The pathogenesis of colibacillosis in broilers infected with virulent or vaccine strains of infectious bronchitis virus

De pathogenese van colibacillose in vleeskuikens geïnfecteerd met virulente en vaccin stammen van het infectieuze bronchitis virus

(met een samenvatting in het Nederlands)

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Voor Albert van Achter,

mijn grootvader zaliger, die mij, tegen de wens van mijn ouders in,
(mama en papa vonden een studie rechten of economie geschikter voor mij)
heeft vergezeld bij mijn inschrijving aan de faculteit diergeneeskunde.

Voor Jo (Van Eck) en Marius (Dwars),

de éminences grises van de Nederlandse pluimveewetenschappen,
die mij naast hun fenomenale kennis over pluimvee,
een schat aan levenswijsheid hebben bijgebracht.

Nihil volentibus arduum

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Chapter 1

General introduction

Broiler industry

Poultry is an important source of meat, worldwide, and the consumption of poultry products is still increasing. According to Pearson and Dutson (1997) and De Haan *et al.* (2001) this is due to increasing human population, religion (most world religions do not prohibit the consumption of poultry meat), the western beauty image (low fat percentage in poultry meat) and women working out doors in the western world (poultry meat is fast and easy to cook). Also increasing welfare in countries with enormous human populations like China and India plays an important role. People have more money to spend and, moreover, poultry meat is a rather cheap source of protein.

Broiler breeds are selected for their rapid growth: nowadays, broiler chicks are growing from 50 g at day of hatch to more than 2 kg within 6 weeks. This genetic selection in combination with production in industrialized systems has fulfilled the increasing worldwide demand for poultry meat: over the last 40 years growth rate of broilers has more than doubled (Julian, 1998). This rapid growth, however, poses these birds for high demands. The greater part of ingested energy is invested in growth, and Rauw *et al.* (1998) concluded that broilers, selected for high production efficiency, seem to be more at risk for welfare and health problems. Among other reasons, the rapid growth and high flock density might explain the frequent occurrence of a variety of health problems in broilers (Barlett, 1988). In this context, infectious diseases are important in broiler industry due to increased mortality, growth retardation, and the curative and preventive use of antibiotics and chemotherapeutics. Moreover, economic losses may result from loss of uniformity of the flock and condemnations in the slaughterhouse (Goren, 1991; Vandemaele *et al.,* 2002; McKissick, 2006).

Worldwide, colibacillosis is one of the most important causes of economic losses for broiler industry (Gross, 1991; Yogaratnam, 1995).

Colibacillosis

According to Barnes *et al.* (2003), colibacillosis in chickens refers to any localized or systemic infection caused entirely or partly by *Escherichia coli (E. coli)*. *E. coli* is a gram-negative, flagellated bacterium, which is part of the normal flora in the

digestive tract of chickens. In general *E. coli* is of low pathogenicity for chickens (Hofstad *et al*., 1978; Nakamura *et al*., 1992), but pathogenic avian strains of *E. coli* (APEC) are identified, which belong to a small range of serotypes including O78:K80, O1:K1 and O2:K1 (Hofstad *et al*., 1978; Wray *et al*., 1996; Mead and Griffin, 1998).

Broilers suffering from colibacillosis are depressed, show respiratory distress and growth retardation. Mortality usually remains below 5%, but morbidity often reaches more than 50% (Wray *et al.,* 1996; Vandekerckhove *et al.,* 2004). Several pathological signs due to an infection with an *E. coli* strain can be distinguished in poultry: septicemia, granuloma, inflammation of air sacs, cellulitis, swollen head syndrome, peritonitis, salpingitis, osteomyelitis, panophalmitis and omphalitis/yolk sac infection. More detailed information about *E. coli* infections is reviewed by Landman and Cornelissen (2006). In broilers, colibacillosis mainly results in respiratory infections (airsacculitis) and peritonitis/ pericarditis (Gross, 1961; Goren, 1978; Pourbaksh *et al.,* 1997).

Day-old chicks may become infected via the yolk sac, but in older chicks the infection is considered to be mainly airborne. Young broiler chickens up to three weeks of age are highly susceptible to the disease, but chickens of four weeks and older are considered quite resistant to primary colibacillosis (Goren, 1978). However, various risk factors may increase the susceptibility of broilers to colibacillosis, e.g. respiratory viruses and environmental factors like dust and high concentrations of NH_3 and CO_2. In addition, the *E. coli* concentration in the air of the broiler house is an important factor. In general this concentration is highest in the second half of the production period due to increased production of droppings; and especially in winter due to limited ventilation. Airborne dust particles in broiler houses can contain 10^5-10^6 *E. coli* bacteria/g and these bacteria may persist for long periods (Harry and Hemsley, 1965). As a consequence, colibacillosis most frequently occurs in the second half of the production period in flocks reared in the cold season.

Colibacillosis is mainly treated with antibiotics, but the use of these drugs is costly and the period in which broilers can be treated is limited because of the withdrawal period. Moreover, treatment often does not result in sufficient recovery before slaughter (Goren, 1991). So, colibacillosis is an often reported observation in slaughterhouses, and responsible for a considerable part of condemnations at processing (Yogaratnam, 1995).

As stated, several risk factors for colibacillosis have been identified, but their importance may vary. Often reported predisposing factors are infections of the respiratory tract by pathogens like Infectious Bronchitis virus (IBV), Newcastle disease virus (NDV), Avian pneumovirus (APV) and *Mycoplasma gallisepticum* (Mg) (Goren, 1991; Nakamura *et al.,* 1992; Turpin *et al*., 2002; Vandekerchove *et al.,* 2004). In the Netherlands, IBV is probably the most important triggering factor for colibacillosis in broilers as the virus is isolated frequently in many regions of the country; the latter in contrast to the other respiratory pathogens (J.J. de Wit, personal communication)

Infectious Bronchitis

The term "Infectious bronchitis" (IB) was introduced by Schalk and Hawn in 1931 to describe the clinical and pathological features of a transmissible respiratory disease of chicks in the USA. Beach and Schalm (1936) isolated the aetiological virus, which was unrelated to any other virus known at that time and was named 'infectious bronchitis virus'. The first propagation of the virus in embryonated chicken eggs was reported in 1937 by Beaudette and Huston.

The virus is round to pleomorphic in shape. It has an envelope with club-shaped surface projections named spikes. Based on these spikes, the virus was classified as a coronavirus, from "corona", the Latin word for crown. Initially it was assumed that only a single serotype of IBV existed: the Massachussets serotype represented by the M41 strain. However, Jungherr *et al.* (1956) reported the isolation of another strain i.e. the Connecticut 46 strain, which differed serologically from the M41 strain. Since then, numerous publications from all over the world have shown a wide variety of serologically different IBV strains (variant serotypes) (e.g. Dawson and Gough, 1971; Lohr, 1976; Doi *et al.,* 1982; Locher *et al.,* 1983*a,b*; Cook, 1984; Davelaar *et al*., 1984).

The virus predominantly affects the respiratory tract, but beside it also the intestinal tract, the kidneys and the oviduct. IB may result in reduced feed consumption and weight gain. Young chicks (up to 4-5 weeks of age) show clinical signs after IBV infection: mucous nasal discharge, sneezing, tracheal rales and wet eyes (Hofdstad, 1984). Especially mucous nasal discharge is a typical sign of infection with a virulent IBV strain and often affects 100% of the birds of an infected flock (Matthijs *et al.,* 2003).

Vaccines have been developed to reduce clinical signs and economic losses due to IB and they are widely applied. IBV strains attenuated by chicken embryo passage are used as vaccines. These live vaccines are usually applied by spray and they hardly induce clinical signs, such as mucous discharge (Gough and Alexander, 1979). The vaccines are able to induce good clinical protection against a field virus infection of the same serotype as the vaccine strain, but vaccination may fail when infection with strains of other serotypes occurs (Davelaar *et al*., 1984; Cavanagh and Naqi, 1997)

Generally, broilers are vaccinated at day of hatch or at approximately 14 days of age. Although IB vaccine viruses do not seem to be virulent, they are still able to replicate in respiratory epithelia (Cavanagh, 2003; Cavanagh, 2007). Farsang *et al.* (2002) in Sweden and Meulemans *et al*. (2001) in Belgium isolated IBV vaccine like strains from flocks that had not been vaccinated against IB. These findings suggest that vaccine strains just like virulent strains are able to spread between flocks. This is supported by the results of an inventory study in 10 broiler flocks in The Netherlands by Lambers *et al.* (2000). They found antibodies to IBV in all tested birds, although IB vaccination had not been applied and nasal discharge had not been noticed in these birds. The condemnation rate due to colibacillosis in these broiler flocks was, however, rather high. This might imply that not only virulent IBV could be a predisposing factor for colibacillosis, but also IB vaccine virus.

Furthermore it is important to notice that Dutch broiler farmers vaccinate against IB not only to prevent clinical signs of this disease but also to combat increasement of susceptibility for colibacillosis in case the birds become infected with virulent IBV. The foregoing considerations resulted in three research questions as given in “scope of the thesis” (questions 1, 2 and 3).

Scope of the thesis

The research approach was experimental, to exclude variation due to other factors than IB.
First, the following three questions have been investigated:

1. The first research question (Chapter 2) was to assess the ability of IB vaccine virus to enhance the susceptibility for colibacillosis in comparison with this ability of virulent IBV. In this study an infection was carried out with a virulent IBV strain (D387 or M41) or an IBV vaccine strain (H120 or H52), followed by infection with *E. coli*. Clinical signs and pathological findings after the superinfection were recorded and compared.
2. The second research question to be investigated was whether an IBV vaccine virus (H120) was able to spread in a flock (Chapter 3). Only if vaccine viruses can spread efficiently, they could be a potential risk factor for the occurrence of colibacillosis, as only then they might be able to spread from one flock to another. For this study, a transmission experiment was carried out with a vaccine strain (H120) and a virulent strain (M41). The reproduction ratio was calculated from the number of contact-infections in each group.
3. The third research question was to determine whether vaccination with IBV vaccine was able to protect broilers against an *E. coli* superinfection after infection with a virulent IBV virus (Chapter 4). In this experiment broilers were vaccinated against IB (H120); four weeks later birds were infected with a virulent IBV strain (M41) of the same serotype as the vaccine virus followed by superinfection with *E. coli*. Clinical signs and pathological findings after the superinfection were recorded and compared between vaccinated and unvaccinated birds.

Secondly, underlying mechanisms of the increased susceptibility for colibacillosis after IBV infection were studied. A better understanding of the processes involved in this phenomenon might contribute to develop other types of vaccines, or to develop intervention measures with respect to prevention of colibacillosis. In addition, a suitable animal model in which the pathogen-host interactions can be studied using 'natural' pathogens, might contribute to the understanding of superinfections more in general.

Two presumed mechanisms by which IBV may facilitate the occurrence of colibacillosis were investigated: damage of the epithelium due to replication of IBV and/or by means of an immune reaction of the host. For this purpose, immunological and histological changes following a superinfection with *E. coli* were investigated; more in detail:

4. The development of histological lesions in the respiratory tract after a single *E. coli* infection compared to infection with *E. coli* following previous IBV infection, was studied (Chapter 5).
5. The dynamics of the infection with IBV followed by the *E. coli* infection in combination with the dynamics of the immunological response was determined: various cell types involved in the immune response were stained in histological slides, and the location of the two pathogens in various tissues was determined (Chapter 6).
6. The dynamics of pro-inflammatory cytokines and the functional activity of macrophages were determined (Chapter 7).

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Chapter 2

Ability of Massachusetts type Infectious Bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus

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Summary

The ability of Massachusetts type vaccine virus and virulent infectious bronchitis (IB) field virus to increase colibacillosis susceptibility were compared. In four experiments, female commercial broilers housed in isolators, were infected at 29 days of age intratracheally (i.t.) and oculonasally with IB vaccine strains (H120 and H52) or virulent IB field strains (D387 and M41) (4.8 or 6.8 $\log_{10}$ EID_{50}/broiler). Five days later *E. coli* 506 strain was given i.t. (5.6 to 8.8 $\log_{10}$ cfu/broiler). The incidence of nasal discharge at 3 and 5 days after IB virus infection was the parameter used to assess the clinical effect of the IB infection, while mortality, body weight uniformity and *E. coli* lesions at 7 days following *E. coli* inoculation were used as parameters for colibacillosis. Nasal discharge was observed in 6/117 (5%), 26/119 (22%), 35/119 (29%) and 115/120 (96%) of broilers infected with H120, H52, D387 and M41 virus respectively. Apart from H52 and D387, differences between IBV strains were significant. In contrast, generally IB vaccine viruses and virulent IB viruses did not differ significantly in their ability to induce colibacillosis susceptibility. Mean colibacillosis lesion score (MLS) of H52 virus infected birds (range: 3.7 to 5.4) even significantly exceeded the MLS of birds infected with the other IB viruses (range: 1.9 to 4.2). The ability of H120 virus to induce colibacillosis susceptibility tended to be the weakest. The practical consequences of these findings are discussed.

