# Immunopathogenesis of Avian Metapneumovirus in the Turkeys

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To My Parents,
Dr. Woen Suep Cha and Ms. Young Hea Jung

## Abstract

Avian metapneumovirus subtype C (aMPV/C) causes a severe upper respiratory tract (URT) disease in turkeys. The disease is characterized by viral replication and extensive lymphoid cell infiltrations in the URT. The identity of infiltrating cells and their possible involvement in the immunopathogenesis of the disease are not known. The role of local mucosal immunity in viral defense has not been examined for aMPV/C. The overall objective of the study was to examine the immunopathogenesis of aMPV/C *in ovo* and hatched turkeys, with emphasis on the involvement of local mucosal immunity in viral defense. Three specific objectives were pursued. First, the immune cells, especially mucosal T cells that infiltrate the URT of turkeys following aMPV/C exposure were characterized. Two-week-old aMPV/C antibody-free turkeys were inoculated oculonasally (O/N) with live aMPV/C. At 5 and 7 days post inoculation (DPI), lymphoid cells infiltrating the mucosal lining of the turbinates of the virus-exposed and untreated control turkeys were isolated by enzymatic treatment. In the URT, aMPV/C exposure increased the proportion of CD8+ T cells but not of CD4+ T cells. In addition, CD8 gene expression was upregulated after virus exposure whereas CD4 gene expression remained unchanged. At 5 and 7 DPI, aMPV/C-exposed turkeys showed upregulated gene

expression of IFN-γ and IL-10 in the turbinate tissue. These results suggested that aMPV/C modulated local cellular immunity in the URT of turkeys.

Secondly, the ability of an adjuvanted inactivated aMPV/C (Ad-iaMPV/C) inoculated by the respiratory route to induce protective mucosal immunity in the URT was examined. aMPV/C antibody-free turkeys were inoculated via the O/N route with inactivated virus adjuvanted with synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid (Poly IC). Ad-iaMPV/C immunized turkeys showed an increased number of mucosal IgA+ cells in the URT and increased levels of virus-specific IgG and IgA in the lachrymal fluid and serum. After 7 or 21 days post immunization, turkeys were challenged with pathogenic aMPV/C via the O/N route. Turkeys immunized with Ad-iaMPV/C were protected against microscopic lesions and the replication of the challenge virus in the URT. These observations revealed that inactivated aMPV/C administered by the respiratory route induced protective immunity against challenge with the pathogenic virus.

As the last objective, we studied the immunopathogensis and protective immunity of aMPV/C in turkeys following *in ovo* exposure. aMPV/C was inoculated into commercial aMPV/C antibody-free turkey eggs via the amniotic route at embyronation day (ED) 24. Hatchability of eggs was not affected by the virus inoculation. At the day of

hatch (ED 28) (4DPI) and 5 days post hatch (9DPI), the virus genome was detected by qRT-PCR in the turbinate, trachea and lung but not in the thymus or the spleen. Turbinate mucosa had mild lymphoid cell infiltration, and there were no detectable lesions in the lung. Spleen cells and thymus cells from virus-exposed turkeys responded poorly to T cell mitogens. In addition, IFN-γ and IL-10 gene expression was increased in the turbinate tissue of virus-exposed turkeys. *In ovo* virus exposure increased the levels of aMPV/C-specific IgG in the serum and the lachrymal fluid. At 3 weeks of age, the *in ovo* immunized turkeys were protected against a challenge with pathogenic aMPV/C. These data indicated that *in ovo* vaccination may be used in turkeys to control aMPV/C.

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# GENERAL INTRODUCTION

Avian metapneumovirus (aMPV), an important respiratory pathogen of turkeys, causes an acute, highly contagious, upper respiratory tract (URT) infection and immunosuppression. In the United States, the subtype C of aMPV (aMPV/C) is endemic in Minnesota and several neighboring Midwestern states (Goyal et al., 2000; Jirjis et al., 2000; Senne, 1997). aMPV/C isolates from the U.S. show low sequence homology with subtypes A (aMPV/A) or B (aMPV/B) that are prevalent in Europe and other countries (Seal, 2000; Shin et al., 2002). In this study, we focused on aMPV/C. aMPV/C-infected turkeys typically show clinical signs such as respiratory distress, depression, coughing, sinusitis, airsacculitis, and mortality (Goyal et al., 2000). The virus replicates in the URT of turkeys, especially in the ciliated epithelial cells of nasal turbinates and infraorbital sinuses (Jirjis et al., 2001). Usually, the main economic loss due to aMPV/C is attributed to immunosuppression and poor flock performance.

Since aMPV/C infection is acquired through the respiratory tract and the virus remains localized in the URT, local mucosal immunity likely plays an important role in the pathogenesis of the virus (Liew et al., 1984; Oien et al., 1994; Zanvit et al., 2005). We and others have noted that large numbers of lymphoid cells infiltrate the respiratory mucosa during the acute phase of infection with aMPV/C (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2001). Little is known about the phenotypes of the infiltrating cells

and their possible involvement in mucosal immunity. Recently, we identified the presence of B cells and aMPV/C-specific antibodies in the respiratory mucosa of turkeys exposed to aMPV/C (Cha et al., 2007). Quantitation of the relative proportions of mucosal IgA+, IgG+ and IgM+ cells revealed a significant increase ($P<0.05$) in the number of infiltrating IgA+ but not of IgG+ and IgM+ cells after virus exposure. Further, elevated levels of aMPV/C-specific IgA were detected in the nasal secretions and the bile of virus-exposed birds. These results indicated that local mucosal immunoglobulins were involved in the immunopathogenesis of aMPV/C in turkeys. The presence of T cells in aMPV/C-induced cellular infiltration in URT and the possible role of these cells in viral pathogenesis have not been examined. In Objective 1, we determined the phenotype (CD4/CD8) of the mucosal T cells in the aMPV/C-induced lymphoid cell infiltration and examined turbinate tissue for cytokine gene expression.

A live attenuated aMPV/C vaccine is currently being used to control aMPV/C in the Midwestern states of the U.S. although the long-term efficacy of the vaccine is not known. Efforts are likely to continue to develop more effective vaccines (Bennett et al., 2005; Chary et al., 2005; Kapczynski, 2004; Kapczynski and Sellers, 2003; Patnayak et al., 2003). In Objective 2, we examined the possibility of inducing local mucosal immunity by vaccinating turkeys with adjuvanted, inactivated aMPV/C (Ad-iaMPV/C)

administered by the respiratory route. Mucosal administration of inactivated vaccines has previously been shown to be effective against other respiratory viruses such as RSV and influenza (Etchart et al., 2006; Ichinohe et al., 2005; Oien et al., 1994; Zanvit et al., 2005). Inactivated vaccines are preferred to live vaccines which replicate in the host and are shed into the environment. Because of lack of virus shedding, inactivated vaccines may facilitate virus eradication.

*In ovo* vaccination has been used widely to administer avian vaccines both experimentally and commercially. After the first successful *in ovo* vaccination was reported for Marek's disease in 1982 (Sharma and Burmester, 1982), the technique has been used to protect birds against a number of viral infections (Ahmad and Sharma, 1992; Sharma, 1985; Sharma et al., 2002; Stone et al., 1997; Stone, 1993; Wakenell et al., 1995; Whitfill et al., 1995). The advantages of *in ovo* vaccination include early immunity, reduction in bird stress, precise and uniform dosage, ease of handling and reduced labor cost. Studies have shown that *in ovo* vaccination with aMPV/A and aMPV/B was safe and effective in both chickens and turkeys (Hess et al., 2004; Tarpey and Huggins, 2007; Worthington et al., 2003). However, *in ovo* vaccination with aMPV/C has not been examined. In addition, viral pathogenesis following *in ovo* exposure has not been studied with any subtype of aMPV. In objective 3, we examined the immunopathogenesis of

aMPV/C following *in ovo* exposure. Additionally, *in ovo* immunized turkeys were tested for protection against challenge with pathogenic virus.

# **CHAPTER I**. Literature review

## I. Avian metapneumovirus (aMPV)

### I-1 General Characteristics

#### Classification and Morphology

aMPV is classified as a member of the genus *metapneumovirus* within subfamily *pneumoviridae* of the family *paramyxoviridae.* The subfamily *pneumoviridae* consists of two genera: pneumovirus and metapneumovirus. Human respiratory syncytial virus (RSV) and mouse pneumovirus are the members of the genus pneumovirus. The genus metapneumovirus comprises human metapneumovirus (hMPV) and aMPV.

aMPV is a non-segmented, single stranded, negative sense RNA virus. The aMPV viral genome is approximately 14kb with an approximate molecular weight of 500 x $10^6$. The virus is pleomorphic, usually roughly spherical and has a diameter of 80-200nm. aMPV has the following genes: neucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), surface glycoprotein (G), and viral RNA-dependent RNA polymerase (L) (3'-N-P-M-F-M2-SH-G-L-5') (Ling et al., 1992; Lwamba et al., 2005).

#### Subtypes of aMPV

aMPV is classified into four subtypes based on nucleotide and deduced amino acid sequence data (Pringle, 1998). Subtype A (aMPV/A) or B (aMPV/B) are prevalent in

Europe and a number of other countries (Cook, 2000; Gough and Collins, 1989; Jones et al., 1988; Juhasz and Easton, 1994); subtype C (aMPV/C) is present in the U.S. and recently in South Korea (Lee et al., 2007; Seal, 2000; Shin et al., 2002). A phylogenetic study revealed that aMPV/A and aMPV/B were more closely related to each other than to aMPV/C (Seal, 1998). Subtypes D (aMPV/D) isolated in France showed low G gene sequence identity with aMPV/A, aMPV/B and aMPV/C subtypes (Bayon-Auboyer et al., 2000). Sequence data have revealed that hMPV is more closely related to aMPV/C than to the other subtypes (Toquin et al., 2003). In addition, when the four subtypes were compared in the phylogenetic analysis, aMPV/A, aMPV/B and aMPV/D were more closely related to each other than to subtype C (Bayon-Auboyer et al., 2000; Lwamba et al., 2005).

**Physical and Biochemical Properties**

An isolate of aMPV/C from turkeys in Minnesota was stable at pH 5 to 9 for one hour and lost viability after less than 12 weeks at 4°C, four weeks at 20°C, two days at 37°C, and six hours at 50°C. The virus remained viable after seven days of drying at room temperature. Disinfectants such as quaternary ammonia, ethanol, iodophor, a phenol derivative, and sodium hypochlorite (bleach) effectively decreased the viability of the virus (Jirjis et al., 2000). Similar findings were reported with the early European turkey

isolates of aMPV (aMPV/A and aMPV/B) which were stable at pH 3 to 9, inactivated at 56°C after 30 minutes and sensitive to a lipid solvent (Zhang et al., 2002). When the survival of aMPV/C in the turkey litter at various temperature was examined, the virus survived for up to 60 days at a temperature of -12°C and viral RNA was detectable in the litter kept at 8°C for over 90 days (Velayudhan et al., 2003).

**I-2 History**

aMPV was first isolated in 1978 from sinus exudates of turkeys in South Africa (Buys et al., 1989). Subsequently, aMPV isolations were reported in United Kingdom (Jones et al., 1988; McDougall and Cook, 1986), Germany (Naylor and Johns, 1993), France (Giraud et al., 1986), Netherlands (Cook et al., 1993), Brazil (Ferreira et al., 2007), Mexico (Decanini et al., 1991) and other countries around the world (Buys et al., 1989; EL Houadfi et al., 1991; Lee et al., 2007; Tanaka et al., 1995; Weisman et al., 1988). aMPV is also referred to as turkey rhinotracheitis virus (TRTV), swollen head syndrome (SHS) in chicken and avian rhinotracheitis (ART). In the United States, the first aMPV/C outbreaks were reported in Colorado in 1996 (Kleven, 1996; Senne, 1997) but they were successfully controlled by intense biosecurity measures (Kleven, 1996). In 1997, aMPV/C was later detected in Minnesota (Goyal et al., 2000). The virus became endemic

in Minnesota with an infection rate of 35-57% in commercial turkey flocks (Lauer, 2005). The first aMPV/C isolation in Minnesota (aMPV/MN/turkey/1-a/97) was made by serial passage of infected tissues from clinically sick turkeys in chicken embryo fibroblasts followed by adaptation in Vero cells (Goyal et al., 2000). aMPV/C infections in Minnesota have demonstrated a bimodal seasonal trend, with a high incidence in spring and fall (Shin et al., 2002). Within the last few year, aMPV/C has spread from Minnesota to the neighboring states of North Dakota, South Dakota, Wisconsin and Iowa (Bennett et al., 2004). Certain recent isolates of aMPV/C (MN19) were more virulent than the isolates recovered from earlier outbreaks (Velayudhan et al., 2005). In addition, aMPV/C has been isolated from wild geese indicating the possibility of possible transmission of the virus from wild birds to turkeys (Bennett et al., 2005). aMPV/C antibodies and viral genome have been detected in wild birds in Georgia, South Carolina, Arkansas and Ohio (Turpin et al., 2008).

## I-3 Pathogenesis

### Pathology

aMPV causes an acute, highly contagious URT infection in turkeys. Clinical signs of aMPV infection in turkeys consist of difficulty in breathing, sneezing and coughing, nasal

discharge, conjunctivitis, sinusitis, airsacculitis, and tracheal rales (Cook, 2000; Goyal et al., 2000; Lister and Alexander, 1986; Naylor and Johns, 1993). The virus also causes poor egg quality and a drop in egg production in breeding turkeys (Cook, 2000; Cook et al., 2000; Jones et al., 1988; Stuart, 1989). Lesions caused by aMPV are restricted to the URT (Catelli et al., 1998; Cha et al., 2007; Chary et al., 2002a; Cook et al., 1991; Cook, 2005; Jirjis et al., 2000; Jirjis et al., 2002a; Majo et al., 1995; Panigrahy et al., 2000). Histologically, all subtype of aMPV cause severe lymphoid cell infiltration in submucosa and damage to the epithelial layer in the turbinates (Cha et al., 2007; Chary et al., 2002a; Majo et al., 1995; Naylor and Johns, 1993). aMPV/A and aMPV/B but not aMPV/C have been shown to cause substantial damage to the trachea (Catelli et al., 1998; Cha et al., 2007; Chary et al., 2002a; Cook et al., 1991; Cook, 2005; Jirjis et al., 2000; Jirjis et al., 2002a; Majo et al., 1995; Njenga et al., 2003; Panigrahy et al., 2000; Stuart, 1989). aMPV/C antigen was detected in the ciliated epithelial cells of nasal turbinate and infraorbital sinuses (Catelli et al., 1998; Cha et al., 2007; Chary et al., 2002a; Cook et al., 1991; Cook, 2005; Jirjis et al., 2000; Jirjis et al., 2002a; Majo et al., 1995; Panigrahy et al., 2000; Stuart, 1989). When aMPV/C infection occurs, the morbidity can reach 100% and mortality in uncomplicated cases range from 1-5% (Jirjis et al., 2001; Jirjis et al., 2000; Shin et al., 2002). It has been shown that aMPV infection in turkeys promotes

secondary bacterial complications such as *Pasteurella spp.* and *Mycoplasma gallisepticum* infections (Cook et al., 1991; Naylor et al., 1992). Turkeys given aMPV/C along with other viral or bacterial pathogens also developed more severe clinical signs and mortality than those given virus alone (Alkhalaf et al., 2002; Jirjis et al., 2004; Shin et al., 2002). Co-infection with aMPV/C and *Bordetella avium* alone or with *Escherichia coli* and *Ornithobacterium rhinotracheale* induced a severe aMPV/C-like illness in turkeys characterized by well pronounced signs and microscopic lesions (Jirjis et al., 2004). Similarly, aMPV/C co-infected with Newcastle disease virus resulted in increased morbidity and swollen infraorbital sinuses (Shin et al., 2002).

