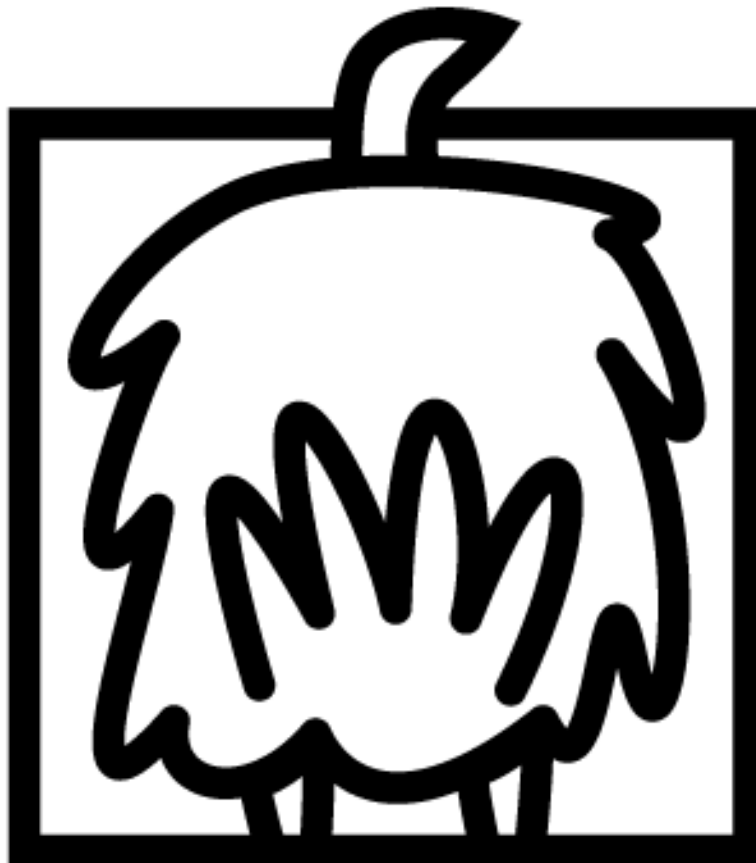# Mixed respiratory infections in turkeys, with emphasis on avian metapneumovirus, *Ornithobacterium rhinotracheale*, *Escherichia coli* and *Mycoplasma gallisepticum*

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Mixed respiratory infections in turkeys, with emphasis on avian metapneumovirus, *Ornithobacterium rhinotracheale*, *Escherichia coli* and *Mycoplasma gallisepticum*

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LIST OF ABBREVIATIONS

AGP: agar gel precipitation

AIV: avian influenza virus

APC: antigen presenting cell

APEC: avian pathogenic *E. coli*

APV: avian metapneumovirus

ARP: avian respiratory phagocyte

AUC: area under the curve

BHI: brain heart infusion

CD_{50}:   50% ciliostatic dose

CEF: chicken embryo fibroblasts

CEL: chicken embryo liver

CO_2:   carbon dioxide

cfu: colony forming units

DNA: desoxyribonucleic acid

dpbi: days post bacterial inoculation

dpmi: days post *M. gallisepticum* inoculation

dpvi: days post viral inoculation

ELISA: enzyme-linked immunosorbent assay

HEPA: high efficiency particulate air

HI: hemagglutination inhibition

HPLC: high performance liquid chromatography

Ig: immunoglobulin

ME: Mycoplasma Experience

MHC: major histocompatibility complex

MIC: minimal inhibitory concentration

MIC_{50}:   minimal inhibitory concentration required to inhibit the growth of 50%
                of the organisms

MIC_{99} :   minimum inhibitory concentration required to inhibit the growth of
                90% of the organisms

ml: milliliter

MPC: mutant prevention concentration

NDV: Newcastle disease virus

NK: natural killer

O_2 :   oxygen

PAS: Periodic Acid Schiff

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

Q-PCR: quantitative PCR

QRDR: quinolone resistance-determining region

RSA: rapid slide agglutination

SN: seroneutralization

SPA: serum plate agglutination

SPF: specified pathogen free

$TCID_{50}$:   tissue culture infectious dose with a 50% endpoint

TOC: tracheal organ cultures

$Vd_{(ss)}$:   apparent volume of distribution at steady state

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Chapter 1: GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1. 1. The avian respiratory system

1.1.1. Anatomy and physiology

The principal function of the respiratory system in birds is exchanging oxygen (O_2) and carbon dioxide (CO_2) between atmosphere and blood, but also temperature regulation and phonation. In complete contrast to the tidally ventilated mammalian respiratory system, where fresh inhaled air is mixed with residual stale air in the respiratory airways, the avian lung is a flow-through system (Reese *et al*., 2006). The respiratory tract begins at the nares, consists of passages between conchae in the head and subsequently leads inhaled gas to the larynx. The trachea extends from the larynx, and branches into two extrapulmonary primary bronchi. From each primary bronchus, four groups of secondary bronchi (medioventral, mediodorsal, lateroventral and laterodorsal) arise and from the secondary bronchi multiple parabronchi (Fedde, 1998). In birds, the body cavity is not divided by a diaphragm and ventilation is achieved through a unique way of air transport which requires the action of the air sacs. As a consequence, the avian lung is rigid, fixed at the thoracic walls and comprises a highly complex structure. Air sacs occupy every available space in the body coelom not occupied by other viscera and most birds have nine air sacs: paired cervical air sacs, an unpaired clavicular air sac that is connected to each lung, paired cranial thoracic air sacs, paired caudal thoracic air sacs, and paired abdominal air sacs. The cervical, clavicular, and cranial thoracic air sacs arise from the medioventral secondary bronchi, and they are often called the cranial air sacs. The caudal thoracic and abdominal air sacs (the caudal air sacs) arise from the lateroventral and mediodorsal secondary bronchi and from the continuation of the intrapulmonary primary bronchus. The air sacs are auxiliary structures that pump air through the respiratory tract, but do not contribute to the gas exchange with the blood (Fedde, 1998; Reese *et al*., 2006). The O_2 and CO_2 exchange only occurs in the lungs. During inspiration, active contraction of some muscles of the body wall causes an increase in the volume of the air sacs which results in pressure in the air sacs less than that in the atmosphere and gas moves through the lungs into the air sacs. The inspired air completely bypasses the cranially lying openings of the medioventral secondary bronchi, a process which is called inspiratory aerodynamic valving (Reese *et al*.,

2006). In contrast, during the inspiratory phase as well as the expiratory phase, air flows in the mediodorsal and lateroventral secondary bronchi. About one half of the inspired volume passes through the paleopulmonic parabronchi and in this way in the cranial air sacs, and the remainder passes through the much smaller neopulmonic parabronchial network to the caudal air sacs, and through the direct connection from the intrapulmonary primary bronchus to the abdominal air sacs. During expiration, reduction in coelomic volume (decrease in the volume of the air sacs) increases the pressure in the air sacs and air moves out of the air sacs. Some of the air from the caudal air sacs again traverses the neopulmonic parabronchi and most of the air enters the paleopulmonic parabronchi, travelling in the same direction as during inspiration. Air from the cranial air sacs flows through the medioventral secondary bronchi to exit the lung without contacting any parabronchial gas exchanging surfaces. Thus exchange of O_2 and CO_2 between air and blood occurs both during inspiration and expiration in birds and nearly all of the air that was inhaled, has passed over paleopulmonic parabronchial gas exchanging surfaces during some part of the respiratory cycle. The walls of the parabronchi are perforated by numerous openings that lead to the respiratory atria. Funnel-shaped infundibulae arise from the atria and open into the air capillaries. The inhaled air flows through the parabronchial lumen and then into the exchange tissue through the atria, the infundibulae, and the network of air capillaries. The air capillaries are closely surrounded by a network of blood capillaries, which together constitute the most efficient gas exchanger unit among air-breathing vertebrates (Reese *et al.*, 2006). The blood-gas barrier in the avian lung is approximately 56-67% thinner than that of a mammal of the same body mass and the respiratory surface area is approximately 15% greater (Maina *et al.*, 1989). While large surface area and thin tissue barrier enhance respiratory efficiency, these structural features predispose birds to pulmonary injury from environmental toxicants and invasion by pathogenic organisms (Reese *et al.*, 2006).
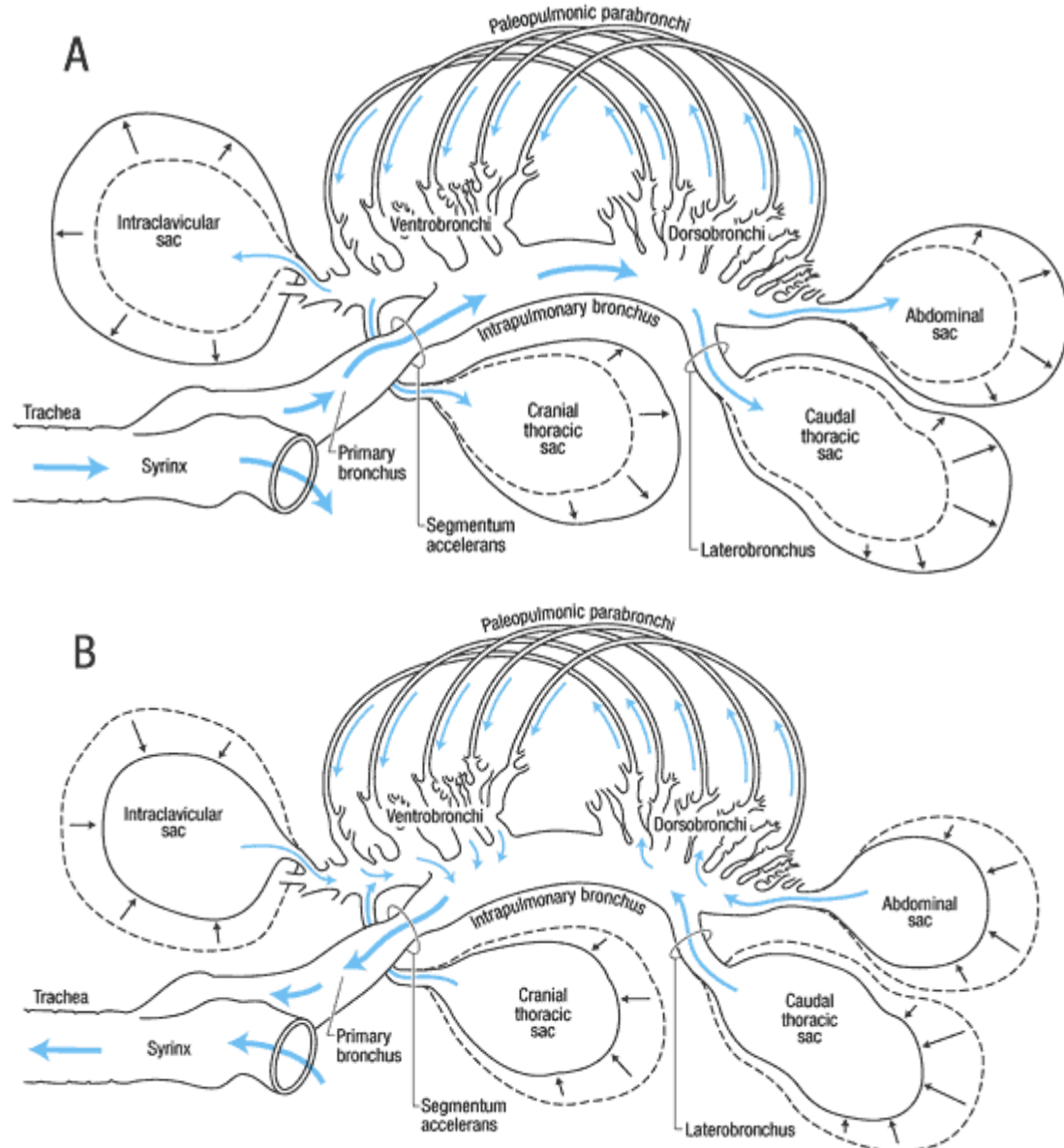
Figure 1. Schematic representation of the right paleopulmonic lung and air sacs of a bird and the pathway of gas flow through the pulmonary system during inspiration and expiration. For purposes of clarity, the neopulmonic lung is not shown. A: Inspiration. B : Expiration.

(Source: http://www.people.eku.edu/ritchisong/birdrespiration.html)

1.1.2. Defense mechanisms

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

1.1.2.1. Innate resistance

The initial line of defense for the airway is the nasal and tracheal epithelium, which prevents pathogens from entering the body. Multiple mucous glands within the pseudostratified ciliated columnar epithelium produce mucus which forms a layer on top of the cilia of the epithelial surface. Particulate material that is caught in the mucus gets transported by the movement of the cilia in an oral direction, where it is swallowed and digested or excreted by coughing and sneezing (Koch, 1991; Sharma, 2003). Furthermore, mucus contains antibacterial enzymes which impede the attempts of pathogens to colonize.

For pathogens that do enter the body, the next line of defense is provided by other innate immune mechanisms, such as phagocytic cells that include heterophils and macrophages, and natural killer (NK) cells. Monocytes-macrophages, cells belonging to the mononuclear phagocytic system, are considered to be the first line of immunological defense. These cells originate from the bone marrow and subsequently enter the blood circulation. Upon migration to various tissues, monocytes mature and differentiate into tissue macrophages (Dietert *et al.*, 1991; Qureshi *et al.*, 2000). Macrophages then get involved in innate and acquired immunity (Qureshi *et al.*, 2000). Since the respiratory surface is in proportion much larger than that of mammals and the tissue barrier is much thinner (Maina, 1989; Maina *et al.*, 1989), one can expect that, as stated above, the avian respiratory tract is relatively more easily attacked by pathogens than the mammalian one (Nganpiep and Maina, 2002). One would hence assume that for a similar defense competence, more residing avian respiratory phagocytes (ARP = macrophages and polymorphonuclear leukocytes such as heterophils) would occur on the surface of avian lungs. Paradoxically, the normal, steady-state avian respiratory system has very low numbers of residing ARP in comparison to the mammalian system, and as a consequence birds must rely heavily on the influx of ARP into the site of infection for non-specific defense against bacteria and other pathogens (Ficken *et al.*, 1986; Qureshi *et al.*, 1994; Klika *et al.*, 1996; Lorz and Lopez, 1997; Qureshi *et al.*, 2000; Toth, 2000). Interestingly, ARPs were never found on the surface of the air capillaries (respiratory surface) which represent the functional equivalent to the mammalian alveoli but were regularly present on the surfaces of the atria and the infundibulae, structural units immediate to the air capillaries (Nganpiep and Maina, 2002; Reese *et al.*, 2006). Thus, macrophages seem to be located at strategic check points were fresh

air is distributed into the gas exchange areas and where particles can be trapped and removed. The paucity and even lack of ARPs in birds has been used to explain a purported high susceptibility of poultry to respiratory diseases. Nganpiep and Maina (2002), however, showed that a composite defense armament has additionally developed in the avian respiratory system. A highly lytic upper airway epithelium endowed with lysosomes (apparently lacking in mammals), generally robust ARPs, and efficient translocation of subepithelial macrophages onto the respiratory surface, play a role in the protection of the respiratory system (Nganpiep and Maina, 2002). In the air sacs, being thin walled and lacking an elaborate ciliated epithelium, particle clearance is largely accomplished by phagocytic cells albeit significantly lower than in the lungs (Nganpiep and Maina, 2002; Reese *et al*., 2006).

1.1.2.2. Adaptive immunity

When pathogens cannot be withheld by physical barriers nor controlled by innate immune defense mechanisms, adaptive immunity (specific immune response) is required to specifically focus defense mechanisms on that particular antigen resulting not only in the elimination of the pathogen but also in protecting in case of a repeat encounter with the same pathogen (memory). Adaptive immunity is mediated by a variety of cells, of which T lymphocytes, B lymphocytes, and macrophages are the most important.

In poultry, adaptive immunity, including the cell-mediated and humoral arm, is critically dependent on regulation by T lymphocytes (T cells), the coordinators of the immune response. Maturation of the T cells takes place in the thymus, a feature shared with mammalian species (Arstila *et al*., 1994). Before T cells can initiate and participate in an adaptive immune response to a pathogen, the antigen has to be presented by host cells in the context of their major histocompatibility complex (MHC) molecules, i.e., as an antigenic peptide bound to the MHC molecule. The MHC molecules come in two forms: the MHC class I is expressed by essentially all nucleated cells, whereas the MHC class II is expressed mainly by cells of the immune system, the so-called antigen presenting cells (APC) such as macrophages, dendritic cells and B lymphocytes (B cells). These APC also deliver other signals equally important to the T cell activation, the so-called second or costimulatory signals (Arstila *et al*., 1994). Activation of T cells results in proliferation of the activated T cells and their differentiation into subpopulations of diverse effector cells, helper T

cells (CD4+), suppressor T cells, and cytotoxic T cells (CD8+), or memory cells. Effector functions of T helper cells primarily involve production of cytokines (soluble molecules secreted to the extracellular space), and expression of membrane-bound cell-surface molecules, all affecting other cells of the immune system. The cytotoxic T cells, in contrast, are mostly killers that are specialized in the elimination of intracellular antigens. The latter include those that have entered cells via the endocytic pathway (exogenous antigens; e.g., phagocytosed bacteria) or were produced within the cell such as viral proteins and proteins resulting from neoplastic transformation of the cell (endogenous antigen) (Erf, 2004). Another lineage of T cells exists (γδ T cells), but their physiological significance remains largely a matter of speculation.

Besides the T lymphocytes, other cells important to the cellular immune response include macrophages, dendritic cells, NK cells, and effector cells of antibody dependent cellular toxicity (Sharma, 1991). NK cells can also be regarded an effector cell of specific cell-mediated immunity as they greatly benefit from T helper mediated activity (Erf, 2004).

Unlike mammals, birds have a special organ, the bursa of Fabricius, where the development of B lymphocytes (B cells) from their immature precursors takes place. For humoral immunity, B cells differentiate into plasma cells that secrete antigen-specific antibodies. Antibodies can prevent disease caused by pathogens and provide protection, but they are primarily effective in preventing entry of pathogens through mucosal surfaces (e.g., secretory IgA) and in eliminating extracellular antigens (Koch, 1991). Most organisms stimulate both cell-mediated immunity and humoral immunity, although the type of immunity most critical for defense may vary with the organism (Vandaveer *et al.*, 2001; Sharma, 2003; Erf, 2004).

1.2. Respiratory disease in turkeys

Respiratory disorders are one of the important diseases affecting turkeys and are continuing to cause high economic losses in many areas world-wide due to reduced growth, increased feed conversion rates, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter (van Empel and Hafez, 1999). They may be induced by various viral and bacterial agents, either alone or in combination. Besides these infectious organisms, non-infectious factors, such as climatic conditions (e.g. inadequate ventilation, high ammonia levels, too high or too

low relative humidity) and management-related problems, may also contribute to the occurrence or the severity of respiratory problems in turkeys (van Empel and Hafez, 1999).

Different respiratory viruses such as influenza virus type A, paramyxovirus types 1, 2, 3 and 6, and avian metapneumovirus (APV), have been shown to be able to elicit respiratory problems (Van de Zande, 2001). Viral agents are mostly being attributed a triggering role, since the clinical signs following experimental inoculation with these viruses are less severe than those observed in the field. Viral infections generally cause rather acute respiratory problems from which birds usually can recover fairly easily. The problems, however, become more critical when bacterial pathogens are involved. Implicated bacteria include *Escherichia coli*, *Pasteurella multocida*, *Bordetella avium*, *Ornithobacterium rhinotracheale*, *Mycoplasma gallisepticum*, *M. synoviae*, *M. iowae*, *M. meleagridis*, *M. imitans*, *Chlamydophila psittaci* and *Riemerella anatipestifer*. With these bacterial agents, it is not always straightforward to reproduce clinical signs following experimental infection. This has led to a still contemporary discussion point whether the different bacterial agents are primary or rather secondary pathogens.

In the present thesis, the experimental research focuses on four important turkey pathogens, i.e. APV, *O. rhinotracheale*, *E. coli*, and *M. gallisepticum*. Hence, the most important literature data on these agents will be discussed below.

1.2.1. Avian metapneumovirus

1.2.1.1. Etiology, epidemiology and pathogenesis

APV (avian metapneumovirus, avian pneumovirus, turkey rhinotracheitis virus) is a member of the family *Paramyxoviridae*, subfamily *Pneumovirinae* and genus *Metapneumovirus* (Pringle, 1998; 1999). The disease was reported initially in turkeys and shortly thereafter in chickens in South Africa in the late 1970s (Buys *et al*., 1989a; Buys *et al*., 1989b). Later, the disease was reported in Europe, with the etiological agent being isolated in the United Kingdom, France and Germany (Giraud *et al*., 1986; Wilding, 1986; Hafez and Weiland, 1990). At present, the disease is a major problem in most turkey and chicken producing countries worldwide (Van de Zande, 2001).

The APV isolates that exist worldwide are currently classified into four subgroups, namely, subgroups A, B, C, and D, according to their antigenic and molecular variations. The U.S. strains of APV belong to subgroup C (which have been shown to be more related to human metapneumovirus than other APV subgroups), while the strains in other parts of the world, especially the European countries, belong to the other three subgroups (Govindarajan *et al*., 2006; Guionie *et al*., 2007).

The two most important species susceptible to APV infection are turkeys and chickens, although pheasants, guinea fowl and ducks may also become infected. APV antibodies have occasionally been reported in ostriches and in sea gulls, but the virus has not been isolated from these species. Geese and pigeons appear to be refractory to APV. In both turkeys and chickens the primary site of replication appears to be the respiratory tract (mainly turbinates and trachea, but also lungs and air sacs), and ciliated epithelial cells are the target cells (Jones *et al*., 1988; Majó *et al*., 1995). Rapid transmission occurs horizontally, mainly by direct contact, although fomites are possibly involved. There is no published evidence that APV may be vertically transmitted, even though the virus can be detected in the reproductive tract of laying birds (Jones *et al*., 1988).

1.2.1.2. Clinical findings and lesions

A primary APV infection may result in severe respiratory disease in susceptible turkeys, though mild or even subclinical infections occur as well. All ages are predisposed to APV infections, but with a different clinical outcome. Young poults seem to be more susceptible than older ones and the severity of disease and mortality is higher (Alexander *et al*., 1986). APV outbreaks can be characterized by sneezing, depression, tracheal rales, swollen infraorbital sinuses and nasal and often frothy, ocular discharge, snicking, dyspnoea, and head shaking. The nasal discharge may become thicker and mucopurulent as a result of secondary bacterial infection. In laying birds, there may be a drop in egg production with an increased incidence of poor shell quality and peritonitis (Gough, 2003). The onset of signs is rapid and the infection may spread through a flock within 24h. The morbidity reaches 100% and mortality varies between 2 and 50%, with the highest percentage in young birds. Secondary infections and bad management conditions increase the mortality rate and

prolong the disease, and this is probably the reason why the disease in the field is much more severe than under experimental conditions (Cook, 2000; Gough, 2003).

1.2.1.3. Diagnosis

The clinical picture of an APV outbreak can be indicative, but laboratory diagnosis is necessary to confirm an APV infection. Virus can be isolated from suspensions of turbinates and trachea or from tracheal swabs using tracheal organ cultures (TOC) prepared from chick or turkey embryos (Cook *et al*., 1976), since subgroups A and B viruses cause ciliostasis, this in contrast to subgroup C. APV can also be isolated in chicken embryo yolk sac and different cell lines such as chicken embryo fibroblasts (CEF), monkey kidney cells (VERO) and chicken embryo liver (CEL). Virus isolation should be attempted at the very first sign of clinical disease, since the virus shedding takes place for a very short period (Cook and Cavanagh, 2002). Detection of the virus can be done by direct and indirect immunofluorescence staining and PCR. Still diagnosis of APV is usually done serologically. The seroneutralization (SN) test, which can be performed in a variety of systems, including TOC, CEF, CEL or VERO cultures, is sometimes used. However, the ELISA is the most commonly used test since it shows similar sensitivity and is much less time consuming compared with the SN test (Grant *et al*., 1987; Hafez and Löhren, 1990; Cook and Cavanagh, 2002).

1.2.1.4. Treatment and control

For treatment and control, one can say that good management practices and good biosecurity are important in helping to prevent infection and minimize the effects. Although APV infections themselves cannot be treated, antimicrobials are used to control secondary bacterial infections (Hafez *et al*., 1990). Quality vaccines are available and APV infections can be prevented by vaccination (Cook, 2000). Today, several live-attenuated and inactivated vaccines, developed by different companies, have been licensed.

1.2.2. *Ornithobacterium rhinotracheale*

1.2.2.1. *Etiology, epidemiology and pathogenesis*

Already in 1991, a new respiratory disease was observed in broiler chickens in South Africa by J. Du Preez (van Beek *et al*., 1994), but *O. rhinotracheale* was actually first characterized in 1993 by Charlton *et al*. Within a relatively short period of time, a world-wide spread of *O. rhinotracheale* was seen. *O. rhinotracheale* is a Gram-negative, non-motile, pleomorphic, rod-shaped, non-sporulating bacterium of the rRNA superfamily V.

O. rhinotracheale has been isolated throughout the world from numerous bird species, including turkey, chicken, (chukar) partridge, duck, goose, guinea fowl, gull, ostrich, pheasant, pigeon, quail, and rook (Hafez, 2002; Chin *et al*., 2003).
Up to date, 18 serotypes (A through R) of *O. rhinotracheale* have been determined. Serotyping has revealed that the majority of chicken isolates are of serotype A (94%). Serotype A is also the most prevalent serotype among turkey isolates (57%). In turkeys, 97% of the strains isolated belong to the four major serotypes A, B, D, and E (van Empel, 1997; van Empel and Hafez, 1999).

Although it has been proven that *O. rhinotracheale* is highly sensitive to different chemical disinfectants (Hafez, 2002; Hafez and Schulze, 2003), it seems difficult to eradicate it. *O. rhinotracheale* infection can affect every new restocking even in previously cleaned and disinfected houses, especially in areas with intensive poultry production as well as in multiple age farms. *O. rhinotracheale* appears to have become endemic (Hafez, 2002; Chin *et al*., 2003). *O. rhinotracheale* spreads horizontally by direct and indirect contact through aerosols or drinking water, and there is circumstantial evidence that vertical transmission occurs (van Empel, 1998; van Empel and Hafez, 1999; Chin *et al*., 2003).

1.2.2.2. *Clinical findings and lesions*

By now, it is clear that *O. rhinotracheale* can cause acute, highly contagious disease in poultry, but the severity of clinical signs, duration of the disease and mortality of confirmed *O. rhinotracheale* outbreaks have been found to be extremely variable (van Empel and Hafez, 1999; Chin *et al*., 2003). In many cases, young poults are affected between two and eight weeks of age (Chin *et al*., 2003). Initial symptoms are coughing, sneezing and nasal discharge followed, in some cases, by severe

respiratory distress, dyspnoea, prostration, and sinusitis. These symptoms are accompanied with a reduction in feed consumption and water intake. In turkey breeder flocks, there can also be a decrease in egg production and in the number of hatchable eggs (De Rosa *et al.*, 1996; van Empel and Hafez, 1999; Chin *et al.*, 2003). Normal mortality ranges between 1 and 15% during the acute phase (8 days post infection), but infections can be accompanied with mortality rates of up to 50% (Chin *et al.*, 2003). The pathological lesions can include rhinitis, tracheitis, airsacculitis, oedema, uni- or bilateral consolidation of the lungs with fibrinopurulent exudates in the pleura (Hinz *et al.*, 1994; van Empel *et al.*, 1996; Sprenger *et al.*, 1998). Pericarditis, peritonitis and enteritis can also be detected (van Empel and Hafez, 1999). In some cases, swelling of the liver and spleen as well as degeneration of heart muscles have been observed. After infection of the respiratory tract, the bacterium can also disseminate to other sites of the body resulting in local pathology such as hepatitis, meningitis and joint-infections (Sprenger *et al.*, 1998; van Empel and Hafez, 1999; Chin *et al.*, 2003).

1.2.2.3. Diagnosis

It is difficult to make a presumptive diagnosis based on clinical signs and necropsy findings. *O. rhinotracheale* can usually be isolated from trachea, tracheal swabs, lungs and air sacs. The infraorbital sinus and nasal cavity are also suitable sites for culture, but *O. rhinotracheale* can be masked easily by the overgrowth of other bacterial species. Optimal growth of the organism is obtained by incubation on 5% sheep blood agar for at least 48 h under micro-aerophilic conditions (5 to 10% CO_2). A good selective medium is not yet available, but to suppress overgrowth by fast-growing bacteria (e.g. *E. coli*, *Proteus* sp., *Pseudomonas* sp.) in contaminated samples, gentamicin and polymyxin (both 5 µg/ml) can be added to the sheep blood agar (van Empel, 1997). As only about 90% of *O. rhinotracheale* strains are resistant to both these antimicrobials, sheep blood agar without these additives should always be included (van Empel and Hafez, 1999). Further identification can be done by the different biochemical properties of *O. rhinotracheale* as included in the API-20NE and API-ZYM systems (BioMèrieux, France) or fatty acid profile (Charlton *et al.*, 1993). Other tests for identification include rapid slide agglutination (RSA) test, agar gel precipitation (AGP) test, PCR assays (van Empel, 1998; Hung and Alvarado, 2001) and immunohistochemical staining.

Serology is useful for flock monitoring or as an aid in the diagnosis of *O. rhinotracheale* infection, and can be carried out using slide agglutination test prepared from different serotypes, ELISA tests (commercially available), or DOT-immunobinding assays (Hafez, 2002).

1.2.2.4. Treatment and control

In practice *O. rhinotracheale* infections are mostly dealt with using different antimicrobials such as amoxicillin, ampicillin, doxycycline, tetracycline, trimethoprim/sulphonamide, chlortetracycline, enrofloxacin and florfenicol. Treatment of *O. rhinotracheale* infections with antimicrobials is being compromised by the variable susceptibility of strains. *O. rhinotracheale* can acquire resistance against antimicrobials such as doxycycline, enrofloxacin, flumequine, lincomycin, trimethoprim, sulfachloropyridazine and tylosin (Devriese *et al*., 1995; Chin *et al*., 2003). The sensitivity of *O. rhinotracheale* to antimicrobials is very inconsistent and appears to depend upon the source of the strain. In Germany, 90% of strains are resistant to enrofloxacin (Hafez, 1996), while those isolated in France and Belgium are almost always very sensitive to this antimicrobial (Devriese *et al*., 1995; Dudouyt *et al*., 1995; Roger and Léorat, 1997; Devriese *et al*., 2001).

In the past, vaccines based on inactivated whole-cell formulations have been developed and shown to induce protective immunity in both chickens and turkeys (van Empel and Hafez, 1999; Schuijffel *et al*., 2006). For chickens, serotype A is the most important serotype, but for turkeys protection against more serotypes is needed. Both in experimental studies and in the field, it was found that cross-protection is not always induced by vaccination with bacterins in oil adjuvant (van Empel and Hafez, 1999). Very recently, Schuijffel *et al*. (2005) used an alternative strategy in order to identify cross-protective vaccine targets: sera from live vaccinated and cross-protected birds were used for immunoscreening of an *O. rhinotracheale* serotype G expression library. Based on further obtained results, it appears they identified a good candidate for the development of a cross-protective vaccine against *O. rhinotracheale* infections. Vaccination with live *O. rhinotracheale* has been investigated and shown to be feasible (van Empel and van den Bosch, 1998) although in practice it is not yet possible because, until now, all the investigated *O. rhinotracheale* strains have been pathogenic after viral priming.

1.2.3. *Escherichia coli*

1.2.3.1. Etiology, epidemiology and pathogenesis

E. coli is a Gram-negative, non-sporeforming, rod-shaped bacterium, of the family *Enterobacteriaceae*. Most strains are motile and have petrichous flagella (Barnes *et al.*, 2003).

Although *E. coli* is present in the normal microbiota of the intestinal tract, other host mucosal surfaces and in the bird's environment, only a certain number of these strains possessing specific virulence attributes, designated as avian pathogenic *E. coli* (APEC), are able to cause disease (Dho-Moulin and Fairbrother, 1999; Vandekerchove, 2004). Since serotyping for the somatic antigen (O-serotyping) is still the most frequently used typing method for diagnostic purposes, the O-type is often used for APEC description. O1, O2 and O78 are reported as the main serotypes in different disease types by several authors (Barnes *et al*., 2003; Vandekerchove, 2004). Many other serotypes have been found less frequently, and some pathogenic isolates do not belong to known serotypes or are untypeable (Barnes *et al*., 2003).

Colibacillosis refers to any localized or systemic infection (e.g. septicemia, peritonitis, cellulitis, salpingitis, osteomyelitis, synovitis, omphalitis, airsacculitis, and coligranuloma) caused entirely or partly by APEC, and is the most frequently reported disease in surveys of poultry diseases or condemnations at slaughter, hence responsible for severe economic losses (Dho-Moulin and Fairbrother, 1999; Barnes *et al*., 2003). Most, if not all avian species, are susceptible, although clinical disease is reported most often in chickens, turkeys and ducks. Susceptibility and severity of infection are greatest in young birds (Barnes *et al*., 2003; Rodriguez-Siek *et al*., 2005).

Horizontal infection with *E. coli* usually occurs through contact with other birds, or through faeces, contaminated water and feed. Natural respiratory tract infection of poultry by APEC is thought to occur via the inhalation of faeces-contaminated dust (Dho-Moulin and Fairbrother, 1999). Carlson and Whenham (1968) have demonstrated that the risk of colibacillosis increases with the level of environmental contamination. Dust in poultry houses may contain 10^5-10^6 colony forming units (cfu) *E. coli*/g. These bacteria may persist for long periods, particularly under dry conditions (Harry, 1964; Barnes *et al*., 2003). Vertical infection results

from the transmission of *E. coli* from breeders, via contaminated shells during hatching, or *in ovo*, as a result of salpingitis.

The virulence mechanisms of avian pathogenic *E. coli* have not been clearly characterized yet. A number of potential virulence factors have been identified in APEC strains isolated from diseased birds, but their role in causing disease is not completely understood (Barnes *et al.*, 2003). Besides bacterial virulence factors, probably also host resistance is a great determinant of colibacillosis occurrence (Barnes *et al.*, 2003). In fact, colibacillosis is usually considered to be a secondary disease, following a primary infection with respiratory pathogens and/or unfavorable environmental conditions (Barnes *et al.*, 2003; Vandekerchove *et al.*, 2004).

1.2.3.2. Clinical findings and lesions

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

1.2.3.3. Diagnosis

The diagnosis of colibacillosis is first suggested by the clinical picture and by the presence of typical macroscopic lesions such as airsacculitis, sometimes associated with pericarditis and perihepatitis. Diagnosis needs to be confirmed by the isolation of pathogenic *E. coli* from the heart blood and affected tissues, like liver, spleen, pericardium or bone marrow, on selective media like McConkey, eosin-methylene blue or drigalki agar. Care must be taken to avoid faecal contamination of samples. Further identification of the isolated colonies is based on biochemical reactions (Dho-Moulin and Fairbrother, 1999). The diagnosis is strengthened if the

isolated culture belongs to a known pathogenic serogroup. Different ELISAs have been developed for detection of antibodies, although they have limited value because they can only detect homologous APEC types (Leitner *et al.*, 1990; Bell *et al.*, 2002). All currently known virulence-associated factors, detected in strains isolated from colibacillosis lesions, can also be detected in faecal isolates from clinically healthy chickens. For this reason, none of these traits can be used for APEC identification.

1.2.3.4. Treatment and control

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

Measures should be taken to prevent introduction of pathogens that promote infections with APEC (Barnes *et al.*, 2003). The housing climate (humidity, ventilation, dust and ammonia) and the stocking density must be kept optimal (Dho-Moulin and Fairbrother, 1999; Vandekerchove, 2004). The great diversity among APEC strains limits the possibilities of vaccination, and vaccines are not used on a large scale (Dho-Moulin and Fairbrother, 1999; Vandekerchove, 2004).

1.2.4. *Mycoplasma gallisepticum*

1.2.4.1. Etiology, epidemiology and pathogenesis

M. gallisepticum is the most pathogenic and economically significant mycoplasma pathogen causing respiratory disease in chickens and turkeys. *M. gallisepticum* is a species of the family *Mycoplasmataceae* (class *Mollicutes*) (Ley, 2003). Mycoplasmas (or mollicutes) are bacteria that lack a conventional bacterial cell wall and are surrounded only by a thin trilaminar membrane (Bradbury, 2005) and they represent the smallest known organisms in nature capable of self-replication

(Razin *et al*., 1998). The price they have to pay for their simplicity includes a slow growth cycle and a considerable dependence upon the host for many nutrients, and hence for survival (Bradbury, 2005).

     M. gallisepticum infections naturally occur primarily in gallinaceous birds, and are commonly known as chronic respiratory disease of chickens and infectious sinusitis of turkeys (Ley, 2003). However, *M. gallisepticum* has also been isolated from naturally occurring infections in pheasants, ducks, geese, chukar partridge, peafowl, bobwhite quail and Japanese quail (Ley, 2003; Levisohn and Kleven, 2000). *M. gallisepticum* probably can infect susceptible birds at any age, although it is stated that young birds are, in general, more susceptible to infection with *M. gallisepticum* (Bradbury and Levisohn, 1996; Ley, 2003).

     Horizontal transmission occurs readily by direct or indirect contact of susceptible birds with infected carriers or contaminated fomites (Levisohn and Kleven, 2000). The upper respiratory tract and/or conjunctiva are portals of entry for the organism in aerosol or droplets (Bradbury and Levisohn, 1996; Levisohn and Kleven, 2000). *M. gallisepticum* is considered to be primarily a surface parasite of the respiratory tract and conjunctiva, although spread to other organs indicates that transient systemic infections occur, resulting in acute and chronic diseases at multiple sites (Ley, 2003). Vertical transmission of *M. gallisepticum* is known to occur in eggs laid by naturally infected hens and has been induced following experimental infections (Ley, 2003). This is suggested to occur as a sequel to acute respiratory infection, due to contiguity of the abdominal air sacs to the oviduct (Levisohn and Kleven, 2000).

1.2.4.2. Clinical findings and lesions

     Clinical signs, morbidity, and mortality associated with *M. gallisepticum* infection in turkeys may be highly variable depending on *M. gallisepticum* strain virulence, complicating infections, and environmental and other stressors (Ley, 2003). Clinical signs attributed to *M. gallisepticum* seen in turkeys include sinusitis, respiratory distress, depression, decreased feed intake, and weight loss. As the disease progresses, tracheal rales, coughing, and laboured breathing may become evident if tracheitis or airsacculitis are present (Ley, 2003). The infection may last for months in untreated flocks and an important characteristic of *M. gallisepticum* is the frequent occurrence of asymptomatic infection (Levisohn and Kleven, 2000). Gross lesions

consist primarily of catarrhal exudates in nasal passages, trachea, bronchi, and air sacs. Sinusitis is usually most prominent in turkeys. Air sacs frequently contain caseous exudates, and some degree of pneumonia may be observed. In severe cases of typical air sac disease, there is the triad of airsacculitis, fibrinous or fibrinopurulent perihepatitis, and adhesive pericarditis resulting in high mortality and extensive condemnations at slaughter. However, these lesions may occur with other pathogens, and are not pathognomonic for *M. gallisepticum* (Ley, 2003). Razin *et al.* (1998) stated that the molecular mechanisms of mycoplasma pathogenicity have remained largely elusive and that the clinical picture of mycoplasma infections was more suggestive of damage due to host immune and inflammatory responses rather than to direct toxic effects by mycoplasmal cell components. Attachment to host cells is considered an important virulence factor, and in order to mediate adherence, *M. gallisepticum* organisms have specialized terminal tip structures (Bradbury, 2005). In spite of their remarkable reduction in genome size, mycoplasmas have a surprisingly great capacity for antigenic variation of major surface antigens, also named phenotypic plasticity (Levisohn *et al.*, 1995; Razin *et al.*, 1998). The ability of mycoplasmas to immunomodulate host immune responsiveness contributes to their pathogenic properties, enabling them to evade or suppress the host defense mechanisms and establish a chronic, persistent infection (carrier state) (Razin *et al.*, 1998; Ley, 2003; Rottem, 2003; Bradbury, 2005; Reinhardt *et al.*, 2005). Another virulence factor is (possibly) the ability to invade cells. Indeed, in tissue culture *M. gallisepticum* was found capable of entering non-phagocytic host cells (Winner *et al.*, 2000; Ley, 2003).

1.2.4.3. Diagnosis

The gold standard for *M. gallisepticum* diagnosis is their isolation and identification using species-specific antibodies and/or PCR for detecting the DNA. For *M. gallisepticum* culture, suspensions of tracheal or air sac exudates, turbinates, lungs, or swabs from trachea and choanal cleft can be inoculated directly to complex media such as mycoplasma broth or agar medium (Ley, 2003). Immunofluorescence or immunoperoxidase procedures may be used for rapid identification of mycoplasma cultures and inoculation of 7-day-old embryonated chicken eggs may be employed as another means of isolating *M. gallisepticum.* Furthermore, various PCR based procedures are available that are relatively rapid, sensitive and specific (Ley, 2003;

Bencina, 2005). Serologic procedures are useful for flock monitoring in *M. gallisepticum* control programs and to aid in diagnosis when infection is suspected. The serum plate agglutination (SPA) test which is commercially available is a quick, inexpensive and relatively sensitive test, but non-specific reactions (false positive) have been frequently observed (Bencina, 2005). The hemagglutination inhibition (HI) test has been commonly used to confirm reactors detected by SPA or ELISA, but it is time-consuming, the reagents are not commercially available, and the test may lack sensitivity. ELISAs were developed to increase testing efficiency and improve sensitivity and specificity of results relative to the SPA and HI tests. In a recently performed study, Feberwee *et al.* (2005) compared the technical performance of different available tests and concluded that it is not advisable to rely completely on one test (system) only. At present, serology is not a valid screening method if any *M. gallisepticum* vaccines have been used, because current serological tests cannot differentiate between antibody responses to vaccine or field strains (Bencina, 2005).