Introduction

Colibacillosis (fibrinous polyserositis) in commercial broilers is of major clinical and economic importance. The disease mainly occurs in the second half of the growing period following respiratory tract damage. Amongst other factors, poor climatological house conditions, infections with respiratory viruses and/or mycoplasmas might cause damage of the respiratory tract, resulting in increased susceptibility to colibacillosis (Goren, 1978; 1991; Gross, 1990; Dho-Moulin and Fairbrother, 1999). Amongst respiratory infections of chickens, infectious bronchitis virus (IBV) plays a dominant role (Yoder *et al*., 1989; Nakamura *et al*., 1992; Cook and Mockett, 1995; Glisson, 1998). Under experimental conditions, both infections with field or vaccine strains of IBV increase colibacillosis susceptibility in chickens (Goren, 1978; Smith *et al*., 1985; Cook *et al*., 1986). As live IBV vaccine strains, such as H120 and H52, are widely used (Sharma, 1999), they might play an important role in the induction of colibacillosis in the field. This seems especially relevant to broilers, as these birds are more sensitive to colibacillosis than layers (Goren, 1991). Therefore, it is of interest to compare the ability of IB vaccine and field viruses to increase susceptibility to colibacillosis.

Although some work has been done previously on *E. coli* susceptibility in chickens in combination with IB vaccine (H120; H52) and virulent IB virus (M41) (Smith *et al*., 1985; Cook *et al*., 1986), we considered it necessary to perform more research in commercial broilers for the following reasons. Smith *et al*. (1985) and Cook *et al*. (1986) inoculated specified pathogen free (SPF) Rhode Island Red (RIR) chickens with vaccine or M41 virus in combination with a pool of *E. coli* strains in order to develop an IBV-*E. coli* infection model and to examine the efficacy of IB vaccination with the aid of this model, rather than to compare vaccine strains with virulent IB strains for their ability to increase colibacillosis susceptibility.

Moreover the result of their studies were conflicting: Smith *et al*. (1985) found no distinct differences in mortality after intranasal infection of 9-day old SPF RIR chicks with mixtures of H120, H52 or M41 virus and a pool of *E. coli* strains, while Cook *et al*. (1986), using the same infection model, observed mortality rates in 25-day-old SPF RIR chicks of 14% and 73% following infection with H120 and M41 virus in combination with the *E. coli* pool, respectively. Additionally, published research on this theme in broilers is lacking and results obtained in SPF RIR chicks cannot be extrapolated to commercial broilers as the strain of chicken

used might be of importance (Smith *et al*., 1985). Finally, as both IBV and *E. coli* are probably mainly airborne (Goren, 1978; 1991; Cook and Mockett, 1995; Glisson, 1998; Dho-Moulin and Fairbrother, 1999), intranasal inoculation alone, as performed by Smith *et al*. (1985) and Cook *et al*. (1986), might result in insufficient exposure of the lower respiratory tract to the pathogens. Therefore in our study IBV was applied intratracheally (i.t.) and oculonasally and *E. coli* was applied i.t.

The present study aimed to compare Massachusetts type IB vaccine and virulent field viruses in their ability to increase colibacillosis susceptibility in a broiler breed of chicken. Two IBV vaccine strains H120 and H52 and two virulent IBV field strains D387 (Davelaar and Kouwenhoven, 1976; Davelaar *et al*., 1984) and M41 were used.

Material and methods

Experimental chickens

For each of 4 experiments, eighteen days incubated hatching eggs originating from a *Mycoplasma gallisepticum* free broiler parent (Cobb) flock, were obtained from a commercial hatchery and subsequently hatched at the Spelderholt Poultry Research Centre in Beekbergen, the Netherlands. At day of hatch (= day 0) chickens were sexed cloacally. As body weight was one of the parameters only female chickens were used.

Housing, feeding and management

From day 1, chicks were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) with a volume of 1.3 m^3 and fitted with a wire floor of 1.05 m^2. Isolators were ventilated at a rate of approximately 30 $m^3 h^{-1}$.

Chickens were fed a commercial ration containing 12.4 MJ of metabolisable energy per Kg and 19.5% crude protein. To diminish leg disorders and hydrops ascites, after a period of *ad libitum* supply, the feed was restricted on 'skip a day base'. Restriction was approximately 15% of the *ad libitum* intake. Skip a day feeding started at day 18 in experiment 1; at day 16 in experiment 2 and at day 15 in experiments 3 and 4.

In experiment 1, birds were fed until removed from the isolators at day 41; in the other experiments feed was withdrawn at day 40 at 16:00 h. Tap water was

provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h, thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35ºC at day 1 to 20ºC at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20ºC.

Inocula

IB vaccines were obtained as commercial freeze-dried 1000 doses vials of H120 (Nobilis IB H120, batch 90046C containing $10^{8.4}$ EID_{50} of virus per vial; Intervet, Boxmeer, The Netherlands) and H52 (Nobilis IB H52, batch 91056B, containing $10^{7.7}$ EID_{50} of virus per vial). Virus titres were kindly provided by Intervet, Boxmeer, The Netherlands.

The IB D387 strain used, is a virulent field strain of the Massachusetts serotype (Davelaar *et al*., 1984; Kusters *et al*., 1987) and was isolated in The Netherlands by Davelaar and Kouwenhoven (1976). Allantoic fluid of SPF eggs, yielding IB D387 virus in its 8th egg passage and with a virus titre of $10^{7.0}$ EID_{50}/ml was prepared by the Dutch Animal Health Service (Deventer, The Netherlands), and stored at –70^{o}C until used. The virulent Massachusetts IB M41 strain was obtained from Intervet as virus-containing allantoic fluid, with a virus titre of $10^{8.3}$ EID_{50}/ml. The virus had undergone 10 passages in SPF embryonated eggs. Allantoic fluid was stored at –30^{o}C until used. All IBV inocula were prepared in *aqua destillata* just prior to use, in such a manner that inocula with virus titres of $10^{4.7}$ EID_{50}/ml or $10^{6.7}$ EID_{50}/ml were obtained.

The *E. coli* strain 506 (O78; K80) is a flumequine resistant strain isolated from an inflamed pericardium of a commercial broiler suffering from natural colibacillosis (Van Eck and Goren, 1991). The ability of the *E. coli* 506 strain to cause colibacillosis in SPF chickens and commercial broilers, which had been exposed to live IB vaccine or Newcastle disease vaccine virus, was demonstrated by Goren (1978) and Van Eck and Goren (1991). Colibacillosis could be induced following i.t. inoculation up to at least 28 days of age.

The inocula were prepared by submersing one frozen bead (-70^{o}C) of a batch containing the *E. coli* 506 strain in a required volume of 0.1% glucose broth. After 20 h of incubation at 37^{o}C tenfold dilutions were made using PBS according to international standards (ISO6887, 1983).

In experiments 1 and 2, cultures diluted 1:10 were used, resulting in concentrations of $10^{7.8}$ and $10^{8.8}$ colony forming units (cfu)/ml respectively. In

experiments 3 and 4, the cultures were diluted 1:1000. The final concentrations were $10^{5.6}$ cfu/ml. Control of the bacterial concentrations was performed according to international standards (ISO7402, 1985).

Experimental design and inoculations

Four experiments were performed. At day 1, female chicks were individually tagged and weighed. Per experiment ten groups, each consisting of fifteen birds, were formed. As body weight was one of the parameters used, groups were given the same body weight distribution at day 1. Each group was housed in a separate isolator.

Data on IBV and *E. coli* inoculations are given in Table 1. At day 29 IBV inoculation was performed by the oculo-nasal route, allowing one droplet with a volume of approximately 0.05 ml in each eye and nostril, and i.t. (1.0 ml per bird). Each of the IBV strains used (H120, H52, D387 and M41) was given to birds in two isolators, where a different dose was used in each isolator (6.8 $\log_{10}$ EID_{50}/broiler or 4.8 $\log_{10}$ EID_{50}/broiler). The *E. coli* 506 strain was administered i.t. at day 34 in a volume of 1.0 ml/bird. In experiments 1 and 2 high *E. coli* doses were administered; whereas a lower dose was given in experiments 3 and 4 (see Table 1). An *E. coli* control group (Table 1; group 9) and a placebo group (Table 1; group 10) were included in each experiment.

I.t. application of IBV and *E. coli* was performed using a 1.0 ml syringe fitted with a blunt-ended pipette tip (Corning, N.Y., cat. no. 4862, USA). Assessment of clinical signs, mortality, body-weight, antibody titres to IB M41 and *E. coli* lesion scores was performed as given below. Experiments ended at day 41.

Clinical observations

Mortality was recorded daily. Dead birds were stored at –20^{o}C until postmortem examination at day 41. Clinical signs resulting from IB infection were determined at days 29, 32, 34 and 41 (at day of IBV inoculation and 3, 5 and 12 days later) by individual scoring of birds with mucous nasal discharge. Hereto mild pressure was applied directed from the nasal bone region towards the nostrils.

Table 1. Experimental design: IBV and E. coli 506 inocula, doses and administration routes used in four experiments to study the effect of IBV on induction of colibacillosis in broiler chickens

IBV oculonasally (one drop in each eye and nostril) and intratracheally (1 ml/bird) at 29 days of age			E. coli 506 intratracheally (CFU [log10]/broiler in 1 ml) at 34 days of age		
Experiments 1 to 4			Experiment 1[b]	Experiment 2[b]	Experiments 3 and 4[c]
Group[a]	IBV strain	EID_{50}/broiler $_{(log10)}$			
1	H120	6.8	7.8	8.8	5.6
2	H120	4.8	7.8	8.8	5.6
3	H52	6.8	7.8	8.8	5.6
4	H52	4.8	7.8	8.8	5.6
5	D387	6.8	7.8	8.8	5.6
6	D387	4.8	7.8	8.8	5.6
7	M41	6.8	7.8	8.8	5.6
8	M41	4.8	7.8	8.8	5.6
9	Distilled water	-	7.8	8.8	5.6
10	Distilled water	-	PBS-diluted glucose broth	PBS-diluted glucose broth	PBS-diluted glucose broth

[a]Number of birds per group = 14 or 15; each group was housed in a separate isolator.
[b]A 20 h glucose broth culture of E. coli 506 diluted 10^{-1} in phosphate-buffered saline (PBS) was used.
[c]A 20 h glucose broth culture of E. coli 506 diluted 10^{-3} in PBS was used.

Clinical inspections were done blindly. A bird was recorded as having nasal discharge due to IBV infection, when this clinical sign was observed on either one or both sides and either on day 32 or day 34 or on both occasions. From day 29 (day of IBV inoculation) feed troughs were checked daily for the presence of feed as feed consumption was used as parameter of disease.

Body weight and body weight uniformity
Birds were weighed individually at day 29 (day of IBV inoculation), at day 34 (day of *E. coli* inoculation) and at day 41 (end of experiment). Then the body weight uniformity was calculated. Body weight uniformity is defined as the percentage of birds with a body weight between mean body weight minus 10% and mean body weight plus 10%.

Serological examination

For each hatch, maternally derived haemagglutination inhibition (HI) antibodies to IBV M41 were determined on 24 surplus chickens at day-old. Blood samples were collected after decapitation. At day 29 (day of IBV inoculation) and day 41 (end of experiment) all broilers were vein punctured (*vena ulnaris* at day 29 and *vena jugularis* at day 41), and blood samples were also examined for HI antibodies against IBV M41. The HI test using 8 haemagglutinating units was performed as described by Alexander and Chettle (1977). Titres are expressed as $\log_2$ of the reciprocal value of the highest serum dilution showing complete HI. Titres exceeding $\log_2$ 4 were considered positive.

Postmortem examination

At day 41 the experiments were ended. Birds were humanely killed and terminal blood samples were collected. Colibacillosis lesions were scored macroscopically in the following organs: right thoracic airsac, left thoracic airsac, pericardium and liver. Thoracic airsac lesions were considered as representative for *E. coli* pathology of the respiratory tract. Birds with pericardium and/or liver lesions are referred to as systemically infected.