**Host Range**

Natural hosts of aMPV are turkeys and chickens. aMPV/A and aMPV/B cause disease in both chicken and turkeys whereas aMPV/C seems to infect only turkeys (Buys et al., 1989; Cook et al., 1993; Cook, 2005; Worthington et al., 2003). Game birds may become serologically positive for aMPV (Gough et al., 2001; Welchman Dde et al., 2002). Guinea fowl and pheasants have been shown to develop swollen head syndrome following exposure to the virus (Litjens, 1980; Ogawa et al., 2001). aMPV antibodies were also detected in farmed ostriches in Zimbabwe (Cadman et al., 1994) and in seagulls in the Baltic (Heffels-Redmann et al., 1998). In United States, aMPV was detected in the

turbinates of sparrows, ducks, geese, swallows, gulls and starlings (Bennett et al., 2004; Lwamba et al., 2002; Shin et al., 2002; Shin et al., 2000). Toquin et al. (Toquin et al., 1999) also reported aMPV/C-like viruses in commercial Muscovy ducks. Following exposure, aMPV/C remained detectable in mice for up to 14 days, 6 days in rats and 21 days in waterfowl without any clinical signs (Nagaraja, 2000). Infectious aMPV/C was isolated from wild geese (Bennett et al., 2005). Several species of wild birds also had aMPV/C antibodies (Turpin et al., 2008).

**Virus Transmission**

Bird-to-bird transmission within flocks seems to occur by direct contact and inhalation of virus-infected respiratory secretions (Alexander et al., 1986; Alkhalaf et al., 2002; Cook et al., 1991; McDougall and Cook, 1986). Epidemiological studies indicate that migratory birds may play a critical role in viral transmission in different geographic regions (Bennett et al., 2004; Cook, 2000; Shin et al., 2000; Velayudhan, 2002). Sporadic reports indicate that the virus may also be transmitted vertically (Jones et al., 1988; Shin et al., 2000).

## I-4 Immune Responses

### Humoral Immunity

Turkeys develop a strong antibody response to aMPV (Cook, 1989a, 1989b). After 1-week-old turkeys were exposed to an attenuated, vaccine strain of aMPV/A, antibodies were detectable by ELISA within 6 days and persisted for at least 14 weeks (Cook, 1989b). A virulent strain of aMPV/A induced neutralizing antibodies within 5 days of the appearance of clinical respiratory disease and antibody levels began to decrease by day 13 (Jones et al., 1988). When 30-week-old laying turkeys were experimentally infected with aMPV, ELISA and serum neutralizing antibodies reached high titers by 12 days post infection and the titers were maintained through 89 days of observation (Jones et al., 1988). In aMPV/C infection, virus-specific antibody was detected in the serum from 1 to 14 weeks post inoculation, with peak levels at 5 to 7 weeks post inoculation (Jirjis et al., 2002a). The attenuated, vaccine strain of aMPV/C (passage 63) has been shown to induce irregular seroconversion in turkeys (Patnayak et al., 2002). Exposure to aMPV/C also induces mucosal IgG and IgA antibodies (Cha et al., 2007).

**Cell-mediated Immunity**

Because antibody levels are not always associated with protection against infection and some antibody-free turkeys show resistance to aMPV, it is assumed that other immune mechanisms, particularly cell-mediated immunity, are involved in viral

defense (Chary et al., 2002a; Jones et al., 1992). A study with TRTV using cyclophosphamide treatment suggested a role of cell-mediated immunity in the host defense. Cyclophosphamide-treated turkeys were resistant to virulent virus challenge despite the absence of ELISA antibodies in the serum (Jones et al., 1992). Recently, Liman *et al.* (Liman and Rautenschlein, 2007) observed both humoral and cell-mediated immune response in turkeys after aMPV/A and aMPV/B exposure. Virus exposure induced circulating virus neutralizing antibodies and increased the number of CD4+ T cells in the spleen and the Harderian gland at 7 and 14 days post infection. Further, virulent aMPV/A and aMPV/B induced increased IFN-γ expression in the Harderian gland.

**Immunosuppression**

Although aMPV-induced immunosuppression is the principal cause of economic loss in commercial turkeys, the mechanism of immunosuppression has not been well examined. TRTV has been shown to cause temporary impairment of humoral and cell-mediated immunity in turkeys during the acute phase of the disease when clinical signs are most pronounced (Timms et al., 1986). The immunosuppressed birds had an increased incidence of secondary bacterial infections. Dual infection of turkeys with *Ornithobacterium rhinotracheale* (ORT) and aMPV/A revealed that the dually infected

group had a longer persistence of ORT in the respiratory tract and enhanced macroscopic and histological lesions compared to the group given either ORT or aMPV/A alone (Marien et al., 2005). aMPV/C caused transient inhibition of *in vitro* response of spleen cells to a T cell mitogen (Chary et al., 2002a). Mitogen-stimulated splenocytes produced increased amounts of Nitric Oxide (NO) which may have contributed to reduced proliferation (Chary et al., 2002a).

**Local Mucosal Immunity**

Local mucosal immunity plays an important role in host defense against most respiratory viral infections, including paramyxoviruses. Both cellular and humoral immune responses are involved (Chang and Braciale, 2002; de Waal et al., 2003; Kimpen et al., 1992; Liew et al., 1984; Oien et al., 1994; Valosky et al., 2005; Weltzin et al., 1996; Zanvit et al., 2005). The role of local immunity in the pathogenesis of aMPV in turkeys has not been extensively examined. It have been shown that aMPV/B induced mucosal IgA in chickens (Ganapathy et al., 2005). A study with aMPV/A and aMPV/B in turkeys also revealed that the proportions of CD4+ T cells were increased in the spleen and Harderian gland at 7 and 14 days post inoculation. In addition, there was an upregulation of IFN-ɣ gene expression in the Harderian gland (Liman and Rautenschlein, 2007). Our recent studies have shown that following respiratory exposure to aMPV, there

was an increase in several populations of B cells (IgA+, IgG+ and IgM+ cells) and virus-specific IgA in the respiratory mucosa (Cha et al., 2007). Elevated levels of aMPV/C-specific IgA were detected in the nasal secretions and the bile of virus-exposed birds (Cha et al., 2007)

### I-5 Diagnosis

### Virus Isolation and Identification

#### *Isolation in Cell Culture*

Generally, the isolation of aMPV has proven to be extremely difficult because the virus has a fastidious nature and virus isolation attempts from clinical cases often result in contamination with secondary organisms (Lister and Alexander, 1986; Worthington et al., 2003). The best material source is from nasal secretions or tissue scraped from the sinuses of affected birds obtained within 6 - 7 days of virus exposure (Cook and Cavanagh, 2002; Gough et al., 1988; Worthington et al., 2003). Tracheal organ culture (TOC) of turkeys and chickens were extensively used to isolate aMPV/A and aMPV/B in Europe (Cook and Cavanagh, 2002). However, the TOC system was not suitable for the isolation of aMPV/C in the U.S. (Cook et al., 1999). Instead, multiple blind passages in chicken embryo fibroblasts (CEF) were successfully used in the primary isolation of virus from

nasal turbinate and choanal/tracheal swabs (Goyal et al., 2000; Jirjis et al., 2001; Shin et al., 2002). Multiple blind passages in a continuous quail tumor cell line (QT-35) cells were also suitable for isolating aMPV/C from infectious tissues. All aMPV/C isolates in CEF or QT-35 cells were adapted to grow in Vero cells (Goyal et al., 2000). The cytopathic effect (CPE) of aMPV in cell cultures was characterized by syncytia formation within 5-7 days of infection. Recently, Kong et al. (Kong et al., 2007) established a non-tumorigenic immortal turkey turbinate cell line (TT-1) to propagate aMPV in high titers. The same authors also revealed that serial passages of aMPV/C in Vero cells but not in TT-1 cells induced large G gene deletions (Kong et al., 2008). Virus growth in the cell cultures was inhibited by RNA interference targeting N or P genes of aMPV (Ferreira et al., 2007; Munir et al., 2006).

***Reverse Transcription-Polymerase Chain Reaction (RT-PCR)***

Molecular identification of aMPV by RT-PCR is widely used as a diagnostic test. It is more sensitive and rapid than any conventional virus isolation techniques. Generally, turbinates and choanal swabs from infected birds are considered most suitable samples for detecting viral genome by RT-PCR. Primer sequences for the RT-PCR have been designed based on several viral genes, including F, M, N and G genes (Bayon-Auboyer et al., 2000; Cavanagh et al., 1999; Cavanagh et al., 1997; Cook and Cavanagh, 2002; Dar et

al., 2001; Jing et al., 1993; Pedersen et al., 2000; Shin et al., 2000; Wu et al., 2005). Quntitative RT-PCR has also been used to determine viral load per sample (Guionie et al., 2007; Munir et al., 2006; Velayudhan et al., 2005).

### *Other Tests*

Several other assays are available to examine aMPV infection in the poultry. Indirect immunofluorescent assay (Cook and Cavanagh, 2002; Jirjis et al., 2002b; Jones et al., 1988; Naylor and Johns, 1993), immunohistochemistry staining (Jirjis et al., 2001; Shin et al., 2002) and *in situ* hybridization (Chary et al., 2002a; Velayudhan et al., 2005) have been used to detect aMPV antigen. Nucleotide sequence analysis may be employed to differentiate subtypes of aMPV (Cook and Cavanagh, 2002).

## Antibody Detection

### *Enzyme Linked Immunosorbent Assay (ELISA)*

Indirect ELISA is used most extensively to detect aMPV antibodies in turkeys and chickens as well as other avian species (Chettle and Wyeth, 1988; Cook and Cavanagh, 2002; Eterradossi et al., 1995; Grant et al., 1987; Majo et al., 1995). A number of commercial and in-house kits are available. The sensitivity of ELISA to detect antibodies varies with the kit and the antigen being used (Eterradossi et al., 1995). An ELISA based on aMPV/A or aMPV/B antigen may not detect antibodies against aMPV/C (Cook et al.,

1999). It had been suggested that antigen-specific ELISA should be used to follow serologic response in animals (Cook et al., 1999; Toquin et al., 1996). In the U.S., an ELISA using the Colorado strain of aMPV/C as a coating antigen is most commonly used (Chiang et al., 2000). ELISAs using M or N protein of aMPV/C have also been developed (Gulati et al., 2000; Gulati et al., 2001). aMPV/C-specific IgA may also be detected by ELISA (Cha et al., 2007).

***Virus Neutralization***

Virus neutralization (VN) test has been used to detect aMPV antibodies of all subtypes using susceptible cell cultures (Cook et al., 1993; Jones et al., 1988; Kapczynski et al., 2008). The VN test is time-consuming and expensive and has not been adapted for mass serological screening. aMPV/A and aMPV/B cross react in the VN test and VN antibody detection shows good correlation with ELISA and other serological tests (Alkahalaf et al., 2002; Cook and Cavanagh, 2002; Jones et al., 1988).

### I-6 Disease Control by Vaccination

For the control of aMPV/A and aMPV/B, live attenuated vaccines are commonly used and are commercially available for chicken and turkeys. A variety of cell cultures have been used to attenuate the virus (Buys et al., 1989; Cook and Ellis, 1990; Cook et al.,

1989a; Cook et al., 1989b; Jones et al., 1988). In the U. S., a live attenuated aMPV/C vaccine was developed in 2001 (Patnayak et al., 2002) and approved by the USDA. This vaccine is currently being used to control aMPV/C in the Midwestern states but the long-term efficacy of the vaccine is still questionable. Efforts are continuing to develop more efficient vaccines to control aMPV/C in the field. Cold-adapted aMPV/C has been shown to induce protective immunity in turkeys (Patnayak et al., 2003). aMPV isolated from wild geese with a large G gene was also protective in turkeys against turkey-derived aMPV/C (Bennett et al., 2005). A DNA vaccine using a bacterial vector expressing the F but not the N gene showed appreciable protection against aMPV/C in turkeys (Kapczynski and Sellers, 2003). A mixture of recombinant aMPV/C M and N proteins (Chary et al., 2005) and virosome preparations (Kapczynski, 2004) of the virus induced protective immunity in turkeys.

Inactivated oil emulsion vaccines for aMPV/A and aMPV/B are commercially available in Europe and are used widely in chickens and turkeys. The efficacy of these vaccines has not been extensively examined and much of the scientific information is provided by the manufacturers. Typically, turkey breeders are primed with live aMPV vaccine and subsequently injected parenterally with inactivated vaccines. In industry trials, when turkey breeders were given an attenuated live aMPV vaccine at 7 days of age

and injected with an inactivated vaccine at 30 weeks, the egg production drop was effectively prevented following challenge at 38 weeks. In chickens, live attenuated aMPV vaccine is given at 10-12 weeks of age and inactivated vaccine at 18-20 weeks. The use of inactivated vaccines to control aMPV/C has not been adequately examined. Recently, Kapcznski et al. (Kapczynski et al., 2008) reported that intranasal delivery of inactivated aMPV/C vaccine failed to protect turkeys from challenge.

Several investigators examined *in ovo* vaccination with aMPV/A and aMPV/B in turkeys and chickens (Hess et al., 2004; Tarpey and Huggins, 2007; Worthington et al., 2003). Inoculation of turkey eggs at 24 days of incubation with a commercial live attenuated aMPV/A vaccine did not affect hatchability of eggs. Turkeys hatching from vaccine-injected eggs developed anti-aMPV/A antibodies and resisted a challenge with virulent aMPV/A (Worthington et al., 2003). Similarly, in the chicken, *in ovo* administration of live attenuated aMPV/B did not affect hatchability and induced protective immunity (Hess et al., 2004). The use of aMPV/C as an *in ovo* vaccine has not been reported.

**CHAPTER II** Isolation and Phenotypic Analysis of T cells Infiltrating Respiratory Mucosa in Turkeys Exposed to Avian Metapneumovirus Subtype C (aMPV/C).

## II-1 Introduction

Local mucosal immunity plays an important role in host defense against respiratory and enteric viral infections (Asahi-Ozaki et al., 2006; Flynn et al., 1999; Ichinohe et al., 2005; Kiyono and Fukuyama, 2004; Liew et al., 1984; MacDonald, 2003; Oien et al., 1994), including infections with paramyxoviruses (Chang and Braciale, 2002; de Waal et al., 2003; Kimpen et al., 1992; Weltzin et al., 1996). In general, a well developed mucosal immune response prevents replication and transmission of viruses. Both cellular and humoral responses may be involved.

