, providing numerous opportunities for the spread of multiple common viral respiratory diseases (CVRDs) such as Newcastle disease (ND), infectious bronchitis (IB), swollen head syndrome (SHS), infectious laryngotracheitis (ILT) and low pathogenic avian influenza (LPAI) infections caused by Newcastle diseases virus (NDV), infectious bronchitis virus (IBV), avian metapneumovirus (aMPV), infectious laryngotracheitis virus (ILTV) and avian influenza virus (AIV), respectively [5,9–11]. These are highly contagious diseases of poultry, distributed worldwide and they have serious economic impacts on the poultry industry. The causative agents of these diseases affect chickens of all ages except ILTV, which normally does not affect chickens before three weeks of age. These pathogens interact with bacterial agents such as *Escherichia coli*, *Bordetella* and *Pasteurella*, resulting in morbidity in the infected chickens [12]. The continuous emergence of new virulent genotypes from global epidemics and the frequent changes observed in the genomic sequence of these viruses sometimes lead to ineffective

diagnostic and control measures. Outbreaks of some of CVRDs such as IB and SHS are not reported to the ministry of livestock and poultry production. Consequently, the distribution pattern of such chicken diseases is not clear in Pakistan. Moreover, some CVRDs, such as LPAI (H9N2) infections are of great significance to public health [13]. Therefore, it is important to investigate the distribution pattern of CVRDs in different regions and types of chicken flocks to develop scientific and risk based prevention measures of poultry diseases.

The aim of this study was to detect and to characterize chicken respiratory viruses in commercial poultry in Pakistan, which is the first step necessary before control measures can be implemented. Five major chicken respiratory viruses were looked for: AIV, NDV, IBV, aMPV and infectious laryngotracheitis virus (ILTV).

Methods

Samples collection

Between July 2014 and January 2016 a total of 89 commercial poultry farms (broiler and layers) from different locations in Pakistan were sampled. Sampling area was chosen on the basis of poultry population: the selected sampling area is the main Pakistan poultry production region (with more than 50% of the country's poultry farms). Oropharyngeal swab or tissue impression smear (trachea, lungs) were applied directly to Finders Technology Associates (FTA®) sampling cards (Whatman, Inc., Clifton, NJ) from birds showing respiratory signs and allowed to air dry, and kept at 4°C until further processing. Age, flock size and health status are summarized in Table 1. For each flock, 1 or 2 pools of 10 swabs were spotted on each FTA card (one sample number refers to a pool of 10 swabs from symptomatic birds in a given flock, Table 1) in order to detect a prevalence ≥0.14 to ≥0.28% for each virus per flock (for flocks of 10000 birds to 2500 birds with 95% confidence level). According to the farmer's information, some of the farms were vaccinated against Newcastle disease, infectious bursal disease and/or

infectious bronchitis but none of the farms visited vaccinated against aMPV and ILTV (Table 1).

RNA isolation

Punches from FTA cards were incubated at 4°C for 24 hours in 1 mL of Phosphate buffer saline (PBS). Viral RNA and DNA from swabs were extracted using the QIAamp® viral RNA isolation kit (Qiagen Germany) according to the instructions of manufacturer. RNA/DNA was eluted in 50μL elution buffer and stored at −80°C until further use.

Polymerase Chain Reactions (PCR)

After extracting RNA, complementary DNA was generated for the RNA viruses from 5 µL of RNA using RevertAid first strand cDNA synthesis kit (RevertAid First Strand cDNA Synthesis kit, ThermoFisher Scientific, Carlsbad, CA) following the manufacturer's protocol. Briefly, 5μL of total RNA was mixed with random hexamers as primer (0.3 µg/µL) and incubated for 5 min at 65°C. Then, 4μL of 5x reaction buffer (ThermoFisher Scientific, Carlsbad, CA), 0.5 µL of RNase Out (Life Technologies, Carlsbad, CA), 2 µL of 10 mmol/L deoxynucleoside triphosphate (dNTP) solution (Finnzymes, Espoo, Finland), and 1 µL of RevertAid reverse transcriptase (ThermoFisher Scientific, Carlsbad, CA) were added to this mixture at the same time. The reaction volume was completed to 20 µL with distilled water. The RT reaction was performed at 25°C for 10 minutes, followed by 42°C for 60 minutes and finally 70°C for 10 minutes. cDNA was used as the template for PCR amplification. The primers and PCR conditions used for the detection of NDV, IBV, aMPV (types A and B), ILTV, and AIV have been listed in Table 2. Two different Taq DNA Polymerases were used in this study (Qiagen Taq DNA polymerase, Germany and Kapa biosystems, Inc. MA). All programmed cycling was performed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, USA). PCR amplicons were analyzed in a 1.5% agarose gel (Ultrapure, Invitrogen, Merelbeke, Belgium), containing nucleic acid stain (SYBR® Safe DNA gel stain, ThermoFisher Scientific, Carlsbad,

CA) using 1×TBE as electrophoresis running buffer. Bands are compared to a commercially available 100 bp ladder (Bioline HyperLadder™ 100bp) and a positive control.

Sequencing

PCR products were purified (NucleoSpin®Gel and PCR Clean-up kit, Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Purified products were quantified with Qubit® 2.0 fluorometer (ThermoFisher Scientific, Waltham, CA). Ten ng DNA were used for sequencing in both directions with the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Bio-systems) on a capillary sequencer (model 3100 avant, Applied Bio-systems) using the PCR primers as sequencing primers. In case of nucleotide ambiguity, sequencing was repeated. Sequences generated in the present study were submitted to the EMBL/GeneBank database under the accession numbers LT599493 to LT599497.

Data analysis and phylogeny

Assembly and analysis of sequence data were conducted using the BioEdit Software version 5.0.9 [14]. This program was also used to read the sequencing electrophoregrams and to exclude nucleotide ambiguity. To ensure the reliability of sequences, forward and reverse sequences were aligned with ClustalW [15]. Phylogenetic analysis and tree construction were generated using the maximum likelihood method with 500 bootstrap replicates with MEGA software Version 5.05 program [16], and bootstrap values above 50 were labelled on major tree branches for reference. The nucleotide sequences of partial segment of the S1, G, HA, and F genes of IBV, aMPV, AIV, and NDV, respectively were compared with the first 10 blast hits sequences and reference sequences of the same genes from GenBank. Recent classifications for NDV and IBV genotypes were used [10,17].

Results

Virus prevalence and co-infections in Pakistanis farms

Samples were screened for viral respiratory pathogens nucleic acids in Pakistan within the commercial broiler and layer hen populations. We found that the viral respiratory diseases are common even though vaccines are being used on short scale in the country. In total, 161 pools samples from 89 flocks were collected during 2014-2016 from different areas of Pakistan. Among these flocks, 15 flocks (16.8%) were positive for AIV (H9), 14 flocks for NDV (15.7%), 4 flocks with IBV (4.4%), and two flocks with aMPV (2.2%). No ILTV positive sample was detected in our study. A map of Pakistan with collection sites and prevalence for the different chicken viruses tested is presented on Figure 1. Co-infections were common for AIV and NDV (9 AIV/NDV co-infected flocks) but less common for the other tested chicken viruses (Table 1). Briefly, 9 flocks (6,21,23,38,41,42,66,67,68) were found positive for AIV (H9) and NDV coinfections and NDV+aMPV, AIV+aMPV and NDV+IBV coinfections were detected only in flock number 69, 70 and 80 respectively.

Molecular epidemiology of avian influenza virus in Pakistan

Twenty-two of the 161 samples collected were AIV positive by PCR. Partial HA sequences were obtained for 19 Pakistanis strains; they were all identical but one and all clustered with H9 influenza viruses. A/chicken/Pakistan/17/2014 and A/chicken/Pakistan/74/2015 were selected as representative sequences of 2014-2016 Pakistanis samples. They clustered with G1-like viruses and were closely related to strains from Libya, Tunisia, Saudi Arabia, and Pakistan collected from 2005 through 2015 (Fig 2).

Molecular epidemiology of Newcastle disease virus in Pakistan

Nineteen of the 161 samples collected were NDV positive by PCR. A total of 9 partial F gene sequences were phylogenetically compared with representatives of the 18 known genotypes of APMV-1 circulating worldwide. Phylogenetic analysis was based on the full F gene sequence for reference viruses and a 280 nucleotide (nt) sequence of the Pakistanis APMV-1 F gene (nt 267-545) obtained with primers FIP1 and FIP2 (Table 2). The 9 Pakistani NDV sequences were

identical and NDV/chicken/Pakistan/11/2014 was thus included in the analysis as a representative sequence. Deduced amino acid sequences of the F protein cleavage site of Pakistanis NDV strains, GRRQKR*F (aa 111-117), was indicative of a high virulence for these viruses. In the phylogenetic tree, NDV/chicken/Pakistan/11/2014 clearly clustered with sequences of genotype VII viruses (supported by a 100 bootstrap value, Fig 3).

Molecular epidemiology of infectious bronchitis virus in Pakistan

Eight of the 161 samples collected were IBV positive by PCR. A total of 8 partial S1 gene sequences were phylogenetically compared with representatives of the 32 distinct viral lineages known genotypes of IBV circulating worldwide. Phylogenetic analysis was based on the full S1 gene sequence for reference viruses and a 700 nucleotide (nt) sequence of the Pakistanis IBV S1 gene (nt 1-700) obtained with primers CK2 and S15 (Table 2). As our 8 Pakistanis IBV sequences were identical, we selected γCoV/chicken/Pakistan/142/2015 as a representative strain. It clustered with genotype 1 lineage 13 viruses, previously called 793/B or 4/91 genotype, and this grouping was supported by a very high bootstrap value (100) (Fig 4).