1.2.4.4. Treatment and control

Because *M. gallisepticum* can be egg transmitted, maintaining breeder flocks free of *M. gallisepticum* is only possible by starting with stocks known to be free of the infection and then rearing them with adequate biosecurity to avoid introduction of the organism, something that has been proven very difficult. Vaccination with bacterins has been shown to reduce, but usually not eliminate colonization by *M. gallisepticum* following challenge and generally is felt to be of minimal value in long-term control of infection (Ley, 2003). Live *M. gallisepticum* vaccines (strains F, ts-11 and 6/95) are used rather frequently, particularly in multi-age commercial operations (Ley, 2003). However, they should be used only in jurisdictions where they are approved, administered with strict adherence to the manufacturer's instructions, and with careful consideration for the safety on non-target flocks (Bencina, 2005). Currently available *M. gallisepticum* vaccines, however, have shown little potential for use in turkeys. *M. gallisepticum* has shown sensitivity *in vitro* and *in vivo* to several antimicrobials including macrolides (e.g. erythromycin), tetracyclines, pleuromutilins (e.g. tiamulin), fluoroquinolones (e.g. enrofloxacin), aminoglycosides (e.g. streptomycin) and others, but is intrinsically resistant to β-lactams (e.g. penicillins) since these work by inhibiting cell wall synthesis. Also acquired resistance has been noted (Ley, 2003; Bencina, 2005). Treatment can reduce severity

of disease, economic losses and shedding of *M. gallisepticum*, but does not eliminate *M. gallisepticum* from the infected poultry flock (Bencina, 2005). Egg injection or egg dipping were used to introduce antimicrobials into hatching eggs with the aim to obtain mycoplasma-free progeny flocks (Kleven, 2003; Ley, 2003). In general, these methods sometimes did not completely eliminate egg transmission, but it has made it possible to obtain sufficient *M. gallisepticum* -free chicken and turkey breeder flocks in the US (Ley, 2003). Egg heating (±46°C), an alternative method of reducing egg transmission, has also been practiced. Complete elimination of *M. gallisepticum* from all birds in an infected flock by mass antimicrobial therapy is an unrealistic expectation, and treatment should be regarded as a method for short-term amelioration of disease and economic effects, rather than as a long-term solution to the problem.

1.2.5. Multicausal respiratory disease

Although much is known about the individual agents responsible for respiratory diseases in poultry, uncomplicated infections with single agents are the exception. Under commercial conditions, complicated infections involving multiple etiologies with viruses, mycoplasmas and other bacteria, immunosuppressive agents, and unfavourable environmental conditions are more commonly observed than single infections.

In turkeys, only a few studies have been performed to elucidate the effects of combined action of viruses and other micro-organisms. The virus being regarded as having a very important role in turkeys is APV, and as a consequence, most studies have included APV as triggering agent. Cook *et al*. (1991) demonstrated that *B. avium* and *Pasteurella*-like organisms were able to colonize after an APV infection. Infection was somewhat more severe (slightly more severe clinical symptoms and thickened air sacs) when bacteria were included in the inoculum, but no poults in any of the experiments appeared sick and no mortality was recorded. In 1992, Naylor *et al*. demonstrated that an infection with APV accelerated the colonization of the lower tract by *M. gallisepticum* and that simultaneous infection resulted in respiratory disease of greater morbidity than following infection with either agent alone. However, for the affected birds, the severity of disease in the mixed infection group was not greater than that in the *M. gallisepticum* group. In an experiment to investigate the possible pathogenicity of *M. imitans* for turkeys, Ganapathy *et al*.

(1998) showed that in 1-day-old turkey poults, the presence of APV enhanced the ability of *M. imitans* to invade and colonize. *M. imitans* was only isolated from the upper respiratory tract in single infection, but was recovered also from lung and air sacs in the presence of the virus. After dual infection, they saw a significant increase in clinical signs and lesions, although these still remained relatively mild. On the contrary, dual infection of turkey poults with APV and *M. synoviae* did not result in detectable synergism, i.e. no increase in severity of clinical disease, nor gross and microscopic lesions due to APV (Khehra *et al.*, 1999). Several *O. rhinotracheale* strains, isolated from turkey, chicken or partridge, were used for aerosol challenge of turkeys of various ages (van Empel *et al.*, 1996). In turkeys, infection was aggravated by the prior administration of APV or Newcastle disease virus (NDV). In these studies, no airsacculitis nor pneumonia were seen in the absence of virus. Van de Zande *et al.* (2001) reported that APV/*E. coli* dual infection in turkey poults results in respiratory disease with a higher morbidity, higher incidence of lesions, and higher isolation of *E. coli* from inoculated poults compared with groups given single infections. Clinical symptoms such as depression and anorexia were only seen with dually infected birds and correlated well with the high incidence of gross lesions such as pneumonia, airsacculitis, perihepatitis and pericarditis. Mortality, however, as often seen in the field in APV/*E. coli* infected birds, was not encountered in their trial. In 13-wk-old turkeys, dual infection with APV and *E. coli* resulted in more severe clinical signs compared with single infection (Van de Zande *et al.*, 2002). In a study with a US APV isolate (Colorado strain), a dual infection in turkeys with either a turkey Newcastle disease virus isolate or broiler *E. coli* isolate resulted in increased morbidity rates and gross lesions compared with single infection, and more synergism was observed with the viral Newcastle infection than with *E. coli* (Turpin *et al.*, 2002). Most poults receiving APV/*E. coli* exhibited mild clinical signs (mild depression) early during infection, but swelling of sinuses, as frequently reported in the field, was not observed. In the APV/NDV infected birds more severe symptoms were found, ranging from decreased food consumption and in most of the birds nasal exudates with infraorbital, periocular and submandibular swelling. Very recently, Jirjis *et al.* (2004) used an APV subgroup C strain present in the US, to experimentally inoculate turkey poults together with different bacterial species. They found that infection was more severe (increase in severity or incidence of clinical scores, nasal discharge, swollen sinuses, microscopic inflammatory changes in both

upper and lower respiratory tract, and gross lesions in air sacs and lungs) in the turkey poults inoculated with APV when *B. avium* was administered either alone or in combination with *E. coli* and *O. rhinotracheale*. They concluded that *B. avium* had an additive effect on APV infection in turkeys, but this effect was not seen with APV in combination with *E. coli* or *O. rhinotracheale*. Very recently, Van Loock *et al*. (2006) examined the pathogenicity of an APV superinfection in *C. psittaci* predisposed turkeys. APV infection during the acute phase of a *C. psittaci* infection aggravated the severity of clinical signs, macroscopic lesions, pharyngeal APV excretion and histological tracheae lesions. Some of the single APV and single *C. psittaci* infected turkeys excreted nasal exudates with or without swollen sinus, whereas a higher percentage of dually infected turkeys showed similar and more long-lasting symptoms. In contrast, no clear interaction could be established after APV infection in latently *C. psittaci* infected SPF turkeys.

In some studies, other viruses were used in turkeys in order to try to reproduce severe clinical respiratory disease. Back *et al*. (1997) infected SPF turkeys with *O. rhinotracheale* and with *O. rhinotracheale* in combination with Newcastle disease vaccine virus, but were not able to reproduce clinical signs nor mortality. Charles *et al*. (1993) experimented with dual *P. anatipestifer* and NDV infection via different inoculation routes in turkeys, but were unable to reproduce clinical symptoms. They were only able to demonstrate some differences in histopathology. Sivanandan *et al*. (1991) evaluated the effect of an apathogenic avian influenza virus (AIV) subtype (H5N2) on the ability of the respiratory tract of turkeys to clear bacterial infections and suggested that AIV infection contributed to increased numbers and decreased clearance of *P. multocida*. Clinical symptoms were not mentioned. Experiments in turkeys have also been done with two bacterial strains, e.g. De Rosa *et al*. (1997) found that *B. avium* may enhance pathogenicity of *O. rhinotracheale*, although no convincing results were reported, and Droual and Chin (1997) were not able to find a synergistic effect between *O. rhinotracheale* and *E. coli* after intra air sac inoculation. Ficken *et al*. (1986) found that the clearance of *E. coli* from the air sacs was little affected after infection with *B. avium*. Van Alstine and Arp (1987) found in an infection experiment designed to study the effects of *B. avium* infection on the pulmonary clearance of *E. coli* in turkeys, that *B. avium* had no effect on the numbers *E. coli* in the lungs, but was associated with increased numbers of *E. coli* in tracheae. Severe airsacculitis was found more often in *B. avium* pre-infected turkeys.

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

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Chapter 2: AIMS OF THE STUDY

AIMS

Respiratory disease causes important financial losses in the turkey industry worldwide due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter. A variety of respiratory pathogens, both bacterial and viral, and adverse environmental conditions are the main factors contributing to the development of this disease. The pathogenesis of infections with these respiratory disease agents and their mutual interactions, are far from fully unraveled. This is partly responsible for the fact that therapeutic measurements against different bacterial infections are virtually entirely based on the administration of antimicrobials. In the literature, it is frequently mentioned that although different antimicrobial products are available, the clinical effects are very variable in the field. Hitherto, the actual *in vivo* efficacy of different antimicrobials for the treatment of different respiratory infections in poultry has only poorly been investigated. This is to a great extent rooted in the fact that often no suitable infection models are available. To extend our knowledge on the pathogenesis and control of respiratory infections in turkeys, the present thesis was initiated. Single and combined infections with avian metapneumovirus, *Ornithobacterium rhinotracheale*, *Escherichia coli* and *Mycoplasma gallisepticum*, all being important respiratory pathogens, were studied. Our main objective was to develop experimental infection models using these agents and subsequently use these models to study the pathogenesis and to compare the clinical efficacy of different antimicrobial compounds.

More specific goals of the study were:
- To develop *in vivo* experimental infection models for the reproduction of clinical respiratory disease in turkeys using different respiratory pathogens.
- To evaluate the efficacy of different antimicrobials and/or antimicrobial treatment schemes using the above developed infection models in order to allow more judicious antimicrobial treatments.
- To gain more insights in the pathogenesis of the different poultry pathogens and about the possible synergistic action between them.

Chapter 3: EXPERIMENTAL STUDIES

3.1. Development of *in vivo* infection models for the reproduction of clinical respiratory disease in turkeys

3.1.1. Synergy between avian metapneumovirus and *Ornithobacterium rhinotracheale* in turkeys

3.1.2. The influence of *Escherichia coli* and *Ornithobacterium rhinotracheale* on avian metapneumovirus infection in turkeys

3.1.3. Pathogenic interactions between *Mycoplasma gallisepticum*, avian metapneumovirus, *Escherichia coli* and *Ornithobacterium rhinotracheale* infections in turkeys

3.1.1. Synergy between avian metapneumovirus and *Ornithobacterium rhinotracheale* in turkeys

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Summary

The purpose of this study was to assess the possible synergism between *Ornithobacterium rhinotracheale* and avian metapneumovirus (APV), inoculated into turkeys via the natural route, for the reproduction of respiratory disease. Three-week-old SPF turkeys were inoculated oculonasally with either APV subtype A, *O. rhinotracheale* or both agents using two different time intervals (three or five days) between APV and *O. rhinotracheale*. The birds were observed clinically on a daily basis and swabbed intratracheally at short, regular intervals. They were euthanised at 1, 3, 5, 8 and 15 days post single or dual inoculation and examined for gross lesions at necropsy. Samples of the turbinates, trachea, lungs, air sacs, heart, pericardium and liver were taken for bacteriological and/or histological examination. Combined APV/*O. rhinotracheale* infections resulted in overt clinical signs and a longer persistence of *O. rhinotracheale* in the respiratory tract and aggravated the macroscopic and histological lesions in comparison to the groups given single infections. In all *O. rhinotracheale* challenged turkeys, *O. rhinotracheale* was isolated from the turbinates, trachea and lungs, but in turkeys infected with both agents, *O. rhinotracheale* was frequently found in the air sacs and on a single occasion in the heart and pericardium. The time interval between APV and *O. rhinotracheale* inoculation did not have a significant effect on the outcome of the dual infection. A conspicuous important feature was the attachment of *O. rhinotracheale* to the cilia of the epithelium of the turbinates and trachea of both *O. rhinotracheale* and APV/*O. rhinotracheale* infected birds. In conclusion, the results show that *O. rhinotracheale* is able to adhere to and colonize the respiratory tract, but without viral priming is not capable of inducing respiratory disease, under the circumstances used in this study.

Introduction

Viral and bacterial infections of the respiratory tract frequently result in disease in turkeys of all ages and may cause considerable financial losses due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at processing.

Several pathogens play an important role in respiratory disease, alone, in synergy or triggered by non-infectious factors. The respiratory viruses influenza virus type A, paramyxovirus types 1, 2 and 3 and avian metapneumovirus (avian pneumovirus, turkey rhinotracheitis virus) (APV) have been shown to be able to elicit respiratory problems. Involved bacteria are *Escherichia coli*, *Pasteurella* spp., *Bordetella avium*, *Ornithobacterium rhinotracheale*, *Mycoplasma* spp., *Chlamydophila psittaci* and *Riemerella anatipestifer*. These bacterial agents differ from the viral pathogens in that it is not always straightforward to reproduce clinical signs following experimental infection. *O. rhinotracheale*, an emerging infectious pathogen in poultry, illustrates this apparent paradox. This organism is generally believed to be a facultative pathogen (Back *et al.*, 1997; Droual and Chin, 1997; Back *et al.*, 1998; Franz *et al.*, 1997; van Empel *et al.*, 1999). However there are indications that *O. rhinotracheale* may be of primary importance (Ryll *et al.*, 1996; Travers *et al.*, 1996; van Empel *et al.*, 1996; Sprenger *et al.*, 1998). Some researchers believe that the different clinical outcome of *O. rhinotracheale* infections may be attributed to a variation in virulence (Travers *et al.*, 1996). However, very little is known about the pathogenesis of *O. rhinotracheale* infections in turkeys and the virulence determinants of this agent.

Respiratory disease in poultry is a multifactorial problem, with viral and bacterial respiratory pathogens often concurrently present and most probably influencing one another. Recently, in a longitudinal study performed by Van Loock *et al.* (2005), it was shown that both APV and *O. rhinotracheale* infections often occur between production onset and slaughter. Whether this virus, which belongs to the genus *Metapneumovirus* and this Gram-negative bacterium merely act separately or in a synergistic or additive way remains to be elucidated.

Only a few experimental studies have been undertaken both in chickens and turkeys to study possible mutual interactions between viruses and *O. rhinotracheale*. In chickens, *O. rhinotracheale* infection and/or the resulting clinical signs were shown to be aggravated by administration of Newcastle disease virus, infectious

bronchitis virus and APV virus (van Empel *et al.*, 1996; Franz *et al.*, 1997). In turkeys, aerosol or intra-air sac inoculation with APV (European isolate, type A) five days prior to *O. rhinotracheale* administration, elicited a worse clinical picture than single administration of *O. rhinotracheale* (van Empel *et al.*, 1996). More recently the research group of Jirjis *et al.* (2004) were not able to demonstrate the formerly noted additive effect between APV and *O. rhinotracheale* using oculonasal inoculation, although it should be noted that in contrast with the former study, a type C APV isolate present in the USA was used.

The present study was undertaken to develop a dual infection model for APV and *O. rhinotracheale* in turkeys using the natural oculonasal inoculation route. Two time intervals were tested between infections, with clinical signs, gross lesions, histology and bacterial titration as parameters for evaluating possible synergistic potential between both agents.

Materials and Methods

Turkeys

Seventy-five SPF turkeys (AFSSA, Ploufragan, France), hatched in our facilities, were used in this study. The birds received 16 hours of light per day, were housed on litter in separate HEPA-filtered isolation rooms and had free access to food and water.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titre of 5.5 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacterium

The *O. rhinotracheale* strain LMG 9086[T], originally isolated from a turkey with a respiratory tract infection, was used. The strain was serotyped as type A in an agar gel precipitation test (Hafez and Sting, 1999) by Professor H.M. Hafez (Institute of Poultry Diseases, Free University Berlin, Germany). The strain was stored at -70°C. The organism was cultured for 48h at 37°C on Columbia agar (Oxoid LTD., Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. The *O. rhinotracheale* bacteria were transferred into 5 ml brain heart infusion (BHI) (Oxoid LTD) for 24h at 37°C with agitation (microaerophilic incubation). The bacterial challenge inoculum was prepared by washing 1 ml cultured bacteria twice in phosphate buffered saline (PBS). A suspension containing 8.6 $\log_{10}$ colony forming units (cfu)/ml was prepared.

Experimental design

Seventy-five SPF turkeys were randomly divided into five experimental groups of 15 birds at one day of age. By two weeks of age, the birds were shown to be free from maternally derived antibodies to *O. rhinotracheale* and APV, respectively by a commercially available ELISA (Biochek, Gouda, Netherlands) and an in-house serum neutralization test. At the age of three weeks, the birds of group V (virus inoculated) and group B (bacterium inoculated) only received APV or *O. rhinotracheale*, respectively. The turkeys of two groups received successively APV (virus, V) and *O. rhinotracheale* (bacterium, B) with two different time intervals: three days for group V/3/B and five days for group V/5/B. Group C was maintained as an uninoculated control group. Each bird in group V, V/3/B and V/5/B was inoculated with APV by the oculonasal route at a dose of 4.4 $\log_{10}CD_{50}$ per bird. *O. rhinotracheale* was administered oculonasally to each bird of group B, V/3/B and V/5/B with a dosage of 8 $\log_{10}$cfu. For inoculation with APV and/or *O. rhinotracheale*, a total of 250 µl was divided equally over the nares and eyes.

All birds were examined clinically on a daily basis until they were sacrificed. The clinical signs were scored as indicated in Table 1. The mean clinical score was calculated for each experimental group.

Table 1. Scoring system for quantification clinical signs (Van de Zande *et al*., 2001)

Score	Clinical signs
0	Absence of clinical signs
1	Clear nasal exudate
2	Turbid nasal exudate
3	Nasal exudate with mildly swollen infra-orbital sinuses
4	Nasal exudate with extremely swollen sinuses
5	Nasal exudate with extremely swollen sinuses and frothy eyes
6	Score 5 + poor general condition
7	Score 6 + anorexia

Tracheal swabs were collected from groups V, V/3/B and V/5/B at three days post viral infection (dpvi) for virus titration. Tracheal swabs were also taken from birds belonging to groups B and C to detect adventitious exposure. Before inoculation with *O. rhinotracheale*, tracheal swabs were taken from all birds in all groups for bacteriological examination, which proved to be negative for *O. rhinotracheale*. Furthermore, tracheal swabs for bacteriological titration were collected from all groups experimentally inoculated with *O. rhinotracheale* (B, V/3/B, V/5/B) at 2, 4, 6 and 7 days post bacterial inoculation (dpbi) and daily from 9 dpbi until 14 dpbi. The tracheal swabs were collected using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc.**,** Corona, USA) and placed in transport medium. The latter consisted of 1 ml PBS supplemented with Ca^{2+} and Mg^{2+} for bacteriological examination and supplemented with 10% fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco) for virus titration. Processing occurred as described below.

In all groups, three birds were sacrificed from control group and at 1, 3, 5, 8 and 15 days post single (groups V and B) or dual inoculation (groups V/3/B and V/5/B). Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The birds were necropsied and examined for gross lesions. Samples of the turbinates, trachea (proximal and distal part) and lungs were collected from all sacrificed birds for bacteriological examination. A 10% suspension in PBS was made from these tissue samples. Air sacs, heart, pericardium and liver were likewise sampled with cotton swabs for bacteriological analysis. These swabs were suspended in 1 ml PBS

supplemented with Ca^{2+} and Mg^{2+}. All samples for bacterial isolation were processed immediately after collection as described below. Finally, samples from the turbinates, trachea, lungs, heart and liver were collected and fixed in 10% neutral buffered formalin for histopathological examination.

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Virological titration of tracheal swabs

After 20 minutes shaking in the transport medium at 4°C, the swabs were removed and the homogenate was centrifuged (5 min at 8800 × g) to sediment cellular debris. The supernatant was harvested in 1 ml aliquots and stored at -70°C until further processing. Serial tenfold dilutions of the supernatant were inoculated into tracheal organ cultures obtained from 26-day-old turkey embryos and individually maintained in tissue culture tubes (Cook *et al.*, 1976). The tracheal organ cultures were incubated with 0.1 ml of supernatant and 0.9 ml organ culture medium, containing minimal essential medium supplemented with hepes buffer 1 M (2%) (Gibco), penicillin (100 U/ml) (Biopharma), streptomycin (0.1 g/ml) (Certa, Braine-l'Alleud, Belgium), kanamycin (0.1 g/ml) (Gibco) and glutamine (0.3 g/ml) (BDH Laboratory Supplies, Poole, England). The tubes were then placed in a roller drum apparatus and were observed for 7 days and examined for ciliostasis. Virus titres are expressed as $\log_{10}CD_{50}$ per g mucus obtained from the tracheal swabs.

Bacteriological titration of tracheal swabs

After 20 minutes shaking in the Ca^{2+} and Mg^{2+} supplemented PBS, the swabs were removed and undiluted supernatant and tenfold serial dilutions were inoculated in duplicate onto 5% sheep blood agar supplemented with 5 μg/ml gentamicin and 5 μg/ml polymyxin to suppress growth of contaminant bacteria (van Empel and Hafez, 1999). After 24-48h of incubation at 37°C in a 5% CO_2 atmosphere, viable counts were performed. The number of cfu/ml was calculated. Bacterial titres were expressed as cfu per g mucus obtained from the tracheal swabs.

Bacteriological titration of tissue suspensions

From samples of the turbinates, proximal trachea, distal trachea and lungs, the number of *O. rhinotracheale* organisms was determined in threefold by incubating

undiluted tissue suspensions and tenfold serial dilutions of tissue suspensions on 5% sheep blood agar supplemented with 5 µg/ml gentamicin and 5 µg/ml polymyxin. After 24-48h of incubation at 37°C in a 5% CO_2 atmosphere, viable counts were performed and the number of cfu/g tissue calculated. The swabs taken from the air sacs, pericardium, heart and liver were shaken for 20 minutes at 4°C in the Ca^{2+} and Mg^{2+} supplemented PBS. Consequently, the swabs were removed and 20 µl of the suspension was inoculated onto 5% sheep blood agar supplemented with 5 µg/ml gentamicin and 5 µg/ml polymyxin. After 24-48h of incubation at 37°C in a 5% CO_2 atmosphere, the number of cfu per plate was counted and scored on a scale from 0 to 3: score 0 = 0 cfu; score 1 = 1 to 10 cfu; score 2 = 11 to 100 cfu; score 3 = more than 100 cfu.

Histopathology

Following fixation in formalin, the tissues were embedded in paraffin, sectioned at 4 µm, mounted on glass slides and stained with hematoxylin and eosin (H&E) using standard procedures. Tissues from the turbinates and upper and lower trachea were additionally stained by the Periodic Acid Schiff (PAS)-reaction. When bacteria attached to the cilia were observed, a Giemsa and Gram-stain were also performed.

Results

Clinical signs

The mean clinical score for each group is shown in Figure 1. Respiratory signs were not detected in birds from groups B or C (uninoculated control). Respiratory signs were seen in all APV-inoculated groups starting from 3 dpvi. In group V, mean clinical scores gradually increased from 3 until 6 dpvi at which time point the highest mean score (2.6) was seen. Then clinical symptoms declined and were absent in all birds at 9 dpvi. In group V/3/B, the mean clinical score augmented from 3 dpvi and reached a high level from 4 until 9 dpvi, with a peak score (2.9) observed at 6 dpvi and 9 dpvi. A gradual decline was noted starting from 9 dpvi with an absence of clinical signs from 12 dpvi onwards. In group V/5/B respiratory signs increased from 3 dpvi onwards and peaked (2.9) at 9 dpvi. Clinical signs in this group could be

observed until 12 dpvi. In group V, V/3/B and V/5/B respiratory symptoms lasted for 8, 11 and 12 days after viral inoculation, respectively.
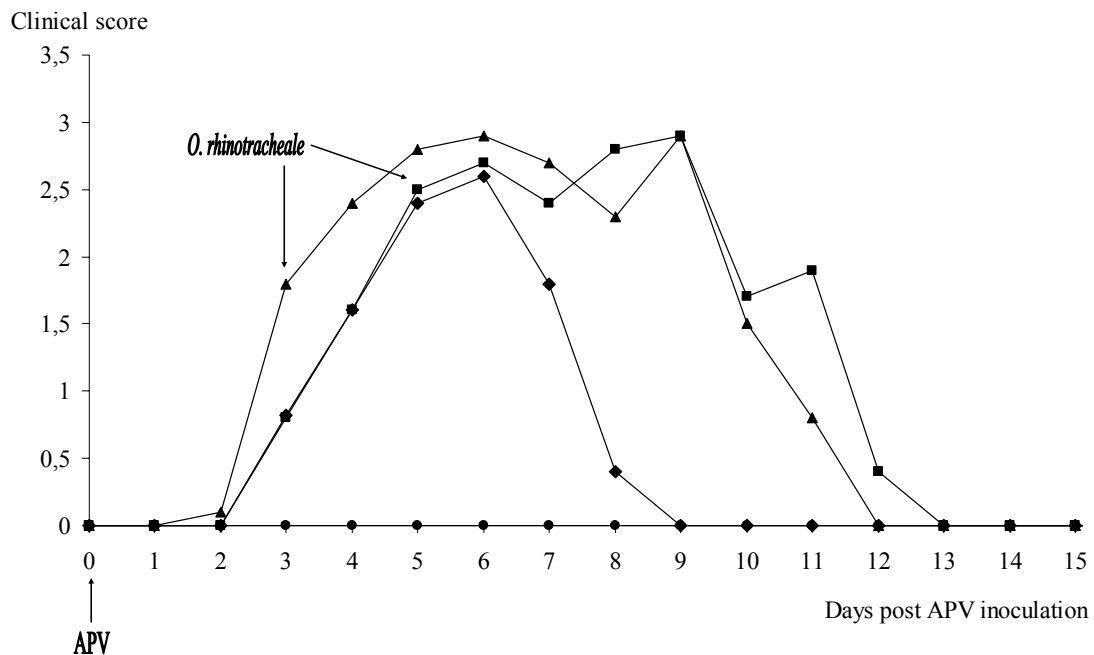
Figure 1. Mean clinical scores in turkeys after single APV inoculation (◆ group V), single *O. rhinotracheale* inoculation (● group B) or dual APV/*O. rhinotracheale* inoculation with interval of three days (▲ group V/3/B) or five days (■ group V/5/B).

Virological titration of tracheal swabs

Confirmatory viral titrations of tracheal swabs collected at 3 dpvi showed that APV was recovered from every individual bird of all APV infected groups. With mean titres of 5.5, 5.7 and 5.5 $\log_{10}CD_{50}$/g mucus for group V, V/3/B and V/5/B, respectively. No viral cross-infection between groups occurred as no APV was recovered from tracheal swabs from group B and C.

Bacteriological titration of tracheal swabs

O. rhinotracheale was not recovered from tracheal swabs from any of the birds of groups V and C. The results of *O. rhinotracheale* titrations of the tracheal swabs of the remaining groups are shown in Figure 2. Mean titres as well as titres for each individual bird are given. In group B the number of *O. rhinotracheale* was the highest (mean of 6.6 $\log_{10}$ cfu/g mucus) at 4 dpbi and after 7 dpbi, *O. rhinotracheale*

could not be isolated anymore. The highest value in group V/5/B was found at 4 dpbi (6.9 $\log_{10}$ cfu/g mucus). In group V/3/B bacterial titres peaked at 6 dpbi (6.7 $\log_{10}$ cfu/g mucus). A high titre was still found at 7 dpbi. For groups V/3/B and V/5/B, *O. rhinotracheale* could not be recovered anymore from 10 dpbi onwards.
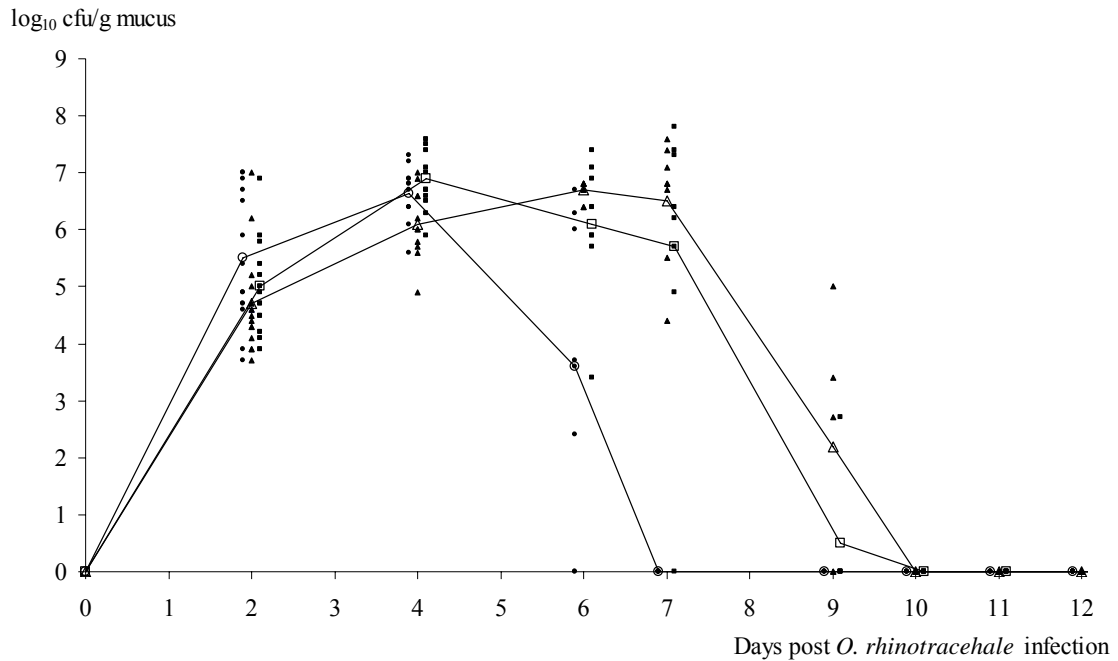
Figure 2. *O. rhinotracheale* titres (in $\log_{10}$cfu/g mucus) in tracheal mucus collected at different time points after *O. rhinotracheale* inoculation of single *O. rhinotracheale* (●,○ group B) and dually APV/*O. rhinotracheale* infected turkeys with an interval of three days (▲,∆ group V/3/B) or five days (■,□ group V/5/B). (Individual values are given with closed patterns; means are given with open patterns)

Bacteriological titration of tissue samples

As expected, *O. rhinotracheale* was never isolated from any of the tissues from the birds of groups V and C. The results of *O. rhinotracheale* titration in the other groups are shown in Table 2.

Table 2. Mean titre of *O. rhinotracheale* ($\log_{10}$cfu/g tissue) isolated from different organs from turkeys inoculated with *O. rhinotracheale* alone (group B) or with APV and *O. rhinotracheale* with an interval of three days (group V/3/B) or five days (group V/5/B).

Organ	Group B					Group V/3/B					Group V/5/B				
	Days post *O. rhinotracheale* inoculation					Days post *O. rhinotracheale* inoculation					Days post *O. rhinotracheale* inoculation				
	1	3	5	8	15	1	3	5	8	15	1	3	5	8	15
Turbinates	7.7/6.2/4.2[a]	7.5/7.5/6.5	9.4/9.0/6.5	0/2.9/0	0/0/0	7.1/6.4/0	8.9/7.5/8.0	8.8/6.6/8.2	3.6/0/8.0	0/0/0	4.8/4.8/.	8.5/8.3/8.1	8.5/7.2/8.3	7.7/7.6/6.7	0/0/0
Trachea (proximal part)	4.0/3.8/2.7	7.6/7.7/7.7	5.9/7.9/7.2	0/0/0	0/0/0	4.0/3.2/2.9	8.3/5.6/5.6	7.4/6.5/4.3	0/0/5.9	2.2/3.5/0	0/0/0	7.6/7.8/8.3	8.0/7.2/7.1	7.3/6.7/0	0/0/0
Trachea (distal part)	2.9/3.9.0	4.1/6.4/2.2	3.6/7.9/7.2	0/0/0	0/0/0	3.9/2.9/2.4	8.1/5.9/6.9	8.3/7.4/7.7	0/0/7.6	0/2.9/0	0/2.2/2.5	8.0/8.1/8.0	7.8/7.4/7.3	8.1/6.6/0	0/0/0
Lungs	0/0/0	0/2.2/3.9	3.2/4.5/3.9	0/0/0	0/0/0	3.5/0/2.8	3.5/5.1/4.1	6.8/6.0/5.8	0/0/5.2	0/0/0	0/0/0	6.3/6.0/6.8	6.9/5.7/6.8	6.5/4.9/4.8	0/0/0
Air sacs[b]	0/0/0[c]	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	3/3/3	0/0/0	0/0/0	0/0/0	2/3/0	2/0/1	0/0/0	0/0/0
Pericardium[b]	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/2	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Heart[b]	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0

a : data from three turkeys ($\log_{10}$ cfu / g tissue)

b : These organs were swabbed. No tissue suspension was made.

c : 0 = 0 colonies / 1 = 1 to 10 colonies / 2 = 11 tot 100 colonies / 3 = more than 100 colonies

In group B, *O. rhinotracheale* could be isolated from the turbinates from 1 dpbi until 8 dpbi, with highest mean titre at 5 dpbi (mean of 8.3 $\log_{10}$ cfu/g tissue). *O. rhinotracheale* was found in the proximal and distal trachea from 1 dpbi until 5 dpbi (peak mean titre at 3 dpbi of 7.7 $\log_{10}$ cfu/g tissue for the proximal trachea and at 5 dpbi of 6.2 $\log_{10}$ cfu/g tissue for distal trachea) and in the lungs from 3 dpbi until 5 dpbi (highest mean titre at 5 dpbi, 3.9 $\log_{10}$ cfu/g tissue). *O. rhinotracheale* was not recovered from any other organ in group B.

In group V/3/B, *O. rhinotracheale* was recovered from the turbinates and lungs between 1 and 8 dpbi (peak mean titre at 3 dpbi of 8.1 $\log_{10}$ cfu/g tissue for the turbinates and at 5 dpbi of 6.2 $\log_{10}$ cfu/g tissue for the lungs), and from the proximal (peak mean titre at 3 dpbi, 6.5 $\log_{10}$ cfu/g tissue) and distal trachea (peak mean titre at 5 dpbi, 7.8 $\log_{10}$ cfu/g tissue) from 1 to 15 dpbi. Furthermore *O. rhinotracheale* was recovered from the air sacs at 3 and 5 dpbi. At 3 dpbi, *O. rhinotracheale* was isolated from the heart of one bird, and at 5 dpbi *O. rhinotracheale* was also found in the pericardium of one animal. *O. rhinotracheale* was never recovered from any of the liver samples.

Finally in group V/5/B, *O. rhinotracheale* could be isolated from turbinates and distal trachea from 1 to 8 dpbi with peak mean titre at 3 dpbi of 8.3 $\log_{10}$ cfu/g tissue and 8 $\log_{10}$ cfu/g tissue respectively, and from the proximal trachea and lungs from 3 to 8 dpbi, with a peak mean titre at 3 dpbi of 7.9 $\log_{10}$ cfu/g tissue and 6.4 $\log_{10}$ cfu/g tissue respectively. Furthermore *O. rhinotracheale* was isolated from the air sacs at 3 and 5 dpbi. *O. rhinotracheale* was never recovered from any of the heart, pericardium or liver samples.

Macroscopic findings

No marked macroscopic lesions were seen in group C. In the animals of group B, at 3 and 5 dpbi, a slight amount of serous to seromucous exudate was found in the turbinates and sinuses of most sacrificed birds. The turbinates, the proximal and on occasion medial trachea were moderately hyperaemic. From 8 dpbi onwards, no lesions were observed. In group V, most birds sacrificed at 4, 6 and 8 dpvi showed seromucous exudate in the turbinates and sinuses. A moderate to severe hyperaemia was also present in the nasal cavities and in the proximal and medial trachea. From 11 dpvi no lesions were apparent. In groups V/3/B and V/5/B, lesions found at necropsy were similar. In birds that were necropsied between 1 and 8 dpbi, exudate was found

in the turbinates and sinuses that evolved from clear to seromucous. The amount of exudate varied from moderate to abundant with an aggravation seen with time. Hyperaemia of the turbinates, the proximal and sometimes medial trachea was prominently present between 3 and 8 dpbi. A moderate hyperaemia of the proximal trachea was seen at 15 dpbi.

Histopathology

In the control group (group C), no marked microscopic changes were observed in the turbinates, trachea or lungs.

In the turbinates and trachea of animals in group V, the most severe lesions were observed at 4, 6 and 8 dpvi. The mucosa was infiltrated with large amounts of heterophils and monomorphonuclear cells. Furthermore, there was multifocal damage to epithelial cells, consisting of cellular necrosis and loss of cilia. In subepithelial mucous glands, a decreased mucus production and several apoptotic bodies were present. A mild, focal, heterophilic infiltration was found in the lungs of one animal at 6 dpvi.

In group B only minor histological abnormalities were observed. A mild infiltration of mixed inflammatory cells was found in the turbinates and trachea at 3 and 5 dpbi and loss of cilia was mildly present in these samples. In one animal euthanised at 5 dpbi, Gram-negative rod-shaped bacteria were seen attached to the cilia of the tracheal epithelium. In the lungs of one animal, a mild, focal infiltration of heterophilic granulocytes was found.

In the dually infected animals, the histological lesions in the samples of the turbinates and trachea were more severe in comparison to the single viral or bacterial infection. In group V/3/B, a severe mixed inflammatory reaction was present in the mucosa of the turbinates and trachea from 3 to 11 dpvi and 3 to 8 dpvi, respectively. From approximately 3 dpvi onwards, loss of cilia and/or necrosis of epithelium, as well as degeneration of mucous glands, were found in the turbinates and trachea. The most severe lesions were observed from 6 to 8 dpvi. Gram-negative, rod-shaped bacteria attaching cilia of tracheal cells were present in one animal 8 dpvi. In lung samples collected at 8 and 11 dpvi, focal areas of suppurative pneumonia, bronchitis and bronchiolitis were observed in one bird.

Compared to group V/3/B similar but slightly more severe tracheal and turbinate lesions were present in group V/5/B. Attachment of Gram-negative bacteria

to cilia of epithelial cells were observed in the trachea of two animals euthanised at 8 dpvi, in the turbinates and/or trachea of two birds at 10 dpvi and in the trachea of two animals at 13 dpvi (Figure 3). Mild lesions in the lungs comparable with those observed in group V/3/B were also present.

A correlation between the presence of bacteria on the cilia, severity of histopathological lesions and clinical scores was not obvious.
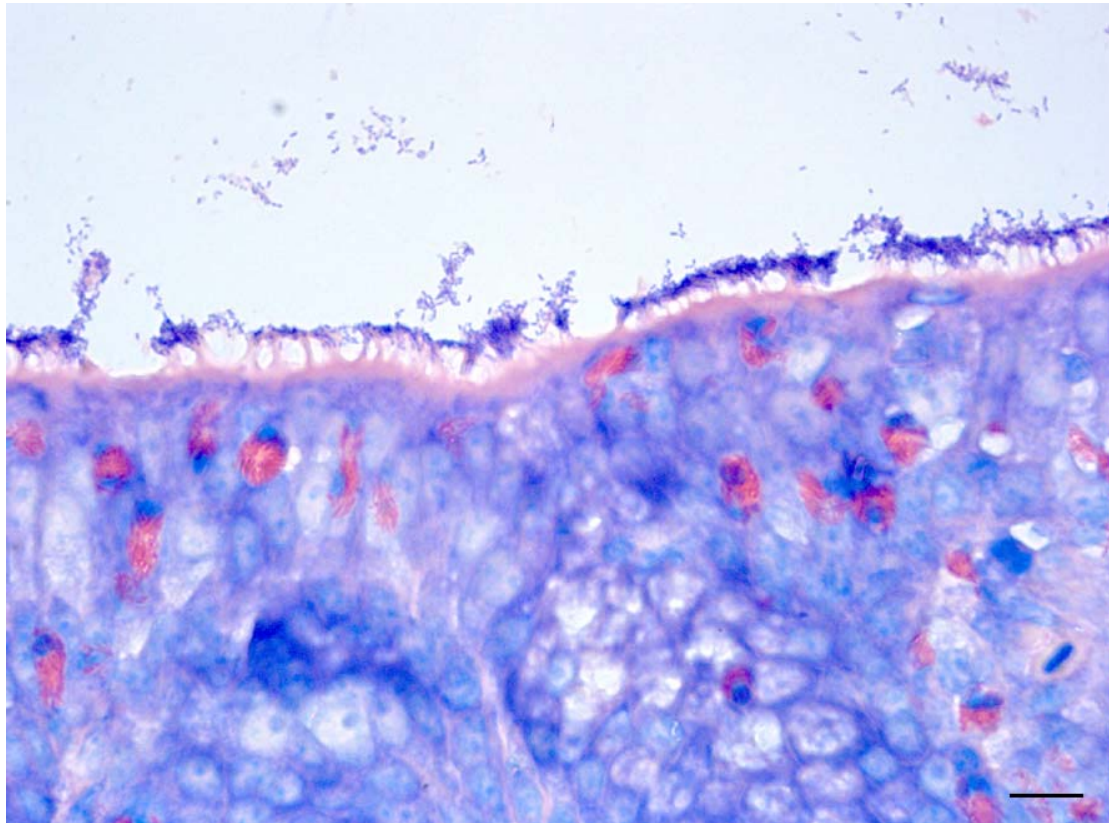
Figure 3. Gram-negative rod-shaped bacteria attached to the cilia of the epithelium of the turbinates in a turkey of group V/5/B. Giemsa. Bar: 10 μm.

Discussion

In the present study, infection of turkeys with *O. rhinotracheale* without a concomitant viral trigger did not provoke overt clinical signs, although at necropsy a slight amount of serous to seromucous exudate was noted in the turbinates and sinuses of most birds, and histological examination revealed minor lesions in the trachea and turbinates. These results corroborate with the findings of several researchers who likewise came across difficulties in producing respiratory signs in

turkeys (Back *et al.*, 1997; De Rosa *et al.*, 1997; Droual and Chin, 1997; Jirjis *et al.*, 2004), but are in contrast with the results of other researchers. Some research groups did reproduce marked disease, occasionally evolving into death, following intratracheal and intrasaccular inoculation of *O. rhinotracheale* (Ryll *et al.*, 1996; Sprenger *et al.*, 1998). Also, van Empel *et al.* (1996) were able to evoke clinical symptoms, albeit fairly mild (growth retardation and airsacculitis), after intra-air sacs and aerosol challenge.