Among paramyxoviruses, mucosal responses to respiratory syncytial virus (RSV), which causes severe lower respiratory infection in human, have been examined in some detail. Infection with RSV resulted in modulation of T cell populations in the lung, bronchoalveolar lavage (BAL) and peripheral blood. Specifically, in the lungs, CD8+ T cells were increased but CD4+ T cells remained unchanged. Both CD8+ and CD4+ T cells were increased in the BAL and blood (Kimpen et al., 1992). Live RSV induced Type 1 (Th1)-mediated immune responses in the lung (Hussell et al., 1996; Tang et al., 1997), whereas the mice immunized with killed RSV vaccine elicited a skewed Type 2 (Th2) cytokine response (Waris et al., 1996). It was shown that different viral proteins of RSV were responsible for different cytokine profile in the lung. Viral fusion (F) protein

induced Th1 cytokines while viral attachment (G) protein induced Th2 cytokines that caused pulmonary eosinophilia (Becker, 2006; Srikiatkhachorn and Braciale, 1997). The effect of RSV on mucosal CD8+ T cells was examined in the mouse by using MHC class I tetramer loaded with M2 protein. Although the proliferation of the naïve RSV-specific CD8+ T cells were normally observed, the virus inhibited the effector activity of activated RSV M2 protein-specific CD8+ T cells infiltrating the lung (Braciale, 2005; Chang and Braciale, 2002). Local IgA responses were also important in RSV pathogenesis (Oien et al., 1994). Monoclonal IgA antibody against RSV F protein administrated intranasally protected rhesus monkeys against virulent RSV challenge (Weltzin et al., 1996).

Avian metapneumovirus subtype C (aMPV/C), a member of the genus *metapneumovirus* in the family *paramyxoviridae*, replicates in the turbinates and infraorbital sinuses (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2001) and causes a severe URT infection in turkeys. Clinical signs include respiratory distress, depression, coughing, sinusitis, airsacculitis and occasionally death (Chary et al., 2002a; Goyal et al., 2000). Virus exposure induces extensive lymphoid cell infiltrations in the URT (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2002a). We have shown previously that the infiltrating cells contained elevated proportions of IgA+ cells. Soluble aMPV/C-specific

IgA was detected in the nasal secretions and the bile of virus-exposed turkeys (Cha et al., 2007).

In the present study, our objective was to examine the presence of T cells in the lymphoid cell infiltrate of the URT. We exposed turkeys with aMPV/C and at the peak of cellular infiltration, we fractionated lymphoid cells from the turbinate tissue by enzymatic treatment. Fractionated single cells were examined for the presence of CD4+ and CD8+ T cells. In addition, we extracted RNA from the turbinate tissue and quantitated the gene expression of IFN-γ and IL-10, two important immune-regulating cytokines.

### II-2 Materials and Methods

**Virus.** The avian metapenumovirus/Minnesota/turkey/1a/1997 isolate of aMPV/C, recovered from a natural outbreak of the virus in Minnesota, was used (Goyal et al., 2000). The virus was adapted to replicate in Vero cell by 41 serial passages. The virus ($10^4$ $TCID_{50}$/bird) was given to the turkeys via the oculonasal (O/N) route.

**Turkeys.** aMPV/C antibody-free Nicolas strain of commercial turkey were obtained from Jenny-O-Turkey Store (Barron, WI). Serum from 1-day-old turkeys was examined by ELISA to ensure the absence of anti-aMPV/C antibodies. Each treatment group was maintained in separate Horsfall isolators under the regulations of the University of

Minnesota Institutional Animal Care and Use Committee. All experimental procedures related to turkeys were approved by this committee.

**Lymphoid cell isolation from turbinate mucosa.** At 5 and 7 DPI, when lymphoid cell infiltration was expected to be at peak (Cha et al., 2007), turbinates (nasal conchae) were collected from turkeys. Turbinate tissue from 3-5 turkeys was pooled, suspended in Hanks balanced salt solution (HBSS) medium supplemented with 5% FBS and cut into small pieces using surgical scissors. Warm 1mM EDTA containing HBSS was added to sheared turbinate tissues and incubated at 37°C for 20-30 minutes in a shaking incubator. After incubation, the supernatant was collected and the remaining turbinate tissue was manually crushed again to collect extra cells. Turbinate cell lysates were washed two times with 5% FBS-containing RPMI. After a final centrifugation, the cell pellet was resuspended again in 1mM EDTA containing HBSS and incubated for 5 minutes in 37°C shaking incubator. The cell lysates were washed two times with 5% FBS containing RPMI ,lymphoid cells were isolated using Ficoll-hypaque separation (Kim et al., 2000) and live cells were enumerated by Trypan blue staining. On average (n=6-7), untreated controls yielded $2.65 \times 10^5 \pm 6.2 \times 10^4$ lymphoid cells per turbinate and virus-exposed turkeys yielded $8.16 \times 10^5 \pm 8.1 \times 10^5$ lymphoid cells per turbinate.

**Histopathology.** For microscopic examination, pieces of turbinate tissue were fixed in

formalin, embedded in paraffin, sliced into 4μm sections and stained with hematoxylin and eosin (H&E). Each H&E stained section was examined at 100x magnification in a blind manner and was given a lesion score of 0-4 depending upon the severity of lymphocyte infiltration in the mucosal layers (0 = no infiltration (less than 20% of the mucosal layer showing lymphoid cell infiltration); 1= minimal (20-40%); 2 = mild (40-60%) ; 3 = moderate (60-80%) ; 4= severe (80-100%)).

**Quantitative real time RT-PCR (qRT-PCR).** qRT-PCR was used to examine gene expression of IFN-γ, IL-10, CD4 and CD8. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacture's instructions. cDNA synthesis was performed with equal amounts of RNA using Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR for turkey cytokines were developed based on the protocols for chicken genes (Hong et al., 2006; Kaiser et al., 2000; Kaiser et al., 2003; Palmquist et al., 2006). Turkey GAPDH was used as an internal control to normalize turkey gene expression. The primers for turkey IL-10, GAPDH, CD4 and CD8 were designed by Primer 3 (http://jura.wi.mit.edu/rozen/papers/rozen-and-skaletsky-2000-primer3.pdf) based on respective gene sequences from Genbank. Chicken IFN-γ primer sequence, showing 100% homology with turkey IFN-γ sequence was designed according to previously published protocols (Kaiser et al., 2000). All cytokine gene

sequences were from turkeys except IFN-γ which were from the chicken. Primer sequences used for qRT-PCR are shown in Table 1. PCR was conducted using Brilliant® II QPCR SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) and amplification and detection were performed in an automated fluorometer (Mxp3005 detection system, Stratagene, La Jolla, CA). Quantitation of cytokine genes were determined by the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). Each sample was run in duplicate and was normalized by the CT of internal control (GAPDH). Mean △CT value of untreated control birds obtained from each experiment was used to calculate fold changes compared to that of virus exposed group. qRT-PCR products of analyzed genes were sequenced to confirm specificity.

**Flow cytometry (FACS).** Flow cytomeric analysis was used to examine the proportion of T cells in the mucosal infiltrates from aMPV/C-exposed and untreated turkeys. Briefly, $5x10^5$ cells were incubated with mouse anti-chicken CD4+ antibodies (clone: CT-4) conjugated to fluorescein (FITC) and mouse anti-chicken CD8+α antibodies (clone: 3-295) conjugated to Phycoerythrin (PE) (Southern Biotech, Birmingham, AL) at 4°C for 30 min. Both antibodies cross-reacted with turkey cells (Liman and Rautenschlein, 2007; Ricks et al., 1999; Suresh and Sharma, 1995). Isotype-matched control antibody was also used to determine the level of background staining in each sample. After incubation, the

cells were washed three times with PBS containing 2% FBS. Positive cells were analyzed by FACSCento flow cytometer, CellQuest Software (Becton Dickinson, Mountain View, CA) and FACS Express software (De Novo Software, Los Angeles, CA). Based on forward and side scatter, live cells were gated and 10,000 events were analyzed for each sample (Kim et al., 2000).

**Experimental plan.** Two-week-old turkeys were divided into two groups, Groups 1 and 2. Turkeys in Group 1 were not treated (untreated control) and those in Group 2 were exposed to aMPV/C. Each turkey received $10^4$ $TCID_{50}$ of the virus via the O/N route. After 5 and 7 DPI, the choanal swabs were collected for viral genome detection by conventional RT-PCR. Consistent detection of viral genome in the choanal swabs of virus-exposed but not in untreated controls confirmed successful virus infection following virus exposure (data not shown). Turbinate tissues within each group were pooled and infiltrating lymphoid cells were isolated. Spleens were also pooled and single cell suspensions were prepared. The phenotypes (CD4/CD8) of T cells in both turbinate and spleen cells were examined by FACS analysis and total RNA extracted from each turbinate was tested for gene expression of turkey CD4 and CD8 molecules by qRT-PCR. In addition, total RNA extracted from individual turbinate tissue from birds in Groups 1 and 2 was examined for gene expression of IFN-γ and IL-10 by qRT-PCR. Three similar

experiments were conducted.

**Statistical analysis**. The Student's t-test for independent sample was performed by using Graphpad Prism 4 (San Diego, CA).

## II-3 Results and Discussion

### Relative proportion of CD4+ and CD8+ T cells and CD4/CD8 gene expression

Consistent with previous reports (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2002a), turbinates from virus-exposed turkeys had extensive lymphoid cell infiltration in the mucosal linings (data not shown). Lymphoid cells recovered from the turbinate mucosa by enzymatic treatment were tested for the presence of T cells by FACS. At 5 and 7 DPI, the proportions of CD8+ T cells but not CD4+ T cells were increased in the turbinates of aMPV/C-exposed turkeys (Figure 1A, Table 2). Virus exposure did not affect the relative proportions of CD8+ and CD4+ T cells in the spleen. Quantitation of turkey CD4 and CD8 gene expression by qRT-PCR confirmed the phenotypic data. As shown in Figure 1B, gene expression of CD8 was upregulated in aMPV/C-exposed birds, whereas CD4 gene expression remained unchanged. These results, for the first time, indicated that aMPV/C exposure promoted the recruitment of CD8+ T cells in the URT.

The presence of increased CD8+ T cells in the mucosal surface of aMPV/C-exposed

turkeys are consistent with similar responses noted with other upper and lower respiratory viruses (Baumgarth et al., 1994; de Bree et al., 2005; Wiley et al., 2001) including paramyxoviruses (Kimpen et al., 1992). Although we did not examine the role of CD8+ T cells may play in the pathogenesis of aMPV/C, studies with other respiratory infections have shown that CD8+ T cells recruited at the site of viral replication are actively engaged in restricting virus spread (Baumgarth et al., 1994; de Bree et al., 2005; Wiley et al., 2001). It is likely that aMPV/C-induced CD8+ T cells may play a similar role in the URT. We also observed that the CD8+ T cell increase noted in the turbinate was not detected in the spleen of virus-exposed turkeys. There were no differences in the relative proportions of CD8+ and CD4+ T cells in the spleen of virus-exposed or untreated control turkeys. In contrast, a study with aMPV subtypes A and B showed an increase in the percentage of CD4+ T cells in the spleen and Harderian gland of turkeys (Liman and Rautenschlein, 2007). Differences in T cell modulation underscore the variation in pathogenic mechanisms of different subtypes of aMPV. (Catelli et al., 1998; Chary et al., 2002a; Cook et al., 1991; Jirjis et al., 2000; Majo et al., 1995; Panigrahy et al., 2000)

**Cytokine gene expression in the turbinate tissue**

aMPV/C exposure increased the gene expression of IFN-γ and IL-10 (Fig 2). IL-10 gene expression was upregulated at both 5 and 7 DPI whereas IFN-γ gene expression was

increased only at 7 DPI. Respiratory viruses have been shown to upregulate Th1 and Th2 cytokines in the respiratory tract (Baumgarth et al., 1994; Hussell et al., 1996; Sun et al., 2009; Tang et al., 1997; Waris et al., 1996). IFN-γ is mainly produced by activated T cells and natural killer cells and is considered an important indicator of Th1 type immunity (Breed et al., 1997; Breed et al., 1999; Lawson et al., 2001). IL-10 has been recognized as a major anti-inflammatory cytokine serving as a negative regulator of the response of both innate and adaptive immune cells (Couper et al., 2008; Moore et al., 2001). Although we did not examine the exact cellular source of these cytokines, speculatively, elevated CD8+ T cells may have participated in the production of both local IFN-γ and IL-10. Activated CD8+ T cells have been shown to produce IFN-γ and IL-10 (Stumhofer et al., 2007; Tanchot et al., 1998). It is likely that mucosal IL-10 may be important in regulating inflammation and tissue injury associated with aMPV/C infection as shown for the influenza virus (Sun et al., 2009).

Table 1 Quantitative RT-PCR Primer Sequences

| Target sequence | Primers | Accession no. |
|---|---|---|
| IFN-γ | | Y07922 |
| Forward | 5’-GTGAAGAAGGTGAAAGATATCATGGA-3’ | |
| Reverse | 5’-GCTTTGCGCTGGATTCTCA-3’ | |
| IL-10 | | AM493432 |
| Forward | 5’-CCGAACTGCTGGATGAATTT-3’ | |
| Reverse | 5’-GCGCTTCATTGTCATCTTCA-3’ | |
| Turkey CD4 | | AM884253 |
| Forward | 5’- GAAGTCGGAGAGCAAACAGC -3’ | |
| Reverse | 5’- AAGGTCTTCATTTGCGAGGA -3’ | |
| Turkey CD8 | | AM884251 |
| Forward | 5’- CACCTACCCAGGGAATGAGA -3’ | |
| Reverse | 5’- CTGGTTGATGTTGGTGATGC -3’ | |
| Turkey GAPDH | | U94327 |
| Forward | 5’-GAGGGTAGTGAAGGCTGCTG -3’ | |
| Reverse | 5’-CATCAAAGGTGGAGGAATGG-3’ | |

Table 2 Phenotypic analysis of CD4+ and CD8+ T cells in the turbinate and spleen of aMPV/C-exposed turkeys

| Organ | Sample[1] | CD4+ T cells (%) | | CD8+ T cells (%) | |
|---|---|---|---|---|---|
| | | Untreated | aMPV/C | Untreated | aMPV/C |
| Spleen | Pool-1[2] | 35.06 | 34.43 | 42.79 | 37.8 |
| | Pool-2 | 36.67 | 40.64 | 33.96 | 41.13 |
| | Pool-3 | 40.64 | 29.41 | 41.13 | 24.91 |
| | Pool-4 | NA | 37.04 | NA | 38.99 |
| | AVE±STD | 37.46±2.87 | 35.38±4.72 | 39.29±4.69 | 35.71±7.33 |
| Turbinate | Pool-1 | 33.63 | 33.70 | 25.5 | 40.45 |
| | Pool-2 | 33.04 | 30.59 | 23.47 | 31.29 |
| | Pool-3 | 33.97 | 37.11 | 22.71 | 33.96 |
| | Pool-4 | 49.11 | 35.48 | 25.24 | 38.04 |
| | AVE±STD | 37.44±7.79 | 34.22±2.79 | 24.23±1.36 | 35.94±4.09* |