Molecular epidemiology of avian metapneumovirus in Pakistan

Four of the 161 samples collected were aMPV positive by PCR. A total of 4 partial G gene sequences were obtained, all identical, and aMPV/chicken/Pakistan/107/2015 (representative Pakistani sequence) was phylogenetically compared with representatives of the 4 known genotypes of aMPV (A to D). It clustered with aMPV type B viruses (Fig 5).

Discussion

Viral infections of poultry cause considerable economic losses and respiratory viruses have been frequently reported as a primary or secondary pathogens of poultry worldwide [18]. Our work focuses on the incidences and molecular epidemiology of viral respiratory pathogens in Pakistan within the commercial broiler and layer hen populations.

AIV have been frequently reported in Pakistan since 1994. LPAI H9N2, LPAI and HPAI H7N3 and HPAI H5N1 viruses have been regularly sequenced and 2 LPAI H4N6 viruses from 2010 and 2011 have been isolated and sequenced. LPAI H9N2 and HPAI H7N3 have been isolated from the same flock in 2003 [19] and since then frequent reassortment events between H7N3, H5N1 and H9N2 have been reported [13,20]. In the present study we detected LPAI H9 virus in several Pakistani farms from 2014 through 2016. As samples were collected on FTA cards it was unfortunately not possible to attempt virus isolation and limited nucleic acid quantities did not allow us for amplifying more than the HA2 part of the genomes. Further molecular characterization would be very interesting to confirm that only H9 was present in studied flocks, link clinical signs and genes constellations and to better understand the role of molecular determinants into field pathogenicity of currently circulating H9 viruses in unvaccinated flocks. Similarly, only partial gene sequences could be obtained for NDV, IBV, and aMPV. It enabled us to subtype/genotype currently circulating chicken viruses in Pakistan: genotype VII NDV, GI-13 (793B) IBV and genotype B aMPV, with no difference from the summer of 2014 through January 2016.

Despite the extensive and unrestricted use of imported vaccines, NDV still remains the main poultry disease in both commercial and rural chickens of Pakistan [21]. Recently, NDV of genotype VII (VIIa, VIIb, VIIe, VIIf) were detected in poultry of Pakistan [22–24]. Similarly, Miller et al. [25] reported that viruses of sub-genotype VIIi have replaced NDV isolates of genotype XIII in Pakistan, which were commonly isolated in 2009–2011, and became the predominant sub-genotype causing ND outbreaks since 2012.

Direct comparison of our IBV and aMPV findings with previous ones in Pakistan is not possible due to the absence of published data on these viruses in the country. There had so far been no genomic characterization of IB viruses circulating in Pakistan but serological evidence of the pathogen. Ahmed et al. [26] indeed showed seroprevalence of IBV including M-41 (88%), D-

274 (40%), D-1466 (52%), and 4-91 (8%) strains in Pakistani poultry. Similarly, high prevalence of IBV in backyard poultry (74%) and commercial poultry of Bangladesh (57%) were reported [27]. Sumi et al. [16] classified Indian IBV isolates on the basis of phylogenetic analysis within the Mass genotype (India/LKW/56/IVRI/08, now called GI-1) and the 793/B genotype (India/NMK/72/IVRI/10, now called GI-13). Moreover, IBV viruses of genotype 793/B like (GI-13) and QX like (GI-19) have been reported recently in poultry of Iran and Iraq [28–30]. Considering the geographic vicinity of the 5 countries and the commercial exchanges, it is therefore not surprising that we detected 793/B (GI-13) strains in Pakistan.

High seroprevalence of aMPV in broiler (48%) and breeder (93%) flocks has been reported in Iran [31]. In a recent study in Ahwaz, in the south west of Iran, [32] reported 55.5% aMPV seropositivity. Similarly, [33] reported seroprevalence of aMPV in breeders (34%) of Tamil Nadu in India. The virus has thus clearly circulated in the region. aMPV of subtype B have been reported in Iran [34,35]. In addition, aMPV of subtypes A as well as of subtype C have been reported in China [9,36,37]. Again, geographic vicinity and commercial exchanges of Pakistan with Iran and India rather than with China may explain the circulation of aMPV type B in Pakistan although the PCR primers used here would not have allowed us for aMPV subtype C detection.

While most of the farms sampled did not vaccinate against any of the chicken viruses we looked for, putative NDV vaccine failures were observed in farms 6, 13, 23, 62, 65, 66, and 80 (Table 1). The NDV vaccines used in Pakistan are strains LaSota (genotype II) and R2B (mesogenic Mukteswar strain, genotype III). The LaSota vaccine is formulated from a lentogenic (low virulence) clone of the LaSota strain and is manufactured in different countries of the world and imported and administered to commercial poultry in Pakistan. However, the R2B strain is manufactured by the local vaccine companies and administered to backyard poultry. It is still a matter of debate whether these vaccine strains (genotypes II and III viruses) are able to elicit a

protective immune response against the prevailing field strains, especially as we observed circulation of genotype VII viruses: the protection should be evaluated in experimental and field conditions. Inability of live vaccines to elicit protective immune response might also be due to improper cold chain supply system, inappropriate route of vaccination, or uneven vaccination schedules. It has been reported that the currently practiced NDV vaccines give better protection against the velogenic NDVs isolated from the 1930s through the 1970s (Herts33/56, California 71) than the ones isolated in past few years [22]. Hence parameters for selection of vaccine strains need to be reconsidered. Homologous strains may be worth a try for immunization against NDV. The optimization of vaccination schedules according to local climate and environmental conditions should also be looked into.

While the present study helped to understand the virus burden in Pakistani poultry production systems, further studies are warranted to fully characterize the virus strains and evaluate vaccines efficacy to counter the different pathogens. Finally, a main piece of information gained from our surveillance project was the frequent AIV/NDV co-infections. This phenomenon has been observed in the past in Bangladesh [38]. Experimental co-infections with the two viruses showed little impact on clinical signs but altered virus shedding (with higher LPAIV than NDV shedding) [39,40].

Conclusions: Our data showed that the above mentioned respiratory viruses were the most important causes of respiratory disease in commercial poultry in Pakistan. The predominance of H9 infection indicates a need for continuous monitoring of AIV among avian species and the awareness against public health risk. Further studies are necessary to assess circulating strains, economic losses caused by infections and coinfections of these pathogens, and the costs and benefits of countermeasures. Farmers need to be educated about the use of vaccines against these pathogens. Further studies are needed to understand the benefit of vaccination against either one of the two pathogens in preventing both diseases. Epidemiology studies on risk

factors in Pakistani farms are also warranted to better assess the putative synergistic effect of the co-infections.

Declarations

Availability of data and materials: All data generated and analyzed during this study are included in this published article

Authors' contributions: SU and HA collected all the required data, SU and AT processed the samples and drafted the manuscript. MD and JLG designed the study, and analyzed and interpreted the data, critically and substantially revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest: authors declare no conflict of interest

Consent for publication: Not applicable

Ethics approval and consent to participate: A local ethics committee ruled that no formal ethics approval was required to conduct this research. Before conducting the research, informed consent was obtained from the owners or managers of the poultry farms used in this study.

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Table 1 Origin of the Pakistani avian samples from which viruses were sequenced

Farm ID /Flock ID	Collection date	Location	Chicken type	Flock size	Age of birds (days)	Health status	Vaccination status	Type of farm	Type of sample	Sample ID	H9	NDV	IBV	aMPV	ILTV
S/6	13/7/14	Kasur	Broiler	3000	10	MRS	V (ND, IB)	open	OP	11	+	+	-	-	-
S/8	17/7/14	Rawalpindi	Broiler	10000	16	MRS	NV	control	OP	12,15	-	+	-	-	-
H/9	19/7/14	Kasur	Broiler	3000	38	MRS	NV	open	OP	17,18	+	-	-	-	-
K/10	22/7/14	Multan	Broiler	3000	20	MRS	NV	open	OP	20	+	-	-	-	-
A/11	25/7/14	Kasur	Broiler	3000	9	MRS	NV	open	OP	21,22	+	-	-	-	-
K/12	27/7/14	Kasur	Broiler	3000	12	MRS	V (ND)	open	OP	23,24	+	-	-	-	-
R/13	29/7/14	Kasur	Broiler	2500	31	MRS	V (ND, IB)	open	OP	26		+	-	-	-
Y/21	13/8/14	Lahore	Layer	3000	80	MRS	NV	open	OP	41,42	+	+	-	-	-
A/22	14/7/14	Kasur	Layer	3000	120	MRS	NV	open	OP	44	+	-	-	-	-
R/23	15/7/14	Lahore	Broiler	10000	31	MRS	V (ND)	control	OP	45,46	+	+	-	-	-
Z/32	23/7/14	Okara	Broiler	3000	10	MRS	NV	open	OP	64,65	-	+	-	-	-
A/38	18/10/14	Kasur	Layer	3000	78	MRS	NV	open	lung swabs	74	+	+	-	-	-
H/41	14/1/14	Multan	Layer	3000	108	MRS	NV	open	OP	77	+	+	-	-	-