Several issues are worth considering to attempt to clarify the divergence in success rate between the different studies. In the challenge experiments mentioned above, various *O. rhinotracheale* strains were used, which may differ in virulence. Apparent virulence differences have indeed been described among *O. rhinotracheale* strains isolated from chickens and turkeys (Ryll *et al.*, 1996; Travers *et al.*, 1996; van Veen *et al.*, 2000b; El-Sukhon *et al.*, 2002). The strain used in the present study originated from a turkey with respiratory disease and therefore was assumed to be at least capable of causing respiratory problems. Furthermore, different inoculation routes applied may explain study discrepancies. In the present study, *O. rhinotracheale* was administered oculonasally, which mimics best the natural disease exposure. In all experiments that were successful in reproducing acute clinical signs in turkeys, intratracheal or intrasaccular infection was applied (Ryll *et al.*, 1996; Sprenger *et al.*, 1998) and in this way the innate defence mechanisms situated at the upper respiratory tract are possibly bypassed. Other possible factors may be host related, with the age and health status as plausible determinants, although the occurrence of the disease does not seem to be correlated to a narrow age group, not in field outbreaks, nor under experimental conditions (De Rosa *et al.*, 1996; Hafez, 1996; Ryll *et al.*, 1996; Travers, 1996; van Empel *et al.*, 1996; Hinz and Hafez, 1997; Sprenger *et al.,* 1998; van Veen *et al.*, 2000a). Noteworthy is the observation that in all successful experiments conventional turkeys were used instead of SPF animals. Possibly, in conventional turkeys, undefined infectious agents lead to synergistic effects upon coinfection with *O. rhinotracheale*. Nonetheless we chose for SPF turkeys in our trial as such turkeys, compared to conventional birds, have a better defined bacterial status with better opportunity to conduct reproducible experiments.

Although in the present study no highly acute disease with mortality was induced in association with *O. rhinotracheale* challenge, *O. rhinotracheale* was shown to be able to adhere to and colonize the upper respiratory tract of turkeys, and

could be recovered from the turbinates and trachea from 1 until 8 dpbi. Microscopic examination revealed Gram-negative, rod-shaped bacteria attached to the cilia of the epithelium of the trachea and turbinates of various infected birds. This is the first study reporting this distinct feature and hence constitutes the *in vivo* confirmation of the data generated by Soriano *et al*. (2002) having demonstrated *in vitro* adherence ability of *O. rhinotracheale* to chicken tracheal epithelial cells. The latter study did not prove actual adhesion to the cilia, again emphasising the value of the present study. The ability of bacteria to adhere to mucosal epithelium is dependent on the expression of adhesive molecules or structures, called adhesins, allowing attachment of the organisms to complementary molecules on mucosal surfaces, the receptors (Jacques and Paradis, 1998). The adherence of bacteria to host tissue is usually regarded as an important prerequisite for colonization and virulence manifestation of a microorganism. There is no information about the mechanism by which *O. rhinotracheale* adheres to the tracheal surface, nor about the alleged receptor. Hence our findings concerning tissue adhesion justify further investigations into adhesion mechanisms.

Infection with APV caused clinical disease with various symptoms including nasal discharge evolving from serous to seromucous, swollen infraorbital sinuses, snicking, open mouth breathing and frothy ocular discharge. Upon histological examination, infiltration of heterophils and monomorphonuclears was noted in the mucosa of the turbinates and trachea. Furthermore, focal damage to the epithelium, loss of cilia, and degeneration of the mucous glands with excretion of mucus in the lumen were evident. All these lesions approximately had the same course, and by 11 dpvi the histological picture was restored to normal. These APV-caused lesions are in agreement with other experiments. Inflammation and deciliation of the trachea and turbinates had already been observed in experiments performed by Jones *et al*. (1986), Randall and Reece (1996), Van de Zande *et al*. (1999), and Jirjis *et al*., (2002, 2004). Degeneration of the mucous gland cells and damage to the respiratory epithelium were likewise previously demonstrated (Randall and Reece, 1996; Van de Zande *et al*., 1999).

In the present study, viral priming with APV was necessary to evoke marked clinical disease. The APV/*O. rhinotracheale* dual infection resulted in a higher morbidity than single infections with either agent. The clinical symptoms in themselves were similar in nature to those caused by APV alone, but they were more

severe and persisted markedly longer. This aggravation of clinical disease was reflected in the necropsy findings in that the lesions found in the respiratory tract were clearly more outspoken in the dually infected birds. Furthermore, microscopical lesions in the birds having received both agents were generally more extensive and more prolonged in comparison to the singly-infected animals. Also, bacterial titres in the turbinates and trachea remained at a higher level for a longer period of time in comparison with the singly infected animals, a phenomenon especially noted in group V/3/B. APV priming appears not to be necessary for *O. rhinotracheale* to be able to infect the lungs, although it seems to facilitate this process with bacteria being isolated at an earlier stage and in larger amounts. Following the combined APV/*O. rhinotracheale* infection, *O. rhinotracheale* was able to infect the air sacs, a feature never encountered in the mono-infected birds. In one group of the dually infected birds (V/3/B), *O. rhinotracheale* was isolated from non-respiratory internal organs (in one bird the pericardium and in one bird the heart). At necropsy however, no signs of septicaemia were noticed. *O. rhinotracheale* has in the past occasionally been isolated from liver, spleen, kidney, ovary, oviduct, joints and brain after experimental infections (Travers *et al*., 1996; Back *et al*., 1998; Sprenger *et al*., 1998; van Veen *et al*., 2000b). Furthermore, pericarditis has been encountered under natural circumstances (Hafez, 1994; van Beek *et al*., 1994; Hafez, 1996). These findings all endorse our results that APV and *O. rhinotracheale* exert a synergistic pathogenic effect if given consecutively, spaced by three or five days, to susceptible turkeys. The results indicated that increasing the interval between APV and *O. rhinotracheale* infection from three to five days has no major influence on the outcome of the disease.

The clinical signs resulting from the dual infection are similar to but generally milder than those seen in field cases (De Rosa *et al*., 1996; Roepke *et al*., 1998). This difference may be attributed to the often inadequate environmental and management conditions (high animal density, inadequate ventilation, high ammonia levels, too high and low a relative humidity) and additional pathogens encountered in the field, exacerbating any disease which has been brought about. The animals used in this study were in contrast, kept in spacious rooms with HEPA-filtered air with no extraneous pathogens interplaying.

In conclusion, it was shown that the *O. rhinotracheale* strain LMG 9086 in itself is able to adhere to and colonize the respiratory tract, but without viral priming

does not induce respiratory disease. In contrast, dual infection with APV and *O. rhinotracheale* resulted in more severe clinical symptoms, macroscopic and histological findings, and longer persistence of *O. rhinotracheale* in the respiratory tract. The established *O. rhinotracheale* single and APV/*O. rhinotracheale* dual infection models can undoubtedly be used to further investigate the mechanism of *O. rhinotracheale* colonization and the APV/*O. rhinotracheale* synergy. Further, the APV/*O. rhinotracheale* dual infection model may be used to test preventive and curative measures to combat the respiratory disease.

Acknowledgements

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3.1.2. The influence of *Escherichia coli* and *Ornithobacterium rhinotracheale* on avian metapneumovirus infection in turkeys

Marien, M., Nauwynck, H., Chiers, K., Froyman, R., and Decostere, A.

Summary

Three-week-old specified pathogen free turkeys were inoculated oculonasally with either avian metapneumovirus (APV), APV/*Ornithobacterium rhinotracheale*, APV/*Escherichia coli*, APV/*E. coli*/*O. rhinotracheale* or APV/*O. rhinotracheale*/*E. coli* with a three days interval between viral and bacterial inoculation and approximately eight hours between the two bacterial inoculations. The animals were observed clinically and swabbed intratracheally on a daily basis. Five and ten birds were euthanised at five and 15 days post bacterial inoculation (dpbi), respectively, and examined for gross lesions at necropsy. Samples of the turbinates, sinuses, trachea, lungs, air sacs, heart, pericardium and liver were taken for bacteriological and/or histological examination.

Combined APV, *E. coli* and *O. rhinotracheale* infections resulted in more severe and longer persisting clinical signs and more severe macroscopic and microscopic lesions compared to dual APV/*O. rhinotracheale* and APV/*E. coli* infections, and the latter produced more severe disease than the single APV infection. The level of *O. rhinotracheale* and *E. coli* multiplication in the respiratory organs did not differ greatly between the dual and triple infection groups, although it should be noted that after triple infection *E. coli* could be isolated from the trachea two days longer in comparison with the dually infected group. Another conspicuous feature was that only in the triple infected birds *O. rhinotracheale* was frequently recovered from the pericardium, and once from the heart and from the liver.

These findings all endorse our conclusions that APV, *E. coli* and *O. rhinotracheale* exercise an additive pathogenic effect in the reproduction of respiratory disease if given consecutively to susceptible turkeys.

Introduction

Respiratory diseases cause financial losses in the turkey industry world-wide due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter (van Empel and Hafez, 1999).

Several pathogens may play an important role in respiratory disease, alone, in synergy or triggered by other infectious pathogens or by non-infectious factors such as climatic conditions and management related problems. Respiratory disease may be produced by respiratory viruses such as influenza virus type A, paramyxovirus types 1, 2 and 3 and avian metapneumovirus (turkey rhinotracheitis virus) (APV). However, clinical signs following experimental inoculation with these viruses are less severe than those observed in the field. It is generally accepted that secondary bacterial pathogens are often involved, with amongst others *Escherichia coli*, *Pasteurella* spp., *Bordetella avium*, *Ornithobacterium rhinotracheale*, *Mycoplasma* spp., *Chlamydophila psittaci* and *Riemerella anatipestifer*.

Van Loock *et al*. (2005) demonstrated in a longitudinal study that both APV and *O. rhinotracheale* infections often occur between production onset and slaughter on Belgian turkey farms. In a previously performed experiment, we were able to demonstrate the synergistic effect between APV and *O. rhinotracheale* for the reproduction of respiratory disease, when inoculated into turkeys via the natural infection route (Marien *et al*., 2005). Nevertheless, the clinical signs resulting from the APV/*O. rhinotracheale* dual infection were similar, but generally milder than those seen in field cases (De Rosa *et al*., 1996; Roepke *et al*., 1998). Likewise, Van de Zande *et al*. (2001) were able to show an additive effect between APV and *E. coli* infection in turkeys, but again less severe symptoms (i.e. no mortality) were seen than those encountered in the field. This difference may be attributed to the often inadequate environmental and management conditions in the field and synchronous infections with several pathogenic agents. The natural presence of *O. rhinotracheale* and *E. coli* has often been demonstrated in poultry (El-Sukhon, 2002; Vandekerchove *et al*., 2004). In contrast to dual infection studies, very few triple infection experiments have been carried out in turkeys and chickens. Jirjis *et al*. (2004) demonstrated that infection of turkey poults with APV followed by a combination of *B. avium*, *E. coli* and *O. rhinotracheale* was more severe compared to the poults inoculated with APV alone. The additive effect was primarily attributed to *B. avium*.

The present study was undertaken to develop a triple infection model for APV, *E. coli* and *O. rhinotracheale* in turkeys using the natural oculonasal inoculation route. The objective was to reproduce respiratory disease with severe clinical symptoms as encountered in the field, more in particular septicaemia as frequently seen in practical conditions, in order to be able to evaluate amongst others antimicrobial treatment schemes and environmental or management factors. Clinical signs, gross lesions, histology and bacterial titration were enclosed as parameters for evaluating possible synergistic effects between the three agents.

Materials and Methods

Turkeys

Seventy-three specified pathogen free (SPF) turkeys (AFSSA, Ploufragan, France), hatched in our facilities, were used in this study. The birds received 16h of light per day, were housed on litter in HEPA-filtered isolation rooms and had free access to food and water.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titre of 5.5 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacteria

The *O. rhinotracheale* strain LMG 9086^{T}, originally isolated from a turkey with a respiratory tract infection, was used. The strain was serotyped as type A in an agar gel precipitation test (Hafez and Sting, 1999) by Professor H. M. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany). The strain was stored at -70°C. The organism was retrieved from the frozen suspension and cultured for 48h at 37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. Ten colonies were transferred into 5 ml brain heart infusion (BHI) broth (Oxoid LTD) for 24h at 37°C with agitation (microaerophilic incubation). The bacterial challenge inoculum was prepared by

washing 1 ml cultured bacteria twice in phosphate buffered saline (PBS). A suspension containing 8.6 $\log_{10}$ colony forming units (cfu)/ml was prepared.

A Belgian *E. coli* isolate retrieved from turkeys with colibacillosis was used. It was identified and serotyped as O2:K1 by Dr. Flemming Scheutz (Statens Serum Institute, The International *Escherichia* and *Klebsiella* Centre, Copenhagen, Denmark). The strain was stored at -70°C. The organism was retrieved from the frozen suspension and cultured overnight at 37°C on MacConkey agar (Oxoid LTD). Three colonies were transferred into BHI broth and cultured for 24h at 37°C. The bacterial challenge inoculum was prepared by washing 1 ml cultured bacteria two times in PBS. A suspension containing 8.6 $\log_{10}$ cfu/ml was prepared.

Experimental design

Seventy-three SPF turkeys were randomly divided into six groups at one day of age. Five groups consisted of thirteen animals and one group of eight animals (control group). At two weeks of age, the birds were shown to be free from maternally derived antibodies to *O. rhinotracheale* and APV, by a commercially available ELISA (Biochek, Gouda, the Netherlands) and an in-house serum neutralization test (Marien *et al.*, 2006), respectively. Before bacterial inoculation, tracheal swabs were taken from all birds of each experimental group for bacteriological examination. All animals were negative for *O. rhinotracheale* and *E. coli*.

At the age of three weeks, turkeys of two groups first received APV followed by *O. rhinotracheale* (A/O) or *E. coli* (A/E) three days later. The animals from the groups A/O/E and A/E/O first received APV, followed by *O. rhinotracheale* and *E. coli* three days after APV inoculation. The animals of group A/O/E were inoculated with *O. rhinotracheale* at 8 am and with *E. coli* at 5 pm. In group A/E/O, the turkeys were inoculated with *E. coli* at 8 am and with *O. rhinotracheale* at 5 pm. In group A, turkeys received only APV and group C was maintained as an uninoculated control group. The inoculation with APV occurred by the oculonasal route at a dose of 4.4 $\log_{10}CD_{50}$/per bird (250µl). *O. rhinotracheale* and *E. coli* were administered oculonasally at a dose of 8 $\log_{10}$ cfu/250µl per bird.

All birds were clinically examined on a daily basis throughout the experiment. The clinical signs were scored as described in Van de Zande *et al.* (2001). Shortly, the clinical condition of each bird was adjudged a score from 0 (absence of clinical signs)

to 7 (nasal exudate with extremely swollen sinuses and frothy eyes, poor general condition and anorexia). The mean clinical score was calculated for each experimental group.

Tracheal swabs were collected from groups A, A/O, A/E, A/O/E and A/E/O at three days post viral infection (dpvi) for virus titration and from birds belonging to group C to demonstrate the absence of an adventitious exposure. Furthermore, tracheal swabs were collected daily from all turkeys for titration of *O. rhinotracheale* and *E. coli*. The tracheal swabs were collected using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc.**,** Corona, USA) and placed in transport medium. The latter consisted of 1 ml PBS supplemented with Ca^{2+} and Mg^{2+} for bacteriological examination and supplemented with 10% fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco) for virus titration. Processing occurred as described below.

Five birds of each group were randomly selected and were sacrificed at 8 dpvi (i.e. 5 days post bacterial infection (dpbi) for groups A/O, A/E, A/O/E and A/E/O). The remaining eight (three for group C) birds of the different groups were sacrificed at 18 dpvi. Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The birds were necropsied and examined for gross lesions. Samples of the turbinates, trachea and lungs were collected from all sacrificed birds for bacteriological examination. A 10% suspension in PBS was made from these tissue samples. The sinuses, air sacs, heart, pericardium and liver were sampled with cotton swabs for bacteriological analysis. These swabs were suspended in 1 ml PBS supplemented with Ca^{2+} and Mg^{2+}. For the animals sacrificed at 18 dpvi the sinuses, turbinates, trachea, lungs, air sacs, heart, pericardium and liver were sampled with cotton swabs for bacteriological analysis. All samples for bacterial isolation were processed immediately after collection as described below. Finally, samples from the air sacs, turbinates, trachea, lungs, heart and liver were collected and fixed in 10% neutral buffered formalin for histopathological examination.

This experiment has been approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Virological and bacteriological titration

Viral and bacterial titrations of swabs and tissue suspensions were done using the procedures described by Marien *et al.* (2005), with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar and incubated for 24h at 37°C. The isolated *E. coli* strains were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency, Surrey, United Kingdom).

Histopathology

Following fixation in formalin, the tissues were embedded in paraffin, sectioned at 4 μm, mounted on glass slides and stained with haematoxylin and eosin using standard procedures. Tissues from the turbinates and trachea were additionally stained by the Periodic Acid Schiff-reaction to visualize the mucus layer.

Results

During the experiment, mortalities did not occur in any of the experimental groups.

Clinical signs

The mean clinical scores for all groups are shown in Figure 1. Respiratory disease was not detected in birds from group C (control group).

Signs of respiratory distress were seen in all APV-inoculated groups starting from 4 dpvi. In group A, peak mean score (1.9) appeared at 6 dpvi, after which a gradual decline was noted, with clinical symptoms lasting until 9 dpvi. Both in groups A/E and A/O, a peak mean score of 2.5 was seen at 3 dpbi, followed by a gradual decline until at 7 dpbi (group A/E) and 8 dpbi (group A/O), no clinical signs were observed. The highest mean score (2.8) was found in group A/E/O at 3 and 6 dpbi. Consequently, a decline was noted with an absence of clinical signs from 9 dpbi onwards. Finally, in group A/O/E, the peak mean score was present at 3, 4 and 6 dpbi (2.6). After 6 dpbi the mean scores dropped rapidly and at 9 dpbi all clinical signs had disappeared.
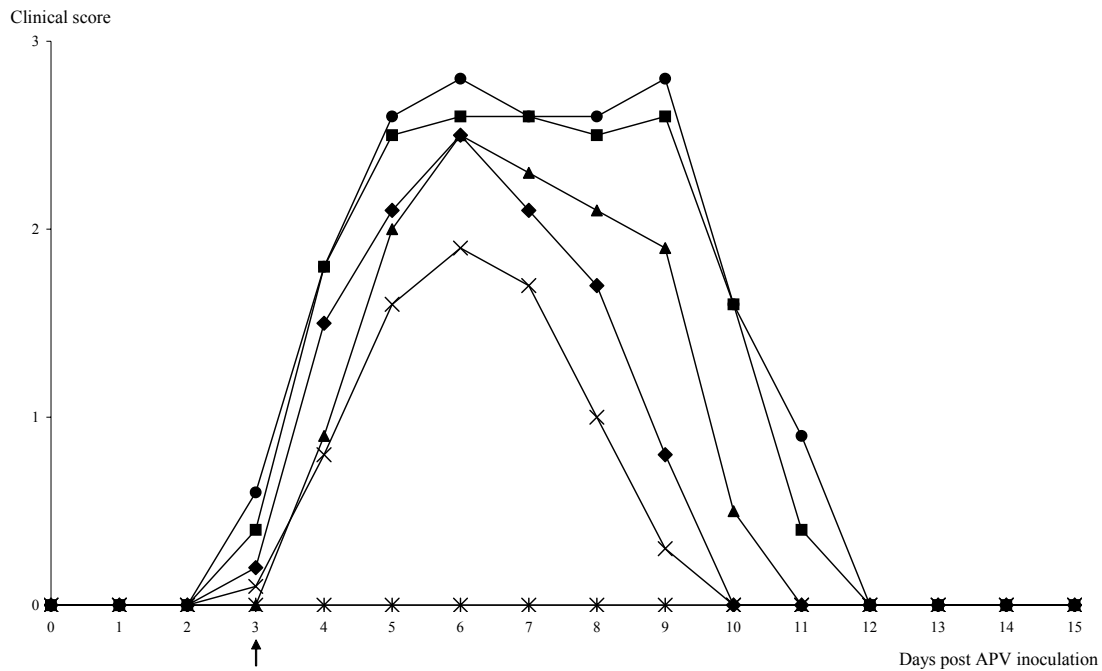
Figure 1. Mean clinical scores in turkeys after single APV inoculation (× group A), dual APV/*O. rhinotracheale* (▲ group A/O) or APV/*E. coli* (◆ group A/E) inoculation with an interval of three days, or triple APV/*O. rhinotracheale*/*E. coli* (■ group A/O/E) or APV/*E. coli*/*O. rhinotracheale* (● group A/E/O) inoculation with an interval of three days between viral and bacterial inoculation, and an interval of 9 hours between the two bacterial inoculations, and in the turkeys of the control group (✳ group C). Arrow indicates day of bacterial inoculation (*O. rhinotracheale* and/or *E. coli*).

Macroscopic findings

Marked macroscopic lesions were absent in group C. In some birds of group A, a slight amount of serous exudate in the sinuses and the turbinates and a slight hyperaemia in the turbinates and trachea were noted. In the groups with dual (groups A/O and A/E) or triple (groups A/O/E and A/E/O) inoculations, the observed macroscopic lesions were comparable, although slightly more severe in the birds with triple infection. Serous to seromucous exudate was found in the turbinates and sinuses, and the amount of mucus varied from moderate to abundant. Hyperaemia of the turbinates and the trachea was frequently encountered. A slightly decreased lucidity of the air sacs was found in one animal of group A/O/E and A/O and in two animals of group A/E/O. A haemorrhage of the lungs was observed in one animal of

group A/O and A/E/O and hyperaemia of the lungs was seen in one bird of group A/O/E. On one occasion in group A/E, the pericardium had a foamy appearance. One animal of group A/E had a mucus plug in the trachea.

Viral titrations of tracheal swabs

Viral titrations of tracheal swabs collected at 3 dpvi showed that APV was recovered from every individual bird of all APV infected groups. Mean titres were 4.0, 4.1, 4.4, 4.2 and 4.6 $\log_{10}CD_{50}$/g mucus for group A, A/O, A/E, A/E/O and A/O/E, respectively. APV was not recovered from tracheal swabs from group C.

Bacterial titration of tracheal swabs

O. rhinotracheale and *E. coli* were not recovered from any of the birds of groups C, A, A/E and groups C, A, A/O, respectively.

The results of *O. rhinotracheale* and *E. coli* titrations of the tracheal swabs of the remaining groups are shown in Figure 2. Mean titres for each experimental group are depicted.

The highest amount of *O. rhinotracheale* in group A/O was found at 6 dpbi (6.5 $\log_{10}$ cfu/g mucus). After the peak, mean titres declined rapidly, until at 9 dpbi no more *O. rhinotracheale* was recovered from tracheal swabs. In groups A/E/O and A/O/E, *O. rhinotracheale* titres peaked at 6 dpbi with titres of 6.7 and 6.4 $\log_{10}$ cfu/g mucus, respectively. Titres remained at a higher level for a longer period of time compared to group A/O, with no more *O. rhinotracheale* bacteria being retrieved from 10 dpbi onwards.

The highest amount of *E. coli* in group A/E was found at 3 dpbi (4.7 $\log_{10}$ cfu/g mucus). Titres remained at approximately the same level until 6 dpbi, after which bacterial titres dropped until at 12 dpbi no more *E. coli* was recovered from tracheal swabs. Although in group A/O/E and A/E/O the peak mean titre (4.1 $\log_{10}$ cfu/g mucus) was seen at 3 dpbi and 8 dpbi, respectively, the isolation rate of *E. coli* out of tracheal mucus in groups A/E/O and A/O/E, approximately had the same course, with a rise to higher mean titres at 3 to 5 dpbi (approximately 4 $\log_{10}$ cfu/g mucus), followed by a gradual decline until at 14 dpbi, no more *E. coli* was isolated.
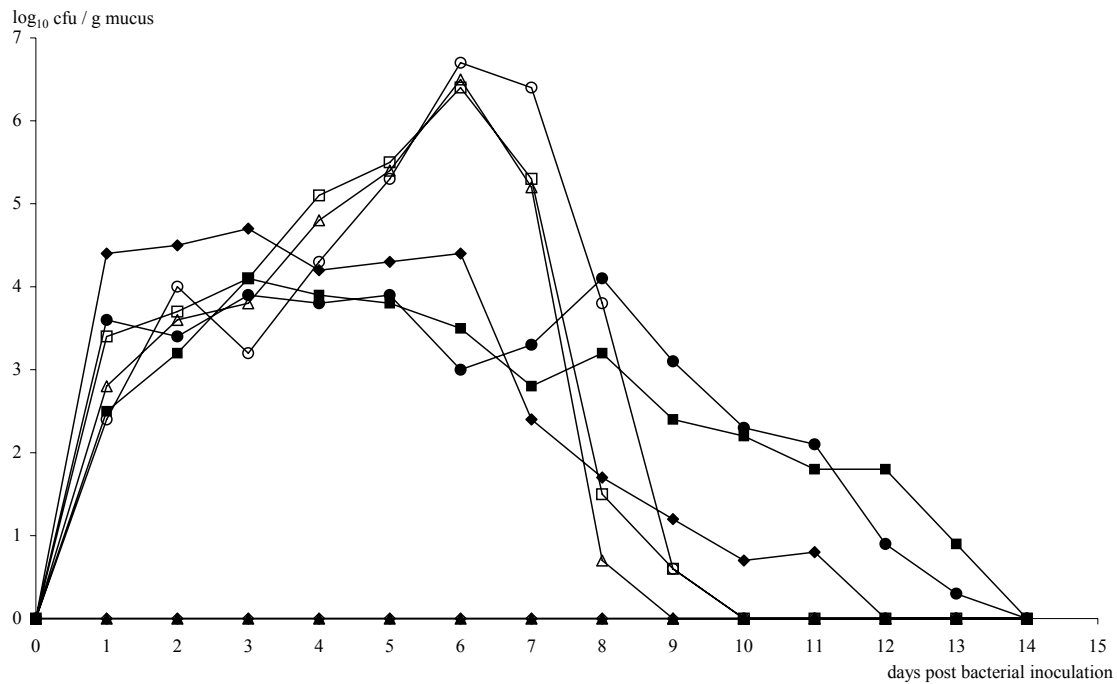
Figure 2. Mean *E. coli* titres (closed symbols) and *O. rhinotracheale* titres (open symbols) (log$_{10}$ cfu/g mucus) in tracheal mucus collected daily after *E. coli* and/or *O. rhinotracheale* inoculation of dually APV/*O. rhinotracheale* (▲, △ group A/O) and APV/*E. coli* (◆, ◇ group A/E) infected turkeys, or triple APV/*O. rhinotracheale* /*E. coli* (■, □ group A/O/E) and APV/*E. coli*/*O. rhinotracheale* (●,○ group A/E/O) infected turkeys.

Bacterial titration of tissue samples

     O. rhinotracheale was never found in any of the tissues from the birds from groups C, A and A/E and *E. coli* was never isolated from any of the tissues from the animals belonging to groups C, A and A/O. The results of *O. rhinotracheale* and *E. coli* titrations in the other groups are shown in Table 1 and 2.

Table 1. Bacterial titre of *O. rhinotracheale* ($\log_{10}$ cfu/g tissue) isolated from different organs of turkeys inoculated with APV followed by *O. rhinotracheale* three days later (group A/O), with APV followed three days later by *O. rhinotracheale* and *E. coli* (A/O/E), or with APV followed three days later by *E. coli* and *O. rhinotracheale* (group A/E/O). Turkeys were euthanised at 5 dpbi.

Organ	*O. rhinotracheale* titre ($\log_{10}$ cfu / g tissue)														
	Group A / O					Group A / O / E					Group A / E / O				
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Turbinates	8.6	7.7	7.9	9.0	6.6	7.5	7.2	7.4	8.7	6.2	10.2	9.0	5.6	9.9	9.0
Trachea	5.9	7.4	7.7	6.0	6.5	7.1	5.9	7.0	8.0	8.1	8.0	7.6	6.7	7.2	7.0
Lungs	2.7	5.4	5.9	6.8	7.2	5.7	5.3	5.8	5.2	6.5	6.1	6.0	5.5	4.8	6.8
Sinus[b]	3[c]	0	3	3	3	1	3	3	1	3	3	3	3	3	3
Air sacs[b]	0	0	0	3	2	0	1	0	1	3	2	3	1	2	1
Pericardium[b]	0	0	0	0	0	1	1	1	0	1	1	1	0	0	1
Heart[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Liver[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2

a: number of turkey
b: these organs were swabbed; no tissue suspensions were made
c: 0 = 0 colonies / 1 = 1 to 10 colonies / 2 = 11 tot 100 colonies / 3 = more than 100 colonies

Table 2. Bacterial titre of *E. coli* ($\log_{10}$ cfu/g tissue) isolated from different organs of turkeys inoculated with APV followed by *E. coli* three days later (group A/E), with APV followed three days later by *O. rhinotracheale* and *E. coli* (A/O/E), or with APV followed three days later by *E. coli* and *O. rhinotracheale* (group A/E/O). Turkeys were euthanised at 5 dpbi.

Organ	*E. coli* titre ($\log_{10}$ cfu / g tissue)														
	Group A / E					Group A / O / E					Group A / E / O				
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Turbinates	9.2	6.8	10	6.7	6.3	4.4	10	8.1	7.7	5.7	9.8	8.4	4.4	9.5	7.5
Trachea	7.0	7.5	8.6	7.5	8.2	4.0	3.6	6.1	7.8	5.0	6.5	3.0	6.3	6.7	3.9
Lungs	0.0	3.4	0.0	4.5	0.0	4.4	0.0	0.0	0.0	4.8	4.3	0.0	0.0	3.3	4.3
Sinus[b]	3[c]	1	1	3	3	3	3	3	0	3	3	2	3	3	3
Air sacs[b]	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Pericardium[b]	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Liver[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

a: number of turkey
b: these organs were swabbed; no tissue suspensions were made
c: 0 = 0 colonies / 1 = 1 to 10 colonies / 2 = 11 tot 100 colonies / 3 = more than 100 colonies

Histopathology

In the control group (group C), no marked microscopic changes were observed in the air sacs, turbinates, trachea or lungs. The histological preparations of the animals of group A displayed mild infiltration of heterophils and monomorphonuclear cells in the mucosa of the turbinates and trachea, degeneration of the mucous glands in the turbinates and trachea, loss of cilia in the trachea, and in one animal mild infiltration of heterophils in the air sacs. No lung lesions were encountered.

Histopathological lesions were similar in groups A/O, A/E, A/O/E and A/E/O: infiltration of heterophils and monomorphonuclear cells in the mucosa of the turbinates and trachea, degeneration of the mucous glands and epithelium in the

turbinates and trachea, loss of cilia in trachea, activation of lymph nodules in the turbinates, suppurative exudate in the lumen of trachea and turbinates, and foci of pneumonia, suppurative bronchitis and bronchiolitis in the lung. Furthermore, infiltration of heterophils and monomorphonuclear cells in the air sacs was noted. In general, it can be stated that all lesions were more severe in the groups with triple infection (group A/O/E and A/E/O), especially the infiltration by heterophils and monomorphonuclear cells in the mucosa of the turbinates and trachea and in the air sacs.

Discussion

In the present study, it was demonstrated that APV, *E. coli* O2:K1 and *O. rhinotracheale* triple infections of turkeys were able to cause similar, but more severe and longer persisting respiratory symptoms compared to dual APV/*O. rhinotracheale* and APV/*E. coli* infections, with the latter causing more severe signs of respiratory distress than single APV infection. It should also be noted that from 4 dpbi onwards, APV/*O. rhinotracheale* dual infection resulted in slightly higher clinical scores in comparison with APV/*E. coli* dual infection. The aggravation of clinical disease by combined action of APV, *O. rhinotracheale* and *E. coli* was reflected in the necropsy findings and the microscopical observations, in that the lesions found in the respiratory tract were slightly more pronounced and extensive in comparison with dual APV/*O. rhinotracheale* and APV/*E. coli* infections, and certainly compared to single APV infection.

Concerning the number of *O. rhinotracheale* and *E. coli* bacteria recovered from the tracheal mucus in the dual and triple infection groups, the most conspicuous observation was that in the triple infection groups, *E. coli* could be isolated from the tracheal mucus to slightly lower numbers from 1 to 7 dpbi, with the reverse situation from 8 dpbi onwards, i.e. *E. coli* being isolated to somewhat higher numbers in the triple infected groups as compared to the dually infected group. Further, *E. coli* could in the triple infected groups be isolated two days longer from the tracheal mucus compared to the dually infected group.

The level of *O. rhinotracheale* multiplication in the respiratory organs of the euthanised animals at 5 dpbi did not show marked differences between the dual and triple infection groups, with bacteria being isolated from turbinates, trachea, lungs,

sinuses and air sacs almost in equal amounts. In most *E. coli* challenged turkeys of the dual and triple infection groups, *E. coli* was isolated from the turbinates, trachea, sinuses and to a lesser extent from the lungs, but could however be isolated from the air sacs of only one animal (group A/O/E).

Thus, both *O. rhinotracheale* and *E. coli* O2:K1 were, after APV priming, able to colonize the upper respiratory tract and invade the lower respiratory tract of turkeys. This is in accordance with experiments performed by Van de Zande *et al.* (2001) and Marien *et al.* (2005). Marien *et al.* (2005) demonstrated that, although *O. rhinotracheale* on itself was able to adhere to and colonize the upper respiratory tract of turkeys, APV priming facilitated infection of the lungs and was necessary to allow *O. rhinotracheale* to infect the air sacs. Van de Zande *et al.* (2001) demonstrated that *E. coli* was only able to colonize the turbinates of turkeys that had been infected previously with APV and hence concluded that *E. coli* takes advantage of the damage caused by the virus to colonize.

Furthermore, this study clearly shows that combined action of *O. rhinotracheale* and *E. coli* enhances the invasion of *O. rhinotracheale* but not of *E. coli* in the non-respiratory internal organs. Only in birds infected with both bacteria could at 5 dpbi *O. rhinotracheale* be isolated out of the pericardium of most of the animals and out of the heart and liver of one bird.

These findings all endorse our results that APV, *E. coli* and *O. rhinotracheale* exert a synergistic pathogenic effect if given consecutively, spaced by three days between the viral and the bacterial challenges, to susceptible turkeys. The results further indicate that the sequence in which the turkeys are infected with *O. rhinotracheale* and *E. coli* has no major influence on the outcome of the disease.

Although in the present study respiratory disease caused by APV inoculation was clearly aggravated by co-infection with *E. coli* and *O. rhinotracheale*, not all the typical disease symptoms of these bacterial infections were observed. One of the most common forms of colibacillosis begins as a respiratory tract infection and may be referred to as airsacculitis. If unattended, this infection may evolve into a bacteraemia and a generalized infection which manifests as a polyserositis (Pourbakhsh *et al.*, 1997a; Dho-Moulin and Fairbrother, 1999; Barnes *et al.*, 2003). In the present study however, no pathological signs of airsacculitis or generalized infection were seen, although the isolate was cultured from a necropsy case with typical polyserositis lesions. The virulence mechanisms of avian pathogenic *E. coli* have not been clearly

characterised yet. In order to produce colisepticemia, *E. coli* must find an appropriate receptor for adhesion, must be able to multiplicate in the tissues and to resist the immunological defenses of the host so it can finally enter and survive in the blood (Dho-Moulin, 1993; Pourbakhsh *et al*., 1997a; 1997b). Previously, it has been shown that the lower respiratory tract (lungs and air sacs) is an important site of entrance of *E. coli* into the bloodstream of birds (Cheville and Arp, 1978; Ackermann and Cheville, 1991; Pourbakhsh *et al*., 1997a). Since the presently adopted *E. coli* strain was able to invade the lungs and the air sacs of some birds but still did not cause typical colisepticemia, the strain was possibly lacking certain characteristics necessary for invasion of the bloodstream. Still we should also keep in mind that differences in severity of clinical disease after experimental infection compared to field cases may also be attributed to the often inadequate environmental and management conditions (high animal density, inadequate ventilation, high ammonia levels, too high and low a relative humidity) and additional pathogens encountered in the field. Avian pathogenic *E. coli* infections for instance are enhanced or initiated by predisposing factors such as environmental conditions and viral or *Mycoplasma* infections (Dho-Moulin and Fairbrother, 1999). The animals used in the present study were in contrast SPF animals kept in spacious rooms with HEPA-filtered air with no extraneous pathogens interplaying. Several studies have been performed to elucidate the effects of the combined action of APV and other microorganisms in turkeys and chickens. In turkeys, following infection with APV, disease may be exacerbated by concurrent infection with various bacteria such as *B. avium* and a *Pasteurella*-like organism (Cook *et al*., 1991; Jirjis *et al*., 2004), with *O. rhinotracheale* (van Empel *et al*., 1996; Marien *et al*., 2005), with *E. coli* (Van de Zande *et al*., 2001; Turpin *et al*., 2002), with *Mycoplasma gallisepticum* (Naylor *et al*., 1992) and with *Mycoplasma imitans* (Ganapathy and Bradbury, 1999). Very recently, Van Loock *et al*. (2006) demonstrated the exacerbating role of APV during acute *Chlamydophila psittaci* infection in young turkeys. In chickens, different researchers observed that the clinical disease due to infection with APV was worsened by *E. coli* (Al-Ankari *et al*., 2001; Majo *et al*., 1997) or *O. rhinotracheale* inoculation (van Empel *et al*., 1996). In these experimental challenge studies, many researchers also came across difficulties in reproducing disease symptoms identical as encountered in the field.

In conclusion, it was shown that in the present experiment, respiratory disease in turkeys was more severe after triple APV, *E. coli* and *O. rhinotracheale* infection

compared to dual APV/*E. coli* and APV/*O. rhinotracheale* infection. The established dual and triple infections models may undoubtedly be used to further investigate the mechanisms of *O. rhinotracheale* and *E. coli* colonization and the synergistic relations between APV, *E. coli* and *O. rhinotracheale*. Furthermore, these models can also be used to test preventive and curative measures to combat respiratory disease.

Acknowledgements

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3.1.3. Pathogenic interactions between *Mycoplasma gallisepticum*, avian metapneumovirus, *Escherichia coli* and *Ornithobacterium rhinotracheale* infections in turkeys

Marien, M., Nauwynck, H., Chiers, K., Froyman, R., Landman, W.J.M., and Decostere, A.

Summary

The purpose of this study was to assess the possible synergism between *Mycoplasma gallisepticum, Ornithobacterium rhinotracheale, Escherichia coli* and avian metapneumovirus (APV) infections in turkeys. The parameters used for the evaluation of synergism were clinical signs, gross lesions, histology and viral and bacterial titration. Eighty-five specified pathogen-free (SPF) turkeys were inoculated with either *M. gallisepticum, M. gallisepticum*/APV, *M. gallisepticum*/APV/*O. rhinotracheale* or *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* with a 10 day interval between *M. gallisepticum* and APV inoculations, a three day interval between viral and *E. coli* and/or *O. rhinotracheale* inoculations, and an 8h interval between *E. coli* and *O. rhinotracheale* inoculations. A non-infected negative group was included as control. The *M. gallisepticum* infection was induced via the intratracheal route, while all other inoculations (APV, *O. rhinotracheale*, *E. coli*) were done via the oculonasal route. Clinical observations of the experimental animals were performed daily (upper and lower respiratory tract symptoms) and intratracheal swabs were performed on a regular basis. Five birds out of a total of 17 birds of each group were sacrificed 18 days after *M. gallisepticum* inoculation and the remaining birds at the end of the experimental period, i.e. 33 days after *M. gallisepticum* inoculation. The birds were examined for gross lesions at necropsy and samples of the turbinates, sinuses, trachea, lungs, air sacs, heart, pericardium and liver were taken for bacteriological and/or histological examination.

After a single *M. gallisepticum* inoculation the birds suffered from very serious respiratory distress, caused by severe airsacculitis, resulting in high mortality rates up to 50% (range 16.7% - 50%). Upper respiratory tract symptoms (nasal exudate and swollen sinuses) were seen in all the *M. gallisepticum*/APV dually infected turkeys, and the severity of the symptoms was exacerbated by inoculation with *E. coli* and/or *O. rhinotracheale*. The peak in the clinical scores, especially in the groups with additional infection with *E. coli* and *O. rhinotracheale*, coincided with a second peak of mortality. The isolation of *M. gallisepticum* from the respiratory organs (tracheae, lungs and air sacs) did not substantially differ between the various *M. gallisepticum* infected groups (average reisolation rate 83.3 %). On the other hand, *M. gallisepticum* seemed to exert an impeding effect on the clearance of *O. rhinotracheale* and *E. coli*, especially from the upper respiratory tract. In general, gross lesions at necropsy were similar in groups *M. gallisepticum*/APV, *M.*

gallisepticum/APV/*O. rhinotracheale* and *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale*. A remarkable finding at necropsy was the presence of septicaemia in the *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* infected poults.

In conclusion, the results obtained in the present study clearly indicate the existence of marked synergism between four distinct respiratory pathogens, i.e. *M. gallisepticum*, APV, *O. rhinotracheale* and *E. coli,* important in commercial poultry. This was evidenced by the higher mortality rates and the aggravated clinical symptoms whenever the birds were infected with an additional pathogen. Similarly necropsy and microscopical lesions were more severe in case two or more pathogens were inoculated. Especially concurrent infection of *M. gallisepticum* and APV infected birds with *O. rhinotracheale* and *E. coli* seemed to aggravate the clinical symptoms and caused higher mortality rates.

Introduction

Respiratory disorders are one of the important diseases affecting turkeys and are continuing to cause high economic losses in many areas world-wide due to reduced growth, increased feed conversion rates, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter (van Empel and Hafez, 1999). Management failures as well as pathogen interplay between various viral and bacterial pathogens are largely involved in this multifactorial disease complex.

In two previously performed experiments, synergistic effects were demonstrated between avian metapneumovirus (APV), *Ornithobacterium rhinotracheale* and *Escherichia coli* for the reproduction of respiratory disease, when inoculated into turkeys via the natural infection route (Marien *et al*., 2005; unpublished results). Nevertheless, the clinical signs resulting from these mixed infections were similar, albeit still generally markedly milder than those seen in field cases (De Rosa *et al*., 1996; Roepke *et al*., 1998). In the latter airsacculitis and septicaemia are frequently noted, resulting in severely diseased chickens and turkeys and even mortalities (van Empel and Hafez, 1999; Vandekerchove, 2004). These features were never encountered in the formerly mentioned experimental trials. This difference may be attributed to the often inadequate environmental and management conditions in the field and synchronous infections with several pathogenic agents, which are very difficult to reproduce experimentally.