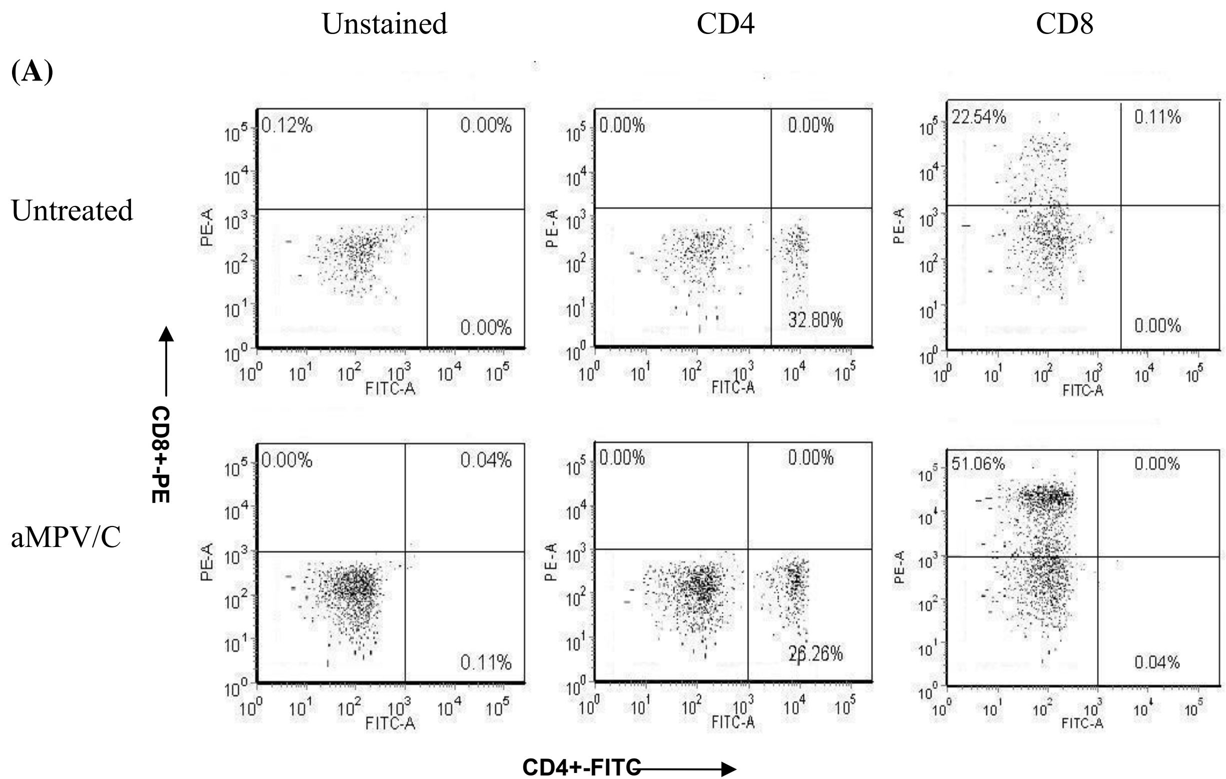
[1] Two-week-old turkeys were inoculated via O/N route with aMPV/C. At 5 and 7 DPI, lymphocytes were isolated from turbinate of untreated and virus-exposed turkeys and examined for CD4+ and CD8+ T cells by FACS analysis. The table represented the combined data of 7 DPI from two experiments.

[2] 1 pool = 3-5 birds

NA=Not Available; AVE±STD= Average ± Standard deviation.

*= $P<0.05$

**(A)**




(B)

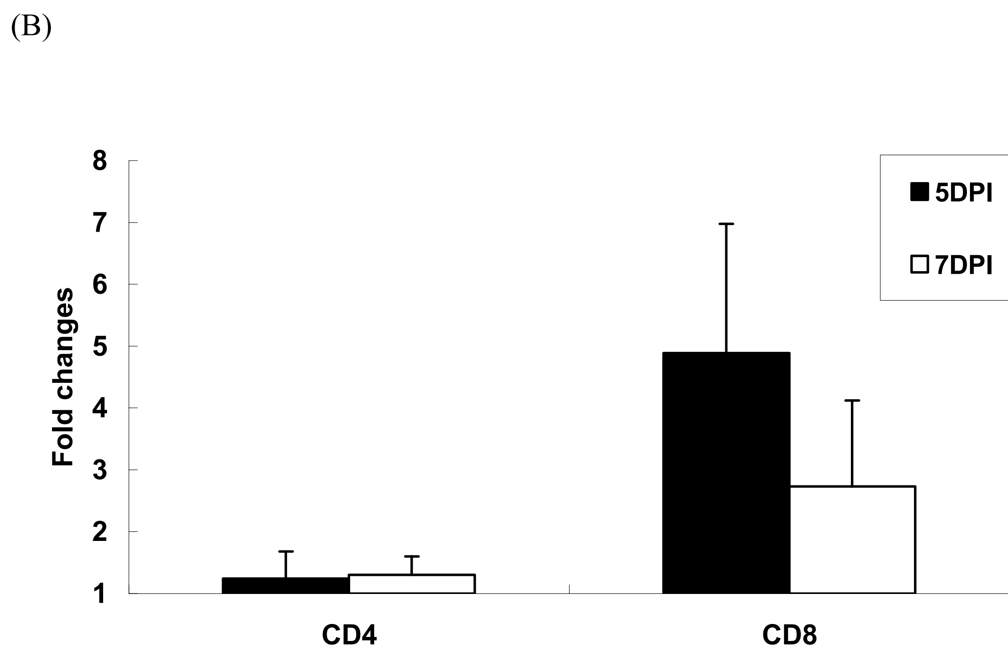



Figure 1 Proportion of CD4+ and CD8+ T cells and CD4/CD8 gene expression in the turbinate tissue. Two-week-old turkeys were inoculated via O/N route with aMPV/C. At 5 and 7 DPI, lymphocytes isolated from turbinate of untreated and virus-exposed turkeys were examined for CD4+ and CD8+ T cells by FACS. Representative dot plot of

turbinate lymphoid cells obtained at 5 DPI is shown (A). Total RNA extracted from the turbinate tissue was examined by qRT-PCR for turkey CD4 and CD8 gene expression (n=3) (B).

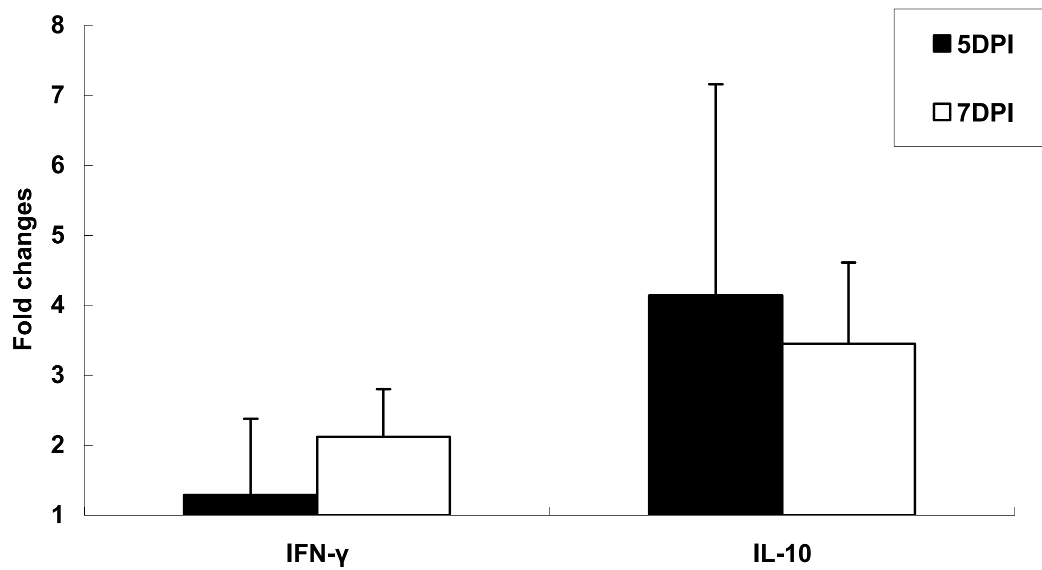



Figure 2 Cytokine gene expressions in the URT. Two-week-old turkeys were inoculated via O/N route with aMPV/C. At 5 and 7DPI, total RNA from the turbinate tissue were tested for gene expression of IFN-γ and IL-10 by qRT-PCR. Values expressed are the means ± Standard Deviation. Data for 5 DPI examined from two experiments (n=6) and 7DPI examine from one experiment (n=3-4).

# **CHAPTER III** Protection against Avian Metapneumovirus Subtype C (aMPV/C) in Turkeys Immunized via the Respiratory Tract with Inactivated Virus.

## III-1 Introduction

Inactivated vaccines administrated via the respiratory mucosa have been shown to induce protective immunity against a number of respiratory viruses including paramyxoviruses, particularly respiratory syncytial virus (RSV). In RSV, intranasal delivery of inactivated RSV increased the frequency and the duration of virus-specific respiratory plasma cell populations, protected mice against live RSV challenge and reduced the Th2 effects routinely seen after challenge of vaccinated animals (Etchart et al., 2006). In another study, intranasal vaccination with RSV chimeric FG glycoprotein induced RSV-specific local IgA and IgG antibodies and protected the URT in mice (Oien et al., 1994). Intranasal administration of inactivated influenza vaccine with a mucosal adjuvant induced cross-protective immune responses against avirulent and highly pathogenic influenza viruses (Asahi-Ozaki et al., 2006; Ichinohe et al., 2005). Mucosal immunization with inactivated influenza virus also resulted in a marked increase of both systemic and mucosal in IgG and IgA and increased systemic and local cytokine production (Zanvit et al., 2005).

Avian metapneumovirus (aMPV) is a member of the genus *metapneumovirus* within family *paramyxoviridae* and a pathogen of economic importance because it causes severe upper respiratory tract (URT) infection in turkeys (Njenga et al., 2003). Infected

turkeys become immunocompromized and perform poorly. There are three subtypes of the virus, A, B and C. In the United States, only subtype C (aMPV/C) has been identified (Bennett et al., 2004; Seal, 2000; Senne, 1997; Shin et al., 2002). Subtype A (aMPV/A) or B (aMPV/B) are prevalent in the Europe and other countries. aMPV/C replicates in the URT and induces lymphoid cell infiltrations in the mucosa (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2001). Current control of aMPV/C in the endemic areas comprises the use of a live attenuated aMPV/C vaccine. The efficacy of this vaccine has not been clearly established and efforts are underway to develop more efficient vaccines (Bennett et al., 2005; Chary et al., 2005; Kapczynski, 2004; Kapczynski and Sellers, 2003; Patnayak et al., 2003). Because the current vaccine contains live virus, vaccinated birds shed the virus in the environment which makes eradication difficult.

Our objective was to examine the possibility of inducing mucosal immunity by vaccinating turkeys with adjuvenated inactivated aMPV/C (Ad-iaMPV/C) administered by the respiratory route. Inactivated vaccines used to control aMPV/A and aMPV/B, are routinely administered via parenteral route.

## III-2 Materials and Methods

**Virus and adjuvant.** The Avian metapenumovirus/Minnesota/turkey/1a/1997 isolate of

aMPV/C at the $41^{st}$ passage in Vero cells (provided by Dr. S. Goyal of the University of Minnesota) was inactivated and used as a vaccine (Goyal et al., 2000). The inactivated virus was given to turkeys via the intranasal (I/N) or the oculonasal (O/N) routes. Formalin inactivation of aMPV/C was performed as previously described with modifications (Oumouna et al., 2005). Briefly, aMPV/C-infected Vero cells were freeze-thawed 2-3 times and the lysate was fractionated by centrifugation for 15 min at 4000 rpm to remove cell debris. Virus titer before inactivation was $10^5$ $TCID_{50}$/ml. For inactivation of the virus, 37% formalin (Sigma, St. Louis, Mo) was added to the clarified virus suspension at a dilution of 1:4000. After 3 days at 37°C, the virus was completely inactivated and did not produce detectable CPE in Vero cells. The inactivated aMPV/C (iaMPV/C) lysate was pelleted by ultra centrifugation for 1hr at 17,600 rpm using a SW32i rotor (Beckman Coulter) and the pellet was resuspended in 1/25 of the original volume in MEM containing 2% FBS. iaMPV/C (200µl/bird) was adjuvanted with 120µl per bird of synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid (Poly IC)) (Sigma, St. Louis, MO) (Ad-iaMPV/C). Total of 320 µl of Ad-iaMPV/C were inoculated per turkey. The safety of Poly IC in turkeys was examined in a preliminary trial in which four groups of one-day-old turkeys each were used. Three groups received 20ug, 40ug and 60ug of Poly IC respectively. The fourth group received PBS. Intranasal

inoculations of Poly IC or PBS were repeated daily for 7 days. Body weight gain was monitored daily and no consistent significant differences were found between groups. Gross or microscopic lesions were not detected in the URT (data not shown). At various intervals following immunization with Ad-iaMPV/C or iaMPV/C, turkeys were challenged with $5x10^3$ $TCID_{50}$ of aMPV/C via the O/N route. The 41st Vero cell passage of aMPV/C was used as the challenge virus. This virus causes extensive lymphoid cell infiltrations in the URT (Cha et al., 2007).

**Turkeys.** Commercial Nicolas strain turkeys were obtained from Jenny-O-Turkey Store (Barron, WI). In Experiments I and II, the commercial turkeys seropositive for anti-aMPV/C antibodies were used. Turkeys used in Experiments III, IV and V were seronegative of such antibodies as confirmed by virus-specific ELISA described below. All experimental groups of turkeys were maintained in separate Horsfall isolators under the regulations of the University of Minnesota Institutional Animal Care and Use Committee (IACUC). All experimental procedures were approved by this committee.

**Enzyme-linked immunosorbent assay (ELISA).** An aMPV/C-specific IgG ELISA was performed as described previously with modifications (Chiang et al., 2000). Blood samples were collected from the wing vein of the turkeys and serum was obtained. Lachrymal fluid were collected by sodium chloride stimulation as described previously

(Ganapathy et al., 2005). The test sera were diluted 1:40 and tears were diluted 1:10 in a dilution/blocking buffer (KPL ,Gaithersburg, MD) and 50μl of samples per well were added to a 96-well plate coated with aMPV/C antigen or control antigen (Vero cell extract). After one hour, HRP-conjugated goat anti-turkey IgG antibody (KPL, Gaithersburg, MD) was added as a secondary antibody. Tetramethylbenzidine (TMB) was used as a developing reagent. An aMPV/C-specific IgA ELISA was performed as described previously (Cha et al., 2007). 50μl of diluted tear sample per well were added to a 96-well plate coated with aMPV/C antigen or control antigen (Vero cell extract). After 1 hour, goat anti-chicken IgA (Bethyl Lab, Montgomery, TX) was added to each well. After one hour incubation, HRP-conjugated rabbit anti-goat IgG antibody (KPL, Gaithersburg, MD) was added as a secondary antibody. TMB was used as a developing signal in each well. The plates were washed 3 times with 0.05% Tween-20 containing PBS between each step. All reactions except TMB treatment were done at 37°C and the TMB treatment was conducted at room temperature.

**Semi-quantitative RT-PCR.** The semi-quantitative RT-PCR was used to examine interferon type I (IFN-I), inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression in the URT as previously described with modification (Cha et al., 2007). Briefly, total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA

synthesis was performed with equal amounts of total RNA from each sample using Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA) following manufacture's instruction. Primers for turkey IFN-I and GAPDH were designed by Primer3 (http://jura.wi.mit.edu/rozen/papers/rozen-and-skaletsky-2000-primer3.pdf) based on respective gene sequences from Genbank. The sequence of IFN-I forward primer used for PCR was 5'-CTGCTGCTCACGCTCCTTAT-3' and reverse primer was 5'-GATGGTGAGGTGAGGGTTGT-3' (product size:399bp). The sequence for GAPDH forward primer was 5'-CTCATCTGAAGGGTGGTGCT-3' and reverse primer was 5'GGATGCTGGGATGATGTTCT-3' (product size: 302bp). Published primer sequences for chicken Cox-2 and iNOS were used (Cha et al., 2007). The standardization for each primer set was conducted to determine the optimal number of cycle needed for product amplification during the exponential phase. Based on this characterization, 33 cycles were used for turkey IFN-I, 22 cycles for turkey GAPDH, 35 cycles for chicken COX-2 and 32 cycles for iNOS. After PCR, 5-6μl of the PCR product were used for electrophoresis on a 1.2% agarose gel, and visualized by ethidium bromide staining for 10-15 minutes. Scanning densitometry was used to determine the maximum OD value of each band in the agarose gel and each sample were normalized by the value from the GAPDH (internal control) counterpart.