H/42	28/11/14	Multan	Layer	2500	145	MRS	NV	open	OP	78	+	+	-	-	-
K/51	15/10/14	Multan	Layer	3000	66	MRS	NV	open	OP	87	-	+	-	-	-
A/59	14/8/15	Rawalpindi	Broiler	3000	14	Open mouth breathing, mucus plug in bronchi	V (ND)	open	OP	98,99	-	-	+	-	-
W/62	17/8/15	Kasur	Broiler	3000	35	Respiratory distress, congested lungs	V (ND, IBD)	open	OP, lung swabs	106,107	-	+	-	+	-
I/64	26/8/15	Rawalpindi	Broiler	3000	25	MRS	V (ND)	open	OP	110,111	-	-	+	-	-
U/65	26/8/15	Mansehra	Layer	3000	208	sickneess, congested lungs, pale carcass	V (ND)	open	OP	112,113	-	+	-	-	-
T/66	29/8/15	Abbottabad	Layer	3000	180	fever, sneezing, gasping, airsacculitis	V (ND)	open	OP, lung swabs	114	+	+	-	-	-

F/67	2/9/15	Kasur	Broiler	3000	33	MRS	V (ND, IBD)	open	OP	115	+	+	-	-	-
S/70	27/9/15	Sheikhupura	Broiler	3000	35	MRS	V (IB, ND)	control	OP	122,123	+	-	-	+	-
K/80	13/12/15	Faisalabad	Broiler	3000	26	MRS	V (ND)	control	OP	142,143	-	+	+	-	-
K/84	2/1/16	Lahore	Broiler	2500	29	MRS	NV	control	OP	150,151	+	+	-	-	-
A/88	9/1/16	Kasur	Layer	3000	120	MRS	NV	open	OP	158,159	-	-	+	-	-

V: vaccinated, NV: non vaccinated, OP: oropharyngeal swab, ND: Newcastle disease, IB: infectious bronchitis, IBD: infectious bursal disease, aMPV: avian metapneumovirus, IBV: infectious bronchitis virus, NDV: Newcastle disease virus, ILTV: infectious laryngotracheitis virus. MRS : Mild respiratory signs: slight opening of the beak and chest movements. "Control" versus "open" farms: environmentally controlled (versus not controlled) poultry farms. Sample numbers: each number corresponds to a pool of 10 swabs from a chicken flock. Data in this table include only those samples and flocks which were found positive for selected viruses.

Table 2 PCR conditions for the detection and genotyping of avian respiratory viruses in samples from Pakistan

	Virus	Primers	Sequences (5-3)	Target	Amplicon size (bp)	PCR conditions	Reference
Detection PCR	**NDV**	FIP-1	5' TACTTTGCTCACCCCCCTT 3'	Fusion gene (F)	280	94 C for 2 min; 40 cycles of 94 C for 30 sec, 58 C for 30 sec, 72 C for 1 min; final extension at 72 C for 5 min	[25]
		FIP-2	5' CATCTTCCCAACTGCCACT 3'				
	IBV	N791	5' GTGATGACAAGATGAATGAGGA 3'	Nucleo-protein gene (N)	380	94 C for 2 min; 40 cycles of 94 C for 30 sec, 54 C for 30 sec, 72 C for 1 min; final extension at 72 C for 5 min	[41]
		N1129	5' CAGCTGAGGTCAATGCTTTATC 3'				
	ILTV	gEU	5' GCTGGGTTCTGGGCTACACAAC 3'	Glyco-protein E gene (gE)	626	94 C for 2 min; 40 cycles of 94 C for 30 sec, 61 C for 30 sec, 72 C for 1 min; final extension at 72 C for 5 min	[42]
		gEL	5' TGCGCGTGACTCGGAGAG 3'				
	aMPV	G1	5' GGGACAAGTATCYMKAT 3'		441	94 C for 2 min; 40 cycles of 94 C for 30 sec, 50 C for 30 sec, 72 C for	[43]

					bp		
		G6	5' CTGACAAATTGGTCCTGATT 3'	Attachment glycoprotein gene (G)		1 min; final extension at 72 C for 5 min	
	AIV	M52C	5' CTTCTAACCGAGGTCGAAAG 3'	Matrix gene (M)	280	95 C for 30 sec; 40 cycles of 95 C for 30 sec, 55 C for 30 sec, 72 C for 30sec; final extension at 72 C for 1 min	[44]
		M253R	5'AGGGCATTTTGGACAAAKCGTCTA 3'				
Genotyping PCR	**AIV**	HA-1134F	5′ GGAATGATHGAYGGNTGGTATG 3′	hemma-gglutinin gene (HA)	600	95 C for 30 sec; 40 cycles of 95 C for 30 sec, 55 C for 30 sec, 72 C for 30sec; final extension at 72 C for 1 min	[45]
		NS-890 R	5′ ATATCGTCTCGTATTAGTAGAAAC AAGG 3′				
	IBV	S15	5' TGAAAACTGAACAAAAGACA 3'	Spike gene (S)	700	95°C for 2 minutes; 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds; final extension of 72°C for 12 minutes	[46]
		CK2	5' CTCGAATTCCNGTRTTRTAYTGRCA 3'				

bp : base pairs

Figures legends.