Lesion scoring was performed as described by Van Eck and Goren (1991) using the following criteria: 0 = no lesions, 0.5 = one yellow/brown pinhead-sized spot indicative of inflammation, 1 = two or more pinhead-sized spots as described at score 0.5, 2 = thin layer of fibrinous exudation on various locations, 3 = thick and extensive layer of fibrinous exudation. The maximum score per bird is twelve. Mean lesion scores per group were calculated. Postmortem examination was performed blind. Birds that died during the experiment were also necropsied.

In each of the experiments, bacteriological analysis was performed of spleen, thoracic airsac, heart and liver of 10 birds. Up to a maximum of 5 birds, that died during the course of the experiment and lacked macroscopical *E. coli* lesions were selected. To complete the 10 birds for bacteriology, the missing chickens were selected from the surviving birds showing *E. coli* pathology at postmortem. Apart from the selection criteria stated, birds for bacteriological examination were collected *ad random*.

Samples were cultured on sheep blood agar plates (ALVOE 009, bioTrading, Mijdrecht, The Netherlands) and incubated overnight (37^{o}C ± 1). Colonies were identified using Bactident® *E. coli* (Merck, no. 13303, Amsterdam). Thereafter the antibiotic sensitivity profile of isolates was established following the ICS guideline

(Ericsson and Sherris, 1971). Isosensitest agar plates (ALVOE 033, bioTrading) and Neo-Sensitabs® (Rosco, no. 21460, Taastrup, Denmark) were used. The following antibiotics were tested: ampicillin, tetracycline, flumequine, enrofloxacine, trimethoprim-sulfa and neomycin. The MIC-breakpoints were established as recommended by the Dutch Working Group on Guidelines for Sensitivity Criteria (Mouton and van Klingeren, 1981).

Statistical analysis
Data were subjected to analysis of variance (ANOVA) in the R programme with treatment as main effect. The experiment was included in the statistical model as an additional independent variable, because the data originated from four experiments. Moreover, interaction terms were also taken into account by the model. Treatment means were tested for statistically significant differences with the use of Turkey HSD at 95% confidence level.

Ethics
Birds were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

Results

As, for each experiment none of the parameters differed significantly between groups inoculated with different doses of the same IBV strain, results of these groups are presented combined.

The number of birds with nasal discharge and M41 HI titres is presented in Table 2; mortality following *E. coli* 506 inoculation, body weight uniformity at day 41 and *E. coli* lesion scores at day 41 is given in Table 3.

Mortality
In the period between IBV and *E. coli* administration (day 29 to 34) only one chick died at 5 days following IBV M41 inoculation due to fibrino-bronchopneumonia. Mortality following *E. coli* 506 administration is given in Table 3.

Table 2. Nasal discharge and mean M41 HI (log_2) titre in female commercial broiler chickens inoculated with IBV vaccine or field virus at 29 days of age followed by *E. coli* 506 administration at 34 days of age.

Group	1+2	3+4	5+6	7+8	9	10
IBV strain	H120	H52	D387	M41	Aq. dest.	Aq. dest.
E. coli 506	+[a]	+	+	+	+	PBS diluted glucose broth
Size of groups at 29 days of age	28 to 30	29 or 30	29 or 30	30	14 or 15	15
Experiment	Broilers with nasal discharge at 32 and/or 34 days of age					
1	0	6	10	25	0	0
2	1	3	5	30	0	0
3	0	7	10	30	0	0
4	5	10	10	30	0	0
Anova[b]	A	B	B	C	A	A
Experiment	Mean M41 HI (log_2) titre ± s.d. at 41 days of age					
1	5.4 ± 1.6	7.9 ± 1.9	6.9 ± 1.5	9.3 ± 1.0	3.0 ± 0.0	3.0 ± 0.0
2	5.0 ± 1.5	7.0 ± 1.0	7.3 ± 1.3	8.6 ± 1.3	3.0 ± 0.0	3.0 ± 0.0
3	4.3 ± 1.4	5.8 ± 0.9	5.7 ± 1.2	5.5 ± 0.9	3.0 ± 0.0	3.0 ± 0.0
4	5.8 ± 1.0	7.4 ± 1.3	7.5 ± 1.5	9.8 ± 0.9	3.1 ± 0.4	3.0 ± 0.0
Anova[c]	A	B	B	C	D	D

[a] *E. coli* 506 inoculated

[b,c] Columns with different capital letter differ significantly ($P < 0.05$).

The mortality in all experiments combined following *E. coli* administration was 10/117 (9%), 10/119 (8%), 4/119 (3%) and 11/119 (9%) in birds inoculated with H120, H52, D387 and M41 respectively. In the *E. coli* control groups (group 9) and the placebo groups (group 10) mortality was 3/59 (5%) and 0/60 (0%) respectively. The total mortality in experiments 1 and 2 (high *E. coli* dose) following *E. coli* inoculation was 24; 6 of these birds died within 48 h of *E. coli* inoculation. In experiments 3 and 4 (lower *E. coli* dose) total mortality following *E. coli* inoculation was 14, of which 7 birds died within 48 h of *E. coli* inoculation. Neither the effect of the IBV treatment nor the effect of the *E. coli* treatment were statistically significant.

Clinical disease signs

At day 29 (day of IBV inoculation) nasal discharge was not observed. The number of broilers with nasal discharge at 32 and/or 34 days of age is given in Table 2. Almost all M41 inoculated birds developed nasal discharge while none or only a low percentage of the birds given H120 did. The number of birds with nasal discharge following H52 or D387 inoculation was in between.

Apart from H52 and D387, the IBV strains differed significantly from each other in their ability to induce nasal discharge. The number of birds with nasal discharge at day 34 was equal to or greater than at day 32. In this regard no obvious differences were found between IBV strains. At day 41 (end of experiments) nasal discharge was not observed anymore. All groups finished their allowance of feed, but IBV and *E. coli* inoculated chickens took longer to do so.

Body weight, body weight uniformity and postmortem examination

The mean body weight of day-old chicks was 43 g in experiments 1, 2 and 4, and 47 g in experiment 3. At day 29, 34 and 41 the mean body weight of groups did not differ significantly within and between experiments. The mean body weight of all groups at day 29, 34 and 41 was in order of magnitude of 1.0, 1.2 and 1.5 kg respectively. The body weight uniformity of all groups at days 29 and 34 ranged from 79 to 100. Differences were not significant. Body weight uniformity and *E. coli* lesion scores at day 41 are given in Table 3.

Depending on the *E. coli* dose, in groups only inoculated with this bacterium (group 9) mean lesion scores varied from 0.1 to 1.2, the percentage of affected birds from 7 to 23 and the percentage of chicks with systemic *E. coli* infection from 0 to 14.

Table 3. Mortality, body weight uniformity and colibacillosis lesions in female commercial broiler chickens inoculated with IBV vaccine or field virus at 29 days of age followed by *E. coli* 506 administration at 34 days of age. In experiments 1 and 2 a high *E. coli* dose was applied; in experiments 3 and 4 a lower dose was given. At 41 days of age the experiments were ended and examinations were performed.

Group	1+2	3+4	5+6	7+8	9	10
IBV strain	H120	H52	D387	M41	Aq. dest.	Aq. dest.
E. coli 506	+[a]	+	+	+	+	PBS diluted glucose broth
Size of groups at 29 days of age	28 to 30	29 or 30	29 or 30	30	14 or 15	15
1	1	2	0	2	2	0
2	6	5	2	3	1	0
3	3	1	1	4	0	0
4	0	2	1	2	0	0
ANOVA[c]	A	A	A	A	A	A
Experiment	Body weight uniformity[d]					
1	79	57	59	44	100	93
2	63	42	57	56	71	100
3	70	83	79	84	100	93
4	63	61	66	71	86	87
ANOVA[e]	A,B	A	A	A	B	B
	Mean colibacillosis lesion score of surviving broilers					
1	1.9	4.6	2.8	3.1	0.5	0.0
2	3.5	5.4	4.2	3.8	1.2	0.0
3	2.1	3.9	2.1	2.5	0.1	0.1
4	2.6	3.7	2.3	3.6	0.1	0.0
ANOVA[f]	A	B	A,C	C	D	D

Group	1+2	3+4	5+6	7+8	9	10
IBV strain	H120	H52	D387	M41	Aq. dest.	Aq. dest.
E. coli 506	+[a]	+	+	+	+	PBS diluted glucose broth
Size of groups at 29 days of age	28 to 30	29 or 30	29 or 30	30	14 or 15	15
			Percentage of surviving broilers with colibacillosis			
1	59	96	83	89	23	0
2	82	100	86	93	21	0
3	81	93	90	96	7	7
4	83	100	86	100	7	0
ANOVA[g]	A	B	A,B	B	C	C
			Percentage of surviving broilers with systemic colibacillosis			
1	4	36	24	19	8	0
2	36	54	50	37	14	0
3	11	17	10	4	0	0
4	23	29	14	29	0	0
ANOVA[h]	A,C	A	A	A,C	B,C	B

[a]*E. coli* 506 inoculated.
[b]In the period between IBV and *E. coli* 506 administration only one chick died (exp. 3; group 7).
[d]Percentage of birds with a body weight between mean body weight plus 10% and mean body weight minus 10%.
[c,e-h]Columns with different capital letter differ significantly ($P < 0.05$).

Body weight uniformity at day 41 varied from 71 to 100 between experiments. The placebo groups (group 10) showed no *E. coli* lesions (except one bird in experiment 3: both thoracic airsacs scored 0.5) and the body weight uniformity ranged from 87 to 100. The *E. coli* control groups (group 9) and the placebo groups (group 10) did not differ significantly in any of the parameters.

All IBV and *E. coli* inoculated groups (groups 1 to 8) showed depressed body weight uniformity, greatly increased mean *E. coli* lesion scores and a high percentage of birds with airsacculitis and systemic colibacillosis in comparison with *E. coli* control groups (group 9) and placebo groups (group 10). Generally, these differences were significant.

No significant differences were observed between IBV strains regarding body weight uniformity and percentage of chicks with systemic colibacillosis. H52 vaccine virus (groups 3 and 4) possessed the strongest ability to increase colibacillosis susceptibility considering all aspects of colibacillosis lesions evaluated. The mean lesion scores were significantly higher than those of other IBV inoculated groups. H120 vaccine virus (groups 1 and 2) seemed to have the least effect on the increase of colibacillosis susceptibility as body weight uniformity and percentage of chicks with systemic colibacillosis did not differ significantly from birds inoculated with *E. coli* alone.

An *E. coli* dose effect was established. Highest doses (experiments 1 and 2) produced lowest body weight uniformity and highest mean lesion scores. Mean lesion scores increased mainly due to an increase in the number of birds with systemic colibacillosis rather than an increase in the percent of affected birds.

In experiment 2 an *E. coli* dose of 8.8 $\log_{10}$ cfu/bird was given (highest dose of all experiments). Body weight uniformity in this experiment was significantly below the uniformity in experiment 3 and mean lesion scores were significantly above those assessed in experiments 1 and 3. Moreover, the number of birds with systemic colibacillosis in this experiment was significantly increased compared to the number of birds with systemic colibacillosis in all other experiments.

Nearly all birds that died within 48 h after *E. coli* inoculation lacked macroscopical *E. coli* pathology, but *E. coli* could be isolated from them. From 14/27 (52%) of surviving chicks with *E. coli* lesions, *E. coli* was cultured. All *E. coli* isolates showed an antibiotic sensitivity pattern identical to that of *E. coli* 506.

Serology

Mean maternally derived M41 HI antibodies ± s.d. at day-old ranged from 5.9 ± 1.4 to 10.3 ± 1.0 between experiments.

At day 29 HI IBV antibodies were not detected in any of the groups (log_2 titres ≤4), this was also the case in non-IBV inoculated groups (groups 9 and 10) at day 41. M41 HI titres at day 41 are presented in Table 2. Except for experiment 3, mean log_2 M41 titres were approximately log_2 8 to 10 in M41 inoculated birds (groups 7 and 8), log_2 7 to 8 in H52 and D387 inoculated birds (groups 3, 4, 5 and 6) and log_2 5 to 6 in H120 inoculated chickens (groups 1 and 2). Titres induced by different IBV strains differed significantly, except for H52 and D387 (groups 3, 4, 5 and 6). In experiment 3 the mean HI titres of all the IBV inoculated groups were significantly below the titres of the corresponding groups in other experiments.