The present study was undertaken to develop a mixed infection model for *M. gallisepticum*, APV, *E. coli* and *O. rhinotracheale* in turkeys, with the objective of reproducing respiratory disease with severe clinical symptoms as noted in the field, in order to be able to evaluate amongst others antimicrobial treatment schemes and environmental or management factors. The parameters that were used for evaluating possible synergistic effects between the four agents were clinical signs, gross lesions, histology and bacterial titration.

Materials and Methods

Turkeys

Eighty-five specified pathogen-free (SPF) turkeys (AFSSA, Ploufragan, France), hatched in our facilities, were used in this study. The birds were housed in separate isolation units of 1.2 m^2 (height: 0.70m) with HEPA (high efficiency particulate air)-filtered air and had free access to food and water.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titre of 5.5 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacteria

The *O. rhinotracheale* type strain LMG 9086^T originally isolated from a turkey with a respiratory tract infection was used. The strain was serotyped as type A in an agar gel precipitation test (Hafez and Sting, 1999) performed by Professor H. M. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany). The strain was stored at -70°C. The organism was cultured for 48h at 37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. The *O. rhinotracheale* bacteria were transferred into brain heart infusion (BHI) broth (Oxoid) for 24h at 37°C with agitation (microaerophilic incubation). The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in phosphate-buffered saline (PBS) followed each time by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was re-suspended in PBS to obtain a final concentration of 8.6 $\log_{10}$ colony-forming units (cfu)/ml.

A Belgian *E. coli* isolate originating from turkeys with colibacillosis was used. It was identified and serotyped as O2:K1 by Dr. Flemming Scheutz (Statens Serum Institute, The International Escherichia and Klebsiella Centre, Copenhagen, Denmark). This strain had been used previously by Van de Zande *et al.* (2001) for the induction of *E. coli* infection in APV infected turkeys and was stored at -70°C. The organism was retrieved from the frozen suspension and cultured overnight at 37°C on MacConkey agar. The *E. coli* bacteria were transferred into BHI and cultured for

another 24h at 37°C. The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in PBS followed each time by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was re-suspended in PBS to obtain a final concentration of 8.6 $\log_{10}$ cfu/ml.

An *M. gallisepticum* field isolate from broilers with respiratory symptoms (chicken/NL/Dev/SP-1608Vin/99) was used as inoculum. It had been isolated on Mycoplasma Experience agar (ME, Mycoplasma Experience, Reigate, Surrey, UK), identified by immunofluorescence test (IFA) (Kiernan, 1990), conventional PCR (IDEXX Laboratories Inc., Schiphol-Rijk, the Netherlands) and stored at -70°C in ME broth (Mekkes and Feberwee, 2005; Feberwee *et al.*, 2005a). One ml of the stored broth (-70°C) was added to 50 ml ME broth, which was then incubated for two days at 37°C until change of colour was observed. At the start and at the end of the inoculations control of the bacterial concentration of the inoculum was performed by means of a quantitative PCR (Q-PCR) as described by Mekkes and Feberwee (2005). This Q-PCR was shown to correlate well with standard counting techniques using culture.

Experimental design

Eighty-five SPF turkeys were randomly divided into five groups of 17 animals at ten days of age. At nine days of age, the birds were shown to be free from maternally derived antibodies to *M. gallisepticum, O. rhinotracheale* and APV, by commercially available ELISA tests (BioChek, Gouda, the Netherlands) for *M. gallisepticum* and *O. rhinotracheale*, and by an in-house serum neutralization test (Marien *et al.*, 2006) for APV.

At ten days of age the birds of groups M (single *M. gallisepticum* inoculation) M/A (dual *M. gallisepticum* and APV inoculation), M/A/O (*M. gallisepticum*, APV and *O. rhinotracheale* inoculation) and M/A/E/O (*M. gallisepticum*, APV, *O. rhinotracheale* and *E. coli* inoculation) were inoculated with *M. gallisepticum* and placed into the HEPA-filtered isolation units. Ten days later, the birds of group M/A, M/A/O and M/A/E/O were inoculated with APV. Three days later, in group M/A/O, turkeys subsequently received *O. rhinotracheale,* and the animals of group M/A/E/O were inoculated with *E. coli* and *O. rhinotracheale*. Inoculation with *O. rhinotracheale* in group M/A/E/O was done approximately eight hours after *E. coli*

inoculation. Group C was used as an uninoculated control group and was kept on litter in an HEPA-filtered isolation room.

All the birds of the respective groups were infected with *M. gallisepticum* by intratracheal inoculation with 0.5 ml ME broth containing approximately 7.8 $\log_{10}$ cfu/ml. APV was inoculated oculonasally at a dose of 4.4 $\log_{10}CD_{50}$, *O. rhinotracheale* and *E. coli* were given at a dosage of 8 $\log_{10}$ cfu per bird. For inoculation with APV, *O. rhinotracheale* and/or *E. coli*, a total of 250 μl was divided equally over the nares and eyes.

All birds were examined clinically on a daily basis throughout the experiment until they were sacrificed. The clinical symptoms were divided into lower respiratory tract and upper respiratory tract symptoms. Dyspnoea was regarded as a sign of lower respiratory tract distress. The clinical signs of the upper respiratory tract (nasal exudate, swollen sinuses and frothy eyes were scored as described by Van de Zande and colleagues (2001). Shortly, the clinical condition of each bird was assigned a score from 0 (absence of clinical signs) to 7 (nasal exudate with extremely swollen sinuses and frothy eyes, poor general condition and anorexia). The mean clinical score was calculated for each experimental group.

All the birds were weighed at the beginning of the experiment (day of *M. gallisepticum* inoculation) and subsequently on the day that they were sacrificed.

Tracheal swabs were collected at 11 days post *M. gallisepticum* inoculation (dpmi) for quantitative *M. gallisepticum* polymerase chain reaction (PCR) and at 3 days post viral infection (dpvi) (13 dpmi) for virus titration. Before *O. rhinotracheale* and *E. coli* inoculation, tracheal swabs were taken from all birds of each experimental group for examination of *O. rhinotracheale* and *E. coli* O2:K1, which proved to be negative.

Tracheal swabs were also collected on 14, 16, 18, 20, 22, 24, 26, 29 and 33 dpmi from all groups for bacteriological titration of *O. rhinotracheale* and *E. coli* and for quantitative *M. gallisepticum* PCR. The tracheal swabs were collected using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc**.,** Corona, USA) and placed in transport medium. The latter consisted of 1.5 ml PBS for *M. gallisepticum*, *O. rhinotracheale* and *E. coli* examination, supplemented with 10% fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco) for virus titration. Processing occurred as described below.

Five birds of each group including group C were sacrificed at 18 dpmi. The remaining birds of each group were sacrificed 15 days later, i.e. 33 dpmi. Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The birds were necropsied and examined for gross lesions.

Samples of the turbinates, trachea and lungs were collected from each of the five birds of each group sacrificed at 18 dpmi for *O. rhinotracheale* and *E. coli* examination. A 10% suspension in PBS was made from these tissue samples. Sinus, air sacs, heart, pericardium and liver were also sampled, however with cotton swabs, for *O. rhinotracheale* and *E. coli* analysis. All samples for *O. rhinotracheale* and *E. coli* isolation were processed immediately after collection (see later). Swabs from the trachea, lungs and air sacs were additionally collected for *M. gallisepticum* culture. The swabs were dipped in Mycoplasma Adler medium (Avian Mycoplasma Liquid Medium; Mycoplasma Experience, Reigate, UK) just prior to sampling. Processing occurred as described below.

For the remaining birds sacrificed at 33 dpmi, turbinates, trachea, lungs, sinuses, air sacs, liver, heart and pericardium were sampled with cotton swabs for *O. rhinotracheale* and *E. coli* analysis and swabs, dipped in Adler, were collected from trachea, lungs and air sacs for culture of *M. gallisepticum*.

From all the birds that spontaneously died during the experiment from 13 dpmi onwards, swabs of the turbinates, trachea, lungs, sinuses, air sacs, pericardium, heart and liver were taken for *O. rhinotracheale* and *E. coli* analysis. No swabs for *M. gallisepticum* culture were taken from these animals.

Finally, samples from the turbinates, trachea and lungs were taken and fixed in 10% neutral buffered formalin for histopathological examination.

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Virological (APV) and bacteriological (*M. gallisepticum, *O. rhinotracheale *and* E. coli*) titration of tracheal swabs

The virus titre in $\log_{10}$ CD$_{50}$ per g mucus and the number of cfu of *O. rhinotracheale* and *E. coli* per g mucus were determined. This was done using the procedures described by Marien and others (2005), with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar

and incubated for 24h at 37°C. The retrieved *E. coli* isolates were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency, Surrey, United Kingdom). Concentration of *M. gallisepticum* in the tracheal samples was performed using the Q-PCR described previously (Mekkes and Feberwee, 2005).

*Bacteriological (*M. gallisepticum, O. rhinotracheale *and* E. coli*) titration of tissue suspensions and swabs*

O. rhinotracheale and *E. coli* numbers were quantified in samples of the turbinates, trachea and lungs of the 25 birds sacrificed at 18 dpmi. The number of cfu of *O. rhinotracheale* or *E. coli* per g tissue was determined as described (Marien *et al.*, 2005) with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar and incubated for 24h at 37°C.

The swabs taken from the sinuses, air sacs, pericardium, heart and liver from the 25 birds sacrificed at 18 dpmi and the swabs from the turbinates, trachea, lungs, sinuses, air sacs, pericardium, heart and liver from the birds sacrificed at 33 dpmi, were inoculated onto 5% sheep blood agar supplemented with 5 μg/ml gentamicin and 5 μg/ml polymyxin for *O. rhinotracheale* isolation and on MacConkey agar for *E. coli* isolation. After 24-48h of incubation at 37°C in a 5% CO_2 atmosphere (*O. rhinotracheale*) and after 24h of incubation at 37°C (*E. coli*), the agar was examined for presence of *O. rhinotracheale* or *E. coli*.

The obtained *E. coli* isolates were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency).

Swabs from the trachea, lungs and air sacs were cultured for *M. gallisepticum* as described by Feberwee and others (2005b) using ME agar plates supplemented with ampicillin (0.2 mg/ml), with the modification that swabs were dipped in Mycoplasma Adler medium before sampling and that a commercial PCR (IDEXX Laboratories Inc.) was used for species identification instead of immunofluorescence.

Histopathology

Following fixation in formalin, the tissues were embedded in paraffin, sectioned at 4 μm, mounted on glass slides and stained with haematoxylin and eosin using standard procedures. Tissues from the turbinates and trachea were additionally stained by the Periodic Acid Schiff-reaction to visualize the mucus layer.

Results

Mortality

During the experiment, high mortality rates were observed in the different experimental groups, except in group C where no mortality was noted. The birds started to die from 5 dpmi onwards. In Figure 1, the cumulative mortality given as a percentage of the total number of animals, is presented. Overall, the highest mortality rates were seen in group M/A/E/O (91.7%) and group M/A/O (83.3%).
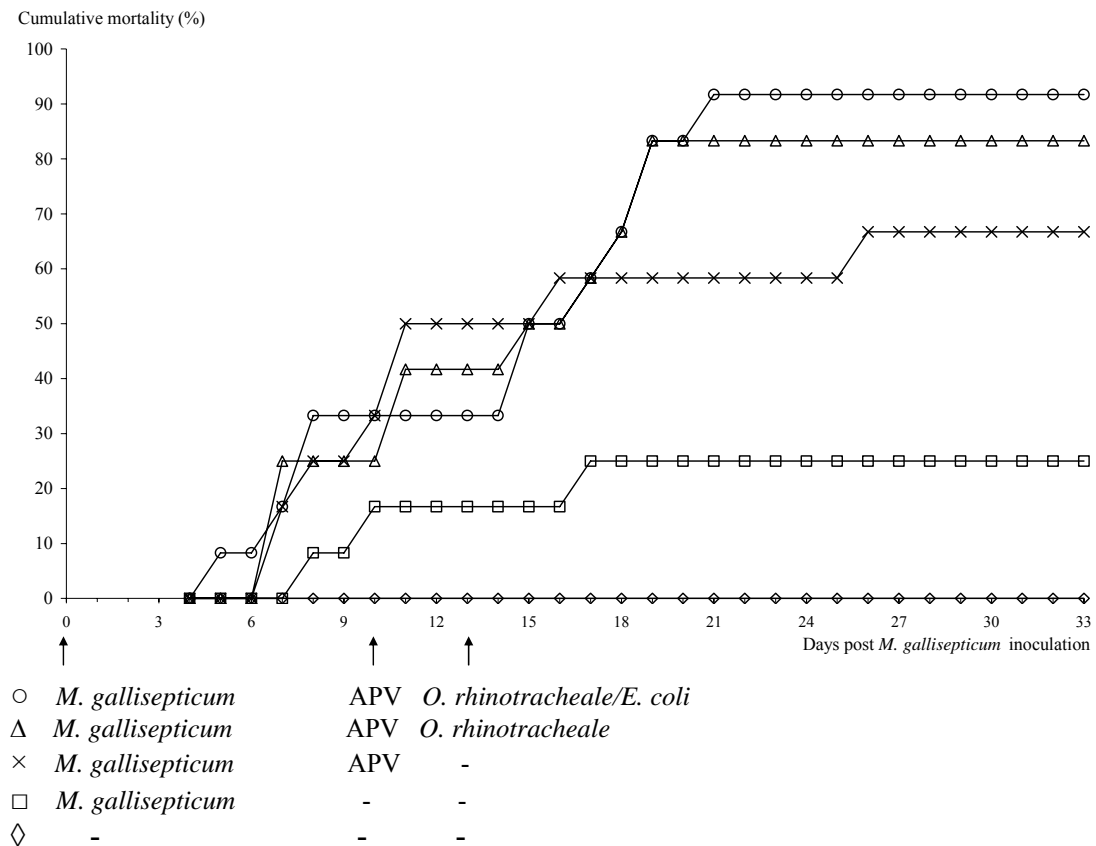
Figure 1. Cumulative mortality with time, given as a percentage of the total number of animals, after single *M. gallisepticum* inoculation (□ group M), dual *M. gallisepticum*/APV inoculation (× group M/A), triple *M. gallisepticum*/APV/*O. rhinotracheale* inoculation (Δ group M/A/O), and *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* inoculation (○ group M/A/E/O), with a ten day interval between *M. gallisepticum* and APV inoculations, a three day interval between APV and *O. rhinotracheale*/*E. coli* inoculations, and an interval of eight hours between the *E. coli* and *O. rhinotracheale* inoculations. No mortalities were seen in the negative control group (◊ group C).

Clinical signs

The mean clinical scores and the number of animals from which the mean clinical score was calculated for each group are presented in Figure 2.

No respiratory symptoms were observed in birds from the uninoculated control group (C).

Lower respiratory tract symptoms were seen in all the *M. gallisepticum* inoculated groups. Severe dyspnoea was noted in a few birds at 5 dpmi and gradually affected all animals. In group M, dyspnoea was apparent during approximately one week after which the symptoms gradually decreased in severity, until at 15 dpmi at which moment the birds only presented dyspnoea after manipulation (i.e. tracheal swabbing). A few days later signs of dyspnoea could not be noticed. In the groups subsequently challenged with APV and in two groups subsequently infected with *E. coli* and/or *O. rhinotracheale*, the signs of dyspnoea lasted several days longer.

Upper respiratory tract symptoms were seen in all APV-inoculated groups starting from 3 dpvi (13 dpmi) but not in groups M and C.

In group M/A, the peak in mean score (2.8) appeared at 6 and 8 dpvi (16 and 18 dpmi), after which a gradual decline was noted, with clinical symptoms lasting until the end of the experiment. The peak in mean score of group M/A/O (4.5) was noted at 9 dpvi (19 dpmi), followed by a gradual diminution with an absence of clinical signs from 22 dpvi (32 dpmi) onwards. The highest mean score (5.0) was found in group M/A/E/O at 11 dpvi (21 dpmi). Thereafter, a decline was noted however clinical signs lasted until the end of the experiment. Poor general condition and anorexia was seen in a number of birds, especially in groups M/A/O and M/A/E/O.
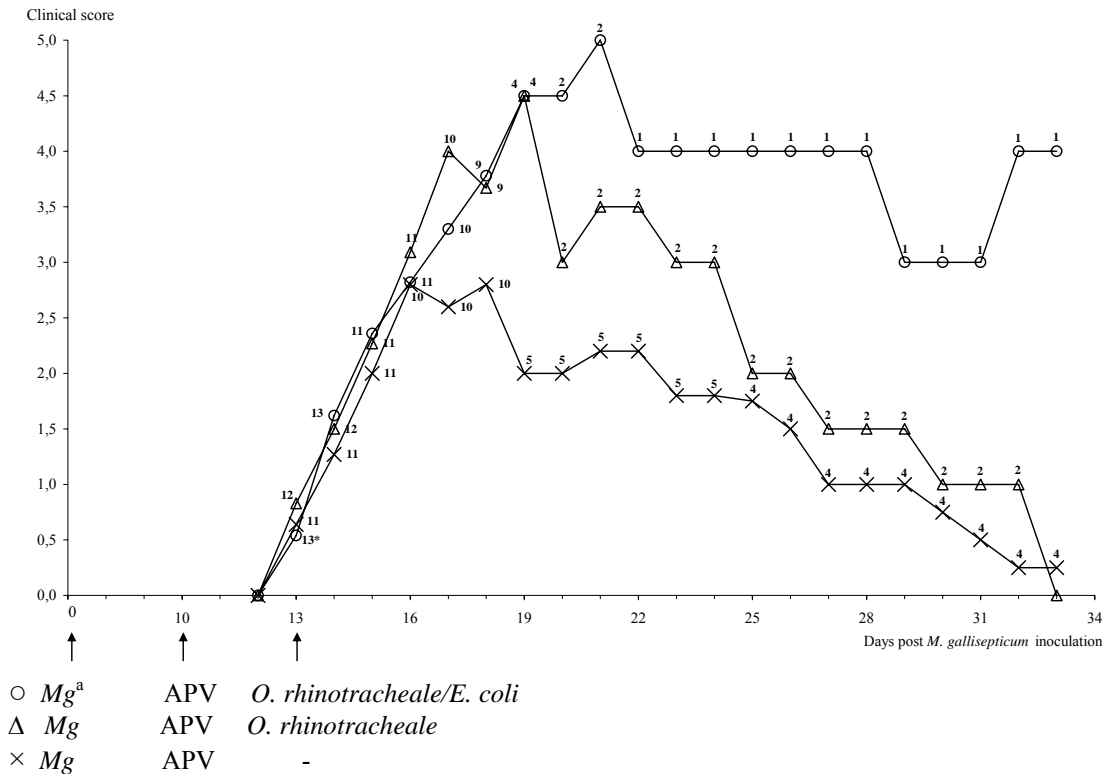
Figure 2. Mean clinical scores (upper respiratory tract symptoms) in turkeys after dual *M. gallisepticum*/APV inoculation (× group M/A), triple *M. gallisepticum*/APV/O. rhinotracheale* inoculation (Δ group M/A/O) or quadruple *M. gallisepticum*/APV/E. coli*/O. rhinotracheale* inoculation (○ group M/A/E/O) with an interval of ten days between *M. gallisepticum* and APV inoculations, an interval of three days between APV and *O. rhinotracheale/E. coli* inoculations, and an interval of eight hours between the *E. coli* and *O. rhinotracheale* inoculations. Because upper respiratory signs were not detected in the turkeys of the single *M. gallisepticum* infected group (group M) and in the negative control group (Group C), these groups are not shown in the figure. The clinical scores are outlined following days after APV inoculation. * For each mean value, the number of animals for which the mean value was calculated, is depicted. [a]: *Mg = M. gallisepticum*.

Macroscopic lesions

Macroscopic lesions were absent in birds of group C during the entire study period.

All the birds that died during the first mortality peak, between 5 and 11 dpmi, were diagnosed with severe airsacculitis as main macroscopic finding.

For the five birds of each experimental group, sacrificed at 18 dpmi, the following macroscopic findings were noted. In group M, severe suppurative airsacculitis and multiple foci of pneumonia were seen. One of the five animals showed no apparent macroscopical lesions. Almost all birds in group M/A, M/A/O and M/A/E/O presented large amounts of serous to seromucous exudate in the turbinates and sinuses, very severe suppurative airsacculitis, and moderate to very severe pneumonia. Hyperaemia of the turbinates and the trachea was also frequently encountered. In one animal of group M/A, serous inflammation of the pericardium was noticed. In group M/A/E/O, the pneumonia was more suppurative in nature. In one animal of group M/A/E/O, severe pericarditis and perihepatitis were noticed and in another animal of the same group a suppurative pericarditis.

In the three animals that died one day after infection with APV, thus before infection with *O. rhinotracheale* and/or *E. coli*, at postmortem only severe airsacculitis was found. Except two animals from group M/A and one animal of group M, five and seven birds of groups M/A/O and M/A/E/O, respectively, died during the time period going from 2 to 8 days after *O. rhinotracheale* and/or *E. coli* infection (15 to 21 dpmi). In all the animals that died in this period, severe seromucous exudate in the turbinates and sinuses, severe suppurative airsacculitis, severe (suppurative) pneumonia and hyperaemia of the turbinates and trachea were observed. Furthermore, in group M/A, in one animal, and in group M/A/E/O in six of the seven dead birds, severe, sometimes suppurative pericarditis and in one animal perihepatitis, were found.

In the animals euthanised at 33 dpmi, the macroscopic lesions were less severe, nevertheless serous to seromucous exudates in turbinates and conchae, airsacculitis, and pneumonia were found in most of the *M. gallisepticum* infected birds. Additionally, serous fluid in the pericardium was found in four of the nine euthanised birds of group M, and in one of the three birds of group M/A. These findings were not seen in the birds of groups M/A/O and M/A/E/O, although it should be noted that in these two groups only two and one animals survived, respectively.

Virological (APV) and bacteriological (*M. gallisepticum, *O. rhinotracheale *and* E. coli*) titration of tracheal swabs

As shown by the virus titrations of tracheal swabs collected at 3 dpvi, APV was recovered from every APV infected bird. Mean titres were 4.1, 4.0 and 3.9 $\log_{10}$ CD_{50}/g mucus for group M/A, M/A/O and M/A/E/O, respectively. APV was not recovered from tracheal swabs from group C and M.

M. gallisepticum DNA was recovered from some of the birds of group C. More specifically, in nine out of the 17 birds *M. gallisepticum* DNA was detected although in low amounts (average of 2.9 $\log_{10}$ cfu/mucus of the positive birds; range 1.7 – 4.3 $\log_{10}$ cfu/g mucus), and from six of these nine birds, the DNA was only found at one sampling point (out of the ten samplings done). In the other three birds, *M. gallisepticum* DNA was found at two sampling points. *O. rhinotracheale* and *E. coli* were not recovered from any of the birds of groups C, M, and M/A and groups C, M, M/A and M/A/O, respectively.

The results of *M. gallisepticum*, *O. rhinotracheale* and *E. coli* titrations of the tracheal swabs of the remaining groups are shown in Figure 3 (a, b and c). The highest mean amount of *O. rhinotracheale* in group M/A/O (7.58 $\log_{10}$ cfu/g mucus) and group M/A/E/O (7.50 $\log_{10}$ cfu/g mucus) was found at 3 days post *O. rhinotracheale* inoculation (16 dpmi). After the peak, mean titres declined gradually until the end of the experiment (20 days post *O. rhinotracheale* inoculation) at which time *O. rhinotracheale* was detected in tracheal swabs with a mean titre of 4.44 $\log_{10}$ cfu/g mucus for group M/A/O and 4.40 $\log_{10}$ cfu/g mucus for group M/A/E/O.

The highest amount of *E. coli* in group M/A/E/O (7.20 $\log_{10}$ cfu/g mucus) was found at 3 days post *E. coli* inoculation (16 dpmi). With the exception of a one log decrease at 7 days post *E. coli* inoculation, titres remained at approximately the same level until the end of the experimental period at 20 days post *E. coli* inoculation (33 dpmi).

The *M. gallisepticum* titres determined by quantitative PCR in the different *M. gallisepticum* inoculated groups remained at a similar level (between 6 and 7 $\log_{10}$ cfu/g mucus) throughout the whole measurement period. It should however be noted that because of the high mortality rates in some groups, the means were based on a variable number of individuals.

Figure 3a

log₁₀ *M.gallisepticum* cfu/g mucus

Days post *M.gallisepticum* inoculation

○ *Mg*ᵃ   APV   *O. rhinotracheale/E. coli*
Δ *Mg*   APV   *O. rhinotracheale*
× *Mg*   APV   -
□ *Mg*   -   -
◊ -   -   -

Figure 3b

log₁₀ *O. rhinotracheale* cfu/g mucus

Days post *M. gallisepticum* inoculation

○ *Mg*ᵃ   APV   *O. rhinotracheale/E. coli*
Δ *Mg*   APV   *O. rhinotracheale*
× *Mg*   APV   -
□ *Mg*   -   -
◊ -   -   -
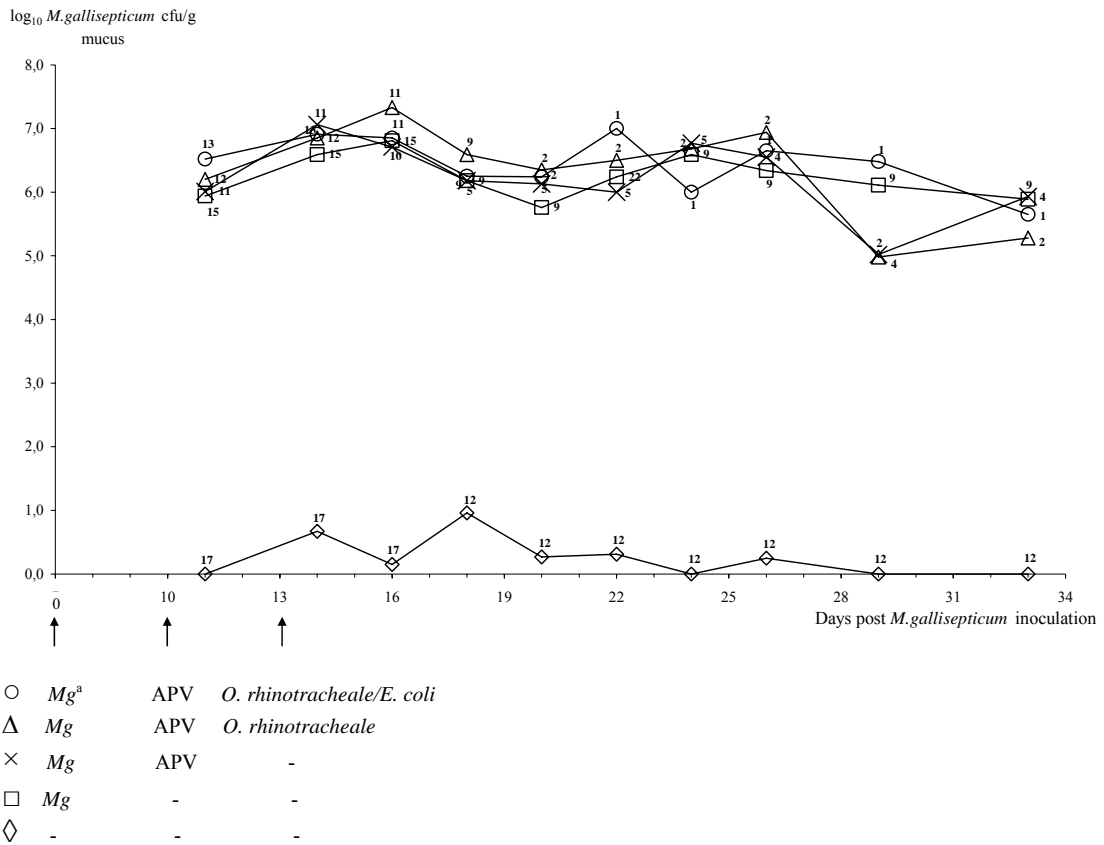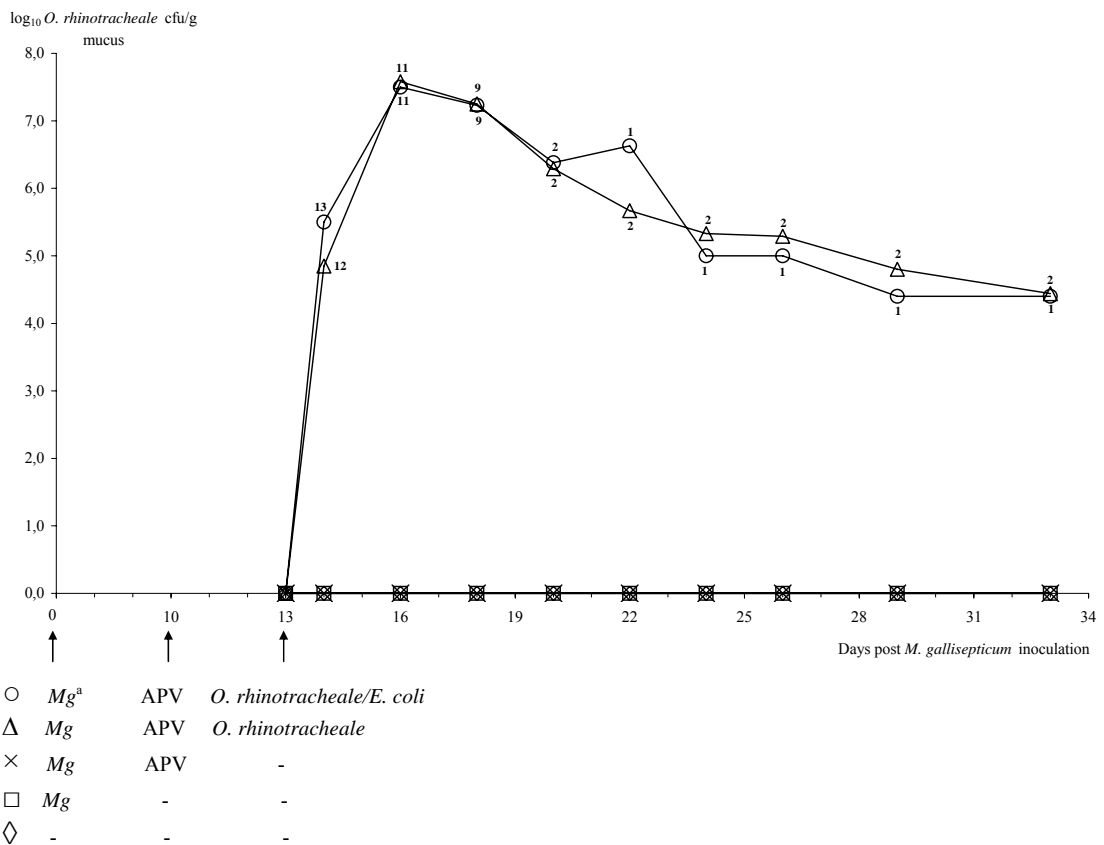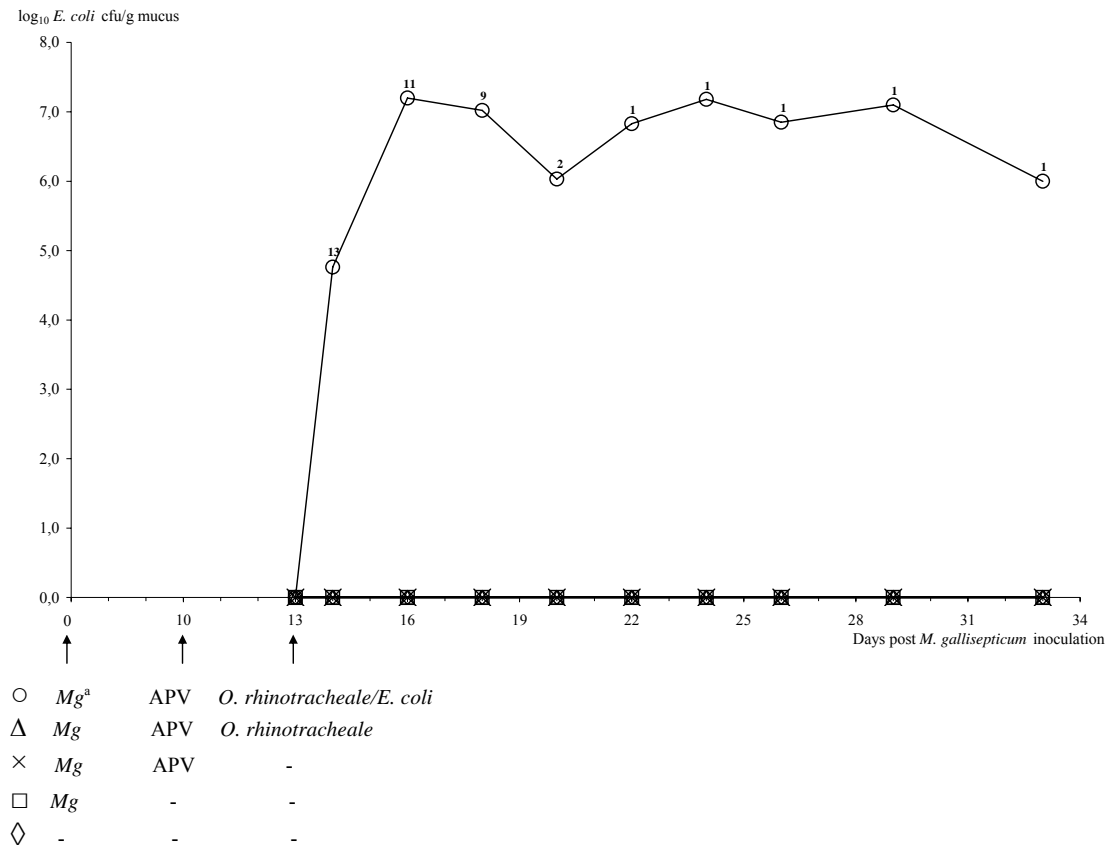
Figure 3c

Figure 3. Mean *M. gallisepticum* (a), *O. rhinotracheale* (b) and *E. coli* (c) titres ($\log_{10}$ cfu/g mucus) (and the number of animals from which the mean titres were calculated for each experimental group) in tracheal mucus collected at different time points after inoculation of single *M. gallisepticum* inoculation (□ group M), dual *M. gallisepticum*/APV inoculation (× group M/A), triple *M. gallisepticum*/APV/*O. rhinotracheale* inoculation (∆ group M/A/O), and quadruple *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* inoculation (○ group M/A/E/O). The negative control group (◊ group C) is included. [a]: *Mg = M. gallisepticum.*

*Bacteriological (***M. gallisepticum, O. rhinotracheale** *and* **E. coli***) titration of tissue suspensions and swabs*

     M. gallisepticum was never isolated from any of the tissues from the animals belonging to group C. This was also the case for *O. rhinotracheale* considering groups C, M, and M/A and for *E. coli* regarding groups C, M, M/A and M/A/O. The results of the *M. gallisepticum* culture of the birds euthanised at 18 dpmi and at 33 dpmi in the different *M. gallisepticum* inoculated groups are shown in Table 1. The results of the *O. rhinotracheale* and *E. coli* titrations of the birds euthanised at 18

dpmi and of the birds that died during the experiment (from 13 dpmi onwards) of groups M/A/O and M/A/E/O, are presented in Table 2. It should be noted that the clinical signs were very similar in nature and severity in all five euthanised (at 18 dpmi) birds of each group. At 33 dpmi, in the two remaining animals of group M/A/O, *O. rhinotracheale* was isolated only from the turbinates and the trachea, and in the only remaining animal of group M/A/E/O, *O. rhinotracheale* and *E. coli* were isolated only from the turbinates and trachea.

Table 1. Culture of *M. gallisepticum* of different organs of turkeys after single *M. gallisepticum* inoculation (group M), dual *M. gallisepticum*/APV inoculation (group M/A), triple *M. gallisepticum*/APV/*O. rhinotracheale* inoculation (group M/A/O), and quadruple *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* inoculation (group M/A/E/O), with a ten day interval between *M. gallisepticum* and APV inoculations, a three day interval between APV and *O. rhinotracheale*/*E. coli* inoculations, and an interval of eight hours between the *E. coli* and *O. rhinotracheale* inoculations

M. gallisepticum isolation																			
Turkeys euthanised 18 days after *M. gallisepticum* inoculation																			
Group		M					M/A					M/A/O					M/A/E/O		
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Trachea	+[b]	+	+	+	+	+	+	+	+	+	+	c	c	+	+	+	+	+	+	+
Lungs	+	-	+	+	+	+	+	+	+	+	+	c	+	+	+	+	+	+	-	-
Air sacs	+	+	+	+	+	+	+	+	c	+	-	+	+	+	+	+	-	+	-	+

Turkeys euthanised 33 days after *M. gallisepticum* inoculation																
Group					M							M/A			M/A/O	M/A/E/O
	1[a]	2	3	4	5	6	7	8	9	1	2	3	4	1	2	1
Trachea	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Lungs	-	-	-	-	-	-	-	+	-	-	-	c	+	-	-	+
Air sacs	-	-	-	-	-	+	+	-	-	-	+	c	-	-	+	+

a: bird number.
b: +: positive for *M. gallisepticum* culture; -: negative for *M. gallisepticum* culture.
c: contamination of medium, no result for *M. gallisepticum* culture.

Table 2. Bacterial isolation of *O. rhinotracheale* and *E. coli* from different organs of turkeys inoculated with *M. gallisepticum*, ten days later with APV, and finally three days later with *O. rhinotracheale* (group M/A/O) or with *E. coli* and *O. rhinotracheale* (group M/A/E/O)

Turkeys (n=5 per group) euthanised at 18 days post *M. gallisepticum* inoculation

	O. rhinotracheale (log$_{10}$ cfu/g tissue)										*E. coli* (log$_{10}$ cfu/g tissue)				
Group	M/A/E/O					M/A/O					M/A/E/O				
	1^{a*}	2	3	4	5*	6	7	8	9	10	1*	2	3	4	5*
Trachea	10.8	7.9	7.2	7.0	3.6	7.4	8.5	7.4	6.5	8.7	11	7.9	6.6	6.1	4.0
Lungs	7.3	8.1	7.1	6.6	6.9	6.6	7.7	7.4	6.4	7.0	6.9	7.8	7.0	5.7	6.2
Air sacs	6.5	4.9	6.2	6.2	4.1	5.7	4.6	7.4	6.0	6.7	6.2	4.7	5.1	4.2	3.7
Sinus^b	+^{c}	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Air sacs^b	+	-	+	+	+	-	-	+	+	+	-	-	+	-	-
Pericardium^b	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-
Heart^b	-	-	+	-	-	-	-	+	-	+	+	+	-	-	-
Liver^b	-	-	+	-	-	-	-	+	-	-	+	-	-	+	-

Turkeys that died during the experiment

	O. rhinotracheale isolation												*E. coli* isolation						
Group	M/A/E/O							M/A/O					M/A/E/O						
	1^{a*}	2	3*	4*	5*	6*	7	8	9	10	11	12	1*	2	3*	4*	5*	6*	7
Days post bacterial inoculation	2	2	4	5	6	6	8	2	4	5	6	6	2	2	4	5	6	6	8
Turbinates^b	+^{c}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trachea^b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lungs^b	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Sinus^b	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	-
Air sacs^b	-	+	+	+	-	+	-	-	-	-	+	-	+	+	+	+	-	+	-
Pericardium^b	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-
Heart^b	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
Liver	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	-

a: bird number.
b: these organs were swabbed and only qualitative bacteriological analysis was performed.
c: +: *O. rhinotracheale* or *E. coli* isolated ; -: no *O. rhinotracheale* or *E. coli* isolated.
*: pericarditis and/or perihepatitis seen at necropsy.

Histopathology

In the control group (C), no marked microscopic changes were observed in the turbinates, trachea or lungs in any of the examined turkeys.

For the five birds of each group, euthanised at 18 dpmi, the following histopathological lesions were found. The histological preparations of the turbinates of animals of group M showed no histological abnormalities. In the tracheae however, mild infiltration of heterophils and monomorphonuclear cells in the mucosa and loss of cilia were seen, and in the lungs, foci of pneumonia were noticed. Histopathological lesions were similar in groups M/A, M/A/O and M/A/E/O, i.e. mostly severe infiltration of heterophils and monomorphonuclear cells in the mucosa of the turbinates and trachea, degeneration of the mucous glands and epithelium in the turbinates and trachea, loss of cilia in trachea and turbinates, activation of lymph nodules in the turbinates, suppurative exudate in the lumen of trachea and turbinates, and foci of pneumonia, suppurative bronchitis and bronchiolitis in the lung. Although, overall the lesions were similar in all mentioned groups, they tended to be more severe in group M/A/E/O, followed by group M/A/O, both compared with group M/A. The histopathological lesions of the animals that died during the experiment from 13 dpmi onwards, were comparable with those of the animals of the same group euthanised at 18 dpmi.

At the end of the experiment period (33 dpmi), the microscopical lesions in the different groups were less severe. In the mucosa of the turbinates and trachea, infiltrations of heterophils and monomorphonuclear cells could still be found, and although epithelial regeneration (hyperplasia) was already seen, regeneration of the cilia had not yet occurred. Recovery of the damaged mucous glands was evidenced by the reappearance of mucus in the mucous glands, an obvious feature in the PAS stained tissue sections. In a few birds some foci of pneumonia could be observed, although markedly less severe.

Weight

At the beginning of the experimental period, the mean weights of each group did not differ greatly. At the end of the experiment the lowest mean weight per group was seen in groups M/A/E/O (1070 g; n=1) and M/A/O (1088 g; n=2), followed by groups M (1198 g; n=9), M/A (1211 g; n=4) and C (1314 g; n=12).

Discussion

The *M. gallisepticum* strain administrated had been used previously in various experimental studies concerning adult chickens. However it caused no or very mild respiratory symptoms (personal communication, Wil Landman, GD Deventer). After single *M. gallisepticum* inoculation in the present study, however, the birds suffered from very serious respiratory distress, caused by severe airsacculitis, resulting in high mortality rates. A number of explanations may clarify the difference in disease expression found between the formerly mentioned studies in adult chickens and the present work. First, the fact that we used turkeys instead of chickens has probably been of major importance, since turkeys are known to be more susceptible to *M. gallisepticum* than chickens, commonly developing more severe clinical signs (Ley, 2003). Secondly, the age of the infected birds may have been of great significance, since it is known that young birds are, in general, more susceptible to infection with *M. gallisepticum* (Bradbury and Levisohn, 1996). Gaunson *et al*. (2006), examined the age-related differences in the immune response to vaccination and infection with *M. gallisepticum*, and stated that it is likely that different cells of the immune system do not fully mature until a number of weeks after hatching and may contribute to the different responses seen in younger birds. According to this research group there is a complex age-related response to *M. gallisepticum* infection, with the age of birds appearing to influence how quickly the bird is able to clear the infection. Finally, the applied inoculation route may have been of influence on the disease outcome. Through intratracheal inoculation, compared with for instance oculonasal inoculation, M. gallisepticum may have bypassed some of the innate defence mechanisms of the upper respiratory tract, possibly resulting in more severe disease.