Figure 1. Map of Pakistan with sampling sites. Areas in Pakistan where samples were collected. The map was drawn using ArcGis and it shows the distribution of positive specimens for respective viruses (AIV in red, NDV in blue, IBV in green and aMPV in grey shades) throughout the country. The pies diameters are proportional to the number of samples collected per district.
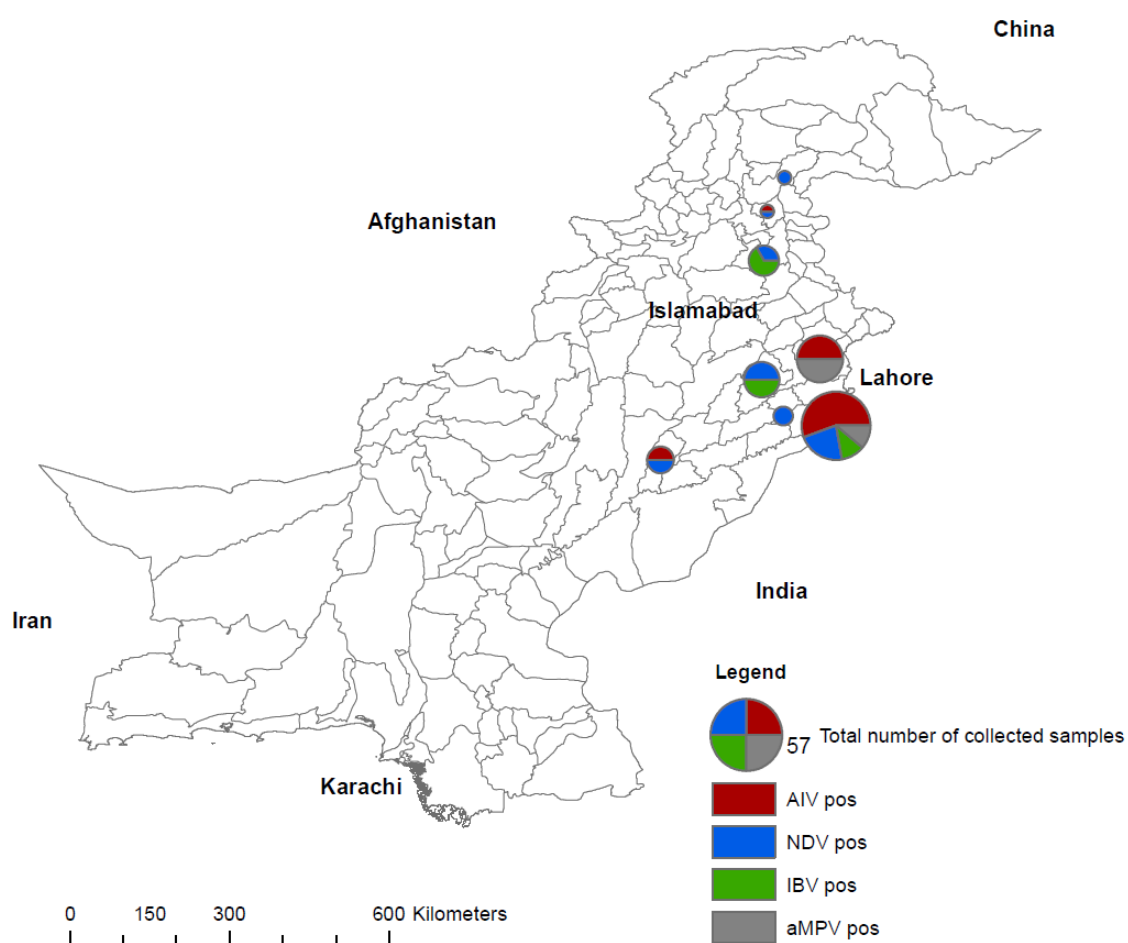
Figure 2. Phylogenetic tree of recent Pakistanis influenza viruses HA genes. Sequences of A/chicken/Pakistan/17/2014 and A/chicken/Pakistan/74/2015 were selected as representative sequences for the present study. The HA2 nucleotide sequences of these 2 viruses (in bold font with a closed circle shaped symbol) were compared with full H9 gene sequences from GenBank and GISAID databases: the first 10 blast hits sequences, reference sequences (including recommended WHO vaccine strains in red font) and previously published Pakistani H9 sequences. *: partial sequence.
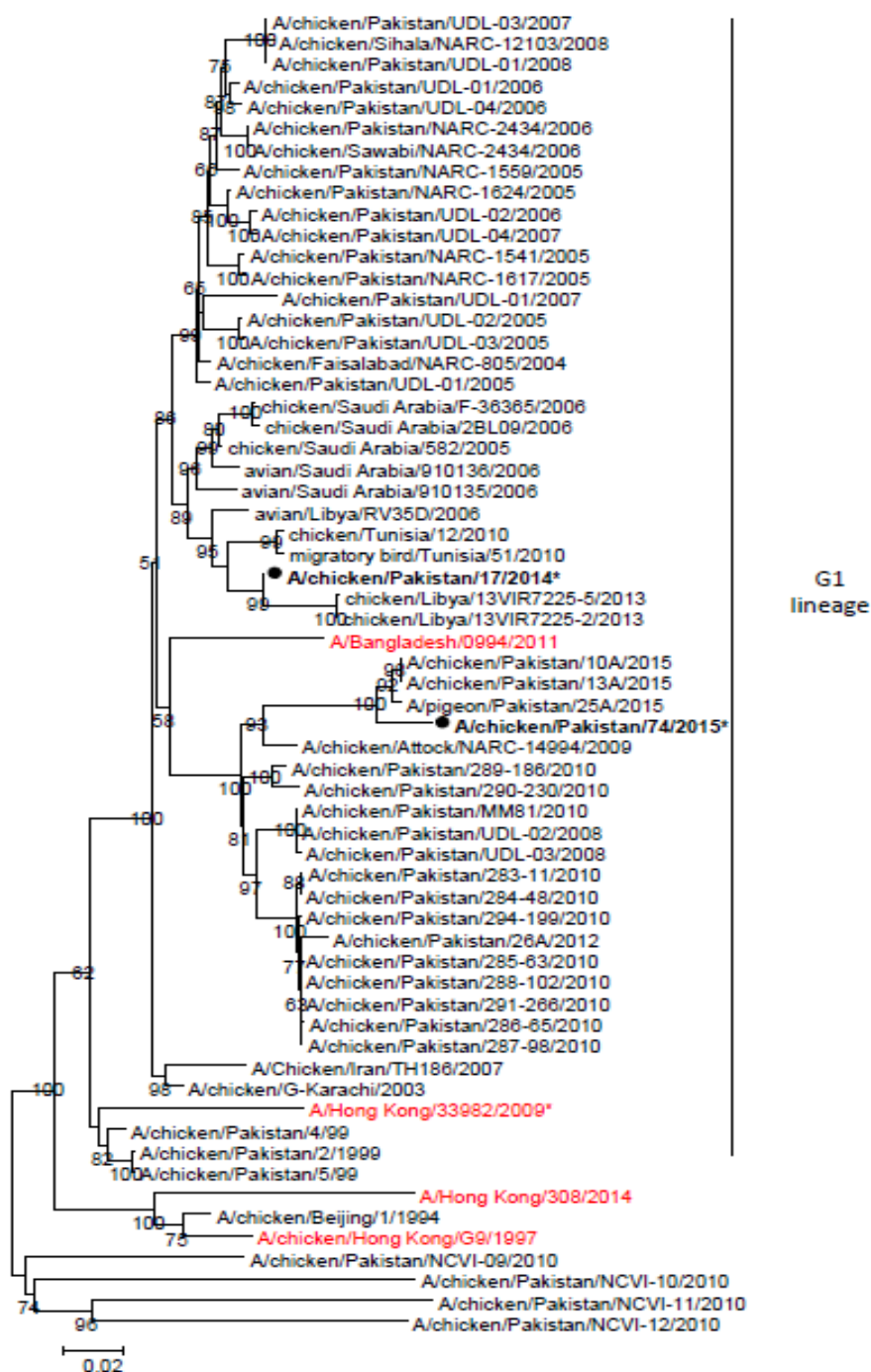
Figure 3. Phylogenetic tree of recent Pakistanis Newcastle disease viruses F genes.
Sequences of Pakistanis NDV were identical and NDV/chicken/Pakistan/11/2014 was selected as a representative strain for the present study. Its partial F gene sequence was compared with its first ten BLAST hits and reference sequences from the 18 known NDV genotypes. *: partial sequence.
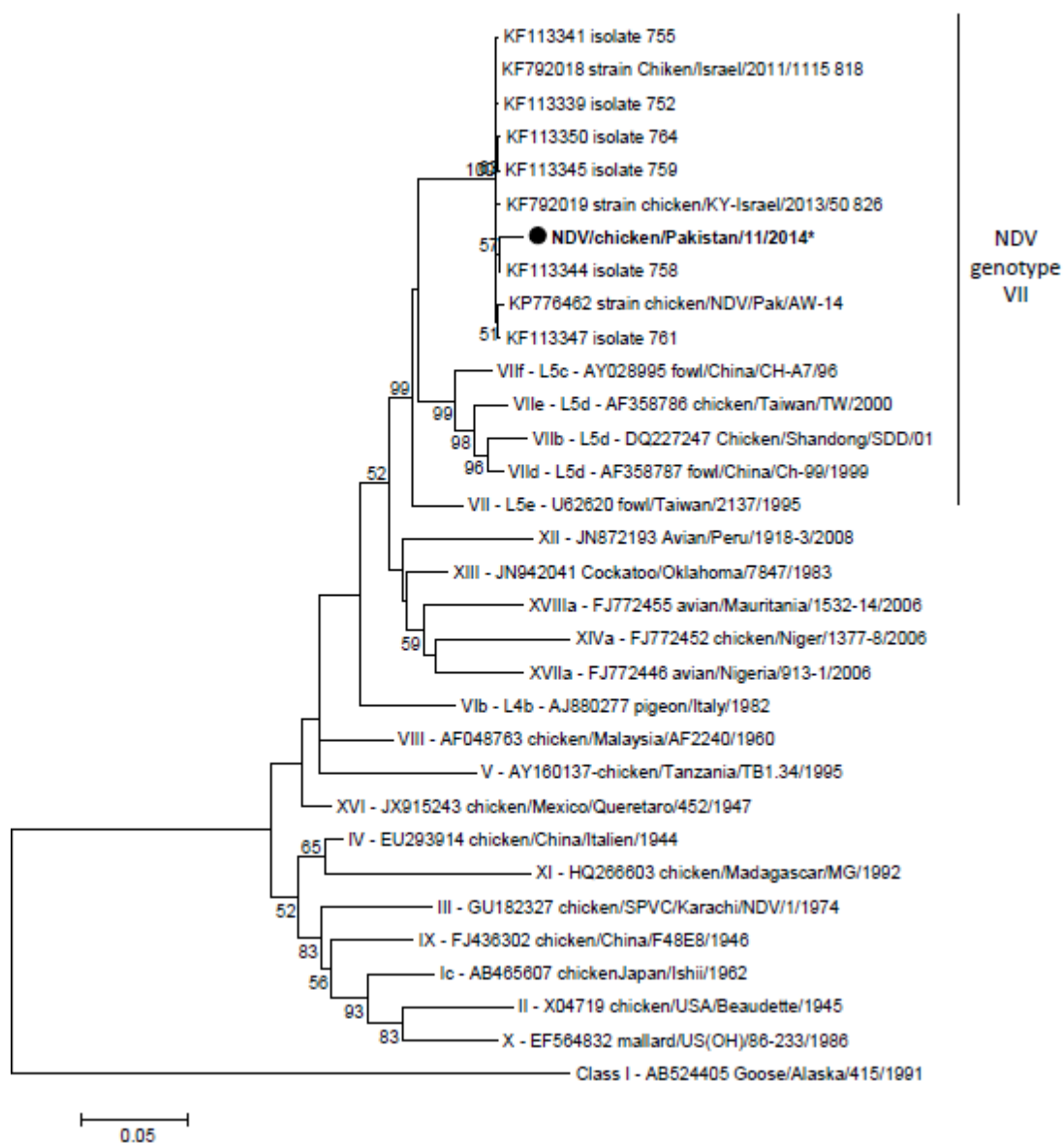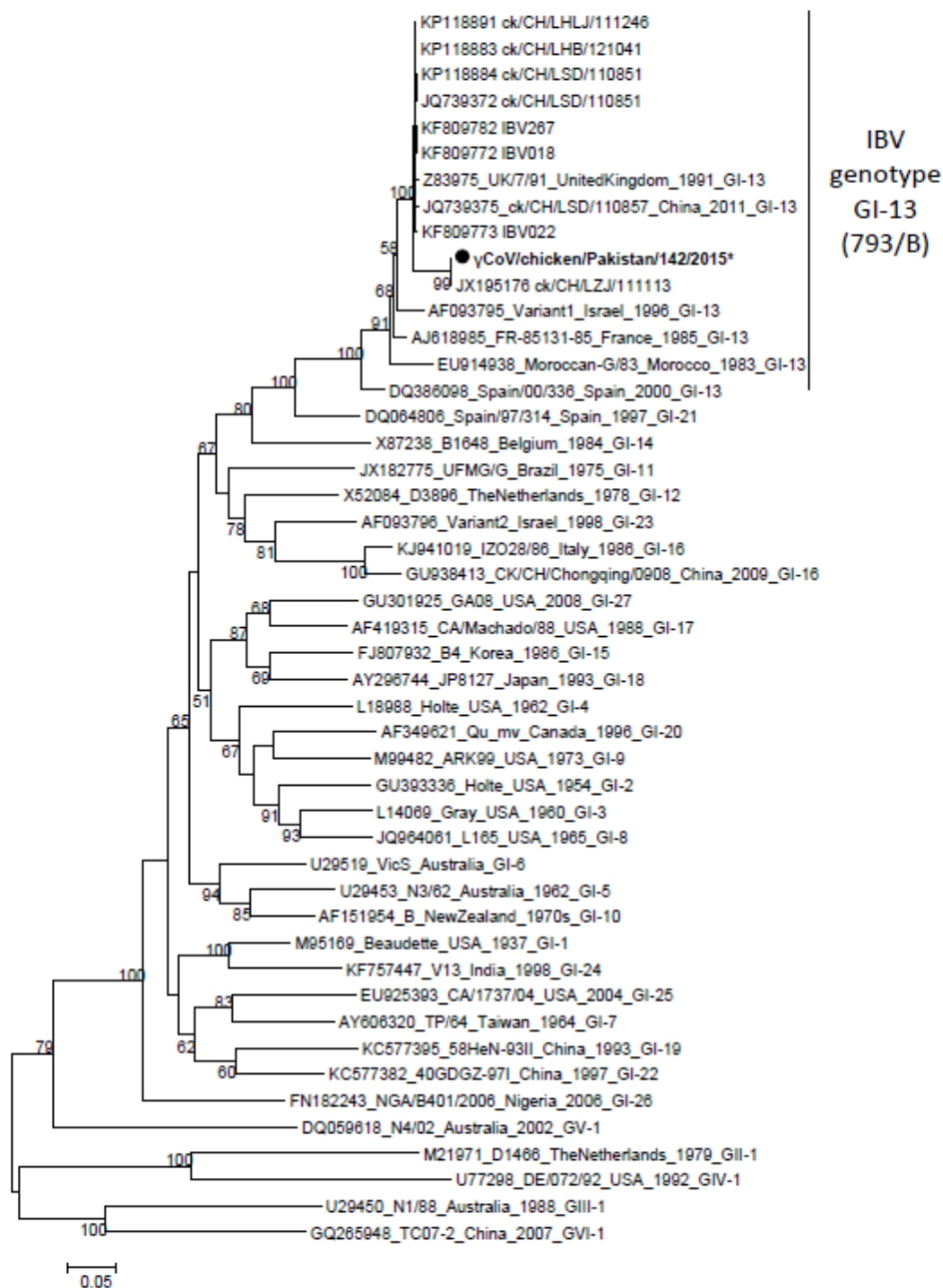
Figure 4. Phylogenetic tree of recent Pakistanis infectious bronchitis viruses spike genes.