Discussion

In the present study, the *E. coli* 506 strain was given i.t. as colibacillosis in broilers is probably mainly airborne (Goren, 1978; 1991; Cook and Mockett, 1995; Glisson, 1998; Dho-Moulin and Fairbrother, 1999). Physical damage as a consequence of i.t. inoculation of chickens previously given IBV by the same route might have influenced the results. However, this is unlikely, as the width of the pipette tip used for i.t. inoculations related to the width of the trachea as well as the volume of the inoculum (1 ml/bird) related to the total capacity of the respiratory system of 34 days old broilers were very small. Nevertheless, an effect of physical damage cannot be ruled out completely. Unfortunately, it is impossible to incorporate a control group to assess such an effect as birds following physical damage of the respiratory tract only will develop colibacillosis in case *E. coli* bacteria are administered as well. Physical damage of the respiratory tract can be avoided by aerosol application. However, from preliminary studies it appeared that *E. coli* doses as used in the present study could not be achieved by far within a reasonable period of time, following aerosol exposure (J.H.H. van Eck, 1994, unpublished results).

The effect of *E. coli* 506 inoculation was assessed 7 days later, as at that time the number of affected birds was expected to be highest as shown previously by Goren (1978). Smith *et al*. (1985) found the interval between the administration of IBV and of *E.coli*, within broad limits, of no importance. In contrast, both Goren (1978)

and Peighambari *et al*. (2000) found maximal effects using an interval of 4 to 6 days. Therefore, in our study an interval of 5 days was used. Since *E. coli* 506 inoculation was performed on a fixed day (5 days after IBV inoculation), results obtained in this study might have been influenced by differences in dynamics of infection between IB viruses i.e. results might have been influenced in case tissue virus titres, and as a consequence tissue damage did not develop synchronically after infection with different IBV strains. However, this is unlikely. Darbyshire (1985) found no significant differences in virus yield in the trachea of SPF RIR chickens four days after i.t. challenge with either H120, H52 or M41 virus. Moreover, in our study all IBV infected groups showed maximum numbers of birds with nasal discharge at the same moment i.e. at five days after inoculation.

Whether or not the *E. coli* doses used in the present study mimic field circumstances in not easy to judge. During the second part of the growing period, total bacterial counts of 10^7 to 10^8 per m^3 house air were obtained in broiler houses, while 10^3 to 10^4 *E. coli* bacteria per m^3 air were detected (Harry and Hemsley, 1965; Carlson and Whenham, 1968). Goren, also isolated 10^3 to 10^4 *E. coli* bacteria per m^3 house air and considered these bacteria as pathogenic as gas production was observed during culture in McConkey medium at 42^{o}C (Goren, Poultry Health Service Doorn, personal communication, 1981). Considering the tidal respiratory volume of a 4- to 5-week-old broiler at 20 l per h (Powell, 2000), an *E. coli* uptake of 10^1 to 10^3 cfu/h per bird was estimated. This dose is below the doses used in the present study ($10^{5.6}$ to $10^{8.8}$ cfu per bird). It should be considered however that under field circumstances the *E. coli* exposure is continuous, while in the present study a single dose was given. Moreover, avian colibacillosis as it is observed under natural conditions, characterised by low to moderate mortality, low to moderate incidence of septicemic lesions and a high percentage of birds with airsacculitis, was reproduced.

While the first experiment was in progress it became clear that as all groups finished their allowance of feed, mean body weight was not a useful parameter, in contrast to body weight uniformity. Body weight uniformity is an important parameter of flock health as disease susceptibility, in general, varies between individuals leading to loss of uniformity when disease occurs. For obvious reasons, feed restriction will additionally depress body weight uniformity. In our study, body weight uniformity proved to be a useful parameter as susceptibility to colibacillosis varied greatly within experimental groups (*E. coli* scores of 0 to 12 were found) and birds were, moreover, feed restricted. In experiment 3,

significantly lower M41 HI titres were obtained than in the other experiments. This is unlikely to be due to an infection failure as clinical signs, body weight uniformity and *E. coli* lesions were in line with the other experiments.

H120 vaccine is widely used in broilers (Sharma, 1999). This vaccine virus is thought to cycle (i.e. pass from vaccine virus infected to susceptible chickens) in commercial broilers (Hopkins and Yoder, 1984) and is detectable up to 3 or 4 weeks of age, occasionally up to older ages, in broilers vaccinated at the hatchery (Cavanagh *et al*., 1999). In the present study, H120 vaccine virus induced colibacillosis susceptibility in 4-week-old broilers almost equal to virulent field viruses. Hopkins and Yoder (1984) moreover demonstrated that chicken passages raised the capacity of H120 vaccine virus to increase the incidence of mycoplasmal airsacculitis in White Rock broilers. A marked increase in the incidence of airsacculitis compared to nonpassaged vaccine virus was found even after one chicken passage.

Although H52 vaccine virus is generally not administered to broilers, it is used at a large scale in layer and reproduction fowl. Meulemans *et al*. (2001) demonstrated that live IB vaccine strains, including H120 vaccine, are able to spread in the field, while a study on the epizootiology of IBV in Sweden revealed a strong probability that vaccination with Massachusetts type vaccine (vaccine strains not specified) had not only lead to the spread of the vaccine virus but in addition, to the appearance of various disease manifestations (Farsang *et al*., 2002). Therefore, H52 vaccine virus might infect commercial broilers. The present study revealed that this vaccine virus increased colibacillosis susceptibility of broilers even more than virulent field strains did.

In view of the foregoing, colibacillosis in IBV susceptible commercial broilers may well be induced by IB vaccine virus and can be as severe as that provoked by virulent IB field virus. Colibacillosis induced by IB vaccine virus, which may also occur during the second half of the growing period, can be considered as a ‘man-made’ disease.

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Chapter 3

Transmissibility of Infectious Bronchitis Virus H120 vaccine strain among broilers under experimental conditions

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Summary

The aim of this study was to quantify transmission of Infectious Bronchitis Virus (IBV) H120 vaccine strain among broilers, and to assess whether birds that have been exposed to vaccine-strain shedding birds were protected against clinical signs after infection with a virulent strain of the same serotype. A transmission experiment and a replicate were carried out, each with six groups of commercial broilers. At day of hatch (n=30) or at 15 days of age (n=20), half of each group was inoculated with either IBV H120 vaccine (H120 group), virulent IBV M41 (M41 group), or mock-infected, thereby contact-exposing the other half of each group. Nasal discharge was recorded, and antibody response and virus shedding were measured. Four weeks after inoculation, all birds in all groups were challenged with IBV M41 to measure clinical protection. The reproduction ratio R (the average number of contact infections caused by one infectious bird) was determined to quantify virus transmission. All contact-exposed birds, except one in one H120 group, became infected with either IBV H120 or IBV M41. Almost all birds contact infected with IBV H120 or IBV M41 were subsequently protected against clinical signs after challenge with IBV M41.The lower limits of the 95% confidence interval (CI) of the reproduction ratio of IBV H120 vaccine and of IBV M41 were significantly above 1. For both IBV H120 and IBV M41 the 95% CI was [2.1-∞] following inoculation at day of hatch and [1.8-∞] after inoculation at 15 days of age. This finding demonstrates that IBV H120 vaccine is able to spread extensively among broilers. This implies that this vaccine strain might be able to become endemically present in the poultry population. It also implies that even if not all birds have received vaccine during spray application, they will most likely be protected against clinical signs after a subsequent field virus infection due to the ability of the vaccine to spread in the flock.

Introduction

Infectious Bronchitis Virus (IBV) infection can cause respiratory signs in broilers, and in addition also predispose broilers for secondary bacterial infections such as *Escherichia coli (E. coli)* infection (Cavanagh and Naqi, 2003; Glisson, 1998; Nakamura *et al.*, 1992). The latter may result in colibacillosis (fibrinous polyserositis), a disease of major clinical and economic importance in broiler industry (Barnes *et al*., 2003).

Vaccines against IBV are often applied to reduce economic losses due to an infection with field strains of IBV. In Europe, IBV H120 vaccine is one of the commonly used live IB vaccines in commercial poultry, and spray vaccination of broilers at 1 day or 15 days of age generally induces good clinical protection upon challenge with a virulent IBV strain of the same serotype (Cavanagh and Naqi, 2003, Davelaar *et al.*, 1984). The vaccine itself is generally considered as safe for use in young chicks, although it has been demonstrated that it sometimes does induce mild signs of Infectious bronchitis (IB) shortly after vaccination (Matthijs *et al*., 2003; Matthijs *et al.,* 2005). More indications of side effects have been shown by Matthijs *et al.* (2003) who demonstrated that the vaccine increased the susceptibility for colibacillosis of four week old broilers to a similar extent as the virulent IBV M41. Colibacillosis in broilers is an airborne disease which mainly occurs in the second half of the production period, when the *E. coli* concentration in the air of broiler houses might be high (Harry and Hemsley, 1965). Thus, infection of broilers with IBV H120 vaccine from approximately 4 weeks of age is undesirable. Such infection might occur in vaccinated broiler flocks due to direct contact exposure of broilers not effectively hit by the vaccine or due to virus spread from vaccinated to unvaccinated flocks. Whether or not this phenomenon occurs in the field has to be determined, but it is clear that it can only occur if the vaccine strain is able to spread from vaccinated to unvaccinated birds or flocks. In addition to this, a transmissible vaccine may be able to persist in a population.

The probability of becoming infected with a live vaccine virus through contact with a vaccinated bird depends on the efficiency of its transmission, which can be expressed by the reproduction ratio R, the average number of contact infections caused by one vaccinated individual in a completely susceptible population (Diekmann *et al*., 1990). If $R<1$, an individual may be infected by contact occasionally, but a major outbreak or epidemic will not occur; if $R>1$, a large

number of individuals may become infected through contact which can result in an epidemic.

In this study we experimentally quantified the transmission of the IBV H120 vaccine strain in commercial broilers. Groups of broilers inoculated with the virulent IBV strain M41 and groups mock inoculated were included in the study as positive and negative control groups respectively. Nasal discharge, antibody response, presence of virus and clinical protection upon challenge with virulent IBV M41 were parameters of infection.

Materials and Methods

Experimental chickens

Eighteen-day-incubated hatching eggs, originating from two broiler parent flocks (Cobb), were obtained from a commercial hatchery and subsequently hatched at the research unit of the Department of Farm Animal Health of Utrecht University. The parent flocks had been vaccinated with IB vaccines H120, H52 and D274.

Housing, feeding and management

From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, the Netherlands) with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^3 h^{-1}. Broilers were fed a commercial ration containing 12.4 MJ of metabolisable energy per kg and 19.5% crude protein, which was restricted to 75% of the *ad libitum* intake on a 'skip a day base' from 15 days of age onwards. Tap water was provided *ad libitum* throughout the experimental period. Light was given during 22 h per day and reduced from 15 days of age to 16 h/day. From 4 days of age onwards red light instead of white light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35ºC at 1 day of age to 20ºC at 31 days of age. From 31 days of age to the end of the experiment isolator temperature was kept at approximately 20ºC.

Inocula

IBV vaccine was obtained as commercial freeze-dried 1000 doses vials of H120 (Nobilis® IB H120; batch number 011406B exp. 02-2004 in the first experiment;

in the replicate experiment batch 043116B exp. 08-2005). The virulent Massachusetts IBV strain M41 was obtained from Intervet (Boxmeer, the Netherlands) as freeze-dried vials, containing $10^{8.3}$ mean egg infectious dose (EID_{50})/ml. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in *aqua destillata* just prior to use.

Experimental design

Two experiments were performed; the second was a replicate of the first except for trachea sampling. In the replicate experiment no trachea samples for assessing virus shedding were taken to make sure that virus transmission was not influenced by tracheal damage due to sampling.

At day of hatch, chicks were individually tagged, and randomly assigned to the different groups. Each experiment was carried out with six groups (A-F); groups A and B consisted of 30 birds, groups C, D, E and F of 20 birds each.

At one day of age, each of the groups A, B and C was randomly divided in two subgroups with an equal number of birds, and subgroups and groups were placed in different isolators. One subgroup of each of the groups A, B and C was inoculated oculo-nasally with 1 dose (at least containing $10^{3.0}$ EID_{50}) of IBV H120 per bird (group A), $10^{4.6}$ EID_{50} IBV M41 per bird (group B), or mock-infected with *aqua destillata* (group C). One droplet with a volume of approximately 0.05ml was applied in each eye and nostril. One day later inoculated subgroups were moved to the isolator of their group mates. At 15 days of age, the same treatment was carried out with groups D, E and F. One day and 15 days of age represent the usual age at which broilers are vaccinated against IB in the field. The dose used for vaccination was a dose commonly used in the field.