Clinical signs attributed to *M. gallisepticum* seen in turkeys include sinusitis, respiratory distress, depression, decreased feed intake, and weight loss. As the disease progresses, tracheal rales and labored breathing may become evident if tracheitis and airsacculitis is present (Ley, 2003). In our study however, turkeys did not seem to suffer from sinusitis. It is mentioned already that turkeys sometimes do not develop sinusitis in experimentally induced infections unless cultures are injected directly into the sinus (Dingfelder *et al*., 1991).

The macroscopical (severe airsacculitis, foci of pneumonia) and microscopical lesions (thickening of mucosa due to heterophilic and monomorphonuclear infiltrations in the

trachea, loss of cilia, focal pneumonia) seen after single *M. gallisepticum* infection, are in accordance with those described in the literature (Ley, 2003).

As mentioned in the results, *M. gallisepticum* DNA was recovered from some of the tracheae of birds in group C. This was regarded as contamination during processing of the samples for different reasons. First, animals which were shown to be positive by the Q-PCR (which has a low detection limit), were not consistently positive, i.e. they proved negative at later time points (data not shown). Secondly, the Q-PCR positive animals showed negative upon *M. gallisepticum* isolation from their organs at post mortem. Finally, serologic examination (ELISA-test BioChek) of serum collected from all euthanised birds, indicated that only the *M. gallisepticum* inoculated birds showed seroconversion, as opposed to the birds in group C which remained negative throughout the trial period (data not shown). However, this last argument can not be considered as conclusive, since seroconversion after infection usually appears after two to three weeks.

Upper respiratory tract symptoms (nasal exudates and swollen sinuses) were only seen in the *M. gallisepticum* infected turkeys after they were inoculated with APV. These upper respiratory symptoms were exacerbated by inoculation with *E. coli* and/or *O. rhinotracheale*. These findings are in accordance with previous experiments executed by our research group (Marien *et al.*, 2005; 2006) in which it was shown that co-infection with *O. rhinotracheale* and/or *E. coli* had a synergistic effect both on the severity and on the duration of clinical symptoms of APV infection. The peak in the clinical scores, especially in the groups with additional infection with *O. rhinotracheale* and *E. coli*, coincided with a second peak of mortality. It appears that by the time the birds were slowly starting to recover from the acute respiratory problems caused by the *M. gallisepticum* inoculation, the additional bacterial infections were too overwhelming for these poults. After this peak, both the mortality rate and the severity of clinical symptoms decreased, although it should be noted that only few animals were left at this time in some of the experimental groups. From 19 dpmi, only 4, 2 and 1 birds were left in group M/A, M/A/O and M/A/E/O, respectively.

Because of the poor general condition and anorexia in some birds, the mean weight in groups M/A/O and M/A/E/O was lower compared with the groups M/A and M, the latter in their turn having lower mean weights compared with the negative control group.

Besides the severity of clinical symptoms and mortality, the isolation of the different bacterial pathogens in the organs of the infected turkeys was also examined. The amount of *M. gallisepticum* bacteria isolated from the tracheae with time did not substantially differ between the various *M. gallisepticum* infected groups. Apparently, co-infection with APV, *E. coli* and/or *O. rhinotracheale* does not seem to have an effect on the *M. gallisepticum* multiplication in the trachea, at least not during the evaluated period. Furthermore, at 18 dpmi, *M. gallisepticum* was cultured from the trachea, lungs and air sacs of almost all *M. gallisepticum* inoculated animals. At 33 dpmi, *M. gallisepticum* was still present in the trachea, but to a lesser extent in the lungs and air sacs of al *M. gallisepticum* inoculated groups, indicating that *M. gallisepticum* clearance in these organs was initiated. The observation that the *M. gallisepticum* titres remained at a high level in the upper respiratory tract throughout the experimental period, is in agreement with the many reports describing the persistent character of *M. gallisepticum* infections in poultry (Razin *et al*., 1998; Ley, 2003; Bradbury, 2005; Reinhardt *et al*., 2005). The ability of mycoplasmas to modulate the host immune responsiveness most probably contributes to their pathogenicity, enabling them to evade or suppress the host defense mechanisms and establish a chronic, persistent infection (Razin *et al*., 1998; Rottem, 2003; Bradbury, 2005). Furthermore, mycoplasmas, including *M. gallisepticum,* are able to vary the expression of surface antigens, thus expressing a continuously changing 'antigenic profile' to the immune system. This is a property of the mycoplasmas which is also named phenotypic plasticity.

Infection with *M. gallisepticum* did seem to have lengthened the presence of *O. rhinotracheale* and *E. coli* in the upper respiratory tract if the outcome of this work is compared with previous experiments in which the same APV, *O. rhinotracheale* and *E. coli* strains (Marien *et al*., 2005; 2006; unpublished results) were used. In those studies *O. rhinotracheale* could not be isolated after 10-12 days post *O. rhinotracheale* inoculation, while *E. coli* was cleared from the trachea at 14 days post *E. coli* inoculation or present only in very low numbers. More specifically, in the present study, *O. rhinotracheale* and *E. coli* remained present in the trachea until 20 days post *O. rhinotracheale*/*E. coli* inoculation, and titres were still at high levels, especially the *O. rhinotracheale* titres (Figure 3b and 3c). This suggests that *M. gallisepticum* exerts an important impeding effect on the clearance of *O. rhinotracheale* and *E. coli*, especially from the upper respiratory tract. Indeed, at the

end of the experiment (20 days post *E. coli/ O. rhinotracheale* inoculation), *O. rhinotracheale* and *E. coli* appeared to be cleared from the lungs, air sacs, and non-respiratory organs, but not from the turbinates and trachea, suggesting that the host's immune response at this level might have been impaired.

In general, gross lesions at necropsy were similar in group M/A, M/A/O and M/A/E/O, with severe airsacculitis, serous to seromucous exudates in the turbinates and sinuses, and moderate to severe pneumonia. A very conspicuous feature, however, was that in almost all birds that died in group M/A/E/O after *O. rhinotracheale* and *E. coli* inoculation, signs of septicaemia were seen. Also, in two out of the five animals euthanised at five days post *E. coli* and *O. rhinotracheale* infection (group M/A/E/O), pericarditis and perihepatitis were seen at necropsy. It is frequently stated in the literature that in poultry, more severe *M. gallisepticum* outbreaks with high morbidity and mortality, frequently result from the involvement of complicating infections and environmental or other stressors (Bradbury and Levisohn, 1995; Levisohn and Kleven, 2000; Ley, 2003). Respiratory-origin colisepticemia is the most common type of colisepticemia in poultry, and it is thought that *E. coli* gains access to the circulation following damage to the respiratory mucosa. As opposed to the present work (applying *M. gallisepticum* inoculation), we were not able in previous trials to experimentally reproduce colibacillosis in turkeys by inoculation with APV, *O. rhinotracheale* and *E. coli*. These findings indicate that compared with bacterial virulence factors, pathogenic interactions are probably at least as important for the induction of colibacillosis (Barnes *et al.*, 2003). Although *O. rhinotracheale* was isolated in some birds from non-respiratory organs, the majority of reisolates were obtained from the respiratory tract, moreover signs of septicemia were never encountered in the M/A/O infected turkeys. The high number of deaths in this group was therefore attributed to the severe respiratory symptoms and anorexia of the birds. In contrast, *E. coli* was isolated most frequently from the non-respiratory organs of the birds diagnosed with septicaemia (Table 2).

In conclusion, the results obtained in the present study clearly indicate the occurrence of a marked synergism between four distinct respiratory pathogens important in poultry, i.e. *M. gallisepticum*, APV, *O. rhinotracheale* and *E. coli*. This was evidenced by the higher mortality rates and the clinical symptoms, which were aggravated whenever the birds were infected with an additional pathogen. This synergism was further confirmed by the more severe necropsy findings and

microscopical lesions found in groups inoculated with more than one pathogen. In this regard a concurrent infection with *M. gallisepticum*, APV, *O. rhinotracheale* and *E. coli* seemed the most detrimental to the birds.

An interesting finding was that we were able to experimentally induce colisepticemia, which has proven to be very difficult in the past (Van de Zande *et al*., 2001). This experimental model could therefore have some important benefits for future research, e.g. the evaluation of antimicrobial strategies or even vaccines.

Acknowledgements

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Chapter 3: EXPERIMENTAL STUDIES

3.2. Evaluation of the efficacy of antimicrobials for the treatment of clinical respiratory disease in turkeys

3.2.1. Comparison of the efficacy of four antimicrobial treatment schemes against experimental *Ornithobacterium rhinotracheale* infection in turkey poults pre-infected with avian metapneumovirus

3.2.2. *In vivo* selection of reduced enrofloxacin susceptibility in *Ornithobacterium rhinotracheale* and its resistance-related mutations in *gyrA*

3.2.3. Efficacy of enrofloxacin, florfenicol and amoxicillin against *Ornithobacterium rhinotracheale* and *Escherichia coli* O2:K1 dual infection in turkeys following avian metapneumovirus priming

3.2.1. Comparison of the efficacy of four antimicrobial treatment schemes against experimental *Ornithobacterium rhinotracheale* infection in turkey poults pre-infected with avian metapneumovirus

Marien, M., Nauwynck, H., Duchateau, L., Martel, A., Chiers, K., Devriese, L., Froyman, F., and Decostere, A.

Summary

The clinical efficacy of drinking-water administration of enrofloxacin for three and five days, amoxicillin for five days and florfenicol for five days for the treatment of respiratory disease induced by an experimental *Ornithobacterium rhinotracheale* infection in turkeys pre-infected with avian metapneumovirus (APV) was assessed based on clinical, bacteriological and histopathological examinations. Experimental groups of 15 susceptible three-week-old turkeys were each inoculated oculonasally with APV subtype A and three days later with susceptible *O. rhinotracheale* bacteria. Antimicrobial treatment started one day after *O. rhinotracheale* inoculation. After infection, the birds were examined and scored for clinical signs, swabbed daily and weighed at different times. Five birds were euthanised and examined for macroscopic lesions at necropsy at five days post bacterial inoculation (dpbi), and the remainder at 15 dpbi. Samples of the turbinates, trachea, lungs, air sacs, heart and pericardium were collected for bacteriological and/or histological examination.

Recovery from respiratory disease caused by an APV/*O. rhinotracheale* dual infection was most successful after enrofloxacin treatment, irrespective of treatment duration, followed by florfenicol. Amoxicillin treatment was not efficacious. Clinical signs and the number of *O. rhinotracheale* organisms re-isolated from the trachea and the different respiratory organs were significantly reduced by enrofloxacin treatment for three and five days. *O. rhinotracheale* bacteria were not re-isolated from the tracheas of the birds treated with enrofloxacin except for one in the five-day group, as early as one day after medication onset. In the group treated with enrofloxacin for five days, *O. rhinotracheale* organisms with a higher minimal inhibitory concentration (MIC) value (x 8) were isolated starting two days following treatment onset initially from a single turkey and subsequently from the other animals.

Introduction

Viral and bacterial respiratory tract infections frequently occur in diseased turkeys of all ages and may cause considerable financial losses due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter (van Empel and Hafez, 1999).

Ornithobacterium rhinotracheale is an infectious pathogen that has been ascribed an etiological role in the respiratory disease complex in turkeys. This Gram-negative bacterium is mostly regarded as a facultatively pathogenic organism, and in field cases, simultaneous isolation of *O. rhinotracheale*, respiratory viruses and/or other bacteria is frequently encountered. One of the viruses judged to have a triggering role is avian metapneumovirus (genus *Metapneumovirus* - MPV) (APV) (van Empel *et al.*, 1996). Recently, in a longitudinal study performed by Van Loock *et al.* (2005), it was shown that both APV and *O. rhinotracheale* infections often occur between hatch and slaughter on Belgian turkey farms. In a previous study using an optimized experimental *in vivo* model, it was demonstrated that *O. rhinotracheale* following APV inoculation in three-week-old turkeys has a synergistic effect on the course of respiratory disease (Marien *et al.*, 2005). Dual infection with APV and *O. rhinotracheale* indeed resulted in more severe clinical signs, macroscopic and histological findings, and a longer persistence of *O. rhinotracheale* in the respiratory tract compared with the single infections.

Disease caused by *O. rhinotracheale* may be reduced by preventing predisposing factors including inadequate ventilation, high ammonia levels, too high or too low relative humidity and infection with additional pathogenic agents (van Empel and Hafez, 1999). In practice, however, *O. rhinotracheale* infections are mostly dealt with using different antimicrobials such as amoxicillin, ampicillin, doxycycline, tetracycline, trimethoprim/sulphonamide, enrofloxacin and florfenicol. Hitherto, the actual *in vivo* efficacy of antimicrobials for the treatment of *O. rhinotracheale* infections in poultry has not yet been investigated. This is to a great extent rooted in the fact that, only until very recently (Marien *et al.*, 2005), no suitable infection model was available. The objectives of the present study are to compare the efficacy of enrofloxacin, amoxicillin and florfenicol for treatment of respiratory disease due to experimental *O. rhinotracheale* infection in three-week-old turkeys following APV challenge. The efficacy was evaluated on the basis of several

parameters, i.e., clinical signs, histopathological findings, re-isolation and titration of the bacterium, and weight gain.

Materials and methods

Turkeys

Seventy-five specific pathogen free (SPF) turkeys (AFSSA, Ploufragan, France) were used in this study. The turkeys were hatched in our facilities. The birds were housed on litter in separate isolation rooms with HEPA-filtered air, had free access to food and water and received 16 hours of light per day. At two weeks of age the birds were shown to be free from maternally-derived antibodies to *O. rhinotracheale* and APV by means of an ELISA available commercially (Biochek, Gouda, the Netherlands) and an in-house serum neutralization test, respectively. Neutralization was tested on Vero cells in 96-well cell culture microplates using standard procedures. Briefly, serial twofold dilutions of the sera were made and incubated for 1 h at 37°C with an equal volume of virus suspension (subtype A), containing 100 tissue culture infectious dose with a 50% endpoint ($TCID_{50}$) APV. The reading was based on the absence or presence of a cytopathic effect over seven days. The SN-titres were the reciprocal of the highest serum dilution that inhibited the cytopathic effect in 50% of the wells.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al*., 1998). The virus stock had a titre of 5.0 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacterium

The *O. rhinotracheale* type strain LMG 9086[T] was used, which was originally isolated from a turkey with a respiratory tract infection. The strain was serotyped as type A in an agar gel precipitation test (Hafez and Sting, 1999) performed by Professor H. M. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany). The strain was stored at -70°C. The organism was cultured for 48h at

37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. The *O. rhinotracheale* bacteria were transferred into brain heart infusion broth (Oxoid) for 24h at 37°C with agitation (microaerophilic incubation). The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in phosphate-buffered saline (PBS) followed each time by five minutes of centrifugation at 2000 × g at 4°C. The resulting pellet was re-suspended in PBS to obtain a final concentration of 8.6 $\log_{10}$ colony-forming units (cfu)/ml. Confirmation of the number of cfu/ml was done by inoculating ten-fold dilutions in PBS on Columbia agar with 5% sheep blood and counting the number of colonies. The minimal inhibitory concentrations (MICs) of amoxicillin, florfenicol and enrofloxacin for this challenge strain were 2 μg/ml, 1 μg/ml and ≤0.03 μg/ml, respectively, as determined according to Devriese *et al*. (2001).

Antimicrobial agents

Three antimicrobial agents were used in this study: enrofloxacin (Baytril[®] 10% oral solution, Bayer, Leverkusen, Germany), amoxicillin (powder form) (Suramox 50[®], soluble powder, Virbac S.A., Carros, France) and florfenicol (soluble form) (Nuflor[®], Schering-Plough S.A., Xochimilco, Mexico) with manufacturer-recommended doses of 10 mg/kg for enrofloxacin and 20 mg/kg for amoxicillin and florfenicol.

Experimental design

Seventy-five SPF turkeys were randomly divided into five groups of 15 birds at one day of age.

In all groups, turkeys first received APV, and subsequently received *O. rhinotracheale* three days later. Each bird was inoculated with APV by the oculonasal route at a dose of 4.4 $\log_{10}CD_{50}$. *O. rhinotracheale* was likewise administered oculonasally with a dosage of 8 $\log_{10}$ cfu. For inoculation with APV and *O. rhinotracheale*, a total of 250 μl was divided equally over the nares and eyes.

Four groups received antimicrobial treatment: enrofloxacin 10 mg/kg for three days (group E3), enrofloxacin 10 mg/kg for five days (group E5), florfenicol 20 mg/kg for five days (group F), and amoxicillin 20 mg/kg for five days (group A). Starting at 24 hours post bacterial inoculation, the drinking water was medicated with the appropriate antimicrobial agent. The birds were continuously dosed and received

their daily medication over a 24h period. To enable correct dosing, the daily water uptake and mean group body weights were determined. All turkeys were weighed immediately before APV inoculation, before *O. rhinotracheale* inoculation, at day five after *O. rhinotracheale* inoculation, and finally at the end of the experiment (15 days post bacterial inoculation (dpbi)). The concentration of each antimicrobial to be administered in the water could be calculated accurately on the basis of the water consumption and body weight data. The fifth group was included as an untreated control group (group C).

All birds were clinically examined on a daily basis throughout the experiment. The clinical signs were scored as described in Van de Zande *et al.* (2001). Briefly, the clinical condition of each bird was assigned a score from 0 (absence of clinical signs) to 7 (nasal exudate with extremely swollen sinuses and frothy eyes, poor general condition and anorexia). The mean clinical score was calculated for each experimental group.

Tracheal swabs were collected from the animals in all groups at 3 days post viral inoculation (dpvi) to confirm infection with APV, and daily until 11 dpbi for *O. rhinotracheale* titration. The tracheal swabs were taken using cotton-tipped aluminium-shafted swabs (Copan Diagnostics Inc., Corona, USA) and placed in 1 ml PBS supplemented with Ca^{2+} and Mg^{2+} and in the case of virus titration an additional 10% fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco). Processing occurred as described below.

Five birds of each group were randomly selected and were sacrificed at 5 dpbi. The remaining ten birds were sacrificed at 15 dpbi. The birds were necropsied and examined for gross lesions. Samples of the turbinates, trachea, lungs, air sacs, heart and pericardium were collected for bacterial isolation and processed immediately as described below. Finally, samples from the turbinates, trachea and lungs were taken and fixed in 10% neutral buffered formalin for histopathological examination.

Virological and bacteriological titration of tracheal swabs

The viral titre in $\log_{10}CD_{50}$ per g mucus and the number of cfu of *O. rhinotracheale* per g mucus were determined. This was done using the procedures described in Marien *et al.* (2005).

Bacteriological titration of tissue suspensions and swabs

Samples of the turbinates, trachea and lungs were titrated for *O. rhinotracheale* from the 25 birds sacrificed at 5 dpbi. The number of cfu of *O. rhinotracheale* per g mucus was determined as described in Marien *et al*. (2005).

The swabs taken from the heart, pericardium and air sacs were inoculated onto 5% sheep blood agar supplemented with 5 µg/ml gentamicin and 5 µg/ml polymyxin. After 24-48h of incubation at 37°C in 5% CO_2 atmosphere, the agar was examined for presence of *O. rhinotracheale*. The same procedure was followed for samples from the turbinates, trachea, lungs, heart, pericardium and air sacs from the birds sacrificed at 15 dpbi.

Histopathology

Following fixation in formalin, the tissues were embedded in paraffin, sectioned at 4 µm, mounted on glass slides and stained with haematoxylin and eosin using standard procedures. Tissues from the turbinates and trachea were additionally stained by the Periodic Acid Schiff-reaction in order to visualize the mucus layer. The degree of severity of the various histological lesions in the trachea and turbinates was assessed, including infiltration of heterophils and/or monomorphonuclear cells, degeneration of the mucous glands in the respiratory epithelium and damage to the ciliated epithelial cells and cilia). Each histological parameter was assigned a score from 0 (absence of particular lesion) to 4 (abundant presence of particular lesion).

MIC of the parent and re-isolated **O. rhinotracheale** *isolates*

The MIC values of 124 *O. rhinotracheale* isolates retrieved from the tracheal swabs before, during and after antimicrobial treatment were determined, specifically for the isolates from enrofloxacin group E3 and E5, for amoxicillin group A and finally for florfenicol group F.

Determination of the MIC values was performed as described by Devriese *et al*. (2001), although modified slightly in that in this study MIC values were determined on Mueller Hinton II agar (BBL, Sparks, MD, USA) supplemented with 5% sheep blood. The antimicrobials used for the antimicrobial susceptibility test were enrofloxacin (Baytril® 10% oral solution, Bayer, Leverkusen, Germany), amoxicillin (Suramox 50®, Virbac S.A., Carros, France) and florfenicol (Nuflor®, Schering-

Plough S.A., Xochimilco, Mexico). Final concentrations ranging from 0.03 to 128 µg/ml (serial doubling dilutions) were tested.

Statistical analyses

The clinical scores and tracheal swabbing data of all animals were first analysed by a fixed-effects model with area under the curve between 0 and 5 dpbi as response variable, and treatment group as fixed effect, to study the early effects of the infection based on all treated animals.

Next, the repeated clinical scores between 0 and 9 dpbi, using only animals that were not euthanised at 5 dpbi, were analysed by a mixed proportional odds model with the clinical score as the response variable, animal as a random effect and treatment group and time as categorical fixed effects.

Tracheal swabbing values between 0 and 9 dpbi, involving only animals that were not euthanised at 5 dpbi, were analysed by a mixed model with $\log_{10}$cfu/g as response variable, animal as a random effect, and treatment group and time as categorical fixed effects. *O. rhinotracheale* titres from the different organs were analysed by a fixed effects model, and weight by a mixed model with animal as random effect and time, treatment group and initial weight as fixed effects. Finally, the histopathological scores were analysed by the Kruskal-Wallis test.

All tests were performed at a global 5% significance level, and the seven relevant pairwise comparisons (C vs E3, C vs E5, E3 vs E5, E5 vs A, E5 vs F, E3 vs A, E3 vs F), were tested at a significance level adjusted using Bonferroni's technique. Only these seven comparisons will be considered throughout the paper.

Results

During the experiment, mortalities did not take place in any of the experimental groups.

Clinical signs

The mean clinical score for each group is shown in Figure 1. Respiratory signs were seen in all APV-inoculated groups starting from 3 dpvi. From 10 dpbi onwards, clinical signs were not detected in any of the animals in the different experimental groups.
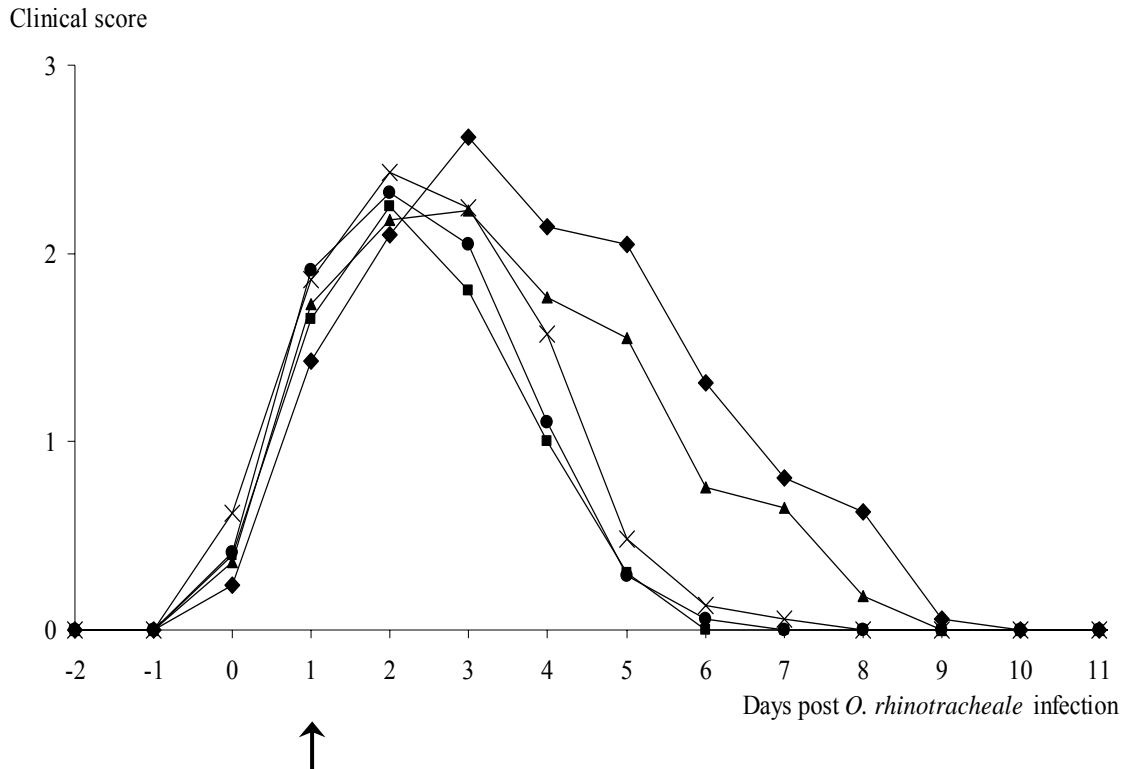
Figure 1. Mean clinical scores in turkeys inoculated with APV and *O. rhinotracheale* and subsequently treated with different antimicrobial agents: ■ group E3, 3 days of enrofloxacin (10 mg/kg); ●group E5, 5 days of enrofloxacin (10 mg/kg); ▲ group A, 5 days of amoxicillin (20 mg/kg); ×group F, 5 days of florfenicol (20 mg/kg) or no treatment = ◆ control group. Arrow indicates first day of antimicrobial treatment.

Using the area under the curve from 0 to 5 dpbi, there is a significant difference between the five treatments (P=0.0007). Group C has the highest total score (10.33), followed by A, F, E5 and E3 (7). Significant differences are shown in Table 2. In the proportional odds model, both a significant change in time (P<0.0001) and a significant difference (P<0.0001) between the treatments were detected. E3 had the lowest odds ratio followed by E5, F, A and C.

Thus, compared with the non-treated control group, clinical signs were significantly reduced by the enrofloxacin treatments, irrespective of treatment duration, but not by treatment with florfenicol or amoxicillin.

Virological titration of tracheal swabs

APV was recovered from tracheal swabs from every individual bird. The mean titres for groups C, E3, E5, A and F were 6.1, 5.9, 6.2, 5.8 and 6.1 $\log_{10}CD_{50}$ / g mucus, respectively.

Bacteriological titration of tracheal swabs

The results of the *O. rhinotracheale* titrations of the tracheal swabs are shown in Figure 2. Mean titres as well as titres for each individual bird are depicted. From 11 dpbi onwards, *O. rhinotracheale* bacteria were not isolated in any of the animals in the different experimental groups.
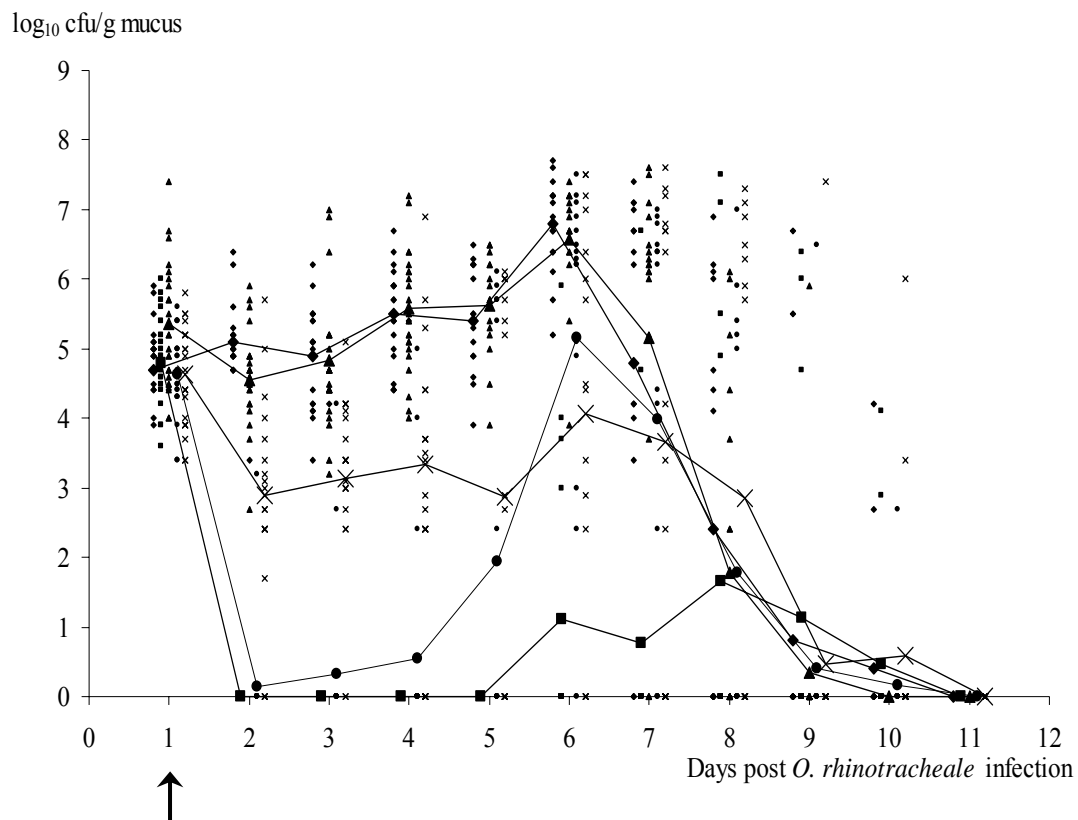
Figure 2. Bacterial titres ($\log_{10}$ cfu/g mucus) in tracheal mucus collected at different times after *O. rhinotracheale* inoculation in APV/O. rhinotracheale dually infected turkeys receiving different antimicrobial treatments: ■ group E3, 3 days of enrofloxacin, (10 mg/kg); ● group E5, 5 days of enrofloxacin (10 mg/kg); ▲ group A, 5 days of amoxicillin (20 mg/kg); × group F, 5 days of florfenicol (20 mg/kg), or no treatment (= ◆ control group). (Individual values are indicated with small symbols; means are indicated with larger symbols.) Arrow indicates first day of antimicrobial treatment.

Using area under the curve in the fixed-effects model, there is a significant difference between the five treatments (P<0.0001), and in the mixed model, both a significant change in time (P<0.0001) and a significant difference between treatments (P<0.0001) were detected. In both analyses, group C has the highest total score followed by groups A, F, E5 and E3 and all seven pairwise comparisons (C vs E3, C vs E5, E3 vs E5, E5 vs A, E5 vs F, E3 vs A, E3 vs F) are significant as indicated in Table 2.

Compared with the non-treated control group, the number of *O. rhinotracheale* organisms in the tracheal mucus was hence significantly reduced by both enrofloxacin treatments (three- and five-day treatment) and by the five-day florfenicol treatment. Compared with the non-treated group, treatment with amoxicillin did not cause a significant reduction in the *O. rhinotracheale* titres in the trachea.

Bacteriological titration of tissue samples

The results of *O. rhinotracheale* titrations in the various organs sampled at 5 dpbi are shown in Table 1. It should be noted that the clinical signs were very similar in terms of severity in all five euthanised birds of one group. For results from the turbinates, trachea and lungs, there were significant differences between the treatments (P<0.0001). Pairwise significant differences are shown in Table 2. Compared with the non-treated control group, the number of *O. rhinotracheale* in the trachea and lungs was significantly reduced by the enrofloxacin treatments and by the florfenicol treatment, as opposed to treatment with amoxicillin. Furthermore, the number of *O. rhinotracheale* isolated from the turbinates was significantly reduced by the 3-day enrofloxacin treatment. For the air sacs, pericardium and heart, very few positive samples were encountered (in group C and group A), and significant differences between the treatments did not occur.

O. rhinotracheale was recovered from none of the organs (turbinates, trachea, lungs, pericardium, and air sacs) of the birds in the five groups swabbed at 15 dpbi.

Table 1. Bacterial titre ($\log_{10}$cfu/g tissue) of *O. rhinotracheale* in different organs of turkeys inoculated with APV and *O. rhinotracheale* with an interval of three days and treated with different antibiotic therapies: group E3, 3 days of enrofloxacin (10 mg/kg); group E5, 5 days of enrofloxacin (10 mg/kg); group A, 5 days of amoxicillin (20 mg/kg); group F, 5 days of florfenicol (20 mg/kg), or no treatment (group C). Turkeys were euthanised at 5 dpbi.

Organ	Mean *O. rhinotracheale* titre ($\log_{10}$cfu / g tissue)																								
	Group C					Group E3					Group E5					Group A					Group F				
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Turbinates	8.5	8.4	6.9	11	8.4	0	0	4.9	0	0	5	7.1	7.5	4.3	4.5	8.4	8.6	8.6	7	6.3	10	8	9.2	6.4	7.3
Trachea	7.9	7.3	7.8	7.5	7.6	0	0	0	0	0	4.2	6.5	6.2	4.5	4.1	7.5	7.5	8.3	6.9	7.6	5.3	7.5	4.8	5.1	6.1
Lungs	6.1	5.8	6	6.7	0	0	0	0	0	0	0	3.2	0	2.8	2.9	6.8	5.5	4.6	6.3	6.4	3.3	0	2.2	3.5	0
Air sacs[b]	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Pericardium[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart[b]	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-

a: number of turkey
b: these organs were swabbed; − no *O. rhinotracheale* was isolated / + at least one cfu of *O. rhinotracheale* was recovered.

Table 2. Statistical analyses of clinical scores, tracheal swabbing, isolation of *O. rhinotracheale* from organs and weight of turkeys inoculated with APV and *O. rhinotracheale* with an interval of three days and treated with different antibiotic therapies: group E3, 3 days of enrofloxacin (10 mg/kg); group E5, 5 days of enrofloxacin (10 mg/kg); group A, 5 days of amoxicillin (20 mg/kg); group F, 5 days of florfenicol (20 mg/kg), or no treatment (group C).

Group	Clinical score		Isolation of *O. rhinotracheale* from tracheal mucus		Isolation of *O. rhinotracheale* ($\log_{10}$cfu/g tissue) at 5 dpbi from			Weight
	Area under the curve (from 0 to 5 dpbi[*1])	Odds ratio (with group C as reference group) (from 0 to 9 dpbi)	Area under the curve (from 0 to 5 dpbi)	$\log_{10}$cfu/g mucus (from 0 to 9 dpbi)	Turbinate	Trachea	Lungs	in g
C	10.33(0.58)[*2a]	1 [a]	25.86(0.61) [a]	4.51(0.23) [a]	8.64(0.71) [a]	7.62(0.34) [a]	4.92(0.75) [a]	578.40(12.54) [a]
A	9.45(0.57) [ab]	0.78(0.32) [a]	25.76(0.59) [a]	4.40(0.22) [a]	7.78(0.71) [a]	7.56(0.34) [a]	5.92(0.34) [a]	582.67(12.09) [a]
F	8.57(0.58) [ac]	0.43(0.16) [ab]	15.82(0.68) [b]	3.05(0.23) [b]	8.22(0.71) [a]	5.76(0.34) [b]	1.80(0.34) [b]	626.19(12.39) [ab]
E5	7.67(0.58) [bc]	0.30(0.11) [bc]	7.94(0.61) [c]	2.14(0.23) [c]	5.68(0.71) [a]	5.10(0.34) [bc]	1.78(0.34) [b]	654.53(12.24) [b]
E3	7.00(0.59) [c]	0.21(0.08) [c]	4.84(0.55) [d]	1.05(0.24) [d]	0.98(0.71) [b]	0.00(0.34) [d]	0.00(0.34) [b]	600.52(13.12) [a]

[*1]: dpbi: days post bacterial inoculation
[*2]: Mean value (standard error)
[abcd]: Treatments sharing a letter do not differ significantly from one another at the 5% global significance level.

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Histopathology

Similar microscopical changes were encountered in all experiment groups. In the mucosa of the turbinates and the trachea, a mixed inflammatory reaction was seen. Furthermore, focal loss of cilia and/or necrosis of the epithelium, as well as degeneration of mucous glands, were found in the turbinates and trachea. In the lung samples focal areas of bronchitis and bronchiolitis were observed.

No significant difference between the five treatments was found for any of the different parameters considered.

Weight

Significant differences occurred between the different treatments (P<0.0001). Weight evolves differently over time from treatment to treatment, i.e., there is a significant interaction between time and treatment (P<0.0001). Pairwise significant differences (0.7%) are shown in Table 2.

Antimicrobial therapy

The theoretically consumed dose of antimicrobials per group of birds was very close (within 10%) to the target dose of 10 mg/kg for enrofloxacin and 20 mg/kg for amoxicillin and florfenicol. The mean actual daily dose (mg/kg) for the total medication period was 10.2 (range: 9.1-11.3) for group E3, 10.2 (range: 9.4-11.9) for group E5, 20.4 (range: 19.0-22.5) for group F, and 19.9 (range: 17.5-22.7) for group A.

MIC of the parent and re-isolated **O. rhinotracheale** *isolates.*

In the *O. rhinotracheale* bacteria re-isolated from birds in groups C, E3, A and F, no changes in MIC values were observed compared with the challenge strain. However, two days after initiation of enrofloxacin treatment onwards, the MICs of the *O. rhinotracheale* bacteria isolated from tracheal swabs from group E5 had values that increased from 0.03 to 0.25 µg/ml.

Discussion

This study is the first to experimentally investigate the clinical efficacy of different antimicrobial therapies for treatment of dual APV/*O. rhinotracheale* infection in turkeys. Statistical analysis of the obtained results revealed that under the circumstances used in this study, recovery from respiratory disease caused by APV/*O. rhinotracheale* dual infection in three-week-old turkeys was most successful overall after enrofloxacin treatment (three or five days of treatment), followed by florfenicol treatment. Clinical signs as well as the number of *O. rhinotracheale* bacteria recovered from the trachea and different respiratory organs (turbinates, trachea and lungs), were significantly reduced by enrofloxacin treatment for three or five days, compared with the non-treated group. Compared with the non-treated group, five-day treatment with amoxicillin did not cause a significant reduction in any of the afore mentioned parameters and although five-day florfenicol treatment significantly diminished the amount of *O. rhinotracheale* isolated out of the trachea and lungs, this did not seem to result in a significant reduction in clinical symptoms. These results are in accordance with data presented by Froyman and Cooper (2003), who demonstrated that enrofloxacin treatment was most efficacious for the treatment of colisepticemia in chickens, followed by florfenicol, and that amoxicillin was not effective.

A possible explanation for the observed differences between antimicrobials relates to their different pharmacokinetic and pharmacodynamic properties. An important pharmacokinetic parameter for assessing the ability of antimicrobial agents to distribute throughout the tissues is the apparent volume of distribution at steady state ($Vd_{(ss)}$). $Vd_{(ss)}$ gives an indication of the diffusion of the active antimicrobial compound in the body tissues. A relatively low $Vd_{(ss)}$ value indicates a drug that is less extensively distributed in extravascular tissues. Different pharmacokinetic studies with enrofloxacin, florfenicol and amoxicillin in either turkeys or chickens have been performed (Carceles *et al.*, 1995; Anadón *et al.*, 1996; Rios *et al.*, 1997; Bugyei *et al.*, 1999; Knoll *et al.*, 1999; Shen *et al.*, 2003; Cox *et* al., 2004; Switala *et al.*, 2004; Dimitrova *et al.*, 2006). From these studies, it may be concluded that the $Vd_{(ss)}$ of amoxicillin (0.042 - 1.52 l/kg) is generally lower than that of florfenicol (1.5 - 4.99 l/kg) and enrofloxacin (2.92 - 3.9 l/kg).

Another perhaps influential parameter may be the elimination half-life of the different antimicrobials. When comparing half-life values determined in different experiments,

it may be concluded that enrofloxacin has long half-life values (Brown, 1996; Bugyei *et al.*, 1999; Garcia Ovando *et al.*, 1999; Knoll *et al.*, 1999; Dimitrova *et al.*, 2006) and that the values for florfenicol are generally lower (Afifi and El-Sooud, 1997; Shen *et al.*, 2002; Shen *et al.*, 2003), with the lowest half-life values noted for amoxicillin (Lashev and Pashov, 1992; Carceles *et al.*, 1995; Anadon *et al.*, 1996). Although both enrofloxacin and amoxicillin have good bactericidal activity, as opposed to florfenicol which is bacteriostatic, e.g., for *E. coli* and *Salmonella* spp. (Graham *et al.*, 1988), it seems that the bactericidal effect of amoxicillin is impaired because it is less efficiently distributed throughout the tissues and in addition is eliminated quickly (Goren *et al.*, 1981). The prominent results for enrofloxacin may be explained by its rapid bactericidal activity at relatively low concentrations (Brown, 1996) and its excellent distribution throughout tissues for longer periods of time (Brown, 1996; McKellar, 1996).

The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) breakpoints for amoxicillin vary greatly according to the bacteria involved. For instance, enterobacteriaceae are considered to be resistant when they have MIC values as high as 32 µg/ml or more, whereas staphylococci are already designated as resistant with MIC values of 0.5 µg/ml or more (CLSI guidelines, 2002). No CLSI breakpoints have been established for *O. rhinotracheale*, but from the results of the present investigation it can be concluded that an infection caused by *O. rhinotracheale* strains with an MIC of 2 µg/ml, does not seem to respond to antimicrobial treatment with amoxicillin. According to Devriese and colleagues (2001) who tested 45 *O. rhinotracheale* strains from poultry, MIC values for ampicillin generally varied between 1 and 8 µg/ml. According to Bush (2003), the antibacterial spectrum of amoxicillin is identical to that of ampicillin and there are few differences in antibacterial activity, especially against Gram-negative bacteria.