Sequences of γCoV/AvCoV/chicken/Pakistan/11/2014, γCoV/AvCoV/chicken/Pakistan/98/2015, γCoV/AvCoV/chicken/Pakistan/99/2015, γCoV/AvCoV/chicken/Pakistan/142/2016, γCoV/AvCoV/chicken/Pakistan/143/2016, and γCoV/AvCoV/chicken/Pakistan/159/2016 were identical so only γCoV/AvCoV/chicken/Pakistan/142/2016 was represented on the tree with a black circle shaped symbol. Its partial S1 gene sequence was compared with its first ten BLAST hits and reference sequences from the 32 known IBV lineages. *: partial sequence.

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Figure 5. Phylogenetic tree of recent Pakistanis avian metapneumoviruses attachment protein genes. Sequences of aMPV/chicken/Pakistan/106/2015, aMPV/chicken/Pakistan/107/2015, aMPV/chicken/Pakistan/122/2016, and aMPV/chicken/Pakistan/123/2016 were identical so only aMPV/chicken/Pakistan/107/2015 was represented on the tree with a black circle shaped symbol. Recent Pakistanis aMPV G gene sequences were compared with their first ten BLAST hits and reference sequences from the 4 genotypes: A, B, C, and D.
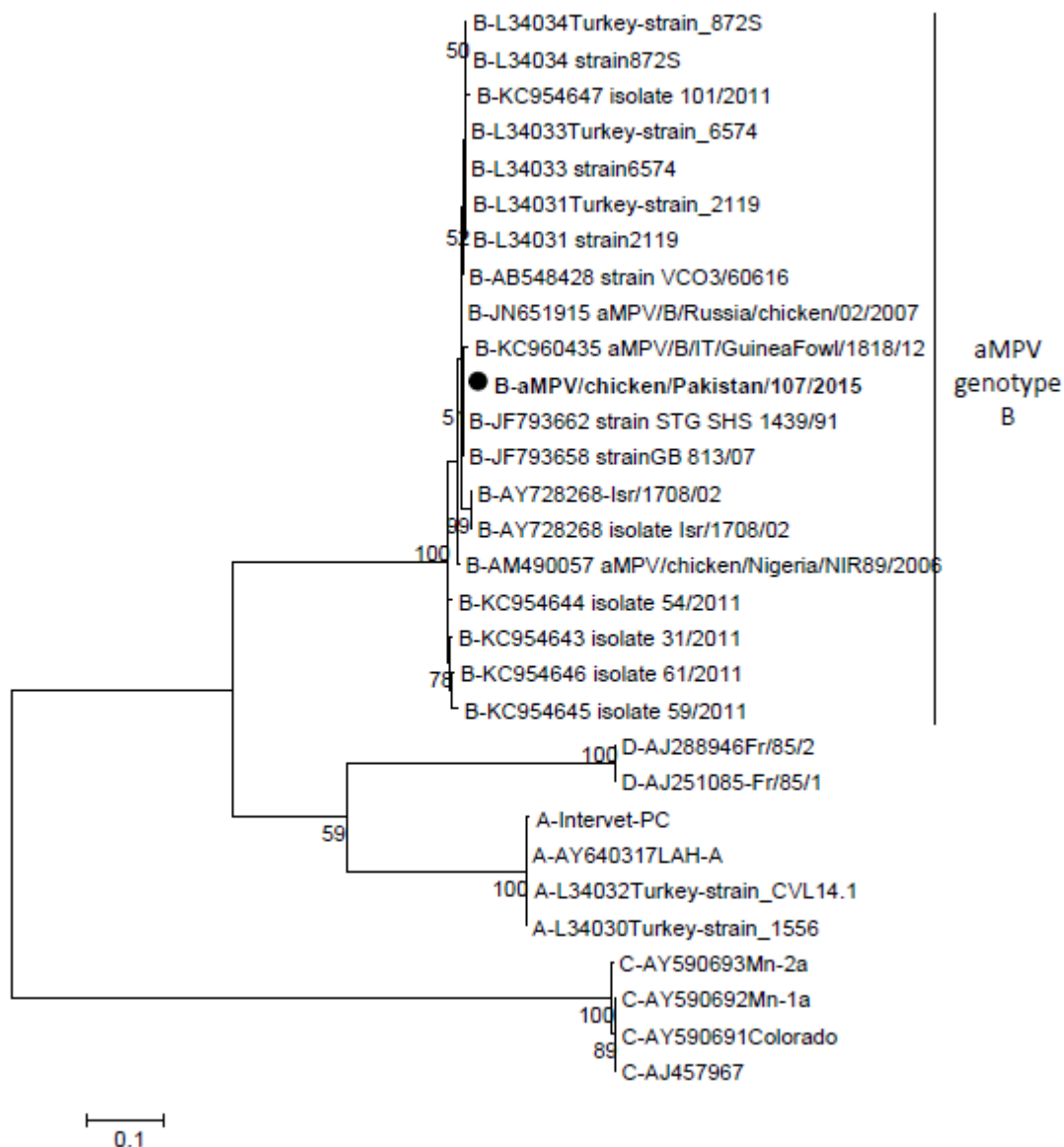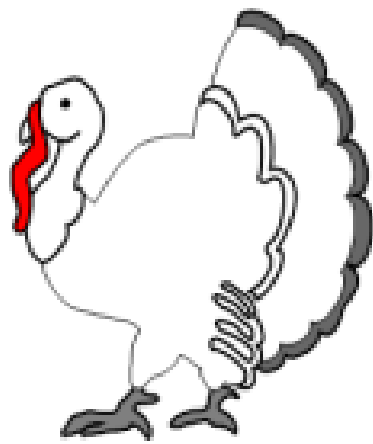
General Discussion & Conclusions

Respiratory diseases in poultry have been reported to be caused by mixed or single infections with several agents (Watanabe *et al.*, 1977; Sakuma *et al.*, 1981; Yashpal *et al.*, 2004) resulting in poor animal welfare, economic losses, and increased antibiotics consumption. It is well appreciated that upper respiratory tract viral infections in poultry are often complicated by more serious bacterial diseases. It is thought that certain pairings of organisms better complement each other than other potential pairings. Co-infections of poultry present a complicated clinical picture confusing the identification and diagnosis and unfortunately little is known on the interactions between co-infecting pathogens (Costa-Hurtado *et al.*, 2014). Influenza virus is commonly thought of in this context along with other respiratory viruses and bacteria.