Four weeks after inoculation (age: 28 days or 42 days), the contact-exposed broilers of groups A, B, D, and E were separated from the vaccinated birds and placed in other isolators, each subgroup in a separate isolator. The broilers from groups C and F were divided randomly over four isolators each containing 10 broilers (now groups C1, C2, F1, F2).

At 29 days of age, the birds of groups A, B and C1 and at 43 days of age the birds of groups D, E and F_1 were challenged with IBV M41 strain intratracheally (1.0 ml per bird containing $10^{5.3}$ EID_{50}/ml) and oculo-nasally (one droplet with a volume of approximately 0.05 ml in each eye and nostril, containing $10^{4.6}$ EID_{50}/0.2ml). The birds in groups C2 and F2 were mock-challenged with *aqua*

destillata. Birds were killed by electrocution and bleeding 6 weeks after initial inoculation.

Birds were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, the Netherlands, in accordance with the Dutch regulation on experimental animals.

Measurements of infection and protection against challenge

Nasal discharge

Nasal discharge is a prominent clinical sign of IB, therefore from the day of initial inoculation the presence of mucous nasal exudate was recorded until at three successive examinations none of the birds showed nasal discharge anymore.

IB vaccination results in protection of the birds against clinical signs of IB, rather than protecting them against infection following challenge with virulent IBV (Cavanagh and Naqi, 2003; De Wit and De Jong, 1998) Therefore protection against IBV M41 challenge was judged in this study on base of nasal discharge only. Scoring was preformed from the day of challenge during two weeks.

Nasal scoring was done individually as described by Matthijs *et al.* (2003) at an interval of one to three days. Clinical inspections were done blindly with respect to inoculation history.

Serology

At day of hatch, serum samples from 24 surplus chicks were collected after decapitation to determine maternally-derived haemagglutination inhibiting (HI) antibodies to IBV. In each experiment, blood samples were taken at 4 weeks after initial inoculation, and at the end of the experiment, 2 weeks after challenge. All broilers were vein punctured (vena ulnaris at 4 weeks after first inoculation and vena jugularis at the end of the experiment). Serum was collected and stored at –20°C until examination.

Serum samples were examined for HI antibodies against IBV M41. The HI test using 8 haemagglutinating units was performed as described by Alexander and Chettle (1977). Titres are expressed as $\log_2$ of the reciprocal value of the highest serum dilution showing complete HI. Titres exceeding $4\log_2$ were considered positive. In addition, all serum samples, except those of day old chicks, were tested in a single dilution (1:500) using a commercial total antibody ELISA system

(IDEXX Corporation, Westbrook, Maine, USA) according to the manufacturer's instructions. From serum-to-positive ratios, individual serum titres, expressed as $\log_2$ values, were calculated using a regression formula (IDEXX software). Titres exceeding $8\log_2$ were considered positive. HI and ELISA tests were performed in one run each.

Virus shedding

In the first experiment, samples were collected from the trachea of all birds using cotton swabs, at days 4, 6, 8, 11, 13 and 15 after the first inoculation to determine virus recovery. Samples were kept in 2 ml Hank's medium at room temperature (De Wit and De Jong, 1998) for approximately one hour. Subsequently, 1 ml of the medium was stored at –80°C until examination. Trachea samples were thawed and 0.5 ml was injected into the allantoic cavity of 10-day-old embryonated SPF eggs (Intervet, Boxmeer, the Netherlands). One day later, the eggs were checked for embryo mortality. Mortality within 24 hours was considered to be non-specific. Seven days after injection, the eggs were harvested and the embryos were checked for the presence of IBV specific abnormalities such as curly toes, dwarfing and thickened amnion (Loomis *et al.*, 1950).

The virus shedding period of the birds is defined as the period in which virus was isolated from tracheal swabs. As a substantial difference between dose of IBV H120 and IBV M41 might exist (see: Discussion), statistical analysis on virus shedding period, using Cox proportional hazards model (Kirkwood and Sterne, 2003), was only performed between groups inoculated with the same virus.

Quantification of virus transmission

The quantification of transmission of virus was based on the number of contact-infected birds. If at least one of the following criteria was fulfilled, a contact-exposed bird was considered being infected by contact: 1) nasal discharge present after contact with inoculated birds, 2) absence of nasal discharge after challenge infection with IBV M41, 3) virus recovery from trachea sample (experiment 1) and 4) antibody titre above the cut off value four weeks after contact with inoculated birds.

A stochastic *SIR* model was used (susceptible-infectious-recovered model) to quantify the transmission of virus. In this model, which has been used for analysis of various transmission experiments (De Jong and Kimman,1994; De Wit and De Jong, 1998; Orsel *et al.*, 2007), S is the total number of susceptible, I the total

number of infectious and *R* the total number of recovered animals. The value of the reproduction ratio that maximizes the likelihood function is called the maximum likelihood estimate (MLE) (Velthuis *et al.*, 2007). With the final size (FS) of infection (the total number of birds that became infected at the end of the experiment) and by means of this MLE, the reproduction ratio can be estimated for each group including a two-sided 95% confidence interval (95% CI).

Results

As the results of experiments 1 and 2 were similar, only those of experiment 1 are presented in detail (Tables 1, 2 and 3).

Clinical observations

Nasal discharge scores in experiment 1 are given in table 1. After first inoculation, in experiment 1 nasal discharge was observed in groups A and D (H120 groups) in 2 out of 15 (2/15) and 3/10 inoculated birds and in 1/15 and 3/10 contact birds respectively. In groups B and E (M41 groups) nasal discharge was observed in 12/14 and 9/9 inoculated birds and in 11/15 and 10/10 contact birds respectively.
In each of groups B an E one broiler died. This mortality was not caused by IBV M41 inoculation.

After first inoculation in experiment 2, mucous nasal exudate was observed in groups A and D (H120 groups) in 4/15 and 9/10 inoculated birds and in 2/15 and 2/10 contact birds respectively. Nasal discharge was observed from two days after inoculation in IBV H120 and IBV M41 groups. The period of nasal discharge after initial inoculation varied from 8 to 14 days in IBV H120 inoculated broilers and from 14 to 18 days in their incontacts (experiments 1 and 2: groups A and D). In IBV M41 inoculated broilers and their incontacts nasal discharge was observed during 6 to 8 days (experiments 1 and 2: groups B and E). Mock inoculated control birds (experiments 1 and 2: groups C and F) were free of nasal discharge, while all had nasal discharge during 5 to 7 days after IBV M41 challenge.

In both experiments, almost all inoculated and contact broilers showed to be protected against the nasal discharge inducing potency of the IBV M41 challenge, performed 4 weeks after initial inoculation (Table 1).

Serology

At day of hatch, nearly all tested surplus birds in both experiments had HI antibody titres against IBV > 4 $\log_2$. The mean maternally derived IBV M41 HI antibody titre was 5.9 (sd 1.4) in experiment 1 and 10.3 (sd 1.0) in experiment 2. Four weeks after initial inoculation, all control birds (groups C and F) in both experiments had HI and ELISA antibody titres below the cut off values. HI and / or ELISA antibodies against IBV were detected in birds in groups A, B, D and E (Table 1). No clear differences were noticed between the serological response of the inoculated birds and their incontacts. Four weeks after inoculation, mean group $\log_2$ HI IBV titres of birds inoculated with IBV H120 at day old and their incontacts (experiments 1 and 2: groups A) were approximately 3 to 4; mean $\log_2$ ELISA IBV titres were 7 to 9. Inoculation at 15 days of age (experiments 1 and 2: groups D), resulted in slightly higher mean titres: $\log_2$ HI titres were approximately 4 to 5; $\log_2$ ELISA titres 9 to 10. Birds inoculated with IBV M41 and their incontacts had higher mean titres, especially higher mean HI titres, than IBV H120 inoculated birds and their contact mates: $\log_2$ HI and $\log_2$ ELISA titres were approximately 6 to 8 and 7 to 10 respectively after inoculation at day old (experiments 1 and 2: groups B) and approximately 8 to 9 and 9 to 10 respectively after inoculation at 15 days of age (experiments 1 and 2: groups E).

After challenge with IBV M41 in all birds a titre increase $\geq 2\log_2$ was demonstrated.

A number of broilers could not be examined serologically due to lack of serum.

Virus shedding

No virus was detected in the trachea samples of the control birds (groups C and F). Results of virus isolation in the other groups is presented in Tables 2 and 3. From at least one sample of each inoculated and each contact exposed bird in groups A, B, D, and E virus was isolated. Virus was detected at day 4 after inoculation in groups A, B, D and E in 2/15, 12/14, 10/10 and 10/10 birds respectively. In group A, 5/14 inoculated and 9/15 contact-exposed birds, and in group B 3/14 inoculated and 2/15 contact-exposed birds were still virus positive at day 15 after inoculation. At day 13 after inoculation in group D, 2/10 inoculated and 1/10 contact-exposed birds were virus positive, while in group E all birds were virus negative at that time. In group D all birds were virus negative on day 15 after inoculation.

Table 1. Experiment 1. Parameters of infection with either IBV H120 vaccine or IBV M41 of inoculated (I) and contact-exposed (S) broilers. Inoculation was done at 1 day or 15 days of age. Represented are the number of broilers with nasal discharge, a positive sero- response (HI and ELISA), and positive virus isolation (VI) test. Also represented are the number of broilers that were clinically protected after challenge with virulent IBV M41 at 4 weeks after first inoculation. Broilers were considered contact-infected if one or more of the parameters of infection were positive. In the last column, the reproduction ratio and its 95% confidence interval (CI) is given.

					Number of positive broilers/number examined						
Group	No. of broilers	**Age**[1]	**IBV strain**	**Treat-ment**	Nasal discharge[2]	HI IBV[3] (>4log$_2$)	ELISA IBV[4] (>8log$_2$)	VI[5]	Protected after challenge with M41[6]	Contact-infected	R[7] [95% CI]
A	30	1	H120	I[8]	2/15	1/14	2/14	15/15	14/14		
				S[9]	1/15	1/15	0/15	15/15	13/15	15/15	3.7 [2.1; ∞]
B	30	1	M41	I	12/14	8/12	0/12	13/13	14/14		
				S	11/15	12/13	1/13	13/13	13/13	13/13	∞ [2.1; ∞]
C	20	1	Mock	I	0/10	0/10	0/10	0/10	0/10		
				S	0/10	0/10	0/10	0/10	-		
D	20	14	H120	I	3/10	4/9	7/8	10/10	10/10		
				S	3/10	1/8	4/7	10/10	10/10	10/10	∞ [1.8; ∞]
E	20	14	M41	I	9/9	5/5	4/5	10/10	8/9		
				S	10/10	10/10	10/10	10/10	10/10	10/10	∞ [1.8; ∞]
F	20	14	Mock	I	0/10	0/10	0/10	0/10	0/10		
				S	0/10	0/10	0/10	0/10	-		

[1]age at IBV inoculation (days).
[2]recorded after initial IBV inoculation.
[3]HI IBV= Haemagglutination Inhibiting antibodies to IBV; measured at 28 days after initial IBV inoculation.
[4]ELISA IBV antibodies were measured at 28 days after initial IBV inoculation
[5]Number positive on any day/number examined
[6]Challenge with virulent IBV M41 at 4 weeks after first inoculation (29 days of age in groups A-C; 43 days of age in groups D-F). Protection was based on the absence of nasal discharge.
[7]R = Reproduction ratio, the estimate is based on the combined outcome of experiments 1and 2.
[8]I = inoculated broilers.
[9]S = susceptible, contact-exposed broilers.

Table 2. Results of virus isolation from trachea's of two groups of broilers (groups A and B) each consisting of 30 birds. At one day of age, half of group A was inoculated with IBV H120 vaccine and half of group B with IBV M41. The other half of the groups served as contact birds. Inoculated broilers were united with their incontacts one day after inoculation. Results are presented on base of individual broilers.