No increase in the amoxicillin or florfenicol MIC value for the bacteria re-isolated from the respective groups was noted. Likewise, no change in antimicrobial sensitivity was observed in *O. rhinotracheale* bacteria reisolated from group E3. In group E5, *O. rhinotracheale* could only be isolated from one bird after the first day after onset of treatment, but on the second day of the five-day treatment course, *O. rhinotracheale* organisms with an eight-fold higher MIC value (from ≤ 0.03 to 0.25 µg/ml) were isolated, first from one turkey, then successively from the other penmates. This increase in enrofloxacin MIC value concurred with a slight *O.*

rhinotracheale re-excretion in the trachea, also reflected in the higher counts in different organs (turbinates and lungs) at 5 dpbi, compared with the almost negative *O. rhinotracheale* counts post-treatment in the turkeys treated for three days.

The re-emergence of *O. rhinotracheale* isolates with increased MIC value was first seen in one animal on day two of antimicrobial treatment, and subsequently, each following day, isolates with increased MIC were found in more birds (data not shown). This may suggest that the less susceptible *O. rhinotracheale* isolate might have spread from one turkey to other penmates, although further epidemiological studies need to be performed to confirm this. Since *O. rhinotracheale* isolates with a rise in MIC value were already found after two days of enrofloxacin treatment, this suggests that this rise in MIC may also have occurred in the three-day treatment group.

Although the MIC of the re-isolated bacteria increased from ≤ 0.03 to 0.25 µg/ml, the *O. rhinotracheale* isolates are not to be considered resistant to enrofloxacin (Devriese *et al.*, 2001; CLSI guidelines, 2002). The term "reduced susceptibility" is more appropriate to characterize the antimicrobial sensitivity status of the isolates with an eight-fold increase in MIC value. It should be noted that in the present study, this emergence of decreased sensitivity did not lead to a clinical relapse. Experimental infection studies, including the fully susceptible *O. rhinotracheale* strain and the *O. rhinotracheale* strain with slightly higher MIC value, would be necessary to be able to conclude anything about the impact of this MIC rise on the *in vivo* efficacy of enrofloxacin. Froyman and Cooper (2003) reported that in chickens, enrofloxacin was less efficacious for the treatment of disease caused by an *E. coli* strain with reduced sensitivity (MIC of 0.5 µg/ml) when compared with the treatment of disease caused by a fully sensitive *E. coli* strain (MIC of 0.015 µg/ml).

O. *rhinotracheale* strain used and Mr. B. Van Dam (BioChek) for supplying the ELISA.

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3.2.2. ***In vivo*** **selection of reduced enrofloxacin susceptibility in** ***Ornithobacterium rhinotracheale*** **and its resistance-related mutations in** ***gyrA***

Marien, M., Decostere, A., Nauwynck, H., Froyman, R., Devriese, L., and Haesebrouck, F.

Summary

This study determines the genetic background of the change in antimicrobial susceptibility to enrofloxacin of *Ornithobacterium rhinotracheale* isolates with increased minimal inhibitory concentration (MIC) values, isolated either from the field or from turkeys treated with enrofloxacin under experimental challenge conditions. In the field *O. rhinotracheale* strains that were either less susceptible or, occasionally, resistant to enrofloxacin, point mutations had occurred in amino acids at positions 83 (serine) or 87 (aspartic acid) of the GyrA subunit.

In the isolates showing reduced susceptibility following experimental enrofloxacin treatment (increase in MIC from ≤0.03 to 0.25 µg/ml), molecular analysis revealed a constantly recurring point mutation (G → T) at nucleic acid position 646 (*E. coli* numbering) of *gyrA* resulting in an amino acid change from aspartic acid to tyrosine at position 87 of the GyrA subunit, which is a known hot spot for fluoroquinolone resistance. This study hence indicates that a single course of enrofloxacin treatment may contribute to the selection of the first mutant with reduced fluoroquinolone susceptibility in *O. rhinotracheale*.

Acquired fluoroquinolone resistance is commonly encountered in *O. rhinotracheale* isolates, and this is the first time that the causal mechanism of fluoroquinolone resistance in *O. rhinotracheale* has been investigated.

Introduction

Ornithobacterium rhinotracheale is an emerging infectious pathogen that has been ascribed an etiological role in the respiratory disease complex in turkeys and chickens, possibly causing considerable financial losses due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter (van Empel and Hafez, 1999).

Generally, *O. rhinotracheale* infections are treated mainly with different antimicrobials, but treatment is becoming more and more difficult as a lot of isolates have acquired resistance to the antimicrobials most commonly used (Devriese *et al.*, 1995; 2001; Malik *et al.*, 2003; Soriano *et al.*, 2003).

A challenge experiment conducted previously was carried out to test the efficacy of four antimicrobial treatment schemes against *O. rhinotracheale* infection in turkeys (Marien *et al.*, 2006). In the experimental group receiving 5 days enrofloxacin treatment, *O. rhinotracheale* organisms with a higher enrofloxacin MIC (0.25 µg/ml) compared to that of the inoculated *O. rhinotracheale* strain ($\leq$ 0.03 µg/ml), were isolated initially from the trachea of one turkey, and subsequently from the other turkeys within that group.

In most Gram-negative organisms, a reduction in the susceptibility to fluoroquinolones is caused by successive point mutations clustered in the quinolone resistance-determining region (QRDR) of the *gyrA* gene (Hooper, 1999; Barnard and Maxwell, 2001; Gibello *et al.*, 2004). However, there are no data whatsoever on the genetic basis for fluoroquinolone resistance in *O. rhinotracheale*. This work was therefore carried out to investigate the resistance mechanism responsible for this *in vivo* selection of reduced enrofloxacin susceptibility in *O. rhinotracheale* following enrofloxacin treatment in turkeys. In addition to the *O. rhinotracheale* isolates derived from the experimentally infected animals, other *O. rhinotracheale* field strains with different *in vitro* sensitivity levels for enrofloxacin, were also included in this study for comparative purposes.

Materials and Methods

Bacterial strains

Twenty-three *O. rhinotracheale* isolates recovered during a previously conducted challenge experiment, designed to test the efficacy of four antimicrobial treatment schemes against *O. rhinotracheale* infection in turkeys, were included in this study (Marien *et al.*, 2006). In brief, four groups of SPF turkeys received continuous drinking water treatment (enrofloxacin, 10 mg/kg, three or five days; florfenicol, 20 mg/kg, five days; amoxicillin, 20 mg/kg, five days) after oculonasal inoculation with avian metapneumovirus and *O. rhinotracheale*. In the experimental group receiving five days enrofloxacin, *O. rhinotracheale* organisms with a higher enrofloxacin MIC (0.25 µg/ml) compared to that of the inoculated *O. rhinotracheale* strain (≤ 0.03 µg/ml), were isolated initially from the trachea of one turkey, and subsequently from the other turkeys within that group.

Additionally, one *O. rhinotracheale* strain with lowered enrofloxacin sensitivity (MIC 0.5 µg/ml), five enrofloxacin sensitive (MIC ≤ 0.03 µg/ml) and 17 strains with unusually high enrofloxacin MIC values (MIC 1 - 2 µg/ml), close to or equal to the clinical breakpoint of 2 µg/ml (CLSI, 2002), all isolated from turkeys in France and broiler chickens in Belgium were likewise part of this study. The strains were stored at -70°C. They were retrieved from the frozen suspension and cultured for 48h at 37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep's blood in a 5% CO_2 atmosphere.

The origins and corresponding enrofloxacin MIC values of the different *O. rhinotracheale* isolates are given in Table 1.

Determination of **gyrA** *sequences*

To determinate the *gyrA* sequences, DNA was extracted using an alkaline lysis method of Baele *et al.* (2000), and the QRDR of the GyrA subunit of *O. rhinotracheale* was first amplified using a consensus degenerate universal primer pair, gyrAF and gyrAR (Maurin *et al.*, 2001). On the basis of the determined sequences of 14 *O. rhinotracheale* isolates and type strain LMG9086, amplified with the universal primers, specific primers for the *O. rhinotracheale gyrA* gene were designed, namely ORTgyrAF (5' CACAGAAGGGTGCTCTATGGG 3') and ORTgyrAR (5' TTCCAGCGGCACCATTTACC 3'). PCR reactions were performed

in a volume of 40µl containing a final primer concentration of 1µM for each of the oligonucleotides, 40 µM of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Puurs, Belgium), 3mM MgCl$_2$, 0.03 U/µl Polymerase Taq platinum (Invitrogen Life Technologies, Merelbeke, Belgium), and 1x PCR buffer (Invitrogen Life Technologies). Two microliters of template DNA were added to the vials. The conditions used for the amplifications were the following: initial denaturation at 95°C for 5 min, 35 cycles of 1 min denaturation at 95°C, 1 min of annealing at 48°C for the PCR with the universal primer pair and at 58°C with the specific primer pair, 1 min of elongation at 72°C, and a final extension of 5 min at 72°C. Five microliters of the PCR products of each sample were mixed with 3 µl of sample buffer 5X (50% glycerol, 1 mM cresol red) and were electrophoresed through a 1.5% agarose gel for 60 minutes at 160 V in 0.5 x TBE (0.45 M Tris-HCl, 0.45 M boric acid, 0.01 M EDTA). After purification of the PCR products with the Qiaquick PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands), the sequences of the PCR-products from the *O. rhinotracheale* strains were determined using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Lennik, Belgium) on an ABI Prism^{TM}310 Genetic Analyser. The electropherograms were exported and converted to Kodon (Applied Maths, Sint-Martens-Latem, Belgium) using Abiconv (Applied Maths).

The obtained amino acid sequences were numbered aligned with the *Escherichia coli gyrA* amino acid sequence for DNA gyrase A subunit (GenBank accession number X06373) and the amino acid sequences of the QRDR of *gyrA* of the *O. rhinotracheale* strains with different enrofloxacin susceptibilities were mutually compared.

Results

Screening **gyrA** *mutations*

The nucleotide and corresponding amino acid sequences of the QRDR of the different *O. rhinotracheale* strains are presented in Figure 1 and the different mutations are summarized in Table 1.

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Aᵃ 517 GTTCACAGAAGGGTGCTCTATGGGATGAATGAAATGGGATTACTCTCTAATCGTGGATACAAAAAATCCGCTCGTGTGGTAGGAGAAGTG
         V  H  R  R  V  L  Y  G  M  N  E  M  G  L  L  S  N  R  G  Y  K  K  S  A  R  V  V  G  E  V
B       GTTCACAGAAGGGTGCTCTATGGGATGAATGAAATGGGATTACTCTCTAATCGTGGATACAAAAAATCCGCTCGTGTGGTAGGAGAAGTG
         V  H  R  R  V  L  Y  G  M  N  E  M  G  L  L  S  N  R  G  Y  K  K  S  A  R  V  V  G  E  V
C       GTTCACAGAAGGGTGCTCTATGGGATGAATGAAATGGGATTACTCTCTAATCGTGGATACAAAAAATCCGCTCGTGTGGTAGGAGAAGTG
         V  H  R  R  V  L  Y  G  M  N  E  M  G  L  L  S  N  R  G  Y  K  K  S  A  R  V  V  G  E  V
D       GTTCACAGAAGGGTGCTCTATGGGATGAATGAAATGGGATTACTCTCTAATCGTGGATACAAAAAATCCGCTCGTGTGGTAGGAGAAGTG
         V  H  R  R  V  L  Y  G  M  N  E  M  G  L  L  S  N  R  G  Y  K  K  S  A  R  V  V  G  E  V
E       GTTCACAGAAGGGTGCTCTATGGGATGAATGAAATGGGATTACTCTCTAATCGTGGATACAAAAAATCCGCTCGTGTGGTAGGAGAAGTG
         V  H  R  R  V  L  Y  G  M  N  E  M  G  L  L  S  N  R  G  Y  K  K  S  A  R  V  V  G  E  V

A  607 ATGGGTAAATTCCACCCTCACGGCGACTCTTCTGTATACGACACTATGGTGCGTATGGCACAAGACTGGTCTATGCGATATATGCTGGTA
         M  G  K  F  H  P  H  G  D  S  S  V  Y  D  T  M  V  R  M  A  Q  D  W  S  M  R  Y  M  L  V
B      ATGGGTAAATTCCACCCTCACGGCGACTCTTCTGTATAC**T**ACACTATGGTGCGTATGGCACAAGACTGGTCTATGCGATATATGCTGGTA
         M  G  K  F  H  P  H  G  D  S  S  V  Y  **Y**  T  M  V  R  M  A  Q  D  W  S  M  R  Y  M  L  V
C      ATGGGTAAATTCCACCCTCACGGCGACTCTTCTGTATAC**A**ACACTATGGTGCGTATGGCACAAGACTGGTCTATGCGATATATGCTGGTA
         M  G  K  F  H  P  H  G  D  S  S  V  Y  **N**  T  M  V  R  M  A  Q  D  W  S  M  R  Y  M  L  V
D      ATGGGTAAATTCCACCCTCACGGCGACT**T**TTCTGTATACGACACTATGGTGCGTATGGCACAAGACTGGTCTATGCGATATATGCTGGTA
         M  G  K  F  H  P  H  G  D  **F**  S  V  Y  D  T  M  V  R  M  A  Q  D  W  S  M  R  Y  M  L  V
E      ATGGGTAAATTCCACCCTCACGGCGACT**A**TTCTGTATACGACACTATGGTGCGTATGGCACAAGACTGGTCTATGCGATATATGCTGGTA
         M  G  K  F  H  P  H  G  D  **Y**  S  V  Y  D  T  M  V  R  M  A  Q  D  W  S  M  R  Y  M  L  V

A  697 GATGGGCAAGGGAACTTTGGTTCTGTGGATGGCGACCCGCCAGCGGCGATGCGTTATACCGAGGCAAGAATGCAAAAAATTTCTGAGGAA
         D  G  Q  G  N  F  G  S  V  D  G  D  P  P  A  A  M  R  Y  T  E  A  R  M  Q  K  I  S  E  E
B      GATGGGCAAGGGAACTTTGGTTCTGTGGATGGCGACCCGCCAGCGGCGATGCGTTATACCGAGGCAAGAATGCAAAAAATTTCTGAGGAA
         D  G  Q  G  N  F  G  S  V  D  G  D  P  P  A  A  M  R  Y  T  E  A  R  M  Q  K  I  S  E  E
C      GATGGGCAAGGGAACTTTGGTTCTGTGGATGGCGACCCGCCAGCGGCGATGCGTTATACCGAGGCAAGAATGCAAAAAATTTCTGAGGAA
         D  G  Q  G  N  F  G  S  V  D  G  D  P  P  A  A  M  R  Y  T  E  A  R  M  Q  K  I  S  E  E
D      GATGGGCAAGGGAACTTTGGTTCTGTGGATGGCGACCCGCCAGCGGCGATGCGTTATACCGAGGCAAGAATGCAAAAAATTTCTGAGGAA
         D  G  Q  G  N  F  G  S  V  D  G  D  P  P  A  A  M  R  Y  T  E  A  R  M  Q  K  I  S  E  E
E      GATGGGCAAGGGAACTTTGGTTCTGTGGATGGCGACCCGCCAGCGGCGATGCGTTATACCGAGGCAAGAATGCAAAAAATTTCTGAGGAA
         D  G  Q  G  N  F  G  S  V  D  G  D  P  P  A  A  M  R  Y  T  E  A  R  M  Q  K  I  S  E  E

A  787 ATGCTTGCCGACATCGACAAAGAAACGGTAGACCAAAAATTAAACTTCGATGATACTTTGTATGAGCCTACTGTACTCCCTACCCGTATT
         M  L  A  D  I  D  K  E  T  V  D  Q  K  L  N  F  D  D  T  L  Y  E  P  T  V  L  P  T  R  I
B      ATGCTTGCCGACATCGACAAAGAAACGGTAGACCAAAAATTAAACTTCGATGATACTTTGTATGAGCCTACTGTACTCCCTACCCGTATT
         M  L  A  D  I  D  K  E  T  V  D  Q  K  L  N  F  D  D  T  L  Y  E  P  T  V  L  P  T  R  I
C      ATGCTTGCCGACATCGACAAAGAAACGGTAGACCAAAAATTAAACTTCGATGATACTTTGTATGAGCCTACTGTACTCCCTACCCGTATT
         M  L  A  D  I  D  K  E  T  V  D  Q  K  L  N  F  D  D  T  L  Y  E  P  T  V  L  P  T  R  I
D      ATGCTTGCCGACATCGACAAAGAAACGGTAGACCAAAAATTAAACTTCGATGATACTTTGTATGAGCCTACTGTACTCCCTACCCGTATT
         M  L  A  D  I  D  K  E  T  V  D  Q  K  L  N  F  D  D  T  L  Y  E  P  T  V  L  P  T  R  I
E      ATGCTTGCCGACATCGACAAAGAAACGGTAGACCAAAAATTAAACTTCGATGATACTTTGTATGAGCCTACTGTACTCCCTACCCGTATT
         M  L  A  D  I  D  K  E  T  V  D  Q  K  L  N  F  D  D  T  L  Y  E  P  T  V  L  P  T  R  I