Natural infections with AIV are more severe than experimental infections, suggesting that secondary agents or other factors play prominent role in the clinical disease process. Natural AIV/ bacterial problems are expected to occur at the same time and have been reported in poultry (Pan *et al.*, 2012; Pu *et al.*, 2012), but the effects of such combinations on the health status of poultry is not well known. In the field, co-infections of AIV do occur with other respiratory pathogens but are not easily detected, and the impact of co-infections on pathobiology is unknown. The mixed AIV infections may provide increased virulence, posing a substantial risk to poultry and public health. The coinfection of *E. coli* and influenza viruses in poultry has been observed in field conditions. During the last decade, the outbreaks of H9N2 influenza virus with severe clinical signs, high mortality (20-65%) and low production (up to 75%) have been reported in commercial poultry farms (Nili & Asasi, 2002, 2003; Bano *et al.*, 2003; Swayne, 2008). Similarly, An outbreak of H9N2 influenza virus infection in chickens in Hong Kong (A/chicken/Hong Kong/739/94) was associated with coughing and respiratory distress in 75% of the birds, with 10% mortality. Treatment with antibiotics reduced the mortality rate, suggesting that bacteria may play some role in the exhibition of the clinical syndrome (Kishida *et al.*, 2004). However, H9N2 viruses in domestic poultry cause mild clinical signs and respiratory diseases with low mortality (less than 5%). It is proposed that

concurrent infection may play a key role in exacerbating mortality in chicken infected with H9N2 influenza virus (Haghighat-Jahromi *et al.*, 2008; Pan *et al.*, 2012; Seifi *et al.*, 2012). It was speculated that severe clinical signs linked to AIV H9N2 infections in the field were, probably due to *E. coli* involvement (Bano *et al.*, 2003). Similarly, these lesions were commonly reported in turkeys during the 1999 outbreak of MP H7N1 AI in Italy in association with secondary bacterial pathogens such as *E. coli*, *Riemerella anatipestifer* and *Pasteurella multocida* (Capua & Marangon, 2000).

For the present PhD thesis work, we intended to link the coinfections observed in Pakistani poultry farms with coinfection studied in experimental conditions. In the present study, we tried to come as close as possible to field conditions in our experiments by choosing commercial turkeys and using aerosol route of inoculation. However, it is still difficult to reproduce field conditions in laboratory settings because many other factors contribute in the production of diseases such as dust, pollution, humidity, temperature, ammonia production, housing stress etc. It was demonstrated that dual infections of turkeys with H6N1 and O78 were able to cause similar, but more severe and longer persisting respiratory symptoms compared to single infections. The AIV H6N1 / *E. coli* (O78) dual infection resulted in a higher morbidity and mortality than single infections with either agent. The clinical symptoms in themselves were similar in nature to those caused by AIV H6N1 alone, but they were more severe and persisted markedly longer. This aggravation of clinical disease was reflected in the necropsy findings in that the lesions found in the respiratory tract were clearly more outspoken in the dually infected birds. Furthermore, microscopic lesions in the birds having received both agents were generally more extensive and more prolonged in comparison to the singly-infected animals. In the poults inoculated with AIV followed by *E. coli* inoculaion, the inflammatory changes, loss of cilia and airsacculitis were more extensive than in poults inoculated with AIV or *E. coli* alone. Our experimental AIV H6N1 caused lesions are in agreement with field outbreak (Corrand *et al.*, 2012). Inflammation and deciliation of the trachea, degeneration of the mucous gland cells and damage to the respiratory epithelium were likewise previously demonstrated (Corrand *et al.*,

2012). Jirjis et al. (2004) reported aggravation of clinical signs and lesions when *E. coli* inoculation followed by aMPV inoculation in turkeys. These findings all endorse our results that AIV H6N1 and *E. coli* (O78) exert an additive or synergistic pathogenic effect if given consecutively, spaced by three days to susceptible turkeys. While we could compare our experimental findings to field observations in France (Corrand *et al.*, 2012), it is difficult to extrapolate to the Pakistani poultry farms situation. In Pakistan, our molecular epidemiology study indeed just targeted avian respiratory viruses. A follow up study is warranted to understand the bacterial co-infections in the field.

In our epidemiological work in Pakistan, we found that the viral respiratory diseases are common even though vaccines are being in some farms. We have characterized four respiratory viruses in our study. There is already published data on AIV and NDV in Pakistan (Iqbal *et al.,* 2009, Munir *et al*., 2013, Miller *et al.,* 2015), however, IBV and aMPV have not been characterized before, although there are some reports on their prevalance based on serological surveys. The information about their genotypes/subtypes will help to better implement control measures. We could not isolate these viruses due to samples on FTA cards and we could not sequence their full genomes due to limited genome quantity on the FTA cards. It will be very interesting to obtain their full genome sequences and look at putative mutations in their genomes. We could not screen and characterize bacterial pathogens in our samples, bacterial pathogens may be possible etiological agents in virus negative samples. Moreover, timing of samples collection is very important. We might have collected samples when there was no virus shedding or very limitrd virus shedding in oropharyngeal excretions: a possible reason for absence of virus detection. In addition, AIV gene constellation should also be studied because reassortant LPAIV H9N2 viruses have been reported in the past (Iqbal *et al*., 2009). Finally, most of the sampled poultry farms lacked proper disinfectants and biological barriers, which favor transmission of infectious diseases. We observed putative vaccine failure for NDV, therefore continous monitoring of NDV in field and evaluation of vaccines should be carried out on regular basis to get maximum protection. Moreover, effect of vaccination prior or during

coinfection should also be evaluated. It is possible that vaccine handling and delivery are incorrect. Vaccines in Pakistan are often delivered by illiterate farmers, who may not follow the manufacturer's instructions regarding vaccine application (withdrawing of water disinfectants from drinking system, appropriate water quality and temperature, appropriate vaccine dose, etc). Therefore, education of farmers will be also an important measure to improve poultry health in Pakistan. Co-infections were common for AIV and NDV but less common for the other tested chicken viruses indicating that these two pathogens could be present at the same time of respiratory disease outbreak and their importance in the field should not be underestimated. In a future study, it would be important to assess the likely exacerbation of clinical disease caused by AIV and NDV co-infections in experimental conditions. It will also be interesting to see which of them infects birds first and how these pathogens interact with each other. Further investigation and characterization of additional respiratory pathogens is required in order to adapt appropriate control strategies in the future. Another future goal for surveillance studies in Pakistan is to detect putative *E. coli* and AIV coinfections, economic losses caused by the coinfections of these pathogens, and the costs and benefits of countermeasures. Furthermore, the effects of reduced antibiotic use on coinfections should be studied. There are some gaps in the knowledge about *E. coli* serotypes in circulation in Pakistan which needs to be filled for better comparison and understanding. During my PhD we carried out both the molecular epidemiology and experimental co-infection studies in parallel and we lacked time to tackle questions raised by the field work in the laboratory settings. This is a clear perspective of my project.

In conclusion, it was shown in experimental conditions that the *E. coli* strain (O78) and AIV H6N1 alone are able to produce mild respiratory infection through adhesion and colonization of the respiratory tract, but without each other help do not induce severe respiratory disease and mortality in suspected turkeys. The results obtained in the present study clearly indicate the occurrence of marked synergistic or additive effects between two distinct respiratory pathogens important in poultry. We have established an experimental infection model of turkey poults

with LPAIV and/or *E. coli* using aerosolization that better mimics field infections than more classical IT inoculations. The established *E. coli* (O78) single and AIV/*E. coli* dual infection models can undoubtedly be used to further investigate the mechanism of *E. coli* colonization and the AIV/*E. coli* synergy. Further, the AIV/*E. coli* dual infection model may be used to test preventive and curative measures to combat the respiratory disease. With consideration of the significant spread of *E. coli* infections as well as LPAIV in poultry, a surveillance of LPAI infection and regular diagnosis of *E. coli* infection and anti-*E. coli* treatment of flocks may help to prevent development of severe clinical disease and economic losses due to such co-infections. Continuous surveillance of AI infection and co-infections studies in experimental poultry models is warranted to find new strategies to control their circulation in domestic and wild poultry. Further studies are also warranted to really assess the cost-benefit of using commercial birds for pathogenesis studies of LPAIV and complicating pathogens. The timing of co-infection would also require further systematic experimental studies to understand the role of prior/post/simultaneous inoculation in disease outcome, pathogenesis and virus shedding pattern. The present study contributes to the unravelling of the multi-factorial respiratory disease complex in turkeys, illustrating that the outcome of AIV infection in acutely *E. coli* predisposed turkeys is aggravated, compared to single AIV infected turkeys. We highlighted the need to reconsider the old dogma of viral infections facilitating bacterial infections and thus causing severe disease. The order of viral / bacterial infections may indeed be inverted in the field as commensal bacteria often may become pathogenic after viral superinfections. Clarifying the interaction mechanisms between the different pathogens will allow a more precise diagnosis and a better treatment, reducing not only economical complications of respiratory diseases, but also the zoonotic risk.
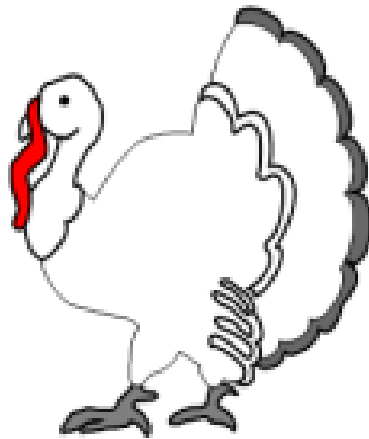
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Annexure

Low pathogenic avian influenza virus experimental infection in the turkey model: effect of the route of inoculation on the course of disease

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Introduction

Low pathogenic avian influenza (LPAI) infections in avian species have become an economic threat to the poultry industry worldwide. An important natural route of avian influenza infection in farms is the inhalation of contaminated dust. Commonly used methods of experimental infection such as intranasal and intratracheal (IT) inoculations bypass the deep air sac access of virus particles. We have developed a reliable aerosol challenge method for reproduction of LPAI infection in turkey poults and compared it with a more conventional intra-tracheal inoculation.