Group	Days post inoculation[1]												
	4	6	8	11	13	15		4	6	8	11	13	15
	-	+	+	+	+	+		+	+	+	+	+	+
	-	+	-	+	-	+		-	+	+	+	+	+
	-	mv[2]	mv	mv	mv	mv		-	+	+	+	-	-
	-	-	+	-	-	+		-	-	-	-	-	+
	-	+	+	-	-	-		-	+	-	-	-	-
A	+	-	-	+	-	-		-	+	+	+	-	+
	-	+	+	+	+	+		-	-	+	+	+	+
Inoculates	+	+	+	+	+	-	Incontacts	-	-	+	+	+	+
	-	+	+	+	+	-		-	-	+	-	+	-
	-	-	-	+	-	-		-	+	+	+	-	+
	-	+	+	+	-	-		-	-	+	+	-	-
	-	+	+	+	-	-		-	-	+	-	+	+
	-	+	+	+	+	+		-	-	-	+	+	-
	-	+	+	-	+	-		-	+	+	+	-	-
	-	-	+	-	+	-		+	-	+	+	+	+
	+	-	+	mv	+	+		+	+	+	-	+	-
	+	+	+	mv	-	-		-	-	+	+	+	-
	+	-	+	mv	-	-		+	+	-	+	+	-
	+	+	+	mv	+	+		-	-	-	mv	+	-
B	-	+	+	mv	-	-		+	+	+	mv	+	+
	+	+	+	mv	+	-		+	+	+	mv	+	-
Inoculates	+	+	+	mv	-	-	Incontacts	+	+	+	mv	+	+
	-	+	-	mv	-	-		-	-	-	mv	-	-
	+	+	-	+	-	-		+	+	+	mv	+	-
	+	-	+	-	-	-		+	+	+	mv	-	-
	+	+	+	+	-	-		+	+	+	mv	+	-
	+	+	+	+	+	+		+	+	+	mv	-	-
	+	+	+	-	-	-		+	+	+	mv	mv	-
	+	+	+	+	-	-		+	-	+	mv	+	-
	mv	mv	mv	mv	mv	mv		+	+	-	mv	+	-

[1]Birds were inoculated oculo-nasally with 1 dose (at least containing $10^{3.0}$ EID_{50}) of IBV H120 vaccine per bird (group A) or $10^{4.6}$ EID_{50} of IBV M41 per bird (group B). [2]mv is missing value.

Table 3. Results of virus isolation from trachea's of two groups of broilers (groups D and E) each consisting of 20 birds. At 15 days of age, half of group D was inoculated with IBV H120 vaccine and half of group E with IBV M41 . The other half of the groups served as contact birds. Inoculated broilers were united with their incontacts one day after inoculation. Results are presented on base of individual broilers.

Group	Days post inoculation[1]													
	4	6	8	11	13	15		4	6	8	11	13	15
	+	+	+	+	-	-		+	+	+	+	-	-
	+	+	+	+	-	-		+	+	+	+	-	-
	+	+	+	-	-	-		+	+	+	-	-	-
	+	+	+	-	-	-		+	+	+	-	-	-
	+	+	+	-	-	-		+	+	+	-	-	-
D	+	+	+	-	-	-		+	+	+	-	-	-
	+	+	+	-	-	-		+	+	+	-	-	-
Inoculates	+	+	+	-	-	-	Incontacts	+	+	+	-	-	-
	+	+	+	-	+	-		+	+	+	-	-	-
	+	+	+	-	+	-		+	+	+	-	+	-
	+	+	+	-	-	-		+	+	+	-	-	-
	+	+	-	-	-	-		+	+	-	-	-	-
	+	+	-	-	-	-		+	+	+	-	-	-
	+	+	-	-	-	-		+	+	-	-	-	-
E	+	+	-	+	-	-		+	+	-	-	-	-
	+	+	+	-	-	-		+	+	+	-	-	-
Inoculates	+	+	+	-	-	-	Incontacts	+	+	-	+	-	-
	+	+	+	+	-	-		+	+	-	-	-	-
	+	+	-	+	-	-		+	+	-	+	-	-
	+	+	+	-	-	-		+	+	-	+	-	-

[1]Birds were inoculated oculo-nasally with 1 dose (at least containing $10^{3.0}$ EID_{50}) of IBV H120 vaccine per bird (group D) or $10^{4.6}$ EID_{50} of IBV M41per bird (group E).

There was no significant difference in duration of virus shedding period between inoculated and contact-infected birds of either group; in other words, inoculated birds did not differ from their contact-infected pen mates with respect to duration of virus shedding, indicating that the groups were homogeneous with respect to that aspect. Birds inoculated at 1 day of age (with either H120 or M41) shed virus significantly longer than birds inoculated at 15 days of age ($p < 0.001$). The hazard ratio was 4.7 with a 95% CI [2.8-7.7].

Quantification of transmission

Based on the measures of infection, all broilers contact exposed to birds inoculated with IBV H120 (groups A and D) or to birds inoculated with IBV M41 (groups B and E) became infected by contact (Table 1), except for one bird in group A (IBV H120 inoculated at day old) of experiment 2. So, the number of contact birds that became infected in experiments 1 and 2 was practically equal. Therefore, the reproduction ratio was estimated for each treatment group using the combined data of both experiments. Reproduction ratio and 95% CI of each treatment group is given in Table 1. Depending on group size, the lower limit of the 95% CI differed slightly but was above 1 for all treatment groups. For both IBV H120 and IBV M41 groups the 95% CI was [2.1-∞] following inoculation at day of hatch and [1.8-∞] after inoculation at 15 days of age.

Discussion

The aim of this study was to quantify transmissibility of IBV H120 vaccine when applied in a dose used under field conditions. The reproduction ratio R of IBV H120 vaccine appeared to be significantly above 1, which implies that this IBV vaccine strain is able to spread extensively within groups of susceptible broilers. This finding might have two implications. First, IBV H120 vaccine might spread between flocks and might have even the potential to become endemically present in the broiler population. Secondly, birds that are not successfully vaccinated by spray will probably become contact-vaccinated, and this contact-vaccination will probably induce sufficient immunity to be clinically protected against an infection with virulent IBV of the Massachusetts serotype. This does not imply, however, that these birds are also protected against increased susceptibility for colibacillosis

induced by virulent IBV infection. Matthijs *et al*. (2005) demonstrated that, following infection with virulent IBV, vaccinated broilers were as sensitive for systemic colibacillosis as unvaccinated ones.

The hypothesis of vaccine spreading is substantiated by findings of Farsang *et al.* (2002) and Meulemans *et al.* (2001) who isolated IBV vaccine virus from unvaccinated poultry populations in Sweden and Belgium, respectively, indicating that transmission of IB vaccine virus from vaccinated to unvaccinated susceptible flocks can occur. This may be beneficial as a 'natural' vaccination against IB and protect these birds against clinical signs after a subsequent infection with a virulent field strain. The transmissibility of IBV H120 vaccine might also be a disadvantage. If vaccine virus is introduced in a broiler flock after the age of approximately 4 weeks, colibacillosis might occur. It is not likely that vaccination with IBV H120 vaccine at day of hatch or at 15 days of age will cause increased susceptibility for colibacillosis in the second half of the growing period, since the vaccine virus spreads rapidly, as demonstrated from the virus excretion data in experiment 1.

All broilers infected with IBV H120 vaccine or IBV M41 at 15 days of age seemed to clear the virus within 13 to 15 days, which is significant faster than virus clearance by birds inoculated at 1 day of age, suggesting a more efficient clearance mechanism in elder broilers due to maturation of the immune system and loss of maternal IBV antibodies.

At 5 days of age (4 days after inoculation) a striking difference existed in tracheal virus recovery between birds inoculated with IBV H120 and those inoculated with IBV M41: 2/15 and 12/14 broilers were virus positive respectively (Table 2). This difference might be due to different pathogenicity of the viruses or due to different inoculation doses. One field dose of IBV H120, containing at least $10^{3.0}$ EID_{50} of virus, was inoculated per bird while $10^{4.6}$ EID_{50} of IBV M41 was given per bird. As a rule, however, a dose of IBV H120 contains 10^{4}-10^{5} EID_{50} of virus (Matthijs *et al*., 2003). So, although the above stated difference is not very likely due to different doses of the viruses used, it can not be ruled out completely as titration of the IB H120 vaccine was not performed.

As commercial broilers, originating from different parent flocks, were used in the present study, maternal HI IBV M41 antibody levels differed substantially between the two experiments (mean HI IBV M41 titre ± sd at day old in experiment 1: 5.9 ± 1.4 and 10.3 ± 1.0 in experiment 2). The effect of these different maternal antibody levels on IBV transmission rate could not be established as virus isolation was only

performed in experiment 1. However, maternal antibodies seemed not to interfere with the proportion of infected contact birds as, except for one bird in experiment 2, all contact birds became infected during the 4 weeks of contact with inoculated birds. IBV replication in epithelial cells of the respiratory and digestive tract is mainly hampered by local immunity provided by IgA, while maternal antibodies belong to the IgG class of immunoglobulins which provide the birds with systemic immunity rather than local immunity (Jeurissen *et al.,* 1994). Therefore excretion and transmission of IBV is likely rather independent of the level of maternal antibodies.

Sampling of the trachea was ended at 15 days after inoculation, and, consequently, in this study it could not be determined how long virus shedding continued. This shedding period might be longer as shown by Naqi *et al*. (2003) who demonstrated that IBV vaccine exposure of 1-day old chicks with maternal antibodies led to periodic virus shedding from trachea and cloaca in all chicks until 77 days post inoculation.

Arguably, sampling the trachea as performed, which was carried out in experiment 1, facilitated the transmission process, as the swab may have caused local tissue damage. The replicate experiment, however, showed that also without tracheal sampling transmission occurred. So, the influence of the trachea swabbing, if at all, did not affect the transmission process substantially.

Our results show that IBV H120 vaccine is able to spread extensively within groups of broilers vaccinated at day of hatch or at 15 days of age. Broilers infected with IBV H120 vaccine via contact are protected against nasal discharge after infection with the virulent IBV M41 strain. Considering the reproduction ratio, it seems reasonable to assume that IBV H120 vaccine is also able to spread between flocks, and might have the potency to become endemically present in the broiler population.

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Chapter 4

Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with a virulent Massachusetts-type IBV strain

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Summary

Vaccination against infectious bronchitis (IB) is aimed to protect against clinical IB. The question is, however, whether vaccinated birds are also protected against predisposure for colibacillosis after a subsequent IBV infection. We examined this research question in four experiments. One-day-old commercial broilers, housed in isolators, were vaccinated with IB vaccine strain H120 by coarse spray or ocularly. Twenty-eight days after vaccination, broilers were challenged with the virulent IBV strain M41. Five days later, broilers were inoculated with *Escherichia coli* strain 506. Body weight uniformity, severity of *E. coli* airsacculitis, and systemic *E. coli* infection at 7 days following *E. coli* inoculation were used as parameters for colibacillosis. IBV vaccination reduced both the number of broilers with *E. coli* airsacculitis as well as the severity of airsacculitis significantly after challenge with IBV-M41 and *E. coli* 506. However, in spray-vaccinated groups, no significant reduction of the number of birds with systemic colibacillosis or the severity of this infection was obtained, and body weight uniformity was not significantly improved compared with nonvaccinated, IBV-M41, and *E. coli* 506-challenged groups. Eye-drop vaccination resulted in conflicting results.

Introduction

Infectious bronchitis (IB) is a highly contagious respiratory disease of broilers (Cavanagh and Naqi, 2003; Nakamura *et al*., 1992), caused by IB virus (IBV). Although signs of IB are usually rather mild, IBV also predisposes broilers for severe bacterial infections (Cook *et al*., 1986; Ginns *et al*., 1998; Glisson, 1998; Matthijs *et al*., 2003), commonly *Escherichia coli* infections. This bacterial infection results in (systemic) colibacillosis, which is of major clinical and economic importance. It is the most frequently reported disease in surveys of poultry diseases and the main cause of condemnation at processing (Barnes *et al*., 2003).

Vaccination of broilers against IB is widely applied and a main reason to do so is to prevent colibacillosis following IBV field challenge. Most vaccines induce good clinical protection against virus belonging to the same serotype as the vaccine virus. However, vaccination generally does not prevent replication of field strains completely (De Wit *et al.,* 1997), and vaccinated broilers can still become infected with IBV field strains. This might imply that the vaccination will not reduce the increased susceptibility of broilers for colibacillosis after an IBV infection, and consequently, IBV might still predispose broilers for colibacillosis despite vaccination.

Because colibacillosis is a major problem in the broiler industry (Yogaratnam, 1995), it might be useful to examine vaccines not only for their ability to protect against IB but also for their ability to protect against predisposure for colibacillosis after IBV infection. Cook *et al.* (1999) carried out a simultaneous infection with IBV and *E. coli* to examine this effect. Extrapolation of their results to the field, however, might be difficult because they used specific- pathogen-free (SPF) Rhode Island Red chickens, which differ substantially from commercial broilers with respect to immune status (maternally derived) and genetics. Moreover, they applied an intranasal inoculation, which might result in insufficient exposure of the lower respiratory tract to the pathogens, as both IBV and *E. coli* are probably mainly airborne (Cook and Mockett, 1995; Dho-Moulin and Fairbrother, 1999; Goren, 1991). Finally, they used mortality as parameter, and Matthijs *et al*. (2003) showed that parameters other than mortality might be more indicative for vaccine efficacy. Moreover, colibacillosis in broiler flocks is attended by only low to moderate mortality.