**Histopathology.** Turbinate tissues were fixed in formalin, embedded in paraffin, and sliced into 4μm sections. Sections were stained with hematoxylin and eosin (H&E) to examine microscopic lesions. Each H&E slide was examined at 100x magnification in a blind manner and was given a score of 0-4 depending upon the severity of lymphocyte infiltration in the mucosal layers (0 = no infiltration (less than 20% of the mucosal layer showing lymphoid cell infiltration) 1= minimal (20-40%); 2 =mild (40-60%) ; 3 = moderate (60-80%) ; 4= severe (80-100%)).

**Immunohistochemistry (IHC).** IgA+ cells in paraffin embedded sections were identified by IHC staining as previously described with some modifications (Cha et al., 2007; Ohshima and Hiramatsu, 2002). Briefly, Goat anti-chicken polyclonal IgA antibody (Bethyl Lab, Montgomery, TX) and goat anti-turkey polyclonal IgG antibody (Sourthern Biotech, Birmingham, AL) were used as the primary antibodies at a dilution of 1:500. Previous studies have shown that the anti-chicken IgA and IgM antibodies cross-react with turkey immunoglobulin (Koci et al., 2004; Loock et al., 2006; Van Nerom et al., 1997). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Bethyl Lab, Montgomery, TX) was used as the secondary antibody at a dilution of 1:250. To quantify the number of IgA+ cells per tissue, 10 random microscopic fields (600X) of mucosal lining were counted for positively stained cells in each tissue sections and the numbers

were added to represent the counts for each turkey.

**Quantitative real time RT-PCR (qRT-PCR).** qRT-PCR using aMPV/C M protein was used to quantify aMPV/C viral load (Munir et al., 2006; Velayudhan et al., 2005). Briefly, viral RNA was extracted from choanal swabs with the Viral RNA Extraction Kit (Qiagen, Valencia, CA) following manufacture's directions. cDNA synthesis were performed with equal amounts of RNA using Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The primers for aMPV/C M protein were designed by Primer 3 (http://jura.wi.mit.edu/rozen/papers/rozen-and-skaletsky-2000-primer3.pdf) based on respective gene sequences information from Genbank. Real time quantitative RT-PCR were conducted using Brilliant® II QPCR SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) and amplification and detection were performed in an automated fluorometer (Mxp3005 detection system, Stratagene). For aMPV/C gene expression, viral copy number was calculated by the standard curve established by viral RNA extracted from the series of 10-fold dilution of previously titrated aMPV/C. Each quantitative RT-PCR product was sequenced to confirm specificity. The aMPV/C-M-forward primer sequence used was 5'-CGGGAAGAAACACACGATT-3' and aMPV/C-M-reverse primer sequence was 5'-CTTGATCTGCCTCCCCACTA-3' (product size: 155bp).

**Experimental design.** Two-week-old turkeys were inoculated with iaMPV/C via the I/N route (Exp I) or the O/N route (Exp II). In both experiments, the inoculations were repeated two weeks later. At 7 days following the second inoculation, turbinate tissue was examined by IHC for IgA+ cells. In Exp III, two-week-old turkeys were divided into four groups (Groups 1-4) and treated as follows: Group 1: untreated, Group 2: iaMPV/C, Group 3: Poly IC alone, and Group 4: Ad-iaMPV/C. Groups 2-4 received each dose via the O/N route. At 1 day post inoculation (DPI), turbinate and spleen tissue were collected and tissue homogenates were examined for gene expressions of IFN-I, Cox-2 and iNOS by semi-quantitative RT-PCR.

Exps IV and V were similar in design. One-day-old turkeys, free of antibodies against aMPV/C, were divided into three groups and treated as follows: Group 1: untreated, Group 2: Poly IC alone, Group 3: Ad-iaMPV/C. In Groups 2 and 3, turkeys were given three consecutive inoculations at 1, 10 and 17 days of age via the O/N route. Preliminary experiments indicated that three consecutive inoculations were needed to obtain best immune response. At 21 (Exp. IV) or 7 (Exp. V) days after the final inoculation, each turkey was challenged by the O/N route with 5000 $TCID_{50}$ of aMPV/C. Serum and lachrymal fluid obtained before challenge were examined by ELISA for aMPV/C-specific IgG and IgA antibodies. Protection against challenge virus was

determined at 5 days post challenge. Immunized turkeys were examined for their ability to resist detectable lesion formations in the URT and the replication of the challenge virus in the respiratory mucosa. Virus replication was examined by qRT-PCR.

**Statistical analysis.** The Student t-test for independent sample was performed by using Graphpad Prism 4 (San Diego, CA).

## III-3 Results

### Induction of IgA+ cells in the respiratory mucosa by iaMPV/C

Previously, we have shown that live aMPV/C increased the numbers of IgA+ cells infiltrating the turbinate mucosa of turkeys (Cha et al., 2007). The data on the ability of inactivated virus to induce IgA+ cells in the turbinate mucosa are shown in Table 1. In Exp. I, turbinate mucosa of Ad-iaMPV/C-treated birds had significantly higher of IgA+ cells than untreated controls ($P<0.05$). Although Exp. II did not show a statistically significant increase in IgA+ cells ($P>0.05$), when data from Exps I and II were combined, and thus increasing the number of birds at risk, the differences in IgA+ cells between Ad-iaMPV-treated and untreated controls were significant ($P<0.05$). These data indicated that respiratory exposure to Ad-iaMPV/C stimulated a local immune response in the URT.

### Gene activations in the turbinate tissue by Ad-iaMPV/C

Turkeys were given Ad-iaMPV/C by the O/N route. One day later, RNA was extracted from homogenized turbinate tissue and spleen. The data shown in Fig 1 indicated that Ad-aMPV/C upregulated the expression of IFN-I, iNOS and Cox-2 in the turbinate but not in the spleen (spleen data not shown). This result showed that deposition of Ad-aMPV/C in the respiratory tract upregulated the genes commonly associated with immune stimulation.

**Induction of mucosal and systemic antibody by Ad-iaMPV/C**

The data from Exps. IV and V**,** shown in Table 2, indicated that the application of Ad-iaMPV/C in the URT induced aMPV/C-specific IgG which was detected in the serum as well as lachrymal fluid. Ad-iaMPV/C also induced aMPV/C-specific IgA in the lachrymal fluid. Thus, respiratory immunization with the inactivated virus induced specific systemic and local immunity.

**Protection against challenge by mucosal immunization with Ad-iaMPV/C**

Protection against challenge was examined by lesion formation and replication of the challenge virus in the URT at 5 days after challenge. In Exps. IV and V, turkeys given Ad-iaMPV/C via the respiratory tract were protected against challenge with a pathogenic virus. Immunized and challenged turkeys did not develop detectable lymphoid cell infiltration in the turbinate mucosa whereas extensive infiltration was noted in unimmunized and challenges turkeys (Fig. 2). Similarly, at 5 days after challenge, the Ad-

iaMPV/C-immunized group had significantly lower (P<0.05) viral copies in the turbinate tissue than the unimmunized group or the group immunized with Poly IC alone (Table 3). Thus the data from two challenge experiments indicated that respiratory immunization with Ad-iaMPV/C protected the URT against subsequent exposure to virulent virus.

**III-4 Discussion**

This study revealed that Ad-iaMPV/C given by the O/N route induced an infiltration of IgA+ cells in the turbinate mucosa and upregulated IFN-I, iNOS and Cox-2 gene expression in the turbinate tissue. In addition, the vaccine induced virus-specific IgG and IgA in the lachrymal fluid and IgG in the serum. Immunized turkeys also resisted a respiratory challenge with pathogenic aMPV/C. Upon challenge, the immunized group had significantly lower (P<0.05) levels of challenge virus than unimmunized controls. This is the first report showing that an inactivated virus preparation of aMPV/C was effective as a respiratory vaccine against virulent challenge. This method of immunization should be highly desirable in virus eradication efforts because the vaccine virus is non-replicating and vaccinated birds will not shed live virus in the environment. Persistent use of a vaccine similar to Ad-iaMPV/C in successive flocks should reduce environmental contamination with aMPV/C and make eradication possible. Additional studies are needed to examine the optimum vaccine dose needed to induce persistent and

long-lasting immunity.

Mucosal immunization with non-replicating or inactivated vaccines has been shown to successfully protect the host against other respiratory viruses. For example, inactivated RSV given by the intranasal route protected mice against live RSV challenge and reduced the Th2 effect routinely seen after challenge of vaccinated animals (Etchart et al., 2006). Intranasal administration of an adjuvanted RSV chimeric FG glycoprotein also induced protection in the URT (Oien et al., 1994). When inactivated influenza HA vaccine with mucosal adjuvant (Poly IC) was inoculated via the respiratory tract, cross-protective immune responses were induced against both avirulent and highly pathogenic influenza viruses in mice (Asahi-Ozaki et al., 2006; Ichinohe et al., 2005).

In a previous study, inactivated aMPV/C given by the mucosal route did not protect turkeys against intranasal virus challenge (Kapczynski et al., 2008). The reasons for the discrepancy between this study and our results are not known. The two studies had conflicting experimental designs and methods of virus inactivation and presentation. Although both studies used formalin-inactivated aMPV/C, we concentrated inactivated virus 25 times to increase the viral load per administration and mixed the virus with Poly IC, a potent respiratory adjuvant (Asahi-Ozaki et al., 2006; Ichinohe et al., 2005; Sloat and Cui, 2006). Poly IC is a Toll-like receptor 3 agonist and mimics the function of

molecular viral dsRNA produced during their replication (Shin et al., 2002). The efficacy of Poly IC as a mucosal adjuvant has been well documented (Asahi-Ozaki et al., 2006; Ichinohe et al., 2005; Sloat and Cui, 2006). Preliminary studies indicated that CpG and CTB, two commonly used respiratory adjuvants, were not as effective in turkeys as Poly IC.

Although we did not examine the mechanism of protection with inactivated aMPV/C, previous studies with other inactivated viruses have suggested that local antibody responses, particularly IgA, play an important role. Inactivated RSV adjuvanted with bacterial outer membrane vesicles induced protection in mice which was associated with virus-specific mucosal IgA+ cells and high levels of IgA in the lung (Etchart et al., 2006). IgA response in the nasal wash induced by inactivated influenza hemaglutinin vaccine was also associated with protection (Asahi-Ozaki et al., 2006; Ichinohe et al., 2005; Zanvit et al., 2005). Similarly, protective immune responses against human immunodeficiency and simian immunodeficiency viruses with mucosal adjuvenated subunit vaccines were associated with mucosal IgG and IgA antibodies (Albu et al., 2003; Kang et al., 2003). In turkeys immunized with Ad-iaMPV/C, we noted increased levels of aMPV/C-specific IgG and IgA in the serum and the lachrymal fluid. Inactivated virus preparations may also induce cell-mediated immunity which may be important for

protection. A role of T cell immunity in protection induced by inactivated infectious bursal disease virus was clearly shown in T cell-compromised chickens (Sharma et al., 2002). Several mammalian inactivated viruses have been shown to induce virus-specific cytotoxic T cells (Guzman et al., 2008; Jiang et al., 2005; Sawai et al., 2008). Additional studies are needed to identify if Ad-iaMPV induces T cell immunity in turkeys and how antibodies and cellular immune responses are involved in modulating viral pathogenesis.

Table 1 IgA+ cells in the URT induced by mucosal immunization with Ad-iaMPV/C.

| Experiment[1] | Treatment | Mean of IgA+ cells by IHC |
|---|---|---|
| | | Turbinate |
| | | 7 DPI[2] |
| Exp I (n=2-3) | none | 44± 4.04[3,a] |
| | Ad-iaMPV/C | 102± 3.54[b] |
| Exp II (n=4-5) | none | 29.60±4.93[a] |
| | iaMPV/C | 37.33±11.15[a] |
| | Ad-iaMPV/C | 49.50±23.23[a] |
| Exp I+II (n=6-8) | none | 35± 8.76[3,a] |
| | Ad-iaMPV/C | 67± 32.36[b] |

[1] Two-week-old commercial turkeys were inoculated via I/N (Exp I) or O/N (Exp II) route. The second dose was given two weeks later.

[2] Ten random microscopic fields (600X) of mucosal lining were counted for positively stained cells and the numbers were added to represent the counts for each turkey.

[3] Mean IgA+ cells per turkey ± standard deviation.

[a,b] Different superscripts within each experimental group indicate statistical differences ($P<0.05$).

Table 2 aMPV/C-specific IgG and IgA induced by mucosal immunization with Ad-iaMPV/C

| Experiment[1] | Vaccine | Time of collection after last immunization[1] | Mean OD value ± STD (ELISA) | | |
|---|---|---|---|---|---|
| | | | aMPV/C-specific IgG | | aMPV/C-specific IgA |
| | | | Tears | Serum | Tears |
| IV(n=4-5) | none | 21 days | 0.00±0.00[a] | 0.00±0.01[a] | 0.00±0.04[a] |
| | Ad-iaMPV/C | | 0.78±0.49[b] | 0.37±0.33[b] | 0.16±0.11[b] |
| V (n=7-8) | None | 7 days | 0.00±0.01[a] | 0.00±0.00[a] | 0.00±0.07[a] |
| | Adjuvant | | 0.01±0.04[a] | 0.00±0.01[a] | 0.00±0.01[a] |
| | Ad-iaMPV/C | | 0.18±0.10[b] | 0.09±0.09[b] | 0.09±0.12[b] |

[1]: One-day-old aMPV/C-free turkeys were immunized via O/N route with Ad-iaMPV/C and inoculation was given 10 days later and final immunization was given 7 days after second booster. Serum and tear were collected at 7 days or 21 days after final immunizations.

Different superscripts within each experiment indicate statistical differences ($P<0.05$).

Table 3 Protection against challenge in turkeys immunized with Ad-iaMPV/C by the respiratory route

| Experiment | Time of challenge after last vaccination[1] | Treatment | | H&E staining[2] | Real-time RT-PCR[3] |
|---|---|---|---|---|---|
| | | Vaccine | aMPV/C Challenge | Mean lesion Score | Viral copy number/bird |
| IV(n=3-5) | 21days | None | - | 1.0±0.71[a] | 0.00±0.00 [b] |
| | | None | + | 2.2±0.84[b] | $4.9x10^4$± 22933.97[a] |
| | | Ad-iaMPV/C | + | 0.4±0.55[a] | $3.2x10^3$±4170.89[b] |
| V(n=5-8) | 7days | None | - | 1.0±0.82[a] | 6.96±3.77 [b] |
| | | None | + | 2.57±1.27[b] | $4.24x10^3$±1700.64[a] |
| | | Poly IC | + | 2.33±0.87[b] | $4.35x10^3$±3996.34[a] |
| | | Ad-iaMPV/C | + | 1.11±1.05[a] | $7.19x10^2$±657.06[b] |

[1] One-day-old aMPV/C-free turkeys were immunized oculonasally with Ad-iaMPV/C or adjuvant alone. Second inoculation was given 10 days later and final inoculation was given 7 days after second booster. At various days later, turkeys were challenged with 5000 $TCID_{50}$ of aMPV/C via O/N route. Turbinate and choanal swabs on respiratory mucosa were collected after 5 days post challenge for H&E staining and real time quantitative RT-PCR.