Materials and Methods

❖ Two LPAI viruses were used to infect four weeks old turkey poults: A/chicken/Tunisia/12/2010(H9N2) and A/turkey/France/09010-1/2009(H6N1)

❖ Six groups (n=6 to 9) of conventional healthy white turkey poults were obtained from the GFA de Pierpont (Castelnau de Montmirail, France)

❖ Turkeys were inoculated with 10^6 plaque forming units (PFU) of H6N1 or H9N2 virus per bird either IT or in an aerosol (A). Control turkeys received PBS IT or aerosolized

❖ A compressor nebuliser CompAir Pro NE C29 E (OMRON, Japan) was used to aerosolize virus for 20 minutes in poultry isolators

❖ Oropharyngeal swabs were collected daily for 8 days post infection to assess the virus shedding in the upper respiratory tract

❖ Three birds per group were necropsied day 4 post-infection and macroscopic lesions were recorded

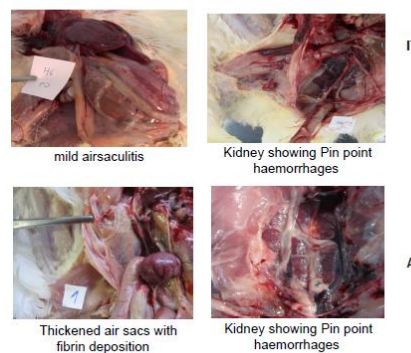❖ Blood was drawn 2 weeks post infection to assess seroconversion by haemagglutination inhibition assay

Nebulizer used for virus aerosolization

Results & Discussion

H9N2 (day 4 post infection)

IT — Turkey poult showing gasping — mild haemorrhages on trachea — mild airsaculitis

A — Kidney showing congestion and haemorrhages — Pin point haemorrhages on trachea — Thickened air sacs with fibrin deposition

H6N1 (day 4 post infection)

IT — mild airsaculitis — Kidney showing Pin point haemorrhages

A — Thickened air sacs with fibrin deposition — Kidney showing Pin point haemorrhages
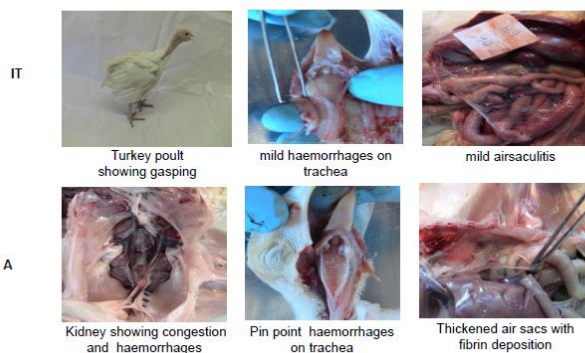
❖ Infected birds showed mild clinical signs (gasping and apathy), while control negative birds remained healthy and active throughout the experiment irrespective of the inoculation route and virus type.

❖ Efficient virus replication was observed in both H6N1 inoculated groups and in the H9N2 aerosol inoculated group for 7 days. H9N2 IT infected birds hardly showed virus replication.

❖ All inoculated birds had seroconverted 14 days post infection.

❖ Gross lesions were observed in infected birds with increased severity for aerosol as compared to IT infected birds.

❖ H9N2 virus aerosol infected turkey poults showed the more severe of gross lesions with fibrin in their air sacs and haemorrhagic kidneys, similar to the necropsy observations reported from the field.
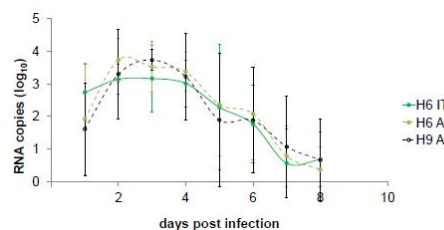
Avian influenza Virus shedding pattern in the turkey model

Conclusion and perspectives

In conclusion, we have established an experimental infection model of turkey poults with LPAI virus using aerosolization that better mimics field infections than IT inoculations. Histological and immunohisto-chemistry analysis are ongoing to assess microscopic lesions and tropism. Further studies in the chicken model are warranted to confirm the benefit of using aerosol inoculation in experimental infections of birds with influenza virus.

Molecular Epidemiology of Avian Infectious Bronchitis and Metapneumoviruses in Pakistan (2014–2016)

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Introduction

Infectious bronchitis virus (IBV) and avian metapneumovirus (aMPV) are very critical pathogens to the respiratory tract of poultry, causing serious economic losses in all countries including Pakistan. Outbreaks of IBV and aMPV are not reported to the ministry of livestock and poultry production. Consequently, the distribution pattern of such avian diseases is not clear in Pakistan. Like other coronaviruses, IBV has a large genome which often accumulates mutations, manifesting in a large number of serotypes of the virus. Further to this, successful vaccination programs rely on current information regarding endemic genotypes.IBV and aMPV infections are often followed by infection with secondary pathogens thus complicating the disease. Therefore, it is imperative to understand the distribution pattern and persisting strains of IBV and aMPV in different regions and types of avian flocks to develop scientific and risk based prevention measures of poultry diseases. The aim of this study was to detect and characterize avian respiratory viruses in commercial poultry in Pakistan, which is the first step necessary before control measures can be implemented.

Materials and Methods

During the study, a total of 161 samples (oropharyngeal swab or tissue impression smear) were collected directly on Finders Technology Associates (FTA®) sampling cards from poultry showing respiratory signs from 2014 through 2016. All IBV and aMPV positive specimens were detected by RT-PCR targeting the S1 or G gene respectively and direct sequencing of positive amplicons was performed to identify the genotype or subtype.
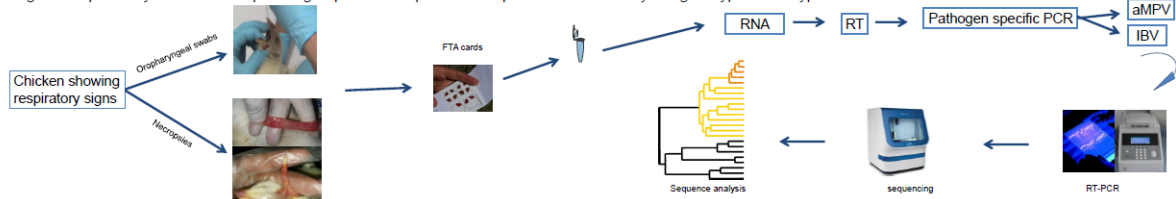
Results & Discussion

Out of 161 tested samples, 8 (4.9%) were positive for IBV and 4 (2.4%) were positive for aMPV which represented 4 (4.4%) and 2 (2.2%) of total examined farms (89) respectively. All aMPV isolates were of a single subtype (subtype B) and similarly, all IBV isolates were categorized into a single genotype (793/B like or GI-13).
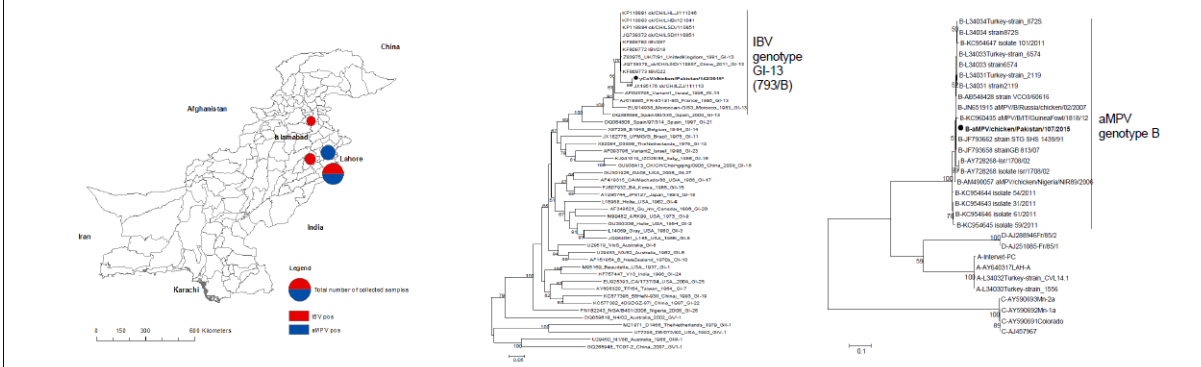
Collections sites and prevalence of aMPV and IBV in Pakistan

Maximum likelihood phylogentic tree based on nucleotides sequences of partial S1 gene of IBV. Representative strain (yCo/V/chicken/Pakistan/142/2015) is shown in bold letters.

Maximum likelihood phylogentic tree based on nucleotides sequences of partial G gene of aMPV.Representative subtype (aMPV/chicken/Pakistan/107/2015) is shown in bold letters.