A  877 CCAAACTTATTGGTAAATGGTGCCGCTGGAATCGCTGTCGGAATGGCA   924
         P  N  L  L  V  N  G  A  A  G  I  A  V  G  M  A
B      CCAAACTTATTGGTAAATGGTGCCGCTGGAATCGCTGTCGGAATGGCA
         P  N  L  L  V  N  G  A  A  G  I  A  V  G  M  A
C      CCAAACTTATTGGTAAATGGTGCCGCTGGAATCGCTGTCGGAATGGCA
         P  N  L  L  V  N  G  A  A  G  I  A  V  G  M  A
D      CCAAACTTATTGGTAAATGGTGCCGCTGGAATCGCTGTCGGAATGGCA
         P  N  L  L  V  N  G  A  A  G  I  A  V  G  M  A
E      CCAAACTTATTGGTAAATGGTGCCGCTGGAATCGCTGTCGGAATGGCA
         P  N  L  L  V  N  G  A  A  G  I  A  V  G  M  A
```
ᵃ: Sequences from strains given in Table 1.

Figure 1. DNA sequence and deduced amino acid sequence of the quinolone resistance determining region (QRDR) and surrounding region of the GyrA subunit of DNA gyrase of the different *O. rhinotracheale* isolates. Differing nucleic acid bases between isolates are depicted in bold, differing amino acids are marked in bold and underlined. Nucleic acid position according to *E. coli* numbering (GenBank accession number X06373).

Table 1. MIC values for enrofloxacin and the nucleic acid changes in *gyrA* in different *O. rhinotracheale* strains.

	Isolate n°	Origin	Sequence[a]	MIC (µg/ml)	Amino acid change (codon) 83[b]	Amino acid change (codon) 87[b]
Isolates challenge study*	1	LMG9086[c]	A	≤ 0.03	-	-
	2-6	Subset 1[d]	A	≤ 0.03	-	-
	7-9	Subset 2[e]	A	≤ 0.03	-	-
	10-23	Subset 3[f]	B	0.25	-	D(GAC) → Y(TAC)
Field isolates from France	24	France[g]	A	≤ 0.03	-	-
	25	France	A	≤ 0.03	-	-
	26	France	A	≤ 0.03	-	-
	27	France	A	≤ 0.03	-	-
	28	France	A	≤ 0.03	-	-
	29	France[h]	C	0.5	-	D(GAC) → N(AAC)
	30	France[i]	D	2	S(TCT) → F(TTT)	-
	31	France	D	2	S(TCT) → F(TTT)	-
	32	France	E	2	S(TCT) → Y(TAT)	-
Field isolates from Belgium	33	Belgium[j]	D	1	S(TCT) → F(TTT)	-
	34	Belgium	D	1	S(TCT) → F(TTT)	-
	35	Belgium	E	1	S(TCT) → Y(TAT)	-
	36	Belgium	E	1	S(TCT) → Y(TAT)	-
	37	Belgium	D	1	S(TCT) → F(TTT)	-
	38	Belgium	E	1	S(TCT) → Y(TAT)	-
	39	Belgium	D	1	S(TCT) → F(TTT)	-
	40	Belgium	D	1	S(TCT) → F(TTT)	-
	41	Belgium	E	1	S(TCT) → Y(TAT)	-
	42	Belgium	D	1	S(TCT) → F(TTT)	-
	43	Belgium	E	1	S(TCT) → Y(TAT)	-
	44	Belgium	D	1	S(TCT) → F(TTT)	-
	45	Belgium	E	1	S(TCT) → Y(TAT)	-
	46	Belgium	A	1	-	-

[a]: A, B, C, D or E: sequences presented in Figure 1.
[b]: amino acid position according to *E. coli* numbering
[c]: reference *O. rhinotracheale* isolate used for inoculation of experimental groups
[d]: *O. rhinotracheale* from 3-day enrofloxacin treatment group, before and after treatment
[e]: *O. rhinotracheale* from 5-day enrofloxacin treatment group, isolated before treatment
[f]: *O. rhinotracheale* from 5-day enrofloxacin treatment group, isolated on different days post treatment
[g]: *O. rhinotracheale* from turkeys in France, enrofloxacin sensitive
[h]: *O. rhinotracheale* from turkeys in France, lowered enrofloxacin sensitivity
[i]: *O. rhinotracheale* from turkeys in France, enrofloxacin resistant
[j]: *O. rhinotracheale* from broiler chickens in Belgium, lowered enrofloxacin sensitivity
*: Reference: Marien *et al.*, 2006

Compared to the sensitive *O. rhinotracheale* strain (MIC ≤ 0.03 µg/ml) used for inoculation in the experimental infection trial (Marien *et al.*, 2006), an acquired G646T transition (corresponding to an amino acid change from aspartic acid to tyrosine at position 87 of the GyrA subunit; *E. coli* numbering) was found in the *gyrA* gene of all the reisolated *O. rhinotracheale* organisms with a rise in enrofloxacin MIC (0.25 µg/ml).

In seven and six of the 14 *O. rhinotracheale* strains with an MIC of 1 μg/ml isolated from broilers in Belgium, an acquired C635T transition and a C635A transition were noted, respectively. This resulted in an amino acid change from serine to phenylalanine and from serine to tyrosine at position 83, respectively. In one isolate with an MIC of 1 μg/ml, no nucleotide change in the QRDR of *gyrA* was found in comparison with the sensitive isolates. No mutations in *gyrA* were found in the enrofloxacin sensitive *O. rhinotracheale* strains from turkeys originating from France. In the three enrofloxacin-resistant strains (MIC of 2 μg/ml), a C635T transition at position 83 (change of serine into phenylalanine) and a C635A transition at position 83 (change of serine into tyrosine) was found in two and one strain(s), respectively. In the *O. rhinotracheale* strain with an MIC value of 0.5 μg/ml, a G646A transition at position 87 (change of aspartic acid into aspargine) was noticed. The nucleotide sequence of the QRDR of *O. rhinotracheale* isolates without point mutations has been submitted to the GenBank database (accession number DQ358690).

Discussion

Resistance to fluoroquinolones primarily occurs by two different mechanisms, that is, either through alterations in the two target enzymes, DNA gyrase and topoisomerase IV, or by inhibiting the drug from reaching its intracellular target through modifications in the outer membrane proteins or by active efflux systems. Recently, plasmid-borne quinolone-resistance was reported in *Klebsiella pneumoniae*, *E. coli* and *Salmonella enterica* serotype Enteritidis (Hooper, 2001; Drlica and Malik, 2003; Cheung *et al*., 2005; Van Bambeke *et al*., 2005). In most Gram-negative organisms, fluoroquinolone resistance is selected by point mutations in the *gyrA* gene, clustered in the QRDR (Hooper, 1999). In *E. coli*, the most extensively studied organism, the QRDR region spans amino acids 51 to 106 (Drlica and Malik, 2003). Molecular analysis of the *O. rhinotracheale* strains with increased MIC value (0.25 μg/ml), as reisolated during the *in vivo* experiment (Marien *et al*., 2006), consistently revealed a point mutation (G→T) at nucleic acid position 646 (*E. coli* numbering) of *gyrA* resulting in an amino acid change from aspartic acid to tyrosine at position 87 of GyrA, a known hot spot for fluoroquinolone resistance (Hooper, 1999).

All point mutations observed in the examined *O. rhinotracheale* field strains occurred in amino acids at positions 83 (serine) or 87 (aspartic acid), two amino acids indeed most commonly mutated in resistant Gram-negative bacteria (Hooper, 1999). In one isolate with MIC 1 μg/ml, no mutation in the QRDR of *gyrA* was found, suggesting that in this strain the decreased enrofloxacin susceptibility is caused by a mutation in another gene such as *parC, gyrB* or *parE* (Hooper, 1999; Jones *et al.*, 2000). To confirm this hypothesis, an option could be to sequence the latter genes. In the present study, however, we decided to focus on mutations in *gyrA*, the gene most frequently mutated in Gram-negative organisms (Hooper, 1999).

Resistance to fluoroquinolones develops progressively, through stepwise mutations that arise spontaneously in bacterial populations, and fluoroquinolone treatment only needs to enrich the mutant subpopulation for resistance to develop (Sanders, 2001; Drlica and Malik, 2003). Usually organisms that are highly susceptible to fluoroquinolones remain within the susceptible range after single-step mutations and require multiple mutations before clinically relevant resistance becomes apparent (Hooper, 2001; Andriole, 2003). The MIC of the reisolated bacteria from the turkeys that had been treated with enrofloxacin increased from ≤0.03 to 0.25 μg/ml and although this is an eight-fold increase, the *O. rhinotracheale* isolates are, according to the current CLSI guidelines, not considered as resistant to enrofloxacin (Devriese *et al.*, 2001; CLSI, 2002). It will be necessary, however, to obtain clinical data or to carry out experimental infection studies including strains of different sensitivities to be able to make a final conclusion about the impact of these mutations on the *in vivo* efficacy of enrofloxacin. Froyman and Cooper reported that in chickens, enrofloxacin was less efficacious for the treatment of disease caused by an *E. coli* strain with reduced susceptibility (MIC 0.5 μg/ml) when being compared with treatment of disease caused by a fully susceptible *E. coli* strain (MIC 0.015 μg/ml) (Froyman and Cooper, 2003).

In conclusion, this study hence indicates that a single course of enrofloxacin treatment may contribute to the selection of the first mutant with reduced fluoroquinolone susceptibility, which could eventually contribute to the emergence of fluoroquinolone resistance in *O. rhinotracheale*.

Acknowledgments

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3.2.3. Efficacy of enrofloxacin, florfenicol and amoxicillin against *Ornithobacterium rhinotracheale* and *Escherichia coli* O2:K1 dual infection in turkeys following avian metapneumovirus priming

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Summary

Experimental groups of 15 susceptible three-week-old turkeys were inoculated oculonasally with avian metapneumovirus (APV) subtype A and susceptible *Escherichia coli* O2:K1 and *Ornithobacterium rhinotracheale* bacteria, with a three days interval between viral and bacterial inoculation and approximately eight hours between the two bacterial inoculations. The aims of the present study were to assess the efficacy of drinking-water administration of enrofloxacin for three and five days, amoxicillin for five days and florfenicol for five days for the treatment of the resulting respiratory disease, based on clinical and bacteriological examinations. Antimicrobial treatment started one day after dual bacterial inoculation. After infection, the birds were examined and scored for clinical signs daily, weighed at different times, and their tracheae swabbed daily. Five birds were euthanised and examined for macroscopic lesions at necropsy at 5 days post bacterial inoculation (dpbi) and the remainder at 15 dpbi. Samples of the turbinates, trachea, lungs, sinuses, air sacs, heart, pericardium and liver were collected for bacteriological examination. Recovery from respiratory disease caused by an APV/*E. coli*/*O. rhinotracheale* triple infection in three-week-old turkey poults was overall most successful after enrofloxacin treatment, irrespective of treatment duration, followed by florfenicol treatment. Compared with the untreated group, clinical signs as well as *O. rhinotracheale* and *E. coli* multiplication in the respiratory tract were significantly reduced by both enrofloxacin treatments and the florfenicol treatment, with the enrofloxacin treatments showing significantly better reductions than the florfenicol treatment. Five-day treatment with amoxicillin, compared with the untreated group, did not cause a significant reduction in any of the aforementioned parameters.

Introduction

Respiratory problems are one of the main disorders leading to economic losses on turkey farms world-wide. They may be induced by various viral and bacterial agents, either alone or in combination. Besides these infectious organisms, non-infectious factors, such as climatic conditions and management-related problems, may also contribute to the occurrence or the severity of respiratory problems in turkeys (van Empel and Hafez, 1999).

Viral agents are mostly being attributed a triggering role, since the clinical signs following experimental inoculation with these viruses are less severe than those observed in the field. Viral infections generally cause rather acute respiratory problems from which birds usually can recover fairly easily. The problems, however, become more critical when bacterial pathogens are involved. Several bacteria such as *Ornithobacterium rhinotracheale*, *Escherichia coli*, *Mycoplasma* spp., *Bordetella avium*, *Pasteurella* spp. and *Chlamydophila psittaci*, have already been proven to make a contribution to the respiratory disease complex in turkeys (Hafez, 2005). When bacterial organisms are involved in an outbreak of respiratory disease, the only option is treatment with antimicrobials. Hitherto, few studies concerning the actual *in vivo* efficacy of antimicrobials for the treatment of bacterial infections in poultry have been performed. Very recently, the efficacy of different antimicrobials for the treatment of respiratory disease caused by avian metapneumovirus (APV) and *O. rhinotracheale* dual infection was evaluated experimentally (Marien *et al.*, 2006).

In a previous study using an optimized experimental *in vivo* model, it was demonstrated that a combined *E. coli* and *O. rhinotracheale* infection following APV-triggering in three-week-old turkeys caused more severe respiratory disease compared with single *E. coli* or *O. rhinotracheale* infection following APV-priming (Marien *et al.*, submitted). The objectives of the present study are to compare the efficacy of enrofloxacin, amoxicillin and florfenicol for the treatment of respiratory disease due to experimental *E. coli* and *O. rhinotracheale* infection in three-week-old turkeys following APV challenge. The efficacy was evaluated on the basis of several parameters, i.e., clinical signs, re-isolation and titration of the bacterial challenge agents, and weight gain.

Materials and methods

Turkeys

Seventy-five specified pathogen-free (SPF) turkeys (AFSSA, Ploufragan, France), hatched in our facilities, were used in this study. The birds received 16h of light per day, were housed on litter in HEPA-filtered isolation rooms and had free access to food and water. At two weeks of age the birds were shown to be free from maternally-derived antibodies to *O. rhinotracheale* and APV by means of an ELISA available commercially (Biochek, Gouda, the Netherlands) and an in-house serum neutralization test (Marien *et al.*, 2006), respectively.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titre of 5.5 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacteria

The *O. rhinotracheale* type strain LMG 9086[T] was used, which was originally isolated from a turkey with a respiratory tract infection. The strain was serotyped as type A in an agar gel precipitation test (Hafez and Sting, 1999) performed by Professor H. M. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany). The strain was stored at -70°C. The organism was retrieved from the frozen suspension and cultured for 48h at 37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. Ten colonies were transferred into 5 ml brain heart infusion broth (BHI) for 24h at 37°C with agitation (microaerophilic incubation). The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in phosphate buffered saline (PBS) followed each time by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was re-suspended in PBS to obtain a final concentration of 8.6 $\log_{10}$ colony-forming units (cfu)/ml. Confirmation of the number of cfu/ml was done by inoculating ten-fold dilutions in PBS on Columbia agar with 5% sheep blood and counting the number of colonies. The Minimal Inhibitory Concentrations (MICs) for

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this challenge strain are as follows: amoxicillin, 2 µg/ml; florfenicol, 1 µg/ml; enrofloxacin, 0.03 µg/ml, as determined according to Devriese *et al*. (2001).

A Belgian *E. coli* isolate retrieved from turkeys with colibacillosis was used. It was identified and serotyped as O2:K1 by Dr. Flemming Scheutz (Statens Serum Institute, The International Escherichia and Klebsiella Centre, Copenhagen, Denmark). This strain was used previously by Van de Zande *et al.* (2001) for establishing an *E. coli* infection in APV-infected turkeys, and is stored at -70°C. The organism was retrieved from the frozen suspension and cultured overnight at 37°C on MacConkey agar. Ten colonies were transferred into 5 ml BHI for 24h at 37°C. The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in PBS followed each time by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was re-suspended in PBS to obtain a final concentration of 8.6 $\log_{10}$ cfu/ml. Confirmation of the number of cfu/ml was done by inoculating ten-fold dilutions in PBS on Columbia agar with 5% sheep blood and counting the number of colonies. The MICs for this challenge strain are as follows: amoxicillin, 2 µg/ml; florfenicol, 1 µg/ml; enrofloxacin, 0.03 µg/ml, as determined according to Devriese *et al*. (2001).

Antimicrobial compounds

Three antimicrobial compounds were used in this study: enrofloxacin (Baytril[®] 10%, Bayer AG, Leverkusen, Germany), amoxicillin (Suramox 50[®], powder form, Virbac S.A., Carros, France) and florfenicol (Nuflor[®] 10% soluble form, Schering-Plough S.A., Xochimilco, Mexico), with manufacturer-recommended doses of 10 mg/kg for enrofloxacin and 20 mg/kg for amoxicillin and florfenicol.

Experiment design

Seventy-five SPF turkeys were randomly divided into five groups at one day of age.

In all groups, turkeys were first infected with APV, and subsequently received *E. coli* and *O. rhinotracheale* three days later. Each bird was inoculated with APV by the oculonasal route at a dosage of 4.4 $\log_{10}CD_{50}$ (250 µl per bird), and *E. coli* and *O. rhinotracheale*, with an interval of approximately eight hours, were likewise administered oculonasally at a dosage of 8 $\log_{10}$ cfu (250 µl per bird) for each bird. Four groups received antimicrobial treatment: group E3 enrofloxacin received 10

mg/kg for three days, group E5 received enrofloxacin 10 mg/kg for five days, group F received florfenicol 20 mg/kg for five days, and group A received amoxicillin 20 mg/kg for five days. Starting at 24 hours post bacterial inoculation, the drinking water was medicated with the appropriate antimicrobial agent. The birds were continuously dosed and received their daily medication over a 24h period. To enable correct dosing, the daily water uptake was determined. Additionally, all animals were weighed immediately before APV inoculation, before bacterial inoculation, at day 5 post bacterial inoculation (dpbi), and finally at 15 dpbi. The concentration of each antimicrobial to be administered in the water could be calculated accurately on the basis of the water consumption and body weight data. The fifth group was included as an untreated control group (group C).

All birds were clinically examined on a daily basis throughout the experiment. The clinical signs were scored as described in Van de Zande *et al.* (2001). Shortly, the clinical condition of each bird was given a score from 0 (absence of clinical signs) to 7 (nasal exudate with extremely swollen sinuses and frothy eyes, poor general condition and anorexia). The mean clinical score was calculated for each experimental group.

Tracheal swabs were collected from the animals in all groups on day 3 post viral inoculation (dpvi) to confirm infection with APV, and daily for *E. coli* and *O. rhinotracheale* analysis. Before bacterial inoculation, tracheal swabs were taken from all birds of each experimental group for bacteriological examination, which proved to be negative for *O. rhinotracheale* and *E. coli* O2:K1. The tracheal swabs were taken using cotton-tipped aluminium-shafted swabs (Copan Diagnostics Inc., Corona, USA) and placed in 1 ml PBS supplemented with Ca^{2+} and Mg^{2+}, and in the case of virus titration also with 10% fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco). Processing occurred as described below.

Five birds of each group were randomly selected and were sacrificed at 5 dpbi. The remaining ten birds were sacrificed at 15 dpbi. Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The birds were necropsied and examined for gross lesions. Samples of the turbinates, trachea and lungs were collected from all sacrificed birds for bacteriological examination. A 10% suspension in PBS was made from these tissue samples. The sinuses, air sacs, heart, pericardium and liver were

sampled with cotton swabs for bacteriological analysis. These swabs were suspended in 1 ml PBS supplemented with Ca^{2+} and Mg^{2+}. For the animals sacrificed at 18 dpvi, the sinuses, turbinates, trachea, lungs, air sacs, heart, pericardium and liver were sampled with cotton swabs for bacteriological analysis. All samples for bacterial isolation were processed immediately after collection as described below.

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Virological and bacteriological titration of tracheal swabs

The viral titre in $\log_{10}CD_{50}$ per g mucus and the number of cfu of *O. rhinotracheale* and *E. coli* per g mucus were determined. This was done using the procedures described in Marien *et al*. (2005), with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar and incubated for 24h at 37°C. The isolated *E. coli* strains were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency, Surrey, United Kingdom).

Bacteriological titration of tissue suspensions and swabs

Samples of the turbinates, trachea and lungs were quantified for *O. rhinotracheale* and *E. coli* from the 25 birds sacrificed at 5 dpbi. The number of cfu of *O. rhinotracheale* or *E. coli* per g tissue was determined as described in Marien *et al*. (2005) with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar and incubated for 24h at 37°C. The swabs taken from the sinuses, air sacs, pericardium, heart and liver were inoculated onto 5% sheep blood agar supplemented with 5 μg/ml gentamicin and 5 μg/ml polymyxin for *O. rhinotracheale* isolation and on MacConkey agar for *E. coli* isolation. After 24-48h of incubation at 37°C in a 5% CO_2 atmosphere (*O. rhinotracheale*) and after 24h of incubation at 37°C (*E. coli*), the agar was examined for presence of *O. rhinotracheale* or *E. coli*.

The swabs from the turbinates, trachea, lungs, sinuses, air sacs, pericardium, heart and liver from the birds sacrificed at 18 dpvi were likewise inoculated and the agars were examined for presence of *O. rhinotracheale* and/or *E. coli*.

The isolated *E. coli* strains were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency).

MIC of the parent and re-isolated **O. rhinotracheale** *isolates*

The MIC values of different *O. rhinotracheale* and *E. coli* isolates retrieved from the tracheal swabs before, during and after antimicrobial treatment were determined. More specifically, the enrofloxacin MICs were determined for the isolates from enrofloxacin groups E3 and E5, the amoxicillin MICs were determined for the isolates from amoxicillin group A, and finally, the florfenicol MICs for the isolates from florfenicol group F.

Determination of the MIC values was performed by sensititre test (Trek Diagnostic Systems, West Sussex, UK).

Statistical analyses

The clinical scores, tracheal swabbing and weight were analysed only from animals that were not euthanised at 5 dpbi. Only the time span between 0 and 9 dpbi was considered in the analysis as it appears from previous experiments (Marien *et al.*, 2005; 2006) that after 9 dpbi most of the response variables, including the clinical score, return to normal. First, the area under the curve (AUC) of the clinical scores and tracheal swabbing between 0 and 9 dpbi was analysed via a fixed-effects model with the treatment group as fixed effect. Next, the repeated clinical scores between 0 and 9 dpbi were analysed using a mixed proportional odds model with the clinical score as the response variable, animal as random effect, and treatment group and time as categorical fixed effects.

E. coli and *O. rhinotracheale* tracheal swabbing values between 0 and 9 dpbi were analysed using a mixed model with $\log_{10}$cfu/g mucus as response variable, animal as random effect, and treatment group and time as categorical fixed effects.

Weight was compared at the end of the trial using a fixed-effects model.

E. coli and *O. rhinotracheale* titres from the turbinates, trachea and lung of the animals euthanised at 5 dpbi were compared between the five treatment groups with the Kruskall-Wallis test, whereas the presence of *E. coli* and *O. rhinotracheale* in the sinus, airsacs, pericardium, liver and heart of the animals euthanised at 5 dpbi were compared between the five treatment groups using the Fisher's exact test (extended version for more than two groups).

All tests were performed at a global 5% significance level, and the seven relevant pairwise comparisons (C vs E3, C vs E5, E3 vs E5, E5 vs A, E5 vs F, E3 vs A, E3 vs F) were tested at a significance level adjusted using Bonferroni's multiple

comparisons technique. Only these seven comparisons will be considered throughout the paper. (Agresti, 2002)

Results

During the experiment, mortalities did not take place in any of the experimental groups.

Clinical signs

The mean clinical score for each group is shown in Figure 1. Respiratory signs were seen in all APV-inoculated groups starting from 3 dpvi. From 10 dpbi onwards, clinical signs were not detected in any of the animals in the different experimental groups.

Using the AUC from 0 to 9 dpbi, there is a significant difference between the five treatments (P<0.0001). Group A has the highest total score, followed by C, F, E3 and E5. Four of the seven analysed pairwise comparisons, namely C vs E3, C vs E5, E3 vs A and E5 vs A, are significant as indicated in Table 1.

In the proportional odds model, both a significant change in time (P<0.0001) and a significant difference (P<0.0001) between the treatments were detected. E5 had the lowest odds ratio, followed by E3, F, C and A. All but one (E3 vs E5) analysed pairwise comparisons are significant as indicated in Table 1.

Thus, compared with the untreated control group, clinical signs were significantly reduced by the enrofloxacin treatments, irrespective of treatment duration, and by the treatment with florfenicol, but not by treatment with amoxicillin. Also, in the enrofloxacin-treated groups, clinical signs disappeared completely three days earlier compared with the untreated group and the amoxicillin-treated group, and two days earlier compared with the florfenicol-treated group.
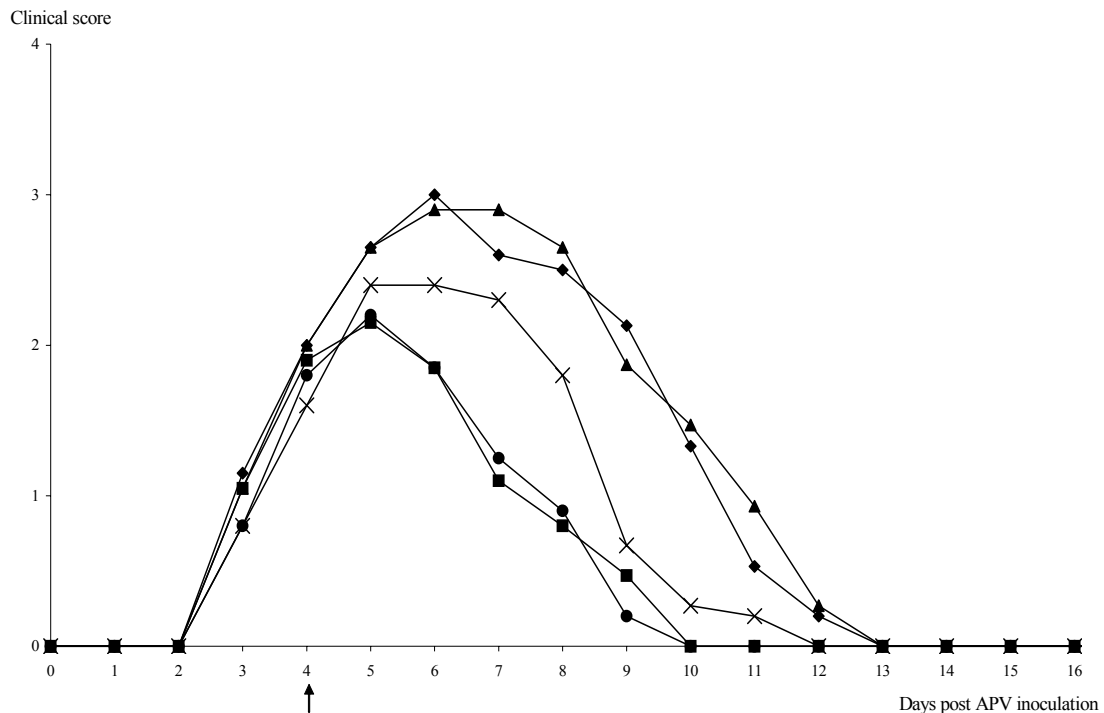
Figure 1. Mean clinical scores in turkeys inoculated with APV, *E. coli* and *O. rhinotracheale* and subsequently treated with different antimicrobial agents: ■ group E3, three days of enrofloxacin (10 mg/kg); ●group E5, five days of enrofloxacin (10 mg/kg); ▲ group A, five days of amoxicillin (20 mg/kg); ×group F, five days of florfenicol (20 mg/kg) or no treatment (◆ control group). Arrow indicates first day of antimicrobial treatment.

Macroscopic findings

Macroscopic findings were comparable in the different experimental groups, with lesions being serous to seromucous exudate in the turbinates and sinuses, and hyperaemia of the turbinates and the trachea. These lesions were found in all groups, but in the groups receiving enrofloxacin treatment and to a lesser extent in the group receiving florfenicol treatment, the observed lesions were less severe.

Viral titrations of tracheal swabs

APV was recovered from tracheal swabs from every individual bird. The mean titres for groups C, E3, E5, A and F were 6.1, 5.9, 6.1, 6.2, and 6.3 $\log_{10}CD_{50}/g$ mucus, respectively.

Bacterial titration of tracheal swabs

       The results of the *O. rhinotracheale* and *E. coli* titrations of the tracheal swabs of the different groups are shown in Figure 2 for *O. rhinotracheale* and Figure 3 for *E. coli*. Mean titres for each experimental group are depicted.
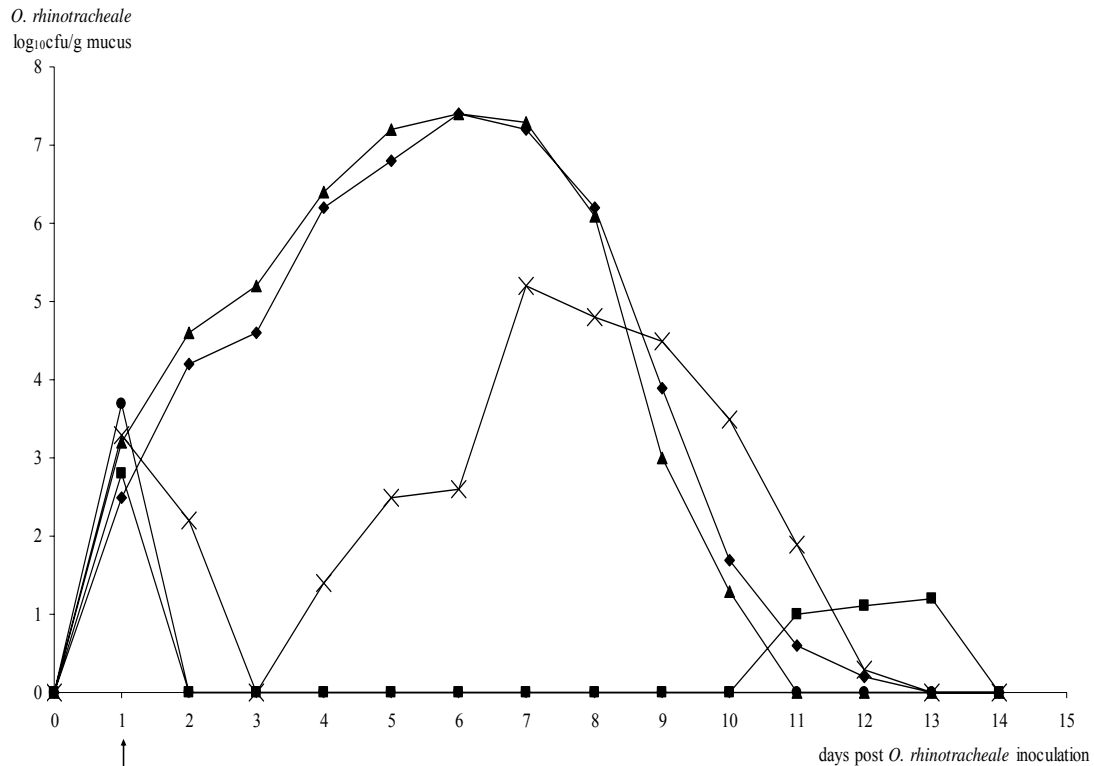

Figure 2. Bacterial *O. rhinotracheale* titres (log$_{10}$cfu/g mucus) in tracheal mucus collected at different time points after *O. rhinotracheale* inoculation in triple APV/*E. coli*/*O. rhinotracheale* infected turkeys, receiving different antimicrobial treatments: ■ group E3, 3 days of enrofloxacin, (10 mg/kg); ● group E5, 5 days of enrofloxacin (10 mg/kg); ▲ group A, 5 days of amoxicillin (20 mg/kg); ✕ group F, 5 days of florfenicol (20 mg/kg), or no treatment (◆ control group). Arrow indicates first day of antimicrobial treatment.

O. rhinotracheale *titres in tracheal mucus*

Using the AUC in the fixed-effects model, there is a significant difference between the five treatments (P<0.0001). The analysis of the repeated counts also reveals a significant difference between treatments (P<0.0001) and a significant change in time (P<0.0001). All of the seven analysed pairwise comparisons, except E3 vs E5, are significant as pointed out in Table 1.

Compared with the untreated control group, the number of *O. rhinotracheale* organisms in the tracheal mucus was hence significantly reduced by both enrofloxacin treatments (three- and five-day treatment) and by the five-day florfenicol treatment, with enrofloxacin treatments showing significantly better reductions than florfenicol treatment. Compared with the untreated group, treatment with amoxicillin did not cause a significant reduction in the *O. rhinotracheale* titres in the trachea.
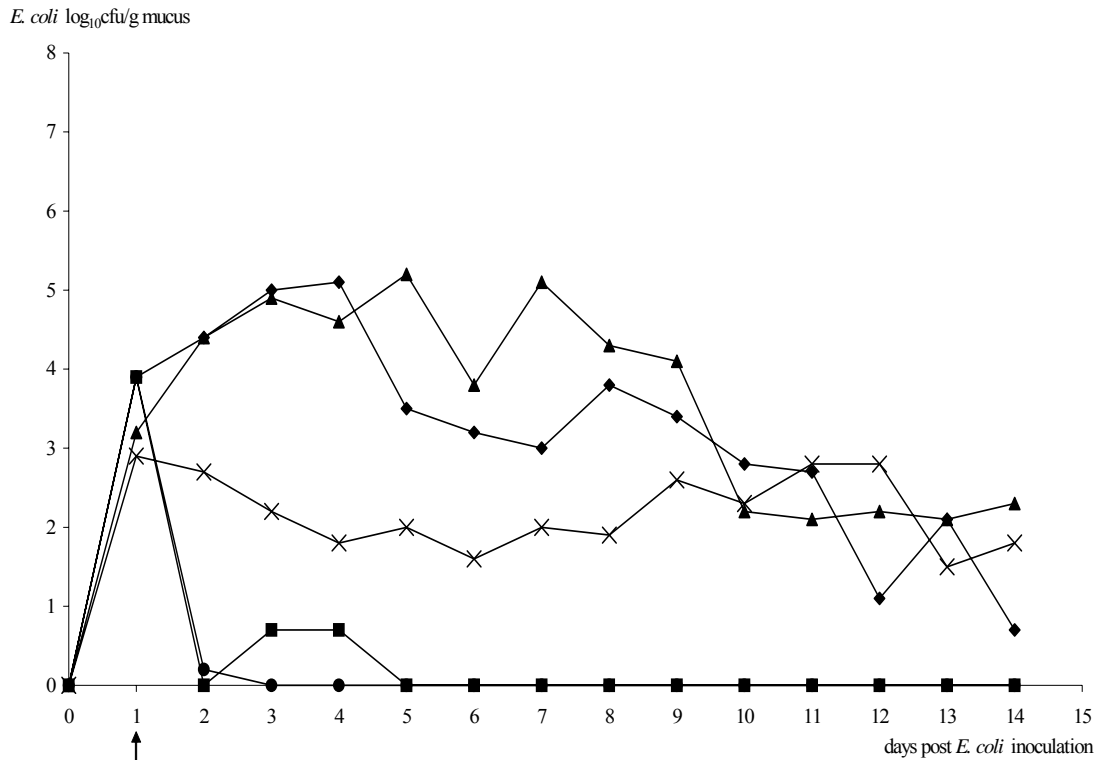
Figure 3. Bacterial *E. coli* titres ($\log_{10}$ cfu/g mucus) in tracheal mucus collected at different time points after *E. coli* inoculation in triple APV/*E. coli*/*O. rhinotracheale* infected turkeys, receiving different antimicrobial treatments: ■ group E3, three days of enrofloxacin, (10 mg/kg); ● group E5, five days of enrofloxacin (10 mg/kg); ▲ group A, five days of amoxicillin (20 mg/kg); ✕ group F, five days of florfenicol (20 mg/kg), or no treatment (◆ control group). Arrow indicates first day of antimicrobial treatment.

E. coli *titres in tracheal mucus*

Using the AUC in the fixed effects model, there is a significant difference between the five treatments (P<0.0001), whereas the analysis of the repeated counts based on the mixed model reveals a significant difference between treatments (P<0.0001) and a significant change in time (P<0.0001). In both analyses, group A has the highest score, followed by C, F, E3 and E5. All of the seven analysed pairwise comparisons, except E3 vs E5 and E3 vs F for the AUC and E3 vs E5 for the repeated counts, are significant as pointed out in Table 2.

Compared with the untreated control group, the number of *E. coli* organisms in the tracheal mucus was hence significantly reduced by both enrofloxacin treatments (three- and five-day treatment) and by the five-day florfenicol treatment, with enrofloxacin treatments showing significantly better reductions than florfenicol treatment. Compared with the untreated group, treatment with amoxicillin did not cause a significant reduction in the *E. coli* titres in the trachea.

Table 1. Clinical scores and isolation of *O. rhinotracheale* out of tracheal mucus and organs (mean value (standard error)) of turkeys inoculated with APV followed by *E. coli* and *O. rhinotracheale* three days later, and treated with different antibiotic therapies: group E3, 3 days of enrofloxacin (10 mg/kg); group E5, 5 days of enrofloxacin (10 mg/kg); group A, 5 days of amoxicillin (20 mg/kg); group F, 5 days of florfenicol (20 mg/kg), or no treatment (group C).

Group	Clinical score[*1] Area under the curve	Odds ratio (group C as reference)	Isolation of *O. rhinotracheale* out of tracheal mucus[*1] Area under the curve	Log_{10}cfu/g mucus	Isolation of *O. rhinotracheale* at 5 dpbi log_{10}cfu/g tissue Turbinates	Trachea	Lungs	Presence (1) or absence (0) Sinus	Air sacs
C	17.20 (1.05) [a]	1 [a]	45.32 (1.20) [a]	5.04 (0.28) [a]	9.88 (1.14) [a]	7.36 (0.61) [a]	5.64 (0.71) [a]	1.00 (0.00) [a]	0.80 (0.45) [a]
A	18.47 (1.33) [a]	1.40 (0.51) [a]	46.71 (1.27) [a]	5.19 (0.27) [a]	9.12 (2.32) [a]	8.12 (1.36) [a]	5.84 (0.93) [a]	1.00 (0.00) [a]	0.80 (0.45) [a]
F	12.07 (0.80) [b]	0.31 (0.09) [b]	23.02 (3.32) [b]	2.56 (0.27) [b]	4.02 (5.52) [ab]	0.54 (1.21) [b]	0.00 (0.00) [b]	0.20 (0.45) [ab]	0.00 (0.00) [a]
E5	8.40 (0.73) [b]	0.10 (0.03) [c]	3.38 (0.24) [c]	0.38 (0.12) [c]	0.00 (0.00) [b]	0.00 (0.00) [b]	0.00 (0.00) [b]	0.00 (0.00) [b]	0.00 (0.00) [a]
E3	8.60 (0.63) [b]	0.11 (0.03) [c]	2.16 (0.50) [c]	0.24 (0.09) [c]	0.00 (0.00) [b]	0.00 (0.00) [b]	0.00 (0.00) [b]	0.00 (0.00) [b]	0.00 (0.00) [a]

[*1]: From 0 to 9 days post bacterial inoculation (dpbi)

[abcd]: Treatments sharing a letter do not differ significantly from each other at the 5% global significance level.

Table 2. Isolation of *E. coli* out of tracheal mucus and organs, and weight (mean value (standard error)) of turkeys inoculated with APV followed by *E. coli* and *O. rhinotracheale* three days later, and treated with different antibiotic therapies: group E3, 3 days of enrofloxacin (10 mg/kg); group E5, 5 days of enrofloxacin (10 mg/kg); group A, 5 days of amoxicillin (20 mg/kg); group F, 5 days of florfenicol (20 mg/kg), or no treatment (group C).

| Group | Isolation of *E. coli* out of tracheal mucus*[1] | | Isolation of *E. coli* at 5 dpbi | | | | Weight at 15 dpbi |
| | | | log_{10}cfu/g tissue | | | Presence (1) or absence (0) | |
	Area under the curve	log_{10}cfu/g mucus	Turbinates	Trachea	Lungs	Sinus	in g
C	33.21 (3.33)[a]	3.69 (0.25)[a]	8.68 (2.16)[a]	3.96 (2.30)[ab]	2.42 (2.30)[a]	1.00 (0.00)[a]	970.47 (38.68)[a]
A	35.78 (3.44)[a]	3.98 (0.24)[a]	9.14 (2.33)[a]	5.90 (1.45)[a]	2.66 (2.44)[a]	0.80 (0.45)[ab]	945.93 (25.76)[a]
F	17.03 (4.21)[b]	1.89 (0.25)[b]	4.28 (5.88)[ab]	2.34 (3.21)[ab]	0.00 (0.00)[a]	0.40 (0.55)[ab]	936.33 (37.82)[a]
E5	4.31 (0.57)[c]	0.48 (0.15)[c]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.00 (0.00)[a]	0.00 (0.00)[b]	941.13 (25.09)[a]
E3	5.46 (1.55)[bc]	0.61 (0.16)[c]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.00 (0.00)[a]	0.00 (0.00)[b]	964.33 (32.57)[a]

*[1]: From 0 to 9 post bacterial inoculation (dpbi)

[abcd]: Treatments sharing a letter do not differ significantly from each other at the 5% global significance level.

Bacterial titration of tissue samples

O. rhinotracheale and *E. coli* were never recovered from any of the organs of the animals in group E3 and E5 sacrificed at 5 and 15 dpbi. For the other groups (group C, A and F), the various *O. rhinotracheale* and *E. coli* titres in the sampled organs at 5 dpbi are shown in Table 3 and Table 4, respectively.

Table 3. Bacterial titre of *O. rhinotracheale* ($\log_{10}$ cfu/g tissue) isolated from different organs from turkeys inoculated turkeys inoculated with APV, *E. coli* and *O. rhinotracheale*, and subsequently treated with different antimicrobial agents: group A, 5 days of amoxicillin (20 mg/kg); group F, 5 days of florfenicol (20 mg/kg) or no treatment, group C. Turkeys were euthanised at 5 dpbi.

Organ	*O. rhinotracheale* titre ($\log_{10}$ cfu/g tissue)
	\multicolumn{5}{c}{Group C}	\multicolumn{5}{c}{Group A}	\multicolumn{5}{c}{Group F}
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Turbinates	8	10.9	10.6	10.2	9.7	9.8	10.2	5	10	10.6	10.6	0	9.5	0	0
Trachea	6.4	8.1	7.4	7.4	7.5	10.5	7.7	7.3	7.2	7.9	2.7	0	0	0	0
Lungs	5.3	4.8	6.7	5.9	5.5	6	5.4	4.5	6.9	6.4	0	0	0	0	0
Sinus[b]	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Air sacs[b]	+	+	+	-	+	+	-	+	+	+	-	-	-	-	-
Pericardium[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver[b]	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-

a: Number of turkey
b: These organs were swabbed; no tissue suspensions were made
c: +: *O. rhinotracheale* was isolated
  -: No *O. rhinotracheale* was isolated

O. rhinotracheale

Significant differences between treatments were observed for *O. rhinotracheale* titrations from the turbinates (P=0.0019), trachea (P=0.0003), lungs

(P=0.0002), sinus (P=0.0003) and air sacs (P=0.002). Pairwise significant differences between the treatments are shown in Table 1.

Compared with the untreated control group, the number of *O. rhinotracheale* in the trachea and lungs was significantly reduced by the enrofloxacin treatments and by the florfenicol treatment, as opposed to treatment with amoxicillin. Furthermore, the number of *O. rhinotracheale* isolated from the turbinates was significantly reduced by the three- and five-day enrofloxacin treatments. For the pericardium, liver and heart, very few positive samples were encountered (in group A), and significant differences between the treatments did not occur.

O. *rhinotracheale* was not recovered from any of the organs (turbinates, trachea, lungs, sinuses, air sacs, pericardium, heart and liver) of the birds in the five groups swabbed at 15 dpbi.

Table 4. Bacterial titre of *E. coli* ($\log_{10}$ cfu/g tissue) isolated from different organs from turkeys inoculated with APV, *E. coli* and *O. rhinotracheale*, and subsequently treated with different antimicrobial agents: group A, 5 days of amoxicillin (20 mg/kg); group F, 5 days of florfenicol (20 mg/kg) or no treatment, group C. Turkeys were euthanised at 5 dpbi.

Organ	*E. coli* O2:K1 titre ($\log_{10}$ cfu/g tissue)														
	Group C					Group A					Group F				
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Turbinates	8.2	5.1	10.3	10	9.8	10.4	9	5.3	11.4	9.6	10	11.4	0	0	0
Trachea	5.3	4.4	5.8	4.3	0	6.5	7.4	4.4	4.3	6.9	5.5	6.2	0	0	0
Lungs	5.1	3.4	3.6	0	0	4.6	0	0	4	4.7	0	0	0	0	0
Sinus[b]	+	+	+	+	+	+	+	+	-	+	+	-	-	+	-
Air sacs[b]	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
Pericardium[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a: Number of turkey
b: These organs were swabbed; no tissue suspensions were made
c: +: *E. coli* was isolated
  -: No *E. coli* was isolated

E. coli

Significant differences between treatments were observed for *E. coli* titrations from the turbinates (P=0.0041), trachea (P=0.0047), lungs (P=0.0252) and sinus (P=0.0028). Pairwise significant differences between the treatments are shown in Table 2. Compared with the untreated control group, none of the treatments caused a significant reduction in the number of *E. coli* isolated from the trachea and lungs. The number of *E. coli* isolated from the turbinates and the sinuses was significantly reduced only by both the enrofloxacin treatments, compared with the untreated group. For the pericardium, liver and heart, no positive samples were encountered, so significant differences between the treatments did not occur.

From the birds swabbed at 15 dpbi, *E. coli* was recovered, respectively, from the sinuses, turbinates and trachea of 2, 8 and 2 of the 10 birds in group C, 3, 6 and 3 of the 10 birds in group A, and 4, 7 and 4 of the 10 birds in group F. *E. coli* was not recovered from any of the organs of the birds in groups E3 and E5 swabbed at 15 dpbi.

MIC of the parent and re-isolated O. rhinotracheale *isolates*

Compared with the challenge strain, no increase was noted in the amoxicillin, florfenicol or enrofloxacin MIC values for the re-isolated bacteria from the respective groups.

Weight

Significant differences were not observed between the treatment groups (P=0.93) as indicated in Table 2.

Antimicrobial therapy

The theoretically consumed dose of antimicrobials per group of birds was close to the target dose of 10 mg/kg for enrofloxacin and 20 mg/kg for amoxicillin and florfenicol. The mean actual daily dose (mg/kg) for the total medication period was 10.2 (range: 9.81 – 10.7) for group E3, 9.9 (range: 9.2 – 10.5) for group E5, 22.7 (range 19.0 – 25.1) for group F, and 21.1 (range 17.3 – 24.4) for group A.

Discussion

This study is the first to investigate experimentally the clinical efficacy of different antimicrobial therapies for the treatment of dual *E. coli*/*O. rhinotracheale* infection after APV challenge in turkeys. Statistical analysis of the obtained results revealed that under the circumstances used in this study, recovery from respiratory disease caused by APV/*E. coli*/*O. rhinotracheale* triple infection in three-week-old turkeys was overall most successful after enrofloxacin treatment (three or five days of treatment), followed by florfenicol treatment. Compared with the untreated control group, clinical signs as well as the *O. rhinotracheale* and *E. coli* multiplication in the respiratory tract were significantly reduced by both the enrofloxacin treatments and the florfenicol treatment, with the enrofloxacin treatments showing significantly better reductions than the florfenicol treatment. Five-day treatment with amoxicillin, compared with the untreated group, did not cause a significant reduction in any of the aforementioned parameters. These results are in accordance with a previously executed experiment in which it was proven that enrofloxacin treatment was significantly better than florfenicol for a reduction in clinical signs and bacterial multiplication in APV/*O. rhinotracheale* -dually infected turkeys and that amoxicillin treatment was not efficacious (Marien *et al.*, 2006). Data presented by Froyman and Cooper (2003), also demonstrated that enrofloxacin treatment was most efficacious for the treatment of colisepticemia in chickens, followed by florfenicol, and that amoxicillin was not effective. Charleston *et al.* (1998) showed that enrofloxacin was effective for treatment of *E. coli* infection in chickens.

Possible explanations for the observed differences between the different antimicrobial products may be the different pharmacokinetic and pharmacodynamic properties of the applied antimicrobials, including the apparent volume of distribution at steady state ($Vd_{(ss)}$), which gives an indication of the diffusion of the antimicrobials in the body tissues. Data from different pharmacokinetic studies in turkeys and chickens, demonstrate that the $Vd_{(ss)}$ of amoxicillin, compared with that of florfenicol and enrofloxacin is lower, which means that amoxicillin is less extensively distributed in the extravascular tissues (Carceles *et al.*, 1995; Anadon *et al.*, 1996; Rios *et al.*, 1997; Bugyei *et al.*, 1999; Knoll *et al.*, 1999; Shen *et al.*, 2003; Cox *et al.*, 2004; Switala *et al.*, 2004; Dimitrova *et al.*, 2006). Furthermore, it must be stated that enrofloxacin has long half-life values (Brown, 1996; Bugyei *et al.*, 1999; Garcia Ovando *et al.*, 1999; Knoll *et al.*, 1999; Dimitrova *et al.*, 2006) and that the values for

florfenicol are generally lower (Afifi and El-Sooud, 1997; Shen *et al.*, 2002; Shen *et al.*, 2003), with the lowest half-life values noted for amoxicillin (Lashev and Pashov, 1992; Carceles *et al.*, 1995; Anadon *et al.*, 1996). It is likely that the bactericidal effect of amoxicillin is being diminished by its less efficient distribution throughout tissues and its relatively fast elimination (Goren *et al.*, 1981). Enrofloxacin, however, is rapidly bactericidal at relatively low concentrations (Brown, 1996) and is distributed very well throughout tissues (Brown, 1996; McKellar, 1996), which may explain its favourable results in the present experiment.

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Chapter 4: GENERAL DISCUSSION

GENERAL DISCUSSION

Studying all the possible interactions between the different respiratory pathogens of importance to turkeys in one doctoral research, would be an unrealistic goal, therefore it was decided to focus on four relevant respiratory pathogens as pointed out by scientific literature and personal communications by veterinary practitioners.

Avian metapneumovirus (APV) was the first agent to be included in our research, since a prevalence study performed in Belgium by Van de Zande *et al.* (1997), revealed that APV had circulated on 100% of the examined turkey farms. Several years later, despite intensive vaccination schemes against APV, antibodies indicative for natural APV infections were still frequently detected by Van Loock *et al.* (2005) in Belgian turkey farms. In the field, *Escherichia coli* and *Ornithobacterium rhinotracheale* are bacterial agents that have frequently been isolated following APV infections, alone or in combination with one another (De Rosa *et al.*, 1996; El-Sukhon *et al.*, 2002; Vandekerchove *et al.*, 2004). In 2005, Van Loock *et al.* reported that five of the eight examined Belgian turkey farms had experienced an *O. rhinotracheale* infection. Since isolation of *O. rhinotracheale* is not always straightforward using routine diagnostic methods and van Empel and Hafez (1999) reported that *O. rhinotracheale* antibody titres decline quite rapidly after infection, infections with *O. rhinotracheale* may be underestimated. *O. rhinotracheale* is believed to play an important role in the respiratory problems in the Belgian turkey industry, which is comparable with the situation in our neighbouring countries (Balloy, 2005; Lister, 2005). Based on results of routine analyses of clinically healthy, diseased and dead birds, Vandemaele *et al.* (2002) concluded that avian pathogenic *E. coli* (APEC) is circulating intensively in Belgian poultry, and appears to be a major contributor to respiratory poultry disease. Finally, an important turkey pathogen able to attribute to the respiratory disease complex in turkeys is *Mycoplasma gallisepticum* (Kleven, 1998; Levisohn and Kleven, 2000). In spite of national programmes for *Mycoplasma* control, *M. gallisepticum* remains a threat to the poultry industry (Lister, 2005). Albeit at very low frequencies, layer flocks in Belgium are still occasionally found to be positive for *M. gallisepticum*.

From the different experimental studies included, new insights in the pathogenesis of *O. rhinotracheale* and *E. coli* infections in turkeys were obtained. It

became apparent that, although both *O. rhinotracheale* and *E. coli* are respiratory pathogens that are able to cause similar disease symptoms and lesions, they intrinsically have different ways of acting. First of all, *O. rhinotracheale* has a very clear tropism for the intact respiratory system, since it was able to adhere to the tracheal epithelium and colonize the turbinates and lungs without previous infection with APV. This is in contrast with *E. coli*, which specifically needs an APV-infected respiratory system in order to sustain its presence. Without prior infection with APV, *E. coli* indeed is not able to colonize the respiratory tract (Van de Zande, 2001; Van de Zande *et al*., 2001). The reasoning for this may be two-fold. Firstly, APV directly interferes with the mucociliary clearance of bacteria by destroying ciliated cells and reducing the number of mucus-secreting goblet cells. Secondly, the degenerative and regenerative changes during APV replication may be responsible for the induction of surface receptors for *E. coli* that allow colonisation. The normal mucus and epithelial cells may actually lack binding sites for this organism. Destruction of the epithelial cells by APV may expose the basement membrane and in this way also uncover certain receptors (Ramphal *et al*., 1980). Ramphal *et al*. (1980) reported that different *Pseudomonas aeruginosa* strains adhered only to desquamating cells of influenza-infected tracheas, but not to normal mucosa, the basal cell layer, or the regenerating epithelium, and only to injured cells and to areas of exposed basement membrane after endotracheal intubation, a phenomenon they call "opportunistic adherence". Van de Zande (2001) was, in APV-primed turkeys, able to demonstrate a large number of *E. coli* in the mucus layer and in the mucus 'clots' present in the lumen of the trachea. It was impossible to determine whether this was a result of specific adherence and colonisation or whether the bacteria were merely retained in a passive way. Most bacterial species that have been studied adhere avidly to mucus, by specific (adhesin-receptor) and non-specific interactions (Dowling and Wilson 1998: Ramphal *et al*., 1991; Barsum *et al*., 1995). Although *O. rhinotracheale* was able to colonize the undamaged trachea, turbinates and lungs, priming with APV still had an exacerbating effect, since it facilitated infection of the lungs and was necessary to allow the bacterial pathogen to infect the air sacs. Apparently infection with APV allows *O. rhinotracheale* to better reach the lower respiratory tissues.

In the present thesis, it also became clear that pre-infection with *M. gallisepticum* has an important effect on the duration of the presence of *O. rhinotracheale* and *E. coli* in the upper respiratory tract (trachea and turbinates). In

the experimental studies without *M. gallisepticum* infection, *O. rhinotracheale* could only be isolated from the trachea until ten to twelve days and *E. coli* until approximately fourteen days post inoculation. When the turkeys were infected first with *M. gallisepticum*, however, *O. rhinotracheale* and *E. coli* remained in the trachea at rather high levels until at least 20 days post *O. rhinotracheale/E. coli* inoculation (end of experimental period). Apparently *M. gallisepticum* exerts an important impeding effect on the clearance of *O. rhinotracheale* and *E. coli*, especially from the upper respiratory tract. Indeed, at the end of the experiment (20 days post *E. coli/O. rhinotracheale* inoculation), *O. rhinotracheale* and *E. coli* were cleared from the lungs, air sacs, and non-respiratory organs, but not from the turbinates and trachea. This might indicate that the host's immune response at this level was affected.

Although *O. rhinotracheale* was able to colonize the healthy respiratory tract, actual respiratory symptoms could not be elicited by single infection with this agent. Indeed, *O. rhinotracheale* was not able to cause primary respiratory symptoms, but was able to exacerbate the symptoms caused by the APV infection. After dual infection, the clinical symptoms in themselves were similar in nature to those caused by APV alone, but they were more severe and persisted markedly longer. Since *E. coli* is not able to colonize the respiratory tract without prior damage caused by APV, it is of course not surprisingly that single *E. coli* infection does not cause clinical symptoms (Van de Zande *et al.*, 2001). Only after prior APV infection *E. coli* colonizes the respiratory tract of turkeys and subsequently aggravates the disease symptoms caused by the viral infection.

An important focus in our studies was the reproduction of septicemia and mortality as seen in the field. Numerous studies have focused on comparing extra-intestinal *E. coli* isolates from colibacillosis-affected poultry with faecal isolates from clinically healthy birds, but none of the virulence factors examined (adhesins, invasivity in cell cultures, serum survival, iron uptake systems, haemolysins, colicin production and toxins), was exclusively associated with extra-intestinal isolates even though for some characteristics, significant differences were found (Vandekerchove, 2004). Previously, it has been shown that the lower respiratory tract (lungs and air sacs) is an important site of entrance of *E. coli* into the bloodstream of birds (Cheville and Arp, 1978; Ackermann and Cheville, 1991; Pourbakhsh *et al.*, 1997a). Based on the results of the APV/*E. coli*/*O. rhinotracheale* triple infection model, we concluded

that since *E. coli* was able to invade the lungs and the air sacs of some birds but still was not able to cause colisepticemia, the strain was possibly lacking certain characteristics necessary for invasion of the bloodstream. However, in the *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* mixed infection model, the same *E. coli* strain was in contrast able to cause septicemia. This leads us to believe that, besides bacterial virulence factors, host susceptibility and resistance factors, the interactions with different pathogens are probably at least as important determinants (Barnes *et al.*, 2003, Ley, 2003).

The hypothesis may be forwarded that infection with mycoplasmas is necessary for *E. coli* to cause septicemia. A first possible explanation for the occurrence of colisepticemia may be the debilitating effect of mycoplasmas on the efficiency of mucociliary apparatus, resulting in the impaired clearance of *E. coli*. Further, mycoplasmas appear to induce transient immunosuppression. This has been shown for *M. gallisepticum* in turkeys concurrently infected with APV and *Avibacterium (Haemophilus) gallinarum* (Matsuo *et al.*, 1978; Naylor *et al.*, 1992). Ganapathy and Bradbury (2003) showed that a virulent strain of *M. gallisepticum* causes temporary T cell suppression in infected chickens. Other reports of immunosuppression in turkeys were done for *Mycoplasma meleagridis* (Ortiz *et al.*, 1981) and *Mycoplasma iowae* (Bradbury, 1984). It is also known that mycoplasmas negatively influence a variety of immune responses ranging from complement activation to ingestion by macrophages (Simecka *et al.*, 1992), and this may influence the immune response against other micro-organisms. Another well-known feature of infection with mycoplasmas is the massive attraction of inflammatory cells such as macrophages and heterophils to the site of infection (Deiters and Mühlradt, 1999), cells designed to destroy different pathogens (Dietert *et al.*, 1991). Results from Pourbakhsh *et al.* (1997b) showed that after intrasaccular inoculation of *E. coli* in chickens, apparently viable bacteria of highly pathogenic isolates, but not low pathogenic ones, were often associated with or within macrophages in the air sacs and lungs. They also demonstrated a strong correlation between the *in vivo* pathogenicity and the *in vitro* ability to resist the bactericidal effects of macrophages. Mellata *et al.* (2003) demonstrated that *in vitro* some virulence factors protected *E. coli* to the bactericidal effect of phagocytes, especially heterophils. Since resident macrophages are very scarce in the avian lungs and air sacs, heterophils form the first line of cellular defense against invading pathogens and their function could be to restrict bacterial multiplication until

subsequent host defenses become active (Toth, 2000; Nganpiep and Maina, 2002; Mellata *et al*., 2003). Mellata *et al*. (2003) reason that some *E. coli* strains might possess virulence factors to combat this heterophil activity and consequently are able to invade more easily. It was shown by Miller *et al*. (1990) that chicken macrophages could phagocytose a virulent *E. coli* phenotype more efficiently *in vitro* than an avirulent phenotype, but the latter was in contrast cleared more efficiently from the circulation.