[2] Each H&E section was examined in blind manner given lesion score using scale established the severity of lymphocyte infiltrations (0: <20%; 1: 20-40%; 2: 40-60%; 3: 60-80%; 4: 80-100%).

[3] At 5 days post challenge, choanal swabs were collected for viral RNA extraction, viral load were tested by aMPV/C real time quantitative RT-PCR. Standard curve were established by known titered aMPV/C to calculate viral copy number of each sample.

Different superscripts within each experiment indicate statistically significant differences ($P<0.05$).

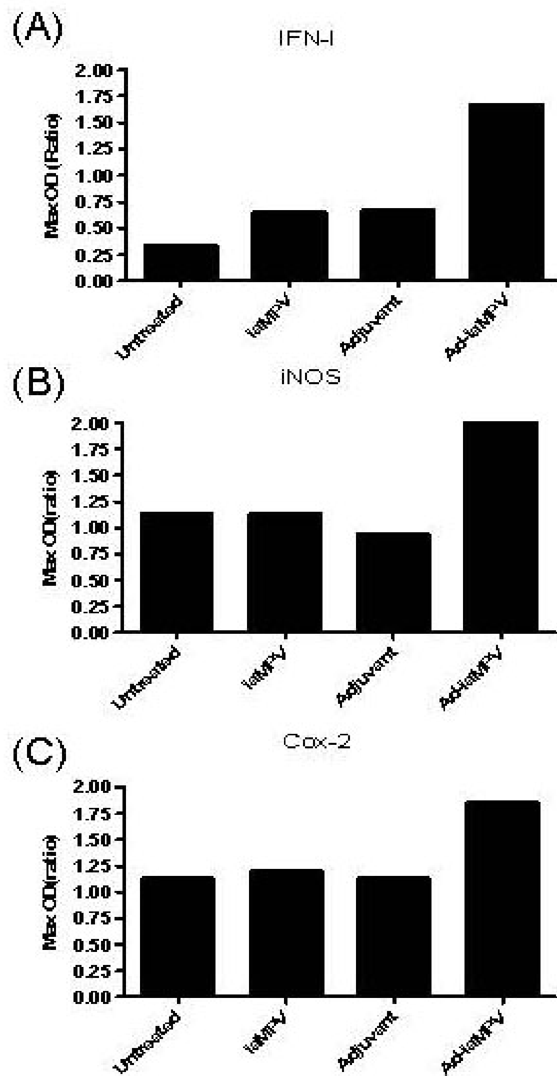



Figure 1 Upregulation of IFN-I, iNOS and Cox-2 gene expressions by Ad-iaMPV/C in the turbinate tissue (Exp III). Two-week-old turkeys were inoculated by the O/N route with iaMPV/C, adjuvant alone or Ad-iaMPV/C. At 1 day after inoculation, total RNA collected from the turbinate tissues were tested for (A) IFN-I, (B) iNOS and (C) Cox-2 gene expression by semi-quantitative RT-PCR. Maximum OD (Max OD) value of each band density were detected by the NIH image software and normalized with the GAPDH counterpart and the data were express as ratio of Max OD value.

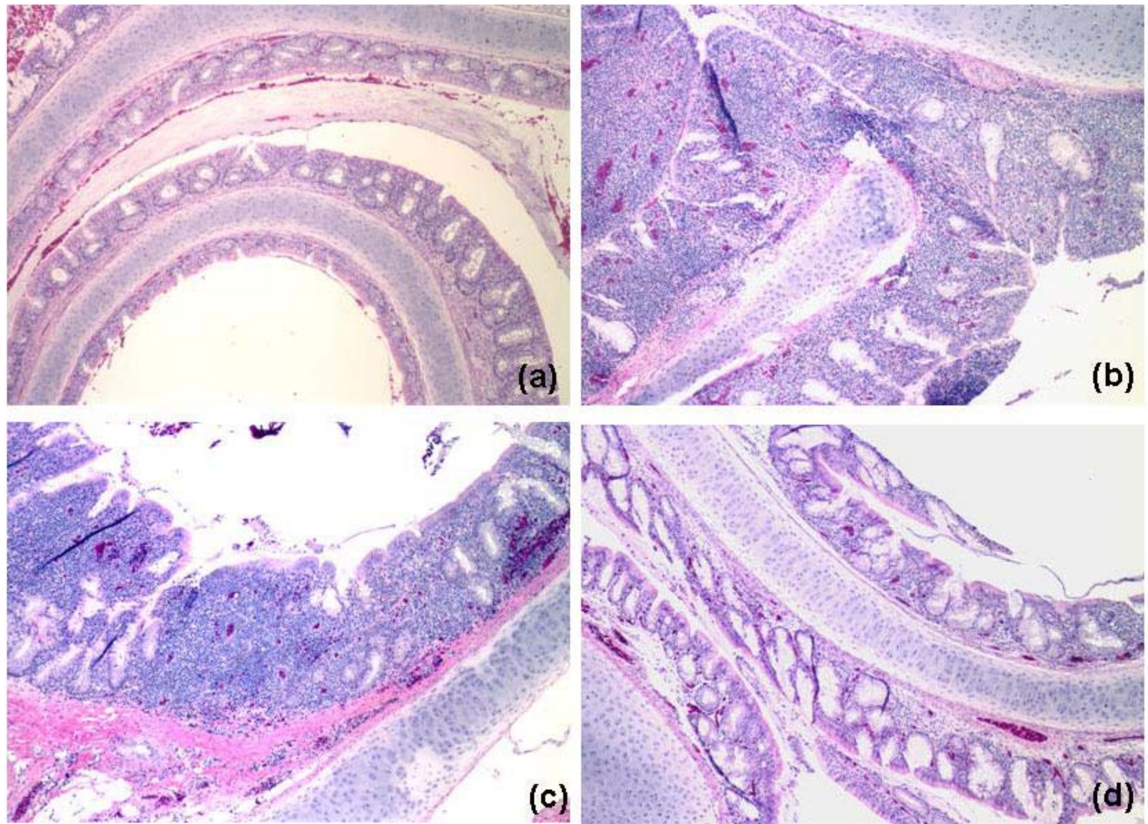



Figure 2 Protection against lesion formation in the URT by the challenge virus.
One-day-old turkeys were given three consecutive O/N inoculations of Ad-iaMPV/C at 1, 10, and 17 day of age respectively. After 7 days post final immunization, birds were challenged with aMPV/C (Exp. IV). At 5 days post challenge, turbinate tissue was examined histologically. (A) Untreated-unchallenged group, (B) Untreated-challenged group. (C) Poly IC-challenged group (D) Ad-iaMPV/C immunized-challenged group. Note extensive lymphoid cell infiltration induced by the challenge virus in unimmunized turkeys in (B) and (C).

# **CHAPTER IV** Pathogenic and immunogenic responses in turkeys following *in ovo* exposure to Avian Metapneumovirus Subtype C

## IV-1 Introduction

*In ovo* vaccination, first reported in 1982 with Marek's disease (Sharma and Burmester, 1982) has been used to protect chickens against a number of viral infections (Ahmad and Sharma, 1992; Sharma, 1985; Sharma et al., 2002; Stone et al., 1997; Stone, 1993; Wakenell et al., 1995; Whitfill et al., 1995). In this procedure, live viral agents are inoculated into the amniotic sac of late-stage embryos. Multiple viral agents may be combined in one injection and induce protection against all the components in the mixture (Sharma et al., 2002). The viral agents inoculated into eggs replicate in a variety of embryonal tissues although the host-viral interactions vary among viruses (Ahmad and Sharma, 1992; Cha et al., 2007; Corley and Giambrone, 2002; Corley et al., 2002; Guo et al., 2004; Hess et al., 2004; Rautenschlein and Haase, 2005; St Hill and Sharma, 2000; St Hill et al., 2004; Worthington et al., 2003; Zhang and Sharma, 2003). *In ovo* vaccination in chickens against Marek's disease virus alone or in combination with other agents such as infectious bursal disease virus has become an industry standard in the U.S. and a number of other countries (Schijns et al., 2008)

Avian metapneumovirus (aMPV) is an important naturally occurring pathogen that causes upper respiratory tract (URT) infection and immunosuppresion in turkeys. There are three principal subtypes of the virus: A, B and C. Subtypes A (aMPV/A) and B

(aMPV/B) are known to be prevalent in Europe and other countries whereas subtype C (aMPV/C) has been detected in the U.S. (Seal, 2000; Shin et al., 2002) and recently in South Korea (Lee et al., 2007). There are major differences in the host range and pathogenesis of aMPV/C in comparison with aMPV/A and aMPV/B. aMPV/C causes natural infection in turkeys but not in chickens; aMPV/A and aMPV/B infect both chickens and turkeys (Buys et al., 1989; Cook et al., 1993; Cook, 2005). Although lesions induced by all three subtypes remain localized in the URT, aMPV/A and aMPV/B but not aMPV/C cause substantial damage to the trachea (Catelli et al., 1998; Chary et al., 2002a; Cook et al., 1991; Cook, 2005; Jirjis et al., 2000; Jirjis et al., 2002a; Majo et al., 1995; Panigrahy et al., 2000).

Currently, aMPV/C in commercial turkey flocks in U.S. is controlled by vaccination with a live attenuated vaccine (Patnayak and Goyal, 2004) . This vaccine is administered to the hatched turkeys in the drinking water. Our objective was to examine the possibility of immunizing commercial turkeys with aMPV/C by administrating the live virus into eggs at ED24. We examined the embryos and newly hatched turkeys for a) virus replication in embryonic tissues; b) presence of lesions in the URT; c) the onset and the duration of aMPV/C-specific IgG in the serum and the lachrymal fluid; d) cytokine gene expression in the URT; and e) *in vitro* mitogenic proliferation of splenocyte and

thymocyte. In addition, we assessed the protective ability of *in ovo* immunization against pathogenic virus challenge.

Our results indicate that, as shown previously with aMPV/A and aMPV/B (Hess et al., 2004; Tarpey and Huggins, 2007; Worthington et al., 2003), turkeys may be immunized against aMPV/C by administering the virus *in ovo.* The embryos responded vigorously to the virus and developed protective immunity.

### IV-2 Materials and Methods

**Virus.** The avian metapenumovirus/Minnesota/turkey/1a/1997 isolate of aMPV/C at the $41^{st}$ passage in Vero cells was kindly provided by Dr. S. Goyal, University of Minnesota. This virus was used for immunization as well as challenge. At ED 24, $5x10^3$ $TCID_{50}$ of the virus were inoculated into each turkey egg via the amniotic route as described previously for chicken and turkey (Ahmad and Sharma, 1992; Hess et al., 2004; Sharma et al., 2002; Tarpey and Huggins, 2007; Worthington et al., 2003). For the challenge, $5x10^3$ $TCID_{50}$ of aMPV/C were given oculonasally (O/N) to each turkey. Although the 41st passage of aMPV/C does not cause clinical disease in turkeys, it induces extensive lymphoid infiltration in the URT (Cha et al., 2007).

**Turkeys.** aMPV/C antibody-free Nicolas strain commercial turkey eggs were obtained

from Jenny-O-Turkey Store (Barron, WI). Serum from 1-day-old turkeys was examined by enzyme-linked immunosorbent assay (ELISA) (Chiang et al., 2000) to ensure absence of anti-aMPV/C antibodies. Turkey eggs were incubated in a clean and disinfected egg incubator and after hatch, each treatment group was maintained in separate Horsfall isolators under the regulations of the University of Minnesota Animal Care and Use Committee. All experimental procedures related to turkeys were also approved by this Committee.

**Enzyme-linked immunosorbent assay (ELISA).** An aMPV/C-specific IgG ELISA was performed as described previously with modifications (Chiang et al., 2000). Serum was obtained from the blood samples collected from the wing vein of the turkeys and the lachrymal fluid was collected by sodium chloride stimulation as described previously (Cha et al., 2007; Ganapathy et al., 2005). The test sera were diluted 1:40 and the lachrymal fluid samples were diluted 1:10 in a dilution/blocking buffer (KPL, Gaithersburg, MD) and 50μl of sample per well was added to a 96-well plate coated with aMPV/C antigen or control antigen (Vero cell extract). After one hour, HRP-conjugated goat anti-turkey IgG antibody (KPL, Gaithersburg, MD) was added as a secondary antibody. Tetramethylbenzidine (TMB) was used as a developing reagent. The plates were washed 3 times with 0.05% Tween-20 containing PBS between each step. All

reactions except TMB treatment were done at 37°C and the TMB treatment was conducted at room temperature.

**Histopathology.** Turbinate tissues were fixed in formalin, embedded in paraffin, and sliced into 4μm sections. Sections were stained with hematoxylin and eosin (H&E). Each tissue section was examined at 100x magnification in a blind manner and was given a lesion score of 0-4 depending upon the severity of lymphocyte infiltration in the mucosal layer (0 = no infiltration (less than 20% of the mucosal layer showing lymphoid cell infiltration); 1 = minimal (20-40%); 2 = mild (40-60%) ; 3 = moderate (60-80%) ; 4 = severe (80-100%)).

**Quantitative real time RT-PCR (qRT-PCR).** Quantitative real time RT-PCR was developed to examine gene expression of IFN-γ, IL-10, IL-18 and aMPV/C. For the cytokine genes, total RNA was extracted from the turbinate tissue using TRIzol regent (Invitrogen, Carlsbad, CA). For the viral genes, the Viral RNA Extraction Kit (Qiagen, Valencia, CA) was used to extract viral RNA from choanal swabs. cDNA synthesis was performed with equal amounts of RNA using Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR for turkey cytokines was similar to that for chicken cytokine genes (Hong et al., 2006; Kaiser et al., 2000; Kaiser et al., 2003; Palmquist et al., 2006). Viral load was quantified by using qRT-PCR for the aMPV/C M protein as

described previously (Munir et al., 2006; Velayudhan et al., 2005)**.** Turkey GAPDH was used as an internal control to normalize turkey gene expression. The primers for turkey IL-10, IL-18, GAPDH and aMPV/C were designed by Primer 3 (http://jura.wi.mit.edu/rozen/papers/rozen-and-skaletsky-2000-primer3.pdf) based on respective gene sequence from Genbank. All primer sequences used for qRT-PCR in the study are shown in Table 1. All cytokine gene sequences were from turkey except the IFN-γ gene sequence which was identical to the chicken IFN-γ sequence (Kaiser et al., 2000). PCR was conducted using Brilliant® II QPCR SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) and amplification and detection was performed in an automated fluorometer (Mxp3005 detection system, Stratagene). Cytokine genes were quantitated by the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). Each sample was run in duplicate and normalized by the CT of the internal control (GAPDH) counterpart. The mean △CT value of untreated control birds obtained from each experiment was used to calculate fold changes compared to each sample of virus-exposed group. For aMPV/C gene expressions, viral copy number was calculated by the standard curve established by viral RNA extracted from a series of 10-fold dilutions of previously titrated aMPV/C. All qRT-PCR products were sequenced to confirm specificity.