Therefore, a study was carried out to assess the effect of IB H120 vaccination of day-old broilers on IBV-related predisposure to colibacillosis. Broilers were infected with a virulent IBV strain and *E. coli*, and clinical signs, mortality, postmortem signs, and body weight were measured to determine the efficacy of IBV vaccination with respect to protection against IB and colibacillosis.

Materials and methods

Birds were housed, handled, and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

Experimental chickens, housing, feeding, and management
Eighteen-days incubated hatching eggs were obtained from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock and subsequently hatched at the Spelderholt Poultry Research Centre in Beekbergen, The Netherlands. Only female chicks were used.

From day 1, chicks were housed in negative-pressure isolators (Beyer and Eggelaar, Utrecht, The Netherlands) with a volume of 1.3 m^3 and fitted with a wire floor of 1.05 m^2. Isolators were ventilated at a rate of approximately 30 m^3/h.
Commercial feed was provided *ad libitum* for 14 days and subsequently restricted on a-skip-a-day basis to 85% of the *ad libitum* intake.

Inocula
IB H120 vaccine (Nobilis IB H120, batch 90046C; Intervet, Boxmeer, The Netherlands) was obtained as commercial freeze-dried vials and was stored at 4°C. The Massachusetts IBV M41 challenge strain was obtained from Intervet International as virus-containing allantoic fluid. The virus had been passaged 10 times in SPF embryonated eggs at the time of inoculation. Virus titrations of IB H120 vaccine and IBV M41 strain were performed in 10-day-old embryonated Specific Pathogen Free White Leghorn (SPF-WL) eggs as described by De Wit *et al.* (1998). The challenge strain was titrated twice, before the start of the experiment and just after ending the last experiment. Doses of vaccine and challenge virus used in the experiments were calculated based on titration results.

The *E. coli* 506 (O78; K80) used in this study, was a flumequine-resistant strain isolated from a commercial broiler suffering from colibacillosis (Van Eck and Goren, 1991). The inocula were prepared as described by Matthijs *et al.* (2003).
Data on vaccine, vaccine dose, vaccination route, IBV M41, and *E. coli* inoculations are given in Table 1.

Experimental design and inoculations
Four experiments were performed consecutively. Each experiment lasted 41 days. The various treatments are shown in Table 1. At day 1, female chicks were individually tagged and weighed. Per experiment, 8-12 groups (see Table 1) of the same body-weight distribution were formed and each group was housed in a separate isolator.

Per experiment, 6 groups were vaccinated with H120 vaccine at day 1 (referred to as vaccine groups): two by eye-drop and four by coarse spray. Eye-drop vaccination was done with one dose of vaccine (0.1 ml; calculated titer, $10^{5.4}$median embryo infective dose [EID_{50}]) per broiler. Coarse spray vaccination was performed with either one dose of vaccine (0.2 ml; calculated titer, $10^{5.4}$ EID_{50}) per bird or 100 doses of vaccine (0.2ml; calculated titre, $10^{7.4}$ EID_{50}) per bird.
At 29 days of age, broilers were inoculated with IBV M41 by eye-nose drop (0.05 ml in each eye and nostril) and intratracheally (i.t.) (1 ml per broiler). In experiment 1, the calculated dose per bird was $10^{4.8}$ (groups 1, 3, 5, 7) and $10^{6.8}$ EID_{50} (groups 2, 4, 6, 8). The calculated dose per bird in experiments 2, 3 and 4 was between $10^{4.7}$ and $10^{5.8}$ EID_{50} (see Table 1).

Escherichia coli 506 was administered i.t. at day 34 in a volume of 1 ml/bird. In experiments 1 and 2, $10^{7.9}$ and $10^{7.5}$ colony forming units (cfu) (further referred to as high *E. coli* dose) were inoculated per broiler, respectively; in experiments 3 and 4, $10^{6.8}$ cfu (further referred to as low *E. coli* dose) were administered per broiler.

In all experiments, sham-vaccinated (coarse spray vaccination with distilled water) control groups were included. Groups 7 and 8 received IBV M41 and *E. coli* 506 (referred to as M41 + *E. coli* groups), groups 9 and 10 were sham IBV M41 inoculated but received *E. coli* 506 (referred to as *E. coli* groups), and groups 11 and 12 were only sham inoculated (referred to as placebo groups) (see Table 1).

Clinical observations
Clinical signs resulting from IB infection were determined at days 29, 32, 34 and 41 by individual scoring of birds with mucous nasal discharge, as described by

Matthijs *et al*. (2003). A bird was recorded as having nasal discharge due to IBV infection, in case this clinical sign was observed on either one or both sides at day 32 and/or day 34 and/or day 41. Mortality was recorded daily. Dead birds were stored at –20^{o}C until postmortem examination at day 41.

Body weight and body weight-uniformity
Birds were weighed individually at day 29 (day of IBV M41 inoculation) and at day 41 (end of experiment). As body weight was a nonvalid parameter (Matthijs *et al.,* 2003), body weight uniformity was used as variable. This variable is defined as the percentage of birds with a body weight between mean body weight minus 10% and mean body weight plus 10%.

Serological examination
Per experiment, hemagglutination-inhibiting (HI) antibodies to IBV-M41 were determined in sera. Sera were collected at day 1, 29 and 41; at day-old from 20 broilers per experiment; and at 29 and 41 days of age of all experimental broilers. Sera were tested by HI IB test as described by Alexander and Chettle (1977). Titres are expressed as $\log_2$ of the reciprocal value of the highest serum dilution showing complete HI. Titres exceeding $\log_2$ 4 were considered positive (De Wit *et al.,* 1998).

Tracheal ciliostasis score
In experiments 2 and 3, five randomly chosen birds from each group were euthanatized at day 34 their tracheas were examined for ciliary activity as described previously (Cook and Mockett, 1995). The mean ciliostasis score was calculated per group starting from mean value per bird.

Postmortem examination
At the end of the experiments, birds were euthanatized. Postmortem examination was performed blindly. Lesion scoring was performed as described by Van Eck and Goren (1991) in the following organs: right thoracic air sac, left thoracic air sac, pericardium, and liver. Lesion scores ranged from 0 to 3: 0 = no lesions, 0.5 = one yellow/brown pinhead-sized spot indicative of inflammation, 1 = two or more pinhead-sized spots as described at score 0.5, 2 = thin layer of fibrinous exudate on various locations, 3 = thick and extensive layer of fibrinous exudate. The maximum score per bird is 12. Birds with *E. coli* pathology of pericardium and/or liver were

referred to as systemically infected. Per group, the percentage of broilers with colibacillosis lesions in the respiratory tract only (airsacculitis as only sign of colibacillosis; further referred to as airsacculitis *sec*), the mean colibacillosis lesion score (MLS) of these birds, the percentage of broilers with systemic colibacillosis, and the MLS of systemically infected broilers were calculated.

In each experiment, bacteriological analysis was performed on samples of spleen, thoracic air sac, heart, and liver of 10 birds. Culturing was performed on sheep blood agar plates and colonies were identified using Bactident® *E. coli* (no. 13303; Merck, Amsterdam, The Netherlands), as described by Matthijs *et al*. (2003).

Statistical analysis and validity of parameters

Because of the different *E. coli* doses, we compared the data obtained in experiments 1 and 2 separately from those obtained in experiments 3 and 4.

Mortality, body weight uniformity, airsacculitis *sec* and systemic colibacillosis were analyzed by a generalized linear model (GLM) using a Poisson error term (McCullagh and Nelder, 1989). In addition, the GLM used a log link function and the natural log of the number of live birds as offset variables. Moreover, experiment and group within the same treatment were included as random effect variables. In addition, nasal discharge was analyzed by use of Fisher Exact test, using a Bonferroni correction to adjust for multiple comparisons. Next, the Kruskal-Wallis analysis of variance was used to analyze the MLS score and the mean tracheal ciliostasis score, variables of the ordinal type, again using a Bonferroni correction to adjust for multiple comparisons. Finally, HI titers were compared by use of a Cox proportional hazards model (Therneau and Grambsch, 2000). All analyses were carried out by the statistical program R, version 1.6.1 (Ihaka and Gentleman, 1996).

Because the power of a statistical analysis is reduced as the number of comparisons increases (Armitage and Berry, 1991), data of the placebo groups (groups 11 and 12) were not included in our analysis of the variables associated with colibacillosis. For the same reason, comparisons between the vaccine groups were not made. Furthermore, if a parameter estimate of the IBV M41 + *E. coli* groups (groups 7 and 8) was not significantly different from that of the *E. coli* groups (groups 9 and 10), the estimates of the same parameter of the vaccine groups were not statistically analyzed, as this parameter was then considered to be nonvalid.

Table 1. Experimental design, nasal discharge, and tracheal ciliostasis score. Commercial female broilers were IB vaccinated at 1 day old and inoculated with IBV strain M41 at 29 days of age and with *E. coli* 506 at 34 days of age.

Groups		1+2[A]	3+4	5[A]+6[A]	7+8	9+10[B]	11+12[C]
IB vaccine		H120	H120	H120	Distilled water	Distilled water	Distilled water
	Application route	Ocular	Coarse spray	Coarse spray	Coarse spray	Coarse spray	Coarse spray
	Dose/broiler	1	1	100	-	-	-
IB M41[D]		+	+	+	+	Distilled water	Distilled water
E. coli 506[E]		+	+	+	+	+	PBS diluted glucose peptone broth
Size of groups at 29 days of age	Exp. 1	30	30	30	30	15	15
	Exp. 2	40	40	40	39	40	40
	Exp. 3	40	40	40	40	40	40
	Exp. 4	15	30	0	30	30	15
		Number of broilers with nasal discharge at 32 and/or 34 days of age					
Exp. 1		0	0	0	30	0	0
Exp. 2		0	0	0	39	0	0
Exp. 3		0	0	0	40	0	0
Exp. 4		0	0	-	30	0	0
Fisher Exact test[F]		A	A	A	B	A	A
		Mean tracheal ciliostasis score (range) at 34 days of age (mean values are based on the examination of ten broilers)					
Exp. 2		2.3 (0-4)	3.0 (0-4)	2.3(0.2-4)	4.0 (4-4)	0.1(0-0.6)	0.2(0-1.4)
Exp. 3		2.1 (0-4)	3.1(0-4)	2.8(0.8-4)	4.0(4-4)	0.0 (0-0)	0.0(0-0.2)
Kruskal-Wallis[G]		A	A	A	B	C	C

[A] Not included in experiment 4.
[B] Not included in experiment 1.
[C] Not included in experiments 1 and 4.
[D] In experiment 1: $\log_{10}$ EID_{50} IBV M41/broiler: calculated titer 4.8 (groups 1, 3, 5 and 7) and 6.8 (groups 2, 4, 6 and 8) and in experiments 2-4: $\log_{10}$ EID_{50} IBV M41/broiler: calculated titer between 4.7-5.8.
[E] *E. coli* 506 cfu [log10]/broiler in 1 ml. Exp. 1: 7.9; exp. 2: 7.5; exps. 3 and 4: 6.8.
[FG] Columns with different capital letter differ significantly ($P < 0.05$)

Table 2. Body-weight uniformity and colibacillosis lesions of commercial female broilers, which were IB vaccinated at 1 day old and inoculated with IBV strain M41 at 29 days of age and with high *E. coli* 506 dose (7.5 to 7.9 $\log_{10}$ cfu/broiler) at 34 days of age. Parameters were assessed at 41 days of age in surviving broilers.