In order to successfully infect the conducting airway, primary colonising bacteria such as *O. rhinotracheale*, must overcome the inherent innate immunity of the respiratory tract. This includes evading the mucociliary escalator and an environment of antimicrobial factors. One of the mechanisms to overcome the mucociliary escalator is to directly bind to cilia on host epithelium and this is a feature which seems important in the pathogenesis of *O. rhinotracheale* infections. Temple *et al*. (1998) found in a trial comparing *in vitro* and *in vivo* data that the *Bordetella avium* strains that were best able to adhere to tracheal cells in culture, were also best capable of colonizing turkey poults *in vivo*. They state that whereas this finding may seem reasonable or even expected, it is remarkable from the standpoint of the number of factors coming into play in the *in vivo* situation, e.g. the onset of the immune response. We also tested the adherence of different *O. rhinotracheale* strains to tracheal organ cultures (TOC) after one, three, six and ten hours of incubation, and found that, although all *O. rhinotracheale* strains appeared to adhere to some extent to the TOCs, there were obvious differences between some of the tested strains (data not shown). We observed differences of about 10^3 cfu/ml between the best adhering strains and the least adhering strains. In a small pilot study we inoculated 15 SPF turkeys with the least *in vitro* adhering *O. rhinotracheale* strain and compared the tracheal adhesion with that of the LMG 9086 type strain which exhibited the best adhesion *in vitro*. Strain LMG 9086 was the strain used in the different experimental studies of the present PhD research. We came to the same conclusions as Temple *et al*. (1998), namely that the *O. rhinotracheale* strain less able to adhere *in vitro* to TOCs also was less capable of colonising live birds. One day after inoculation, the least *in vitro* adhering *O. rhinotracheale* strain could only be isolated from two of the 15 birds at very low cfu/ml. In these latter two birds, *O. rhinotracheale* could only be isolated until three and four days after inoculation (means of 2.1 and 2.5 $\log_{10}$cfu/g mucus). Temple *et al*. (1998) also noticed that the ability of an *O. rhinotracheale*

strain to cause disease *in vivo* fully correlated with its *in vitro* ability to adhere to ciliated tracheal cells. We were, however, not able to evaluate whether the difference in adherence would also mean a difference in the disease-causing ability, since our reference strain was only able to cause obvious clinical symptoms after APV priming. It would be very interesting to identify the adhesins and host-receptors important for *O. rhinotracheale* and whether there are toxins which play a role in the adhesion process as has been described for the CyaA toxin and dermonecrotic toxin of *B. bronchiseptica* (Edwards *et al.*, 2005; Vojtova *et al.*, 2007). The contributions of the individual adhesins and toxins in ciliary attachment should also be evaluated. This might be done by comparing ciliary binding by *O. rhinotracheale* mutant strains deficient in single adhesin or toxin expression. If more insights are obtained in these features of the *O. rhinotracheale* pathogenesis, this could possibly lead to the development of preventive or therapeutic targets against primary colonizing bacteria such as *O. rhinotracheale*.

Based upon the results obtained in our studies it can be assumed that both *O. rhinotracheale* and *E. coli* are secondary pathogens, an issue which in the literature has been a subject for debate. This however does not mean that their importance in the field should be underestimated, since it was shown in our experiments that both are able to exacerbate the clinical picture and especially the duration of the respiratory problems. Dust in poultry houses may contain 10^5-10^6 *E. coli*/g and these bacteria can persist for long periods, particularly under dry conditions (Harry, 1964; Barnes *et al.*, 2003). Also, Lopes *et al.* (2002) studied the ability of *O. rhinotracheale* to remain viable in poultry litter at different temperatures, and their results indicated that this micro-organism survives in poultry litter for some days, especially at lower temperatures. These findings implicate that both these secondary bacterial agents pose a real threat for the poultry industry, keeping in mind that infections and reinfections with APV and *M. gallisepticum* do occur. If an outbreak of APV or *M. gallisepticum* takes place when *E. coli* and/or *O. rhinotracheale* are present in the environment, serious respiratory problems may be encountered with serious economical consequences.

Bacterial respiratory infections in poultry require a quick, feasible and efficacious antimicrobial treatment, preferably via drinking water. Very inconsistent and frequently inadequate results have been obtained with antimicrobial treatment of

O. rhinotracheale and *E. coli* infections in the field (van Empel and Hafez 1999; Chin *et al*., 2003). Several explanations may be cited here, including the variable susceptibility of the different bacterial strains (Devriese *et al*., 1995; Dudouyt *et al*., 1995; Hafez, 1996; Roger and Léorat, 1997; Devriese *et al*., 2001; Barnes *et al*., 2003; Vandekerchove, 2004). It is indeed a fact that emerging antimicrobial resistance is becoming increasingly a problem in most bacterial species. Furthermore, as mentioned above, antimicrobials are frequently used on the off-chance, mainly because not enough information is available. This inconsistency in antimicrobial treatments is to a great extent due to the fact that, at the beginning of this research, no good models were available to evaluate the efficacy of different products. Our objectives thus were to compare the clinical effectiveness of different antimicrobial compounds under identical trial conditions.

The above described experimental infection models undoubtedly represent a number of advantages. *In vitro* antimicrobial sensitivity measurements, preferably minimal inhibitory concentration (MIC) determinations, can be used to predict the clinical outcome of antimicrobial therapy. While these measurements are a good indication of the potency of an antimicrobial, they do not provide the type of information necessary to determine the optimal drug dose or dosing interval. MIC tests additionally tell us hardly anything about the effect of varying drug concentrations over time. Therefore, besides *in vitro* studies, animal infection models are needed, since they are effective means of examining the relationship between pharmacokinetic and pharmacodynamic parameters and *in vivo* efficacy (Wright *et al*., 2000). By assessing the effect of antimicrobials on the bacteria directly in the target organ(s) the overall efficacy of these antimicrobial agents could be evaluated with reasonable accuracy. Also from an ethical view-point and with regard to the 3 R (reduction, refinement and replacement) principle (Russell and Burch, 1959), the optimized models are interesting: because of the daily tracheal swabbing, a lot of data could be obtained from each individual bird without having to sacrifice them, allowing good statistical analysis using only a limited number of animals. Furthermore, the birds apparently did not suffer from the regular tracheal swabbing.

In the two studies performed to evaluate the clinical efficacy of different antimicrobial therapies for the treatment of single *O. rhinotracheale* and dual *E. coli*/*O. rhinotracheale* infection, statistical analysis revealed that recovery from respiratory disease was overall most successful after enrofloxacin treatment (three or

five days of treatment), followed by five-day florfenicol treatment. Five-day treatment with amoxicillin, compared with the untreated group under the circumstances adopted in this study, did not cause a significant reduction in any of the evaluated parameters. Possible explanations for the observed differences between the different antimicrobial products are largely straightforward. Some pharmacokinetic characteristics, such as the apparent volume of distribution at steady state ($Vd_{(ss)}$) and the half-life values of the various compounds, may be highlighted to explain the obtained results. The bactericidal effect of amoxicillin is possibly impaired because of its less efficient distribution throughout the tissues (low $Vd_{(ss)}$) and in addition its quick elimination (low half-life values). Indeed, Prescott reported that although amoxicillin offers pharmacokinetic advantages over ampicillin, it still retains to some degree the difficulty for attaining that concentration in tissues that is necessary to control infections with susceptible Gram-negative bacteria (Prescott, 2000). It appears that the $Vd_{(ss)}$ of florfenicol and enrofloxacin is generally higher than that of amoxicillin, indicating a better distribution throughout the body tissues. Furthermore, the long half-life values for especially enrofloxacin and to a lesser degree for florfenicol attribute to the observed differences between enrofloxacin and florfenicol. Fraatz *et al*. (2005) performed a pharmacokinetic study in growing turkeys medicated in-water with enrofloxacin, florfenicol and amoxicillin. Antimicrobial concentrations were measured in serum, muscle, liver and lung using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and, for enrofloxacin in nose and trachea tissue, with an agar diffusion bioassay. For the amoxicillin product it was shown that it was unstable in the drinking water, leading to a loss of 50% over 24h. Furthermore, amoxicillin was shown to be unstable during sample processing, invalidating the analytical results for this compound. Their results show that in turkeys medicated via drinking water with enrofloxacin, mean enrofloxacin concentrations in the upper (nasal tissues and trachea) and lower (lungs) respiratory tract exceeded MIC_{50} values (MIC_{50} = the average MIC of a susceptible population) for *E. coli* and *P. multocida* by about 5 to 8 log_2 steps ($\times 2^5$ to 2^8) and for mycoplasma and *O. rhinotracheale* by about 2 to 4 steps ($\times 2^2$ to 2^4). In turkeys treated with florfenicol drug penetration in the lower (lungs) respiratory tract appeared to be moderate with concentrations lower than the MIC_{50} values for *E. coli*, mycoplasma and *O. rhinotracheale* and similar to the MIC_{50} for *P. multocida*. They concluded that florfenicol does not distribute as well as enrofloxacin in the target tissues. Other results by Knoll *et al*. (1999) showed

that enrofloxacin concentrations were three-fold higher in the tracheal tissues than in the plasma (Reinhardt *et al.*, 2005).

In different experimental groups treated with enrofloxacin or florfenicol, first bacteria (*O. rhinotracheale* and *E. coli*) appeared to be completely eliminated from the trachea (or the amounts were below the detection limit), after which a flare-up was seen of bacteria without a rise in the MIC value. Whether this temporary rise in bacterial titers in the trachea was due to re-excretion or re-infection from the environment could not be determined. Re-infection from the environment appears possible, since it has been stated that *O. rhinotracheale* is able to survive in poultry litter for a few days (Lopes *et al.*, 2002) and *E. coli* bacteria may persist for long periods, particularly under dry conditions (Harry, 1964; Barnes *et al.*, 2003).

In one of the experiments, *O. rhinotracheale* organisms with an eight-fold higher MIC value (from ≤ 0.03 µg/ml to 0.25 µg/ml) of enrofloxacin were isolated in the group treated with enrofloxacin for five days, starting two days following treatment onset, initially from a single turkey and subsequently from the other animals. Concurrently with this, *O. rhinotracheale* could be re-isolated from the trachea, and was present in higher numbers in the turbinates and lungs at five days post bacterial inoculation, compared with the almost negative *O. rhinotracheale* counts post treatment in the turkeys treated for three days, but without causing a clinical relapse. This represents a typical example of selection of a first-step mutant by fluoroquinolones, as already reported in the literature. The *in vivo* emergence of diminished susceptibility or even resistance to fluoroquinolones has already been described in chickens for, amongst others, *Campylobacter jejuni* (McDermott *et al.*, 2002; Luo *et al.*, 2003; Van Boven *et al.*, 2003), *Campylobacter coli* (Delsol *et al.*, 2004), and a resident *E. coli* microbiota (Barrow *et al.*, 1998). Because of the growing concern for public health regarding the emergence of fluoroquinolones-resistant zoonotic pathogens, such as *Salmonella*, enterohemorrhagic *E. coli*, and *Campylobacter*, it is not surprising that most experiments have focused on these pathogens (Zhao *et al.*, 2005). Resistance to fluoroquinolones arises almost exclusively by *de novo* mutation, and is characterized by the gradual accumulation of mutations that lower intracellular drug concentration and/or sensitivity of the target DNA topoisomerases (Lindgren *et al.*, 2003). With some exceptions, the mutations reported to date have been chromosomal. As wild-type bacteria replicate, mistakes are made in duplicating the DNA. These mistakes are spontaneous mutations that

arise at measurable frequencies usually varying from one in 10^6 (mutation frequency $= 10^{-6}$) to one in 10^9 (mutation frequency $= 10^{-9}$) wild-type cells. Because the number of bacteria in an active infection readily exceeds 10^9, it is likely that in addition to the wild-type cells, in large populations, there are already mutant cells present at the site of the infection (Pourbakhsh *et al.*, 1997; Sanders, 2001; Drlica and Malik, 2003; Wetzstein, 2005). Selection pressure exerted by fluoroquinolone treatment will enrich the mutant subpopulation (Sanders, 2001; Drlica and Malik, 2003). Studies involving well-characterized single-step mutants have shown that each mutation affecting the quinolones most often diminishes susceptibility four- to eight-fold (Sanders, 2001). Usually, organisms that are highly susceptible to fluoroquinolones remain within the susceptible range after single-step mutations and require multiple mutations before clinically relevant resistance becomes apparent (Hooper, 2001; Andriole, 2003). This in consequence means that when the concentration obtained in the tissues is only slightly above the MIC of the wild-type bacteria, there is a reasonable probability that any mutant bacteria will be enriched. This knowledge seems rather worrying, but the predictable and stepwise evolution of resistance to the quinolones also has a positive side, in that it provides an additional basis on which quinolone may provide effective therapy and have a lower chance of selecting resistance (Sanders, 2001). One approach for restricting resistance is to avoid conditions that enrich resistant bacterial subpopulations. Such conditions have been defined *in vitro* for fluoroquinolones with a variety of bacteria. In general, fluoroquinolone-resistant mutants are enriched when drug concentrations fall within a range called the mutant selection window (Drlica, 2003; Drlica and Malik, 2003). The lower boundary of the window is the minimal concentration required to block the growth of wild-type bacteria (since below that concentration the mutant cells do not have a growth advantage) and is often approximated by the MIC_{99}, the minimal concentration that blocks growth of 99% of the cells in a culture (Drlica, 2003). The upper boundary, the mutant prevention concentration (MPC), which is the MIC of the least susceptible, single-step mutant subpopulation, serves as a guide for antimutant dosing strategies, just as the MIC functions in strategies to clear infection of susceptible pathogens. The idea with the MPC is that above this concentration, two concurrent resistance mutations must be acquired so that bacterial growth could occur, so maintaining drug concentrations above the MPC throughout therapy is expected to restrict mutant outgrowth. Determination of the MPC is done by plating approximately 10^{10} bacteria (10^4-10^5

cfu are usually used for MIC testing) into varying concentrations of the quinolone and determining the concentration at which no visible re-growth occurs (Sanders, 2001; Wetzstein, 2005). In different experiments where both MIC and MPC were measured for different isolates, it was shown that there was a poor correlation between the two (Marcusson *et al.*, 2005; Hansen *et al.*, 2006). Doses of antimicrobial that are inside the window are expected to enrich resistant mutant subpopulations selectively, whereas placing concentrations above the window is expected to restrict selective enrichment. Growth of mutants at concentrations above the MPC would require the concurrent presence of two mutations conferring fluoroquinolone resistance. Such clones may be present in populations of about 10^{14} cfu (Blondeau *et al.*, 2004), which are larger than most bacterial populations in diseased chickens (Wetzstein, 2005). This concept seems contradictory with traditional medical teaching, in which one thinks that resistant mutants are selectively enriched only at concentrations below MIC (Drlica, 2003) in stead of also at concentrations above MIC. By attaining MPC conditions *in vivo*, the enrichment of first-step fluoroquinolone-resistant variants should be minimized. Hence, a delay or (ideally) prevention of the emergence of fluoroquinolone resistant strains in the field may become feasible (Wetzstein, 2005). On the negative side however, it should be noted that MPC have been measured for only a few drug–pathogen combinations. First experimental results obtained in a rabbit mono-infection model with *Streptococcus pneumoniae* provided direct evidence in support of the MPC concept (Etienne *et al.*, 2004; Wetzstein, 2005). This concept provides a hopeful perspective for the future of the fluoroquinolones, being able to profit from their many benefits and not having to deal with the concerning problem of resistance. More research therefore should be invested in finding dosing strategies enabling concentrations above the MPC. This however, may appear difficult in the poultry industry, since the current method of medicating is by treating the entire flock via water, even though relatively few birds may be ill. This practice exposes more organisms to the antimicrobial and therefore is more likely to result in the emergence of resistant strains. Additionally this may result in suboptimal dosing (for instance when diseased birds drink less) increasing the probability of selecting for resistant bacterial strains (Barnes *et al.*, 2003; McDermott *et al.*, 2002). Since fluoroquinolones are concentration-dependent antimicrobials, perhaps pulse-dosing (administration of the daily dose antimicrobial in a two to four hour period) may be an interesting option. If enrofloxacin is given in a pulsed manner, the peak levels in

serum and lungs are three- and fourfold higher, respectively, than the steady-state concentrations observed in birds dosed continuously throughout the day. In spite of results from Charleston *et al.* (1998) and Froyman and Cooper (2003), showing no advantage of pulsed dosing compared with continuous dosing, it still might be interesting to look again at this option since it could possibly reduce the resistance problem associated with fluoroquinolones. Other options may be the use of combinations of agents having different targets or of antimicrobial agents that inhibit two different targets with equal efficacy (it would require a bacterium to acquire two concurrent mutations for growth; only rarely would resistant mutants be recovered). A lot of interesting possibilities lie herein for future research.

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Chapter 5: SUMMARY - SAMENVATTING

SUMMARY

Respiratory problems are one of the main disorders leading to economic losses on turkey farms world-wide due to reduced growth, an increased mortality rate, high medication costs and a higher number of condemnations at slaughter. They may be induced by a variety of viral and bacterial agents, either alone or in combination. The pathogenesis of infections with these respiratory disease agents and their mutual interactions, are very complex matters which are far from fully unraveled.

Therapeutic measurements against different bacterial infections are virtually entirely based on the administration of antimicrobials, although it is frequently observed that the clinical effects of the different antimicrobial products are very variable in the field. Very few experimental studies have been focusing on the actual *in vivo* efficacy of different antimicrobials for the treatment of respiratory infections in poultry, since often no suitable infection models are available. To extend our knowledge on the pathogenesis and control of respiratory infections in turkeys, the present thesis was initiated. *Ornithobacterium rhinotracheale*, *Escherichia coli*, *Mycoplasma gallisepticum* and avian metapneumovirus (APV) were studied, being important respiratory pathogens, with a worldwide distribution.

In the first part (***part 1.1.***) of **chapter 1**, a review is given on the anatomical and physiological properties of the avian respiratory tract and on its defense mechanisms. In the second part of chapter 1 (***part 1.2.***), the most important literature data are given on four important turkey respiratory pathogens, i.e. APV, *O. rhinotracheale*, *E. coli* and *M. gallisepticum*. Different aspects are reviewed for each pathogen, i.e. etiology, epidemiology, pathogenesis, clinical findings and lesions, diagnosis, treatment and control measures. Finally, the experimental studies which have been performed in turkeys to elucidate the effects of combined action of viruses and other micro-organisms are summarized.

Chapter 2 describes the scientific aims of the study. Our main objective was to firstly develop experimental infection models using these agents and to, in a second stage, use these models to study the pathogenesis and to compare the clinical efficacy of different antimicrobial compounds.

Chapter 3 describes the performed experimental studies. In a first part (***part 3.1.***), the development of different *in vivo* infection models for the reproduction of clinical respiratory disease in turkeys is discussed.

In a first study (3.1.1.), three-week-old SPF turkeys were inoculated oculonasally with either APV, *O. rhinotracheale* or both agents using two different time intervals (three or five days) between infections. The birds were observed clinically on a daily basis and swabbed intratracheally at regular intervals for bacteriological examination. Birds were euthanised at different time points, examined for gross lesions at necropsy and samples of the turbinates, trachea, lungs, air sacs, heart, pericardium and liver were taken for bacteriological and/or histological examination.

The results showed that, under the circumstances used in this study, *O. rhinotracheale* on itself was able to adhere to and colonize the upper respiratory tract of turkeys, but without viral priming was not capable of inducing respiratory disease. Combined APV/*O. rhinotracheale* infections, regardless of the time interval between inoculations, resulted in overt clinical signs and a longer persistence of *O. rhinotracheale* in the respiratory tract and aggravated the macroscopic and histological lesions in comparison to the groups given single infections. APV priming facilitated infection of the lungs and was necessary to allow *O. rhinotracheale* to infect the air sacs.

In a second study (3.1.2.), turkeys were inoculated oculonasally with either APV, APV/*O. rhinotracheale*, APV/*E. coli* O2:K1, APV/*E. coli*/*O. rhinotracheale* or APV/*O. rhinotracheale*/*E. coli* with a three days interval between viral and bacterial inoculation and approximately eight hours between the two bacterial inoculations. The animals were observed clinically and swabbed intratracheally on a daily basis. Five and ten birds were euthanised at five and fifteen days post bacterial inoculation (dpbi), respectively, and examined for gross lesions at necropsy. Samples of the turbinates, sinuses, trachea, lungs, air sacs, heart, pericardium and liver were taken for bacteriological and/or histological examination.

It was demonstrated that combined APV, *E. coli* and *O. rhinotracheale* infections resulted in more severe and longer persisting clinical signs and more severe macroscopic and microscopic lesions compared to dual APV/*O. rhinotracheale* and APV/*E. coli* infections, and the latter produced more severe disease than the single APV infection. It was shown that both *O. rhinotracheale* and *E. coli* were, after APV

priming, able to colonize the upper respiratory tract and invade the lower respiratory tract of turkeys, although the level of *O. rhinotracheale* and *E. coli* multiplication in the respiratory organs did not differ greatly between the dual and triple infection groups. However, after triple infection, *E. coli* could be isolated from the trachea two days longer in comparison with the dually infected group. Furthermore, it was shown that combined action of *O. rhinotracheale* and *E. coli* enhanced the invasion of *O. rhinotracheale* but not of *E. coli* in the non-respiratory internal organs, although it should be noted that at necropsy no signs of septicemia were seen. These findings all endorsed our conclusions that *E. coli* and *O. rhinotracheale* exercise an additive pathogenic effect in the reproduction of respiratory disease triggered by APV.

In a third study (3.1.3.), approximately the same set-up was used, but an additional intratracheal priming with *M. gallisepticum* was performed. The turkeys were inoculated with either *M. gallisepticum*, *M. gallisepticum*/APV, *M. gallisepticum*/APV/*O. rhinotracheale* or *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* with a ten days interval between *M. gallisepticum* and APV inoculations, a three days interval between viral and *E. coli* and/or *O. rhinotracheale* inoculations, and an eight hour interval between *E. coli* and *O. rhinotracheale* inoculations. Again, the animals were observed clinically on a daily basis (upper and lower respiratory tract symptoms), swabbed intratracheally on a regular basis. Five birds of each group were sacrificed 18 days after *M. gallisepticum* inoculation and the remaining birds at the end of the experimental period, i.e. 33 days after *M. gallisepticum* inoculation. The birds were examined for gross lesions at necropsy and samples of the turbinates, sinuses, trachea, lungs, air sacs, heart, pericardium and liver were taken for bacteriological and/or histological examination.

The results obtained in this study clearly indicate a marked synergistic connection between *M. gallisepticum*, APV, *O. rhinotracheale* and *E. coli*. Evidences for this statement were the higher mortality rates and the clinical symptoms which were clearly aggravated whenever the birds were infected with an additional pathogen. This was also reflected in the more aggravated necropsy findings and microscopical lesions. *M. gallisepticum* exerted an important impeding effect on the clearance of *O. rhinotracheale* and *E. coli*, especially from the upper respiratory tract. A very conspicuous feature was that in the *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* group, signs of colisepticemia (perihepatitis and/or pericarditis) were seen at necropsy. This was a feature not encountered in the previous infection model using *E.*

coli. This finding endorses the statements that compared with bacterial virulence factors, pathogenic interactions are probably at least as important determinants of colibacillosis occurrence.

In the second part of chapter 3 (***part 3.2.***) we evaluated the clinical efficacy of different antimicrobials (i.e. enrofloxacin, amoxicillin and florfenicol) for treatment of respiratory disease in turkeys. We evaluated this in the APV/*O. rhinotracheale* and APV/*O. rhinotracheale*/*E. coli* infection models, based on clinical, bacteriological and histopathological examinations using similar techniques and criteria. Antimicrobial drinking water treatments (continuous dosing) were initiated at 24 h after the last bacterial challenge.

In the first study (3.2.1.) the efficacy of drinking-water administration of enrofloxacin for three and five days, amoxicillin for five days and florfenicol for five days for the treatment of respiratory disease induced by an experimental APV/*O. rhinotracheale* infection (three day interval) in turkeys was assessed. After inoculation, the birds were scored daily for clinical signs, tracheal swabs were also taken on a daily basis and the animals were weighed at different time points. Five birds were euthanised and examined for macroscopic lesions at necropsy at five dpbi, and the remainder at 15 dpbi. Samples of the turbinates, trachea, lungs, air sacs, heart and pericardium were collected for bacteriological and/or histological examination. Recovery from respiratory disease caused by an APV/*O. rhinotracheale* dual infection was most successful after enrofloxacin treatment, irrespective of treatment duration, followed by florfenicol. Treatment with amoxicillin was not efficacious, since compared with the non-treated group, it did not cause a significant reduction of any of the evaluated parameters. Although florfenicol treatment significantly diminished the *O. rhinotracheale* multiplication in the trachea and lungs, this did not seem to result in a significant reduction of clinical symptoms. Compared with the non-treated control group, clinical signs as well as *O. rhinotracheale* multiplication in the respiratory tract were significantly reduced by enrofloxacin treatment for three and five days. In the group treated with enrofloxacin for five days, *O. rhinotracheale* organisms with an eight-fold higher MIC value (from ≤ 0.03 to 0.25 µg/ml), were isolated starting two days following treatment onset initially from a single turkey and subsequently from the other animals. This increase in enrofloxacin MIC value concurred with a slight *O. rhinotracheale* re-excretion in the trachea, also reflected in the higher counts in different organs (turbinates and lungs) at five dpbi, compared to

the almost negative *O. rhinotracheale* counts post treatment in the turkeys treated for three days.

In an additional study (3.2.2.) we decided to look further into the genetic background of this change in antimicrobial susceptibility. In addition to the *O. rhinotracheale* isolates derived from the experimentally infected animals, other *O. rhinotracheale* field strains with different *in vitro* sensitivity levels for enrofloxacin, were included in this study for comparative purposes. In the isolates showing reduced susceptibility following experimental enrofloxacin treatment (increase in MIC from ≤ 0.03 to 0.25 μg/ml), molecular analysis revealed a constantly recurring point mutation (G → T) at nucleic acid position 646 (*E. coli* numbering) of *gyrA* resulting in an amino acid change from aspartic acid to tyrosine at position 87 of the GyrA subunit, which is a known hot spot for fluoroquinolone resistance. The results obtained in the study hence indicated that a single course of enrofloxacin treatment may contribute to the *in vivo* selection of the first mutant with reduced fluoroquinolone susceptibility in *O. rhinotracheale*. In the field strains that were either less susceptible or, occasionally, resistant to enrofloxacin, point mutations had occurred in amino acids at positions 83 (serine) or 87 (aspartic acid) of the GyrA subunit.

In a final study (3.2.3.) similar techniques and criteria were used in order to assess the efficacy of drinking-water administration of enrofloxacin for three and five days, amoxicillin for five days and florfenicol for five days for the treatment of respiratory disease caused by APV/*E. coli*/*O. rhinotracheale* triple infection. The enrofloxacin treatments again proved to be most efficacious, regardless of treatment duration, followed by the florfenicol treatment. Compared with the non-treated group, clinical signs as well as *E. coli* and *O. rhinotracheale* multiplication in the respiratory tract were significantly reduced by both enrofloxacin treatments and the florfenicol treatment, with the enrofloxacin treatments showing significantly better reductions than the florfenicol treatment. Five-day treatment with amoxicillin, compared with the untreated group, did not cause a significant reduction in any of the evaluated parameters.

Chapter 4: Based upon the results obtained in our studies it can be assumed that both *O. rhinotracheale* and *E. coli* are important respiratory pathogens, since they were both able to markedly exacerbate the clinical picture caused by APV and *M. gallisepticum* infections. Both *O. rhinotracheale* and *E. coli* are respiratory

pathogens that are able to cause similar disease symptoms and lesions, but they intrinsically have different ways of acting. *O. rhinotracheale* has a very clear tropism for the healthy respiratory system, in contrast with *E. coli* which specifically needs an APV-infected respiratory system in order to sustain its presence. Furthermore our observations support the idea that both *O. rhinotracheale* and *E. coli* should be considered more as secondary pathogens. It also became clear that *M. gallisepticum* exerted an important impeding effect on the clearance of *O. rhinotracheale* and *E. coli*, especially from the upper respiratory tract. Also the observation that colisepticemia could only be provoked when infection with *M. gallisepticum* was present, emphasises the statement that compared with bacterial virulence factors, pathogenic interactions are probably at least as important determinants of colibacillosis occurrence. Keeping in mind the triggering effect of APV and *M. gallisepticum* on infection with *O. rhinotracheale* and *E. coli*, and keeping in mind that infections with APV and *M. gallisepticum* do occur, both *O. rhinotracheale* and *E. coli* should remain to be considered as threats to the poultry industry. If an outbreak of APV or *M. gallisepticum* takes place when *E. coli* and/or *O. rhinotracheale* are present in the environment, serious respiratory problems may be encountered. In conclusion, once again the complexity of the respiratory disease complex in turkeys has been indicated.

In the two studies performed to evaluate the clinical efficacy of different antimicrobial therapies for the treatment of single *O. rhinotracheale* and dual *E. coli*/*O. rhinotracheale* infection, it was shown that recovery from respiratory disease was overall most successful after enrofloxacin treatment. Fluoroquinolones, like all antimicrobial compounds, should however be used judicious, amongst others in order to counteract the spread of antimicrobial resistance. In our research it was established that a treatment with enrofloxacin can lead to the selection of an *O. rhinotracheale* mutant with decreased sensitivity to fluoroquinolones. One approach for restricting resistance towards fluoroquinolones is to avoid conditions that enrich these mutants. In this respect it should be strived for that during a treatment, the fluoroquinolone concentrations in the blood and tissues remain sufficiently high, so that also mutants with diminished sensitivity will be inhibited and will not be selected.

SAMENVATTING

Ademhalingsproblemen op kalkoenbedrijven leiden wereldwijd tot grote economische verliezen, omwille van verminderde groei, verhoogde sterftecijfers, hogere medicatiekosten en een hoger aantal afkeuringen bij verwerking. Zij kunnen geïnduceerd worden door diverse virale en bacteriële agentia, ofwel afzonderlijk ofwel in combinatie met elkaar. De pathogenese van infecties met deze respiratoire ziektekiemen en hun onderlinge interacties zijn zeer complex en verre van ontrafeld.

De behandeling van bacteriële infecties van het ademhalingsstelsel bij kalkoenen is bijna volledig gebaseerd op de toediening van antimicrobiële middelen. In de praktijk wordt evenwel opgemerkt dat de klinische effecten van antimicrobiële therapie zeer variabel zijn. Het aantal experimentele studies waarin de *in vivo* efficaciteit van antimicrobiële middelen voor de behandeling van ademhalingsaandoeningen bij pluimvee bestudeerd werd, is zeer beperkt. De belangrijkste reden hiervoor is het ontbreken van geschikte infectiemodellen. Om onze kennis uit te breiden over de pathogenese en de controle van ademhalingsaandoeningen bij pluimvee werd dit thesisonderzoek aangevat. *Ornithobacterium rhinotracheale*, *Escherichia coli*, *Mycoplasma gallisepticum* en aviair metapneumovirus (APV) werden bestudeerd aangezien zij belangrijke respiratoire pathogenen zijn met een wereldwijde verspreiding.

In het eerste deel (*deel 1.1.*) van **hoofdstuk 1** wordt een overzicht gegeven van de anatomie en fysiologie van het ademhalingsstelsel bij vogels. Ook de afweermechanismen die hier aanwezig zijn, worden besproken. Hierbij wordt eerst aandacht besteed aan de aangeboren afweer en daarna wordt ingegaan op verworven immuniteit. In het tweede deel van hoofdstuk 1 (*deel 1.2.*), worden de belangrijkste gegevens uit de literatuur weergegeven betreffende vier belangrijke ademhalingspathogenen bij kalkoenen, namelijk APV, *O. rhinotracheale*, *E. coli* en *M. gallisepticum*. Verschillende aspecten worden besproken voor elke pathogeen agens, namelijk etiologie, epidemiologie, pathogenese, klinische bevindingen, letsels, diagnose, behandeling en preventie. Ten slotte wordt besproken welke experimentele studies er reeds werden uitgevoerd bij kalkoenen om betere inzichten te bekomen in de effecten van gecombineerde actie van virussen en andere micro-organismen.

Hoofdstuk 2 beschrijft de wetenschappelijke doelstellingen van de studie. Een eerste objectief was het op punt stellen van experimentele infectiemodellen waarmee ademhalingsproblemen die veroorzaakt worden door infecties met APV, *O. rhinotracheale*, *E. coli* en *M. gallisepticum*, konden gereproduceerd worden. Deze modellen werden daarna gebruikt om de klinische efficaciteit van verschillende antimicrobiële middelen te vergelijken.

Hoofdstuk 3 beschrijft de uitgevoerde experimentele studies. Hierbij wordt vooreerst (deel *3.1.*) de ontwikkeling besproken van verschillende *in vivo* infectiemodellen voor het reproduceren van klinische ademhalingsproblemen bij kalkoenen.

In een eerste studie (3.1.1.) werden drie weken oude SPF kalkoenen oculonasaal geïnoculeerd met ofwel enkel APV, enkel *O. rhinotracheale* of eerst met APV en drie of vijf dagen later met *O. rhinotracheale*. De dieren werden dagelijks klinisch onderzocht en op regelmatige tijdstippen werden tracheale swabs genomen voor bacteriologisch en virologisch onderzoek. Op verschillende tijdstippen werden kalkoenen geëuthanaseerd. De aanwezigheid van macroscopische letsels werd geëvalueerd en er werden stalen genomen van conchae, trachea, longen, luchtzakken, hart, pericard en lever voor bacteriologisch en/of histologisch onderzoek.

De resultaten toonden aan dat, onder de omstandigheden van deze studie, *O. rhinotracheale* op zichzelf in staat was vast te hechten aan de bovenste ademhalingswegen van kalkoenen en deze ook te koloniseren, maar zonder voorafgaande infectie met APV, werden geen ademhalingsstoornissen waargenomen. Gecombineerde APV/*O. rhinotracheale* infecties, onafhankelijk van het tijdsinterval tussen de inoculaties, resulteerden daarentegen in duidelijke klinische symptomen, een langere persistentie van *O. rhinotracheale* in de ademhalingswegen en in een toename van de macroscopische en histologische letsels in vergelijking met de groepen die slechts enkelvoudige infecties hadden gekregen. Een voorafgaande infectie met APV vergemakkelijkte ook het aanslaan van *O. rhinotracheale* ter hoogte van de longen en bleek noodzakelijk om *O. rhinotracheale* toe te laten de luchtzakken te infecteren.

In een tweede studie (3.1.2.) werden kalkoenen oculonasaal geïnoculeerd met ofwel APV, APV/*O. rhinotracheale*, APV/*E. coli* O2:K1, APV/*E. coli*/*O. rhinotracheale* of APV/*O. rhinotracheale*/*E. coli* met een interval van drie dagen

tussen virale en bacteriële inoculatie en ongeveer 8u tussen de inoculaties met de twee bacteriën. De dieren werden dagelijks klinisch onderzocht en hun tracheas bemonsterd. Op verchillende tijdstippen werden vogels geëuthanaseerd, de macroscopische letsels bekeken bij necropsie en verschillende stalen genomen voor bacteriologisch en/of histologisch onderzoek.

Er werd aangetoond dat gecombineerde APV, *E. coli* en *O. rhinotracheale* infecties resulteerden in ernstigere en langer persisterende klinische symptomen en ernstigere macroscopische en microscopische letsels vergeleken met de dubbele APV/*O. rhinotracheale* en APV/*E. coli* infecties. Deze dubbele infecties veroorzaakten op hun beurt ernstigere ziekte dan de enkelvoudige APV infectie. Zowel *O. rhinotracheale* als *E. coli* waren na een voorafgaande infectie met APV in staat om de bovenste ademhalingswegen van kalkoenen te koloniseren en de diepere luchtwegen te invaderen, alhoewel het niveau van *O. rhinotracheale* en *E. coli* vermeerdering in de respiratoire organen niet veel verschilde tussen de dubbele en driedubbele infectiegroepen. Na drievoudige infectie, kon *E. coli* twee dagen langer geïsoleerd worden uit de trachea in vergelijking met de dubbel geïnfecteerde groep. Voorts werd er aangetoond dat de gecombineerde actie van *O. rhinotracheale* en *E. coli* de invasie van *O. rhinotracheale,* maar niet van *E. coli,* in de niet-respiratoire organen in de hand werkte, alhoewel er bij necropsie geen tekenen van septicemie werden gezien. Al deze bevindingen ondersteunen onze conclusie dat *E. coli* en *O. rhinotracheale* een additief pathogeen effect uitoefenen in de ademhalingsziekte uitgelokt door APV.

In een derde studie (3.1.3.) werd ongeveer dezelfde proefopzet gebruikt, maar een additionele intratracheale inoculatie met *M. gallisepticum* werd uitgevoerd. De kalkoenen werden geïnoculeerd met ofwel *M. gallisepticum*, *M. gallisepticum*/APV, *M. gallisepticum*/APV/*O. rhinotracheale* of *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* met tien dagen interval tussen de *M. gallisepticum* en APV inoculaties, drie dagen interval tussen de virale en *E. coli* en/of *O. rhinotracheale* inoculaties, en acht uur interval tussen de *E. coli* en *O. rhinotracheale* inoculaties. Ook nu werden de dieren dagelijks klinisch onderzocht op het voorkomen van ademhalingsstoornissen, en op regelmatige tijdstippen intratracheaal bemonsterd. Na euthanasie, werden de kalkoenen onderzocht op aanwezigheid van macroscopische letsels en verschillende stalen werden genomen voor bacteriologisch en/of histologisch onderzoek.

De resultaten bekomen in deze studie geven aan dat er een duidelijke synergestische werking bestaat tussen *M. gallisepticum*, APV, *O. rhinotracheale* en *E. coli*. Dit werd aangetoond door de hogere sterftecijfers en ergere klinische symptomen bij dieren die geïnfecteerd werden met een extra pathogeen agens. Dit werd ook gereflecteerd in de ergere bevindingen bij necropsie en ergere microscopische letsels. *M. gallisepticum* oefende een belangrijk belemmerend effect uit op de verwijdering van *O. rhinotracheale* en *E. coli*, voornamelijk uit de bovenste ademhalingswegen. Een zeer opvallende bevinding was dat in de *M. gallisepticum*/APV/*E. coli*/*O. rhinotracheale* groep tekenen van *E. coli* septicemie (perihepatitis en/of pericarditis) gezien werden bij necropsie. Dit werd niet vastgesteld in het vorige infectiemodel met *E. coli*. Deze bevinding ondersteunt de hypothese dat het al dan niet tot uiting komen van colibacillose niet enkel afhankelijk is van de virulentiefactoren van de *E. coli* stam waarmee dieren geïnfecteerd worden, maar dat interacties met andere pathogene agentia op zijn minst even belangrijk zijn.

In het tweede deel van hoofdstuk 3 (***deel 3.2.***) werd de klinische efficaciteit van drinkwatermedicatie met verschillende antimicrobiële middelen (enrofloxacine, amoxicilline en florfenicol) voor de behandeling van ademhalingsziekten bij kalkoenen onderzocht. Hierbij werd gebruik gemaakt van de APV/*O. rhinotracheale* en APV/*O. rhinotracheale*/*E. coli* infectiemodellen. Voor het beoordelen van de efficaciteit werden zowel klinische, bacteriologische en histopathologische parameters gebruikt. De antimicrobiële drinkwaterbehandelingen (continue dosering) werden gestart 24u na de laatste bacteriële inoculatie.

In een eerste studie (3.2.1.) werd de efficaciteit beoordeeld van drinkwatertoediening van enrofloxacine gedurende drie en vijf dagen, amoxicilline gedurende vijf dagen en florfenicol gedurende vijf dagen voor de behandeling van ademhalingsproblemen bij kalkoenen veroorzaakt door een experimentele APV/*O. rhinotracheale* infectie (drie dagen interval). Na de infectie werden van de vogels dagelijks tracheaal swabs verzameld voor bacteriologisch (en virologisch) onderzoek. De dieren werden ook dagelijks gescoord voor de ergheid van de klinische tekenen en op verschillende tijdstippen werden ze gewogen. Vijf dieren werden geëuthanaseerd en onderzocht voor macroscopische letsels op vijf dagen na inoculatie met *O. rhinotracheale* en de overblijvende dieren op vijftien dagen na deze inoculatie. Stalen

van conchae, trachea, longen, luchtzakken, hart en pericard werden verzameld voor bacteriologisch en/of histologisch onderzoek.

Herstel van de respiratoire ziekte veroorzaakt door de APV/*O. rhinotracheale* dubbele infectie was het meest succesvol na behandeling met enrofloxacine, onafhankelijk van de behandelingsduur, gevolgd door behandeling met florfenicol. Behandeling met amoxicilline bleek niet doeltreffend, aangezien vergeleken met de niet-behandelde controle groep, er geen significante reductie werd gezien van de geëvalueerde parameters. Alhoewel behandeling met florfenicol de *O. rhinotracheale* vermeerdering in de trachea en longen significant verminderde, bleek dit toch niet te resulteren in een significante vermindering van de klinische symptomen. Vergeleken met de niet-behandelde controlegroep, werden zowel de klinische symptomen als de *O. rhinotracheale* vermeerdering in de ademhalingswegen significant gereduceerd door behandeling met enrofloxacine gedurende drie en vijf dagen. In de groep behandeld met enrofloxacine gedurende vijf dagen, werd *O. rhinotracheale* terug uitgescheiden in de trachea vanaf twee dagen na de aanvang van de behandeling, eerst bij een enkele kalkoen maar vervolgens ook bij de andere dieren. In deze groep was het aantal kolonie vormende eenheden *O. rhinotracheale* per gram conchae- en longweefsel ook hoger op vijf dagen na bacteriële infectie, vergeleken met de bijna negatieve *O. rhinotracheale* tellingen na behandeling van de kalkoenen met enrofloxacine gedurende drie dagen. Voor de *O. rhinotracheale* organismen die geïsoleerd werden in de groep die gedurende vijf dagen behandeld werd, was de minimum inhibitorische concentratie (MIC) van enrofloxacine achtmaal hoger (0.25μg/ml) dan voor de andere *O. rhinotracheale* isolaten ($\leq$ 0.03μg/ml). Deze toename in enrofloxacine MIC waarde lag welliswaar nog steeds onder het resistentie breekpunt (2 μg/ml), maar duidt toch op opbouw van verworven resistentie.

In een additionele studie (3.2.2.) werd dieper ingegaan op de genetische achtergrond van deze verandering in antimicrobiële gevoeligheid. Naast de *O. rhinotracheale* isolaten verzameld van de experimenteel geïnfecteerde dieren, werden, ter vergelijking, ook andere *O. rhinotracheale* veldstammen ingesloten met verschillende *in vitro* gevoeligheidsniveaus voor enrofloxacine. In deze veldstammen die ofwel veminderd gevoelig ofwel resistent aan enrofloxacine waren, hadden puntmutaties plaatsgevonden in de aminozuren op posities 83 (serine) of 87 (aspargine zuur) van de GyrA subunit. Bij de isolaten met verminderde gevoeligheid na experimentele enrofloxacine behandeling (toename in MIC van $\leq$0.03 tot 0.25

µg/ml), toonde moleculaire analyse een punt mutatie (G → T) aan op nucleïnezuur positie 646 (*E. coli* nummering) van *gyrA* resulterend in een aminozuur verandering van aspargine zuur naar tyrosine op positie 87 van de GyrA subunit. Dit is een gekende "hot spot" voor fluoroquinolone resistentie. De resultaten bekomen in deze studies tonen dus duidelijk aan dat een enkele enrofloxacine behandeling kan bijdragen tot de *in vivo* selectie van een mutant met verminderde fluoroquinolone gevoeligheid in *O. rhinotracheale*.

In een laatste studie (3.2.3.) werden dezelfde technieken en criteria gebruikt om de efficaciteit te beoordelen van drinkwatertoediening van enrofloxacine gedurende drie en vijf dagen, amoxicilline gedurende vijf dagen en florfenicol gedurende vijf dagen voor de behandeling van ademhalingsproblemen veroorzaakt door APV/*E. coli*/*O. rhinotracheale* infecties. De enrofloxacine behandelingen, onafhankelijk van de behandelingsduur, bleken opnieuw het meest doeltreffend, gevolgd door de florfenicol behandeling. Vergeleken met de niet-behandelde groep, werden klinische symptomen, alsook de *E. coli* en *O. rhinotracheale* vermeerdering in de ademhalingswegen significant gereduceerd door beide enrofloxacine behandelingen en de florfenicol behandeling, met bij de enrofloxacine behandelingen significant betere reducties dan bij de florfenicol behandeling. Behandeling met amoxicilline gedurende vijf dagen bleek, in vergelijking met de niet-behandelde controlegroep, geen significante reductie te geven van de geëvalueerde parameters.

In **hoofdstuk 4** worden de resultaten van de verschillende studies globaal bediscussieerd. Uit deze studies blijkt dat zowel *O. rhinotracheale* als *E. coli* belangrijke respiratoire pathogenen zijn bij kalkoenen, aangezien beide in staat waren om zeer duidelijk het klinisch beeld veroorzaakt door APV en *M. gallisepticum* infecties te verergeren. Alhoewel *O. rhinotracheale* en *E. coli* hierbij gelijkaardige ziektesymptomen en lesies veroorzaken, zijn er toch ook duidelijke verschillen. *O. rhinotracheale* heeft klaarblijkelijk een tropisme voor de gezonde ademhalingswegen, in tegenstelling tot *E. coli* die een APV-geïnfecteerd ademhalingsstelsel nodig heeft om aanwezig te kunnen blijven. Voorts ondersteunen onze observaties het idee dat zowel *O. rhinotracheale* als *E. coli* eerder moeten beschouwd worden als secundaire pathogenen. Verder werd het ook duidelijk dat *M. gallisepticum* een belangrijk belemmerend effect uitoefende op de verwijdering van *O. rhinotracheale* en *E. coli*, vooral uit de bovenste ademhalingswegen. De observatie dat een *E. coli* septicemie

enkel kon worden uitgelokt wanneer de dieren ook geïnfecteerd waren met *M. gallisepticum*, duidt er op dat menginfecties met verschillende pathogene agentia een heel belangrijke rol spelen bij het tot uiting komen van colibacillose bij pluimvee. Zowel *O. rhinotracheale* als *E. coli* dienen beschouwd te worden als bedreigingen voor de pluimvee-industrie. Indien deze agentia aanwezig zijn wanneer kalkoenen geïnfecteerd worden met APV of *M. gallisepticum*, kunnen ernstige ademhalingsproblemen worden verwacht. Als algemene conclusie kan gesteld worden dat ademhalingsproblemen bij kalkoenen bijzonder complex zijn.

In de twee studies uitgevoerd om de efficaciteit van verschillende antimicrobiële middelen te evalueren voor de behandeling van *O. rhinotracheale* en *E. coli/O. rhinotracheale* infecties, werden de beste resultaten bekomen met enrofloxacine. Fluoroquinolones, zoals trouwens alle antimicrobiële middelen, moeten evenwel oordeelkundig gebruikt worden, onder andere om verspreiding van antimicrobiële resistentie tegen te gaan. In ons onderzoek werd trouwens vastgesteld dat een behandeling met enrofloxacine kan leiden tot het uitselecteren van een *O. rhinotracheale* mutant met verminderde gevoeligheid voor fluoroquinolones. Een benadering voor het beperken van resistentie tegenover fluoroquinolones is het vermijden van omstandigheden die dergelijke mutanten aanrijken. Hierbij moet er naar gestreefd worden dat gedurende een behandeling, de fluoroquinolone concentraties in het bloed en de weefsels voldoende hoog blijven, zodat ook mutanten met verminderde gevoeligheid toch geïnhibeerd worden en niet geselecteerd worden.

CURRICULUM VITAE

Maja Marien werd op 17 januari 1979 geboren te Wilrijk. Na het beëindigen van haar studies secundair onderwijs, richting Wiskunde-Wetenschappen, aan het Koninklijk Atheneum te Lier, startte zij in 1997 met de studies Diergeneeskundige Wetenschappen aan de Universiteit van Antwerpen (RUCA). In 2000 behaalde zij het diploma van kandidaat in de diergeneeskunde met onderscheiding. De studies werden voortgezet aan de Universiteit van Gent, waar zij in 2003 met grootste onderscheiding afstudeerde als dierenarts.

Vanaf september 2003 tot juli 2006 trad zij in dienst als wetenschappelijk medewerker bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten. Gedurende bijna drie jaar werkte zij aan een onderzoeksproject "Pathogenese van infecties met *Ornithobacterium rhinotracheale*, *Escherichia coli*, *Mycoplasma gallisepticum* en aviair metapneumovirus bij kalkoenen", dat gesteund werd door Bayer HealthCare AG, Animal Health. Gedurende enkele maanden werd zij benoemd als assistent Bacteriologie.

In 2006 voltooide zij haar doctoraatsopleiding in de Diergeneeskundige Wetenschappen en behaalde zij aan de Faculteit Psychologie en Pedagogische Wetenschappen haar diploma in de Academische initiële lerarenopleiding.

Sedert augustus 2006 is zij tewerkgesteld als Technisch Manager Pluimvee bij Alpharma Animal Health.

Maja Marien is auteur of medeauteur van meerdere publicaties in internationale tijdschriften. Zij was ook spreker op verschillende internationale congressen.

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ABSTRACTS AND PROCEEDINGS ON NATIONAL AND INTERNATIONAL MEETINGS

- Efficacy of Baytril® and other antimicrobial products in *ORT* or *ORT*/*E. coli* challenged turkeys using a recently developed challenge model. **Marien, M.**, Decostere, A., and Nauwynck, H. 4[th] International Poultry Symposium. Istanbul, Turkey, august 20, 2005.

- Synergy between avian pneumovirus and *Ornithobacterium rhinotracheale* in turkeys. **Marien, M.**, Decostere, A., Martel, A., Chiers, K., Froyman, R., and Nauwynck, H. 14[th] World Veterinary Poultry Congress. Istanbul, Turkey, august 22-26, 2005.

- Experimental infection with *Escherichia coli* O2:K1, *Ornithobacterium rhinotracheale* or both in turkey poults pre-infected with avian pneumovirus and evaluation of the efficacy of different antimicrobial treatments using this model. **Marien, M.**, Nauwynck, H., Duchateau, L., Chiers, K., Froyman, R., and Decostere, A. 6[th] International Symposium on Turkey Diseases. Berlin, Germany, 11th - 13th May 2006.

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- Efficacy of Baytril® and other antimicrobial products in *ORT* or *ORT*/*E. coli* challenged turkeys using a recently developed challenge model. **Marien, M.**, Decostere, A., and Nauwynck, H. 4[th] International Poultry Symposium. Istanbul, Turkey, august 20, 2005.

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- Experimental infection with *Escherichia coli* O2:K1, *Ornithobacterium rhinotracheale* or both in turkey poults pre-infected with avian pneumovirus and evaluation of the efficacy of different antimicrobial treatments using this model. **Marien, M.**, Nauwynck, H., Duchateau, L., Chiers, K., Froyman, R., and Decostere, A. 6[th] International Symposium on Turkey Diseases. Berlin, Germany, 11th - 13th May 2006.

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