***In vitro* mitogenic assay.** Single cell suspensions from spleen and thymus were prepared

as described previously (Kim et al., 2000) and stimulated in vitro with a mitogen comprising a mixture of Phorbol 12-myristate 13-acetate (PMA) (400ng/ml), inomycin (50ng/ml) and anti-chicken CD28 antibody (1μg/ml) (Southern Biotech, Birmingham, AL) (Peters et al., 2003). After 43-48 hours of incubation at 37°C, 1μCi of $^3$H-thymidine was added to label the cells for 6-14 hours. The cells were harvested and the counts per minute (CPM) values were determined using a Matrix 9600 beta scintillation counter (Packard Instrument Co., Meriden, Conn.).

**Experimental Designs**

*Experiment I. Immune Response of turkey embryos to in ovo exposure to aMPV/C*

aMPV/C antibody-free commercial turkey eggs at ED24 were divided into two groups and each egg was inoculated via the amniotic route as follows: Group 1: Vero cell culture medium and Group 2: 5000 $TCID_{50}$ of aMPV/C. The hatchability of eggs was monitored. At ED 28 (the day of hatch) (4 days post inoculation (DPI)) and 5 days post hatch (DPH)(9DPI), total RNA was extracted from two pools of thymus, spleen, turbinate, trachea and lung and quantitated for viral gene expression. Each pool comprised tissues from 3-6 turkeys. The pools of turbinate tissue were examined for cytokine gene expression and turbinate tissues from individual turkeys were examined for histological lesions. Single cell suspensions of spleen and thymus tissue were examined for

mitogenesis. At the age of 3 weeks, serum and the lachrymal fluid were collected from aMPV/C-exposed turkeys and tested for virus-specific IgG by ELISA. Three similar trials were conducted.

***Experiment II. Protection against challenge following in ovo exposure to aMPV/C***

aMPV/C antibody-free commercial turkey eggs at ED24 were divided into three groups. Groups 1 and 2 were untreated and each egg in Group 3 received 5000 $TCID_{50}$ of aMPV/C. At 3 weeks of age, each turkey in Groups 2 and 3 was challenged with 5000 $TCID_{50}$ of aMPV/C via the O/N route. At 5 days post challenge, turbinate tissue from individual turkey in each of the three groups was examined for histological lesions and choanal swabs were tested by qRT-PCR for the presence of viral genome. Two similar trials were conducted.

**Statistical analysis**. The Student's t-test for independent sample was performed by using Graphpad Prism 4 (San Diego, CA).

## IV-4 Results

### Effects of turkey embryos to aMPV/C exposure (Exp. I.)

***a) Hatchability and Replication of aMPV/C in embryonic tissues***

There was no effect of *in ovo* inoculation of aMPV/C on the hatchability of turkey eggs

(data not shown). We and others have shown previously that upon post-hatch exposure, aMPV/C remained localized in the URT (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2001). As shown in Figure 1, *in ovo* exposure to the virus resulted in the dissemination of the virus in the upper and the lower respiratory tracts of the embryo. Viral copy number in the turbinate and trachea was significantly increased over the untreated control values at ED28 (4DPI) and 5DPH (9DPI) ($P<0.05$). In the lung, the viral copy number was higher than in controls at ED28 ($P<0.05$) but not at 5DPH. No detectable virus replication was observed in the spleen and thymus (Fig 1).

***b) Histological lesions and antibody response***

Lymphoid cell infiltration in the turbinate was quantitated and expressed as mean lesion score. Turbinate mucosa of aMPV/C-treated turkeys had mild lymphoid cell infiltration which was significantly higher than controls in Trial 1 ($P<0.05$) but not in Trial 2 ($P>0.05$) (Table 2). Despite the presence of the viral genome, no detectable lesions were observed in the lung (Data not shown). These data indicated that *in ovo* exposure to aMPV/C induced mild lesions which were localized in the URT. At 3 weeks of age, serum and lachrymal fluid were collected and the presence of aMPV/C-specific IgG and IgA was examined. aMPV/C-exposed turkeys showed significantly higher levels of aMPV/C-specific IgG than untreated control birds in the serum and the lachrymal fluid

(P<0.05) (Table 2). Virus-specific IgA was not detected in the serum or the lachrymal fluid (Data not shown). Theses results indicated that *in ovo* exposure to aMPV/C resulted in both systemic and local antibody responses.

***c) Cytokine gene expression***

Total RNA extracted from turbinate tissue at ED28 and 5DPH was tested for IFN-γ, IL-10 and IL-18 expression. The data shown in Fig 2 indicated that aMPV/C-exposure modulated the gene expression of IFN-ɣ and IL-10. Gene expression of IFN-γ was upregulated at ED28 and IL-10 at 5DPH. *In ovo* exposure to aMPV/C did not cause detectable modulation of IL-18 genes.

***d) T cell mitogenesis***

Thymocytes and splenocytes were examined for T cell mitogenesis. As noted previously in turkeys exposed after hatch (Chary et al., 2002a), aMPV/C given *in ovo* caused significant mitogenic inhibition (P<0.05) of splenocytes and thymocytes at 5DPH (Figure 3).

**Protection against respiratory challenge in turkeys following *in ovo* exposure to aMPV/C (Exp. II)**

The results of two challenge trials are shown in Table 3. Turkeys were immunized *in ovo* at ED24 and challenged with pathogenic aMPV/C at 3 weeks of age. At 5 days after the

challenge, the mean lesion score and viral copy number in aMPV/C-immunized birds were significantly lower than in unimmunized birds ($P<0.05$) (Table 3). The results clearly indicated that *in ovo* immunization with aMPV/C protected the URT against respiratory challenge with pathogenic virus.

**IV-4 Discussion**

In the present study, we examined the immunopathogenesis of aMPV/C in turkeys following *in ovo* exposure to the virus. Although the strain of aMPV/C we used was pathogenic for hatched birds and caused well pronounced lymphoid cell infiltration in turbinate mucosa (Cha et al., 2007; Chary et al., 2002a; Jirjis et al., 2001), the effect on embryos was much less severe. Virus exposure did not affect the hatchability of eggs. In contrast to the extensive virus replication in the URT of the embryos, only mild and transient lymphoid cell infiltration was detected in the embryonic turbinates.

Although aMPV/C caused minimal lesions in turkey embryos, qRT-PCR detected the viral genome in the URT as well as in the lung. This deeper penetration of aMPV/C into the lower respiratory tract has not been reported previously. In turkeys exposed after hatch, it is typical of all three subtypes of aMPV to replicate and remain localized in the URT (Catelli et al., 1998; Chary et al., 2002a; Cook et al., 1991; Cook, 2005; Jirjis et al.,

2000; Jirjis et al., 2002a; Majo et al., 1995; Panigrahy et al., 2000). The presence of the aMPV/C genome in the embryonic lung was inconsequential and did not lead to viral persistence or detectable pathological lesions. Mammalian paramyxoviruses are known to cause a severe lower respiratory tract infections and replicate in the lung epithelium (Aherne et al., 1970; Gardner et al., 1970; Openshaw and Tregoning, 2005; Zhang et al., 2002).

A vigorous immune responses was detected following *in ovo* exposure. In turkeys hatching from virus-exposed eggs, virus-specific IgG was detected in the serum and the lachrymal fluid, as has been shown previously in turkeys exposed to the virus after hatch (Cha et al., 2007; Goyal et al., 2003). Unlike post-hatch exposure, we did not detect virus-specific IgA following *in ovo* exposure (Cha et al., 2007). The levels of IgA may have been too low or the sampling time was inappropriate. IgA was likely produced because *in ovo* exposed turkeys clearly developed protective mucosal immunity and resisted a respiratory challenge with virulent virus. *In ovo* exposure to aMPV/C upregulated IFN-γ and IL-10 gene expression in the URT indicating that both Th I and Th II cellular immune responses were stimulated. IFN-γ is a well known antiviral cytokine produced by immune cells. IL-10 has a role in regulating virus-induced inflammation and tissue damage. A study with aMPV/A and aMPV/B showed an increase of IFN-γ gene

expression in the Harderian gland in turkeys (Liman and Rautenschlein, 2007). Other reparatory viruses have also been shown to induce local Th I and Th II immune responses (de Waal et al., 2003; Sun et al., 2009).

Chicken embryonal thymocytes proliferate vigorously *in vitro* upon stimulation with a mitogen comprising a mixture of anti-CD28 antibody, PMA and ionomycin (Peters et al., 2003). Our results indicated that this mitogen also stimulated turkey thymocytes and splenocytes at 5 days of age. *In ovo* exposure to aMPV/C significantly ($P < 0.05$) reduced mitogenic proliferation of thymocytes and splenocytes. This expression of aMPV/C-induced immunosuppression is consistent with that noted previously in turkeys exposed post-hatch (Chary et al., 2002a). The significance of T cell immunosuppression early in life following *in ovo* virus exposure should be examined further. We have shown that turkeys undergoing aMPV/C-induced immunosuppression may respond poorly to other vaccines (Chary et al., 2002b).

Successful *in ovo* immunization of turkeys against aMPV/C shown in this study may have practical benefits. In endemic areas, turkeys may be protected against early post-hatch challenge with the environmental virus by *in ovo* vaccination. The *in ovo* vaccination equipment currently used in the chickens will have to be modified for turkey eggs before such an option can be considered.

Table 1 Quantitative RT-PCR Primer Sequences

| Target sequence | Primers | Accession no. |
|---|---|---|
| aMPV/C-M protein | | AF262570 |
| Forward | 5’-CGGGAAGAAAACACACGATT-3’ | |
| Reverse | 5’-CTTGATCTGCCTCCCCACTA-3’ | |
| IFN-γ | | Y07922 |
| Forward | 5’-GTGAAGAAGGTGAAAGATATCATGGA-3’ | |
| Reverse | 5’-GCTTTGCGCTGGATTCTCA-3’ | |
| IL-10 | | AM493432 |
| Forward | 5’-CCGAACTGCTGGATGAATTT-3’ | |
| Reverse | 5’-GCGCTTCATTGTCATCTTCA-3’ | |
| IL-18 | | AJ312000 |
| Forward | 5’-TGAAATCTGGCAGTGGAATG-3’ | |
| Reverse | 5’-CAACCATTTTCCCATGCTCT-3’ | |
| Turkey GAPDH | | U94327 |
| Forward | 5’-GAGGGTAGTGAAGGCTGCTG -3’ | |
| Reverse | 5’-CATCAAAGGTGGAGGAATGG-3’ | |

Table 2 Mean lesion score in the URT and aMPV/C-specific IgG following *in ovo* exposure to aMPV/C (Exp. I)

| Trial[1] | *In ovo* treatment | Mean lesion score | | aMPV/C-specific IgG by ELISA | |
|---|---|---|---|---|---|
| | | ED28 (4DPI) | 5DPH (9DPI) | 3-week-old | |
| | | Turbinates | | Serum | Lachrymal Fluid |
| 1 (n=4-7) | None | 0.00±0.00[a,2] | 0.00±0.00[a] | 0.00±0.01[a,3] | 0.00±0.01[a] |
| | aMPV/C | 1.25±0.5[b] | 1.2±0.45[b] | 0.74±0.92[b] | 0.15±0.15[b] |
| 2 (n=3-5) | None | 0.00±0.00[a] | 0.25±0.5[a] | 0.00±0.00[a] | 0.00±0.01[a] |
| | aMPV/C | 0.2±0.45[a] | 0.75±0.5[a] | 0.39±0.10[b] | 0.17±0.10[b] |

[1] aMPV/C antibody-free turkey eggs were inoculated at ED24 with aMPV/C. At ED28 (4DPI) and 5DPH (9DPI), turbinate tissue was collected and examined histologically and mean lesion score was determined. Serum and lachrymal fluid collected at 3 weeks of age were tested for aMPV/C-specific IgG by ELISA.

[2]: Mean Corrected OD value ± Standard deviation (ELISA)

[3]: Mean lesion score ± Standard deviation (H&E staining)

[a,b] Different superscript in each column indicate statistical differences ($P<0.05$).

ND=Not Done

Table 3 Protection against challenge in 3-week-old turkeys given aMPV/C *in ovo* (Exp. II)

| Trial[1] | *In ovo* immunization | aMPV/C challenge | Mean lesion score in turbinates | Mean viral copy number per bird (qRT-PCR) |
|---|---|---|---|---|
| 1 (n=7-8) | None | - | $0.22 \pm 0.49^{a}$ | $1.15 \pm 1.97^{a}$ |
| | None | + | $2.22 \pm 1.30^{b}$ | $1.09 \times 10^{4} \pm 11254.42^{b}$ |
| | aMPV/C | + | $0.56 \pm 0.88^{a}$ | $1.67 \pm 3.29^{a}$ |
| 2 (n=8) | None | - | $0.33 \pm 0.71^{a}$ | $52.4 \pm 58.61^{a}$ |
| | None | + | $2.33 \pm 1.22^{b}$ | $6.44 \times 10^{4} \pm 44893.96^{b}$ |
| | aMPV/C | + | $0.43 \pm 0.53^{a}$ | $40 \pm 60.96^{a}$ |

[1] aMPV/C antibody-free turkey eggs were inoculated via amniotic route at ED24 with aMPV/C. At the age of 3 weeks, turkeys were challenged with 5000 $TCID_{50}$ of aMPV/C via the O/N route. At 5 days post challenge, turbinate mucosa was examined for lymphoid cell infiltration and choanal swabs were examined by qRT-PCR for the presence of the genome of the challenge virus.

[a,b] Different superscripts within a column in each trial indicate significant differences ($P<0.05$).

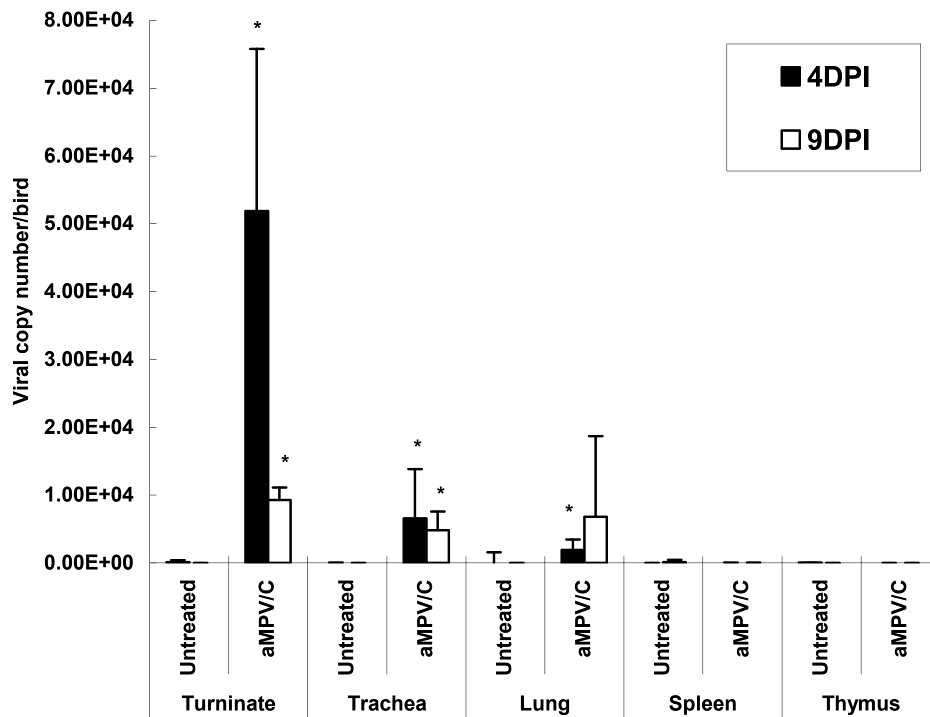



Figure 1 Tissue distribution of aMPV/C following *in ovo* exposure (Exp. I)
aMPV/C antibody-free turkey eggs were inoculated with aMPV/C via the amniotic route at ED24. At ED28 (4DPI) and 5DPH (9DPI), total RNA was collected from the turbinate, trachea, lung, spleen and thymus and tested for the aMPV/C genome by qRT-PCR. Turbinate, trachea and lung had increased viral copy number. The graph represents data from 2-3 trials. In each trial, 2 pools of the turbinate tissue were examined (n=4-6 pools). Each pool comprised tissues from 3-6 turkeys. Asterisks indicated statistical differences between untreated control and aMPV/C groups (P<0.05).