Groups		1+2	3+4	5+6	7+8	9+10	11+12
IB vaccine		H120	H120	H120	Distilled water	Distilled water	Distilled water
	Application route	Ocular	Coarse spray	Coarse spray	Coarse spray	Coarse spray	Coarse spray
	Dose/broiler	1	1	100	-	-	-
IB M41		+[A]	+	+	+	Distilled water	Distilled water
E. coli 506		+[B]	+	+	+	+	PBS diluted glucose peptone broth
Size of groups at 29 days of age	Exp. 1	30	30	30	30	15	15
	Exp. 2	40[C]	40[C]	40[C]	39[C]	40[C]	40[C]
		Body weight uniformity[D]**					
Exp. 1		48	59	46	57	77	73
Exp. 2		62	69	59	35	76	87
GLM[E]		NS, ns	NS, ns	NS, ns			
		Percentage of broilers with airsacculitis as only sign of colibacillosis**					
Exp. 1		24	30	27	64	46	0
Exp. 2		49	50	22	74	8	0
GLM[F]		S, ns	S, ns	S, ns			
		Mean colibacillosis lesion score of broilers with airsacculitis as only sign of colibacillosis**					
Exp. 1		1.2	1.4	1.8	2.3	1.0	-
Exp. 2		1.9	2.3	1.8	3.1	1.0	-
Kruskal-Wallis[G]		S, s	S, s	S, s			

Groups		1+2	3+4	5+6	7+8	9+10	11+12
IB vaccine		H120	H120	H120	Distilled water	Distilled water	Distilled water
	Application route	Ocular	Coarse spray	Coarse spray	Coarse spray	Coarse spray	Coarse spray
	Dose/broiler	1	1	100	-	-	-
IB M41		+[A]	+	+	+	Distilled water	Distilled water
E. coli 506		+[B]	+	+	+	+	PBS diluted glucose peptone broth
Size of groups at 29 days of age	Exp. 1	30	30	30	30	15	15
	Exp. 2	40[C]	40[C]	40[C]	39[C]	40[C]	40[C]
		Percentage of broilers with systemic colibacillosis**					
Exp. 1		8	22	50	36	8	0
Exp. 2		10	19	19	26	12	0
GLM[H]		S, ns	NS, ns	NS, s			
		Mean colibacillosis lesion score of broilers with systemic colibacillosis**					
Exp. 1		3.8	6.9	5.6	8.1	5.0	-
Exp. 2		5.6	7.8	6.7	9.3	4.0	-
Kruskal-Wallis[I]		S, ns	NS, ns	S, ns			

[A]Inoculated with IBV strain M41 by the oculonasal and intratracheal route.
[B]Inoculated with *E. coli* 506 by the intratracheal route.
[C]Ten randomly chosen broilers were killed at 34 days of age to perform the trachea ciliostasis test (see Table 1).
[D]Body-weight uniformity is the percentage of broilers with a body weight between mean body weight plus 10% and mean body weight minus 10%.
mortality occurred in the period from 29 to 34 days of age.
[EFGHI]Columns with capital letter S differ significantly from groups 7 and 8 ($P < 0.05$), columns with capital letters NS do not. Columns with small letter s differ significantly from groups 9 and 10 ($P < 0.05$), columns with small letters ns do not.
[**]Valid parameter (significant difference between values of groups 7 and 8 and groups 9 and 10; $P < 0.05$)

Table 3. Body-weight uniformity and colibacillosis lesions of commercial female broilers, which were IB vaccinated at 1 day old and inoculated with IBV strain M41 at 29 days of age and with low *E. coli* 506 dose (6.8 $\log_{10}$ cfu/broiler) at 34 days of age. Parameters were assessed at 41 days of age in surviving broilers.

Groups		1+2	3+4	5+6	7+8	9+10	11+12
IB vaccine		H120	H120	H120	Distilled water	Distilled water	Distilled water
	Application route	Ocular	Coarse spray	Coarse spray	Coarse spray	Coarse spray	Coarse spray
	Dose/broiler	1	1	100	-	-	-
IB M41		+[A]	+	+	+	Distilled water	Distilled water
E. coli 506		+[B]	+	+	+	+	PBS diluted glucose peptone broth
Size of groups at 29 days of age	Exp. 3	40[C]	40[C]	40[C]	40[C]	40[C]	40[C]
	Exp. 4	15	30	0	30	30	15
		Body weight uniformity[D*]					
Exp. 3		80	73	79	63	79	90
Exp. 4		73	77	-	93	83	87
		Percentage of broilers with airsacculitis as only sign of colibacillosis[**]					
Exp. 3		24	30	42	74	7	0
Exp. 4		4	34	-	94	17	0
GLM[E]		S, ns	S, s	S, s			
		Mean colibacillosis lesion score of broilers with airsacculitis as only sign of colibacillosis[**]					
Exp. 3		1.3	1.7	1.3	2.5	1.0	-
Exp. 4		1.5	1.2	-	2.4	0.8	-
Kruskal-Wallis[F]		S, ns	S, ns	S, ns			

Groups		1+2	3+4	5+6	7+8	9+10	11+12
IB vaccine		H120	H120	H120	Distilled water	Distilled water	Distilled water
	Application route	Ocular	Coarse spray	Coarse spray	Coarse spray	Coarse spray	Coarse spray
	Dose/broiler	1	1	100	-	-	-
IB M41		+[A]	+	+	+	Distilled water	Distilled water
E. coli 506		+[B]	+	+	+	+	PBS diluted glucose peptone broth
Size of groups at 29 days of age	Exp. 3	40[C]	40[C]	40[C]	40[C]	40[C]	40[C]
	Exp. 4	15	30	0	30	30	15
		Percentage of broilers with systemic colibacillosis[**]					
Exp. 3		13	0	3	23	0	0
Exp. 4		13	13	-	3	3	0
GLM[G]		NS, s	NS, ns	NS, ns			
		Mean colibacillosis lesion score of broilers with systemic colibacillosis[*]					
Exp. 3		7.5	-	4.5	6.1	-	-
Exp. 4		6.4	1.5	-	5.0	5.0	-

[A]Inoculated with IBV strain M41 by the oculonasal and intratracheal route.
[B]Inoculated with *E. coli* 506 by the intratracheal route.
[C]Ten randomly chosen broilers were killed at 34 days of age for the trachea ciliostasis test (see Table 1).
[D]Body-weight uniformity is the percentage of broilers with a body weight between mean body weight plus 10% and mean body weight minus 10%.
[EFG]Columns with capital letter S differ significantly from groups 7 and 8 ($P < 0.05$); columns with capital letters NS do not. Columns with small letter s differ significantly from groups 9 and 10 ($P < 0.05$), columns with small letters ns do not.
[*]Nonvalid parameter (no significant difference between values of groups 7 and 8 and groups 9 and 10; $P > 0.05$)
[**]Valid parameter (significant difference between values of groups 7 and 8 and groups 9 and 10; $P < 0.05$)

Per experiment, the variables between groups treated the same way did not differ significantly. Moreover, the variables did not differ between vaccine groups that were inoculated with different IBV M41 doses but otherwise treated the same. Therefore, results of these groups were combined. The results of the vaccine groups were compared with the results of groups 7 and 8 (M41+ *E. coli* groups), and 9 and 10 (*E. coli* groups).

Results

Mortality and serology

In the placebo groups, no mortality was observed. After challenge with the high *E. coli* dose, mortality was 16% in the *E. coli* groups, 11% in the vaccine groups, and 14% in the M41+ *E. coli* groups. After challenge with the low *E. coli* dose, the mortality was below 4% in all groups. In all experiments, mortality showed to be a nonvalid parameter.

All birds had maternally derived antibodies against IBV M41 at day of hatch: mean $\log_2$ HI titers ranged from 6.3 to 8.4 between experiments. At day 29, HI antibodies were not detected in any of the nonvaccinated groups (groups 7-12: $\log_2$ titers ≤ 4), while at that age in groups vaccinated at day old (groups 1-6) $\log_2$ HI antibody titers ranging from 3 to 7 were found. After IBV-M41 challenge, titers of the vaccinated groups (groups 1-6) were significantly below those of the nonvaccinated groups (groups 7 and 8).

Clinical signs and tracheal ciliostasis score

At days 29 and 41, nasal discharge was not observed. None of the birds in the vaccine, *E. coli*, and placebo groups showed nasal discharge, while all birds in the M41+*E. coli* groups did (see Table 1). Ciliostasis was not observed or present at a very low level in the E. coli and placebo groups, while birds of the M41+*E. coli* groups showed complete ciliostasis. Scores of vaccine groups were significantly below those of M41+*E. coli* groups, and significantly above those of *E. coli* and placebo groups. In each of the vaccine groups, birds with complete ciliostasis (score 4) were present. Differences between the vaccine groups were not significant (see Table 1).

Body-weight uniformity and postmortem examination

For the high *E. coli* dose groups (experiments 1 and 2; Table 2), body-weight uniformity of M41+*E. coli* groups was significantly decreased in comparison with the *E. coli* groups. For the low *E. coli* dose groups, (experiments 3 and 4; Table 3), body-weight uniformity proved to be a nonvalid parameter. In the vaccine groups, body-weight uniformity did not differ significantly from the M41+*E. coli* groups or from the *E. coli* groups.

In all vaccine groups in all four experiments, the percentage of birds with airsacculitis *sec* and their MLS were significantly reduced in comparison with the M41+*E. coli* groups. However, spray vaccination (groups 3-6) did not significantly reduce the number of birds with systemic colibacillosis in comparison with the M41+*E. coli* groups in any of the experiments. In the eye-drop vaccine groups in experiments 1 and 2 (high *E. coli* dose), both the percentage of birds with systemic colibacillosis as well as the MLS of these birds were significantly reduced in comparison with the M41+*E. coli* groups, but in experiments 3 and 4 (low *E. coli* dose), these variables were not reduced.

Nearly all birds that died within 48 h after *E. coli* inoculation lacked macroscopic *E. coli* pathology. From all birds that died after *E. coli* inoculation and were cultured bacteriologically, *E. coli* was isolated. *E. coli* was cultured from 11/28 (39%) of surviving chicks with *E. coli* lesions. All isolates of *E. coli* showed an antibiotic sensitivity pattern identical to that of the inoculated.

Discussion

Colibacillosis of the respiratory tract in broilers occurs after *E. coli* is inhaled and the bacterium has defeated local defense mechanisms. Further, systemic colibacillosis, i.e. pericarditis and perihepatitis, develops if the bacterium passes from the respiratory tract to the bloodstream and overwhelms the systemic defense (Hament *et al*., 1999).

Viral infections of the respiratory tract, such as IBV infection, facilitate the pathway of both colibacillosis of the respiratory tract and systemic colibacillosis (Nakamura *et al*., 1992; Peighambari *et al.,* 2000). Likely, complex factors are involved in the development of *E. coli* infection in birds infected with a respiratory disease virus. Tissue damage in the respiratory system due to virus replication is probably of importance as well as immunological mechanisms such as the

predominantly lymphoid response in the respiratory tract, which is not an adequate defense against *E. coli* (Gross, 1990).

In the present study, IB spray vaccinations did reduce *E. coli* airsacculitis *sec* significantly but had no significant effect on systemic colibacillosis. The underlying mechanism of this discrepancy is unclear from our study. IB vaccination reduces the replication of challenge virus (Cook *et al.,* 1986; De Wit *et al*., 1998; Lambrechts *et al.,* 1993; Pensaert and Lambrechts, 1994) and as a consequence likely also reduces tissue damage and the immune response in the respiratory tract following IBV challenge. Obviously, this reduction is sufficient to decrease the sensitivity of the respiratory tract for colibacillosis significantly, but insufficient to prevent the *E. coli* bacterium from becoming blood-borne.

In contrast with *E. coli* airsacculitis *sec*, systemic colibacillosis impairs body weight gain and body weight uniformity (Matthijs *et al.,* 2003; Yamaguchi *et al.,* 2000; Yogaratnam, 1995) and this explains our observation that spray vaccination did not improve body weight uniformity.

Interpretation of results obtained by eye-drop vaccination is difficult as contradictions occurred. Eye-drop vaccination reduced systemic colibacillosis significantly in case the high *E. coli* dose was used, but this effect was not achieved in birds inoculated with the low dose. Moreover, reduction of systemic colibacillosis in birds inoculated with the high *E. coli* dose was not attended by improvement of body weight uniformity.

It had been demonstrated that ocular vaccination is superior to spray vaccination in terms of protection against IB after i.t. challenge (Al-Tarcha *et al.,* 1991; Davelaar and Kouwenhoven, 1977; Toro *et al.,* 1997). In our study however, this effect was not observed since all vaccinations prevented nasal discharge completely and tracheal ciliostasis scores were equally reduced compared with non-vaccinated challenged groups. These contrasting results might be due to the relative high vaccine virus doses used in our study compared to the doses used in the work of others (Al-Tarcha *et al.*, 1991; Davelaar and Kouwenhoven, 1977; Snyder *et al.,* 1983).

In addition to clinical protection to IB, also kinetics of tracheal cilia is considered to be a reliable indicator for protection against IB (Al-Tarcha *et al.*, 1991; Al-Tarcha and Sadoon, 1991; Cook *et