To date, genomic characterization of IB viruses circulating in Pakistan had not been carried out although IBV antibodies had been detected [1]. Ahmed et al (2007) reported antibodies against M-41 (88%), D-274 (40%), D-1466 (52%), and 4-91 (8%) strains in Pakistani poultry. Similarly, high prevalence of IBV in backyard poultry (74%) and commercial poultry of Bangladesh (56.67%) were reported [2]. As far as IBV genotypes in the area are concerned, Mass (India/LKW/56/IVRI/08, now called GI-1) and the 793/B genotypes (India/NMK/72/IVRI/10, now called GI-13) have been reported in India[3]. Moreover, 793/B like and QX like IBV have been reported in Iran and Iraq [4,5]. High seroprevalence of aMPV has also been reported in Iran and India [6,7]. AMPV of subtype B have been reported in Iran [8],which clearly indicates the circulation of these viruses in the region. Considering the geographic vicinity of the 5 countries and the commercial exchanges, it is therefore not surprising that we detected 793/B (GI-13) strains and aMPV subtype B in Pakistan.

Conclusion and perspectives

The current work constitutes the first comprehensive study on the genotyping of IBV and aMPV in Pakistan with useful information regarding the molecular epidemiology of IBV and aMPV. While most of the farms sampled did not vaccinate against any of the avian viruses we looked for, putative vaccine failures were observed in farms. Vaccines against IBV e.g. M41 and H120 are being used to fight the infection at some farms. The putative protective effect of these vaccines against circulating field strains should be evaluated. The present study helped to understand the virus burden in Pakistani poultry production systems but further studies are warranted to fully characterize the virus strains and evaluate vaccines efficacy to counter the different pathogens.

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Protection conferred by H120 vaccine against Moroccan Italy 02 IBV stain in commercial broilers and SPF chicken

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Introduction

Infectious bronchitis (IB) is one of the most contagious diseases that affect poultry worldwide and is responsible for severe economic losses. Clinically, the disease causes respiratory distress, drop in egg production and quality in layers. Phylogenetic analyses confirmed that Moroccan viruses are grouped into three genotypes: Massachusset, Italy 02 and 793/B (Fellahi et al., 2015). Results of previous studies show that the genotype 793/B had almost disappeared from the country while it was predominant in the 80's and 90's (El Houadfi et al., 1986; Alarabi, 2004; El Bouqdaoui et al., 2005). This phenomenon illustrates the continuing evolution and the dynamic changes of IBV, which complicate the development of vaccine strategies. In Morocco vaccination programs largely rely on IBV Massachusetts strain. During the present study, the ability of H120 to protect against a Moroccan-Italy 02 IBV, recently isolated in the country, was investigated in field and experimental conditions.

Materials and Methods

- Broiler and SPF white leg horn chicken were obtained from local hatchery in Morocco and GFA de Pierpont (Castelnau de Montmirail, France).
- Vaccines: H120 + Avinew for commercial broilers (spray, one day old) and H120 for SPF birds (oculonasal inoculation, 1 day old).
- Challenge: 10^3 EID_{50} of Moroccan Italy 02 IBV.
- Clinical signs recorded daily.
- Necropsies 5 days post challenge.
- Oropharyngeal swabs collected 3, 5 and 7 days post challenge (pc).
- ELISA (Synbiotics ProFlock kit and IDEXX kit).


Inoculation of birds through oculonasal route
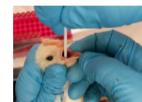
Oropharyngeal swabs

Results

- In commercial broilers: mild respiratory distress to severe gasps, as early as 24 hours pc. Maximum severity of clinical signs: days 3 and 4 pc; 90% of the tracheas (day 5) with hyperplasia and deciliation.
- SPF vaccinated birds: respiratory signs day one pc in 50% of the birds and peak on day 4 pc. In unvaccinated birds: more pronounced respiratory signs as early as day 3 pc and until from 10 days pc.
- Histopathology investigations (day 5 pc): rare lesions in tracheas of vaccinated birds in contrast to deciliation and hyperplasia in unvaccinated chickens.
- Serological analysis: significantly higher antibody titers after than before challenge for commercial and SPF birds.
- SPF birds: virus was detected in 45% of the vaccinated birds on days 3 and 5, while 100% of the swabs were positive on days 3 to 7 pc for unvaccinated chicken.

Experiment 1: Commercial chicken
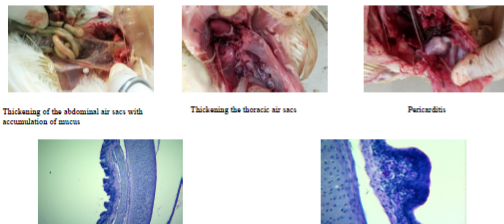
Thickening of the abdominal air sacs with accumulation of mucus

Thickening the thoracic air sacs

Pericarditis

Experiment 2: SPF chicken

Thickening of the abdominal and thoracic air sacs with mucus accumulation


Thickening the abdominal air sacs


Thickening the abdominal air sacs

Histological aspect of a trachea of cross-section from a tested chicken 5 days post infection (deciliation and hyperplasia of the epithelium forming folds)

Histological aspect of a trachea of cross-section from a tested chicken 5 days post infection (deciliation, hyperplasia and a substantial infiltration of the lamina propria with inflammatory cells)

Histological aspect of cross-section of a trachea from a tested chicken 5 days post infection (deciliation, epithelial hyperplasia. Lymphoid infiltration of the lamina propria, exudates)

Histological aspect of a trachea from a tested chicken 5 days post infection (deciliation, epithelial, degeneration, metaplasia, congestion and lymphoid infiltration of the lamina propria)
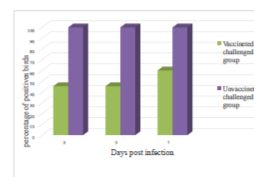

Histological aspect of cross-section of a from a tested chicken 5 days post infection (deciliation metaplasia partial flaky with undifferentiated cells, congestion and lymphoid infiltration of the lamina propria)
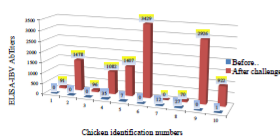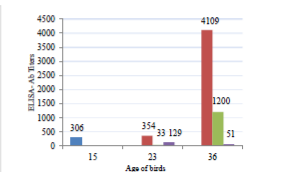

Individual evolution of the ELISA titers before and after challenge for 10 chicken
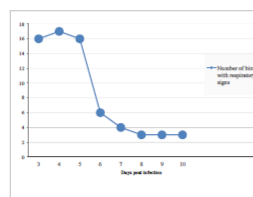
Number of birds with respiratory signs

Kinetics of virus detection by RT-PCR performed on oropharyngeal swabs for each group in days 3, 5 and 7 post infection

Evolution of the average ELISA titers before and after challenge for all groups

Conclusion and perspectives

In conclusion, Moroccan–It02 IBV is highly pathogenic for chicken and the H120 vaccine did not protect birds against clinical signs but contributed to a faster disease recovery. Further studies in the chicken model are warranted to confirm the protection against other circulating variants of IBV in Morocco.

Abstract

Author: Sajid UMAR

Title: Avian influenza and co-infections: investigation of the interactions in the poultry model

Supervisors: Maxence Delverdier & Mariette Ducatez

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The purpose of this study was to assess the burden of co-infections in the field and to better understand the possible synergism between pathogens in a laboratory setting. We focussed on *E. coli* (O78) and low pathogenic avian influenza virus (LPAIV, H6N1) in turkey model and infected the birds via the aerosol route to reproduce respiratory disease. Viral shedding and lesions were more severe and persisted longer during coinfection indicating possible enhancement of pathogenesis for LPAIV by *E. coli* and vice versa. These findings all endorse our conclusions that *E. coli* and LPAIV exercise an additive pathogenic effect in the reproduction of respiratory disease if given simultaneously or spaced by three days between the viral and the bacterial challenges to susceptible turkeys. In parallel, we studied avian respiratory agents circulating in the field in Pakistani farms. There, we focussed on co-infections as well, targeting viruses only as a first study. We observed frequent LPAIV H9 (G1 lineage) and Newcastle disease virus (genotype VII) coinfections in the field.

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Keywords: molecular epidemiology, Pakistan, respiratory viruses, *E. coli*, turkeys, coinfection

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Discipline: Pathology, Toxicology, Genetics and Nutrition

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Résumé

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Titre : Influenza aviaire et co-infections : étude des interactions dans le modèle aviaire

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Ce travail vise à estimer l'impact de co-infections sur le terrain et à mieux comprendre le synergism possible entre agents pathogènes en conditions expérimentales. Nous nous sommes intéressés à *E. coli* (O78) et au virus influenza faiblement pathogène (LPAIV, H6N1) dans le modèle dinde. Les oiseaux ont été infectés par voie aérosole pour reproduire l'infection respiratoire. L'excrétion virale ainsi que les lesions ont été plus importantes lors de la co-infection, ce qui suggère une pathogénicité accrue. Ces résultats montrent que *E. coli* et LPAIV ont un effet additif sur la maladie respiratoire lors qu'ils ont été inoculés soit simultanément soit en différé (à 3 jours d'intervalle) à des dindes naïves. En parallèle, nous avons étudié les virus respiratoires en circulation dans les élevages parkistanais. Des co-infections avec le LPAIV H9 (lignage G1) et le virus de la maladie de Newcastle (génotype VII) ont été fréquemment observées.

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Mots clés : épidemiologie moleculaire, Pakistan, virus respiratoires, *E. coli*, dinde, coinfection

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Discipline ou spécialité : Pathologie, Toxicologie, Genetique et Nutrition

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