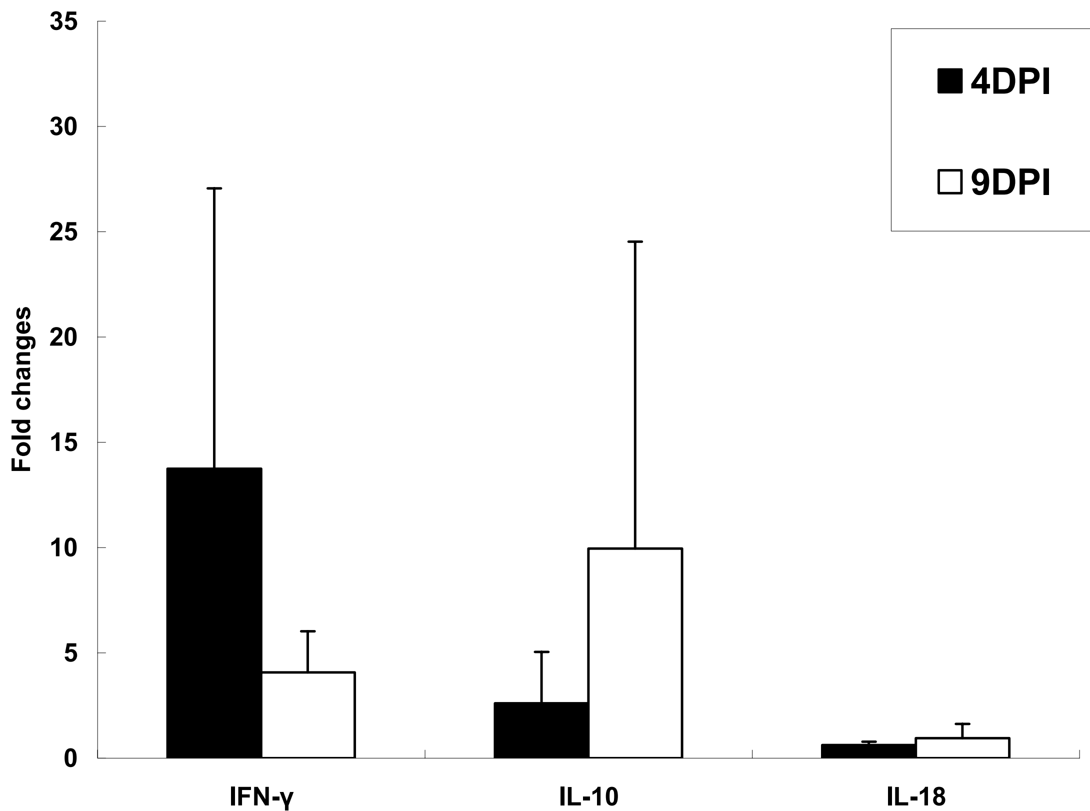



Figure 2 Effect of aMPV/C on cytokine gene expression in the URT (Exp. I)
aMPV/C antibody-free turkey eggs were inoculated via amniotic route at ED24 with aMPV/C. At ED28 (4DPI) and 5DPH (9DPI), total RNA was collected from the turbinate tissue and tested for IFN-γ, IL-10 and IL-18 gene expressions by qRT-PCR. The graph represents the data from 2-3 trials. In each trial, 2 pools of the turbinate tissue were examined (n=4-6 pools). Each pool comprised tissues from 3-6 turkeys. There were no detectable changes in IL-18 gene expression.

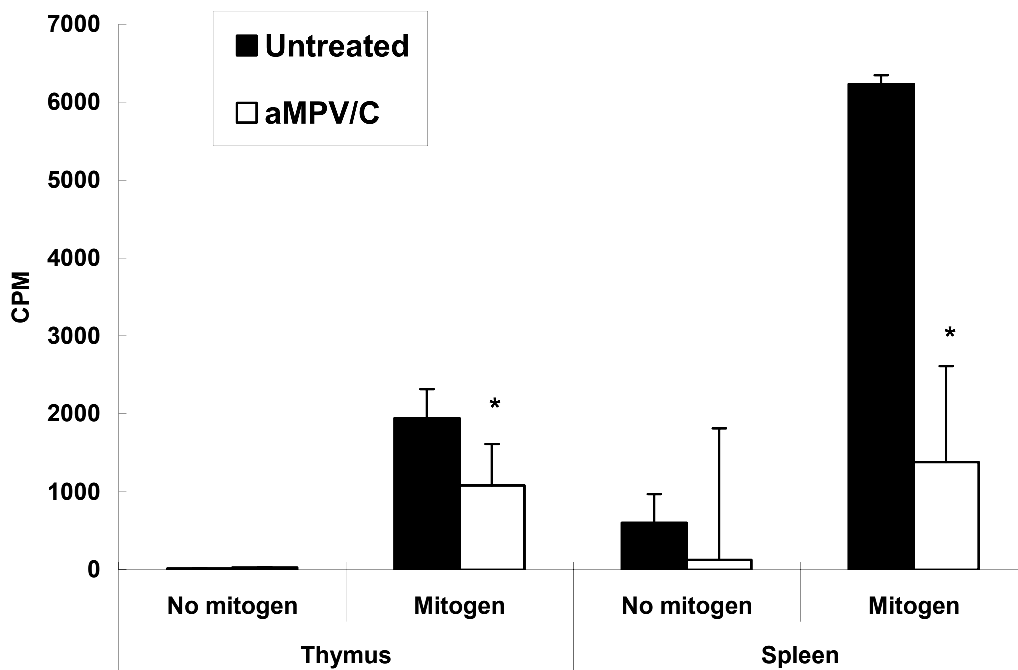



Figure 3 Effect of aMPV/C on mitogenic response of splenocytes and thymocytes (Exp. I)

aMPV/C antibody-free turkey eggs were inoculated via the amniotic route at ED24 with aMPV/C. At 5DPH (9DPI), single cell suspensions of thymus and spleen were tested for mitogenic response. The graph represents mean values from two trials. In each trial, 2 pools of the turbinate tissue were examined (n=3-4 pools). Each pool comprised tissues from 5-6 turkeys. Asterisks indicated statistical differences between the untreated control and the aMPV/C-exposed groups (P<0.05). CPM=Count per minute

# GENERAL DISCUSSION AND FUTURE RESERCH

The overall objective in this study was to examine the immunopathogenesis of aMPV/C in turkeys with a focus on identifying local mucosal immunity. Three specific objectives were pursued.

The first objective was to characterize mucosal T cells in the lymphoid cell infiltration of the URT caused by aMPV/C infection. We isolated lymphoid cells from the turbinate tissue of aMPV/C-exposed turkeys and demonstrated the presence of T cells, especially CD8+ T cells. In addition, RNA extracted from the turbinate tissue revealed an upregulation of gene expression for CD8 molecule, IFN-γ and IL-10 in the URT corresponding to the influx of CD8+ T cells. The presence of increased CD8+ T cells in the mucosal surface of aMPV/C-exposed turkeys is consistent with similar responses noted with other upper and lower respiratory viruses (Baumgarth et al., 1994; de Bree et al., 2005; Kimpen et al., 1992; Wiley et al., 2001). Although we did not examine the role CD8+ T cells may play in the pathogenesis of aMPV/C, studies with other respiratory infections have shown that CD8+ T cells recruited at the site of viral replication are actively engaged in restricting virus spread (Baumgarth et al., 1994; de Bree et al., 2005; Wiley et al., 2001). Respiratory viruses also have been shown to upregulate Th-1 and Th-2 cytokines in the respiratory tract (Baumgarth et al., 1994; Hussell et al., 1996; Sun et al., 2009; Tang et al., 1997; Waris et al., 1996). In addition, activated CD8+ T cells have been

shown to produce IFN-γ and IL-10 (Stumhofer et al., 2007; Tanchot et al., 1998). It is likely that mucosal IL-10 may be important in regulating inflammation and tissue injury associated with aMPV/C infection as shown for influenza virus (Sun et al., 2009). This is the first report to show possible involvement of mucosal CD8+ T cells in the pathogenesis of a respiratory virus in turkeys.

This study provided preliminary evidence of the involvement of mucosal immune cells in the pathogenesis of aMPV/C. The results raise a number of pertinent questions that need further investigation. The questions include the following:

a) How do mucosal B and T cells modulate the pathogenesis of aMPV/C? Because the primary lymphoid organs for B and T cell maturation are located at geographically distant locations in the turkey, surgically-induced B or T cell immunosuppression may be used to examine the role of immune cells in viral pathogenesis. It should be of interest to determine the outcome of virus infection in bursectomized and thymectomized turkeys.

b) What are the subsets of T cells that infiltrate the turbinate mucosa following virus exposure? Are these cells engaged in a virus-specific immune response? What are their cytokine profiles and how cytokines affect viral clearance?

c) What is the origin of lymphoid cells that infiltrate the mucosa following virus

exposure? Do these cells migrate from the circulation or do they proliferate locally? If the cells migrate from the circulation, what chemotactic factors are involved?

Secondly, we examined the ability of an inactivated adjuvanted aMPV/C respiratory vaccine (Ad-iaMPV/C) to induce protective mucosal immunity in the respiratory tract. In this study, we noted that oculonasal administration of Ad-iaMPV/C induced an infiltration of IgA+ cells in the turbinate mucosa and upregulated IFN-I, iNOS and Cox-2 gene expression in the turbinate tissue. Additionally, the vaccine induced virus-specific IgG and IgA in the lachrymal fluid and IgG in the serum. Ad-iaMPV/C immunized turkeys were also protected against respiratory challenge with pathogenic aMPV/C. Upon challenge, the immunized group had significantly lower ($P<0.05$) levels of the challenge virus than unimmunized controls. Mucosal immunization with non-replicating or inactivated vaccines has been shown to successfully protect the host against other respiratory viruses (Asahi-Ozaki et al., 2006; Etchart et al., 2006; Ichinohe et al., 2005; Oien et al., 1994). A recent study showed that inactivated aMPV/C given by the mucosal route did not protect turkeys against intranasal virus challenge (Kapczynski et al., 2008). The reasons for the discrepancy between this study and our results are not known. The two studies had conflicting experimental designs and methods of virus inactivation

and presentation. Although both studies used formalin-inactivated aMPV/C, we concentrated inactivated virus 25 times to increase the viral load per administration and mixed the virus with Poly IC, a potent respiratory adjuvant (Asahi-Ozaki et al., 2006; Ichinohe et al., 2005; Sloat and Cui, 2006). Our data, for the first time, indicated that an inactivated virus preparation of aMPV/C was effective as a respiratory vaccine against pathogenic challenge. This observation opens the door for immunizing commercial turkeys with inactivated respiratory vaccines and thus reducing environmental contamination with live vaccine viruses. The following unanswered questions need further study:

a) What is the optimum dose of Ad-iaMPV/C to induce longest lasting protection? We used only one dose level in this study. Several dose levels should be examined and the duration of immunity at each dose level should be tested in challenge studies.

b) How can vaccine formulation be improved (better adjuvants) to reduce the amount of antigen required to induce protective immunity? Can immunity be improved by incorporating immunomodulators in the vaccine?

c) What is the nature of immunity (humoral or cellular) that is most critical for protection? If most effective protective immunity can be identified, it may be

possible to enhance this immunity and improve protection.

The last aim of this study was to investigate the immunopathogenesis and protective immunity of aMPV/C in turkeys following *in ovo* exposure. *In ovo* exposure to aMPV/C did not affect the hatchability of eggs and elicited a vigorous immune responses in the turkeys hatching from vaccinated eggs. Virus replicated extensively in the URT of the embryos but only mild and transient lymphoid cell infiltration was detected in the turbinate mucosa. The viral genome was present in the embryonic URT as well as in the embryonic lung. This deeper penetration of aMPV/C into the lower respiratory tract has not been reported previously. The presence of aMPV/C genome in the embryonic lung was inconsequential and did not lead to viral persistence or detectable pathological lesions. In turkeys hatching from virus-exposed eggs, virus-specific IgG was detected in the serum and the lachrymal fluid, as has been shown previously in turkeys exposed to the virus after hatch (Cha et al., 2007; Goyal et al., 2003). Unlike the presence of soluble aMPV/C-specific IgA in the nasal secretions and the bile of virus-exposed turkeys exposed after hatch (Cha et al., 2007), virus-specific IgA was not detected in turkeys following *in ovo* exposure. The levels of IgA in the embryos may have been too low or the sampling time was inappropriate. IgA was likely produced because *in ovo* exposed turkeys clearly developed protective mucosal immunity and resisted a respiratory

challenge. *In ovo* exposure to aMPV/C upregulated IFN-γ and IL-10 gene expression in the URT indicating that both Th I and Th II cellular immune responses were stimulated. A study with aMPV/A and aMPV/B showed the increase of IFN-γ gene expression in the Harderian gland in turkeys (Liman and Rautenschlein, 2007). Other reparatory viruses have also been shown to induce local Th I and Th II immune responses (de Waal et al., 2003; Sun et al., 2009). *In ovo* exposure to aMPV/C significant inhibited ($P < 0.05$) the mitogenic proliferation of thymocytes and splenocyte. This expression of aMPV/C-induced immunosuppression is consistent with that noted previously in turkeys exposed post-hatch (Chary et al., 2002a). In addition, *in ovo* immunization with aMPV/C successfully protected the URT against respiratory challenge with pathogenic virus. This result indicated that *in ovo* vaccination may be used under commercial conditions. Future research in this area should be directed to answer the following questions:

a) What is the role of maternal immunity in *in ovo* immunization? We used antibody-negative birds in this study. Under field conditions, most turkeys in endemic areas would be expected to passively transfer anti-aMPV/C antibodies to eggs. The efficacy of *in ovo* vaccination in the face of existing antibodies needs to be examined prior to using this vaccination system in the field.

b) Are *in ovo* immunized birds protected at hatch as in Marek's disease? Because the vaccine is administered several days prior to hatch, it is likely that protective immunity develops prior to hatch. Protection at hatch may be a useful benefit to control the disease in commercial flocks.

c) What are the qualitative and quantitative differences in the immunity responses induced by *in ovo* versus post-hatch protection?

d) How does *in ovo* immunization with live aMPV/C affect immune competence of the embryo and newly hatched poult? This question is important because young turkeys receive vaccines against other diseases and a compromised immune system may interfere with vaccine-induced protection. Studies with chickens have shown that *in ovo* vaccination did not affect responses against routinely used vaccines. Similar studies needs to be conducted in turkeys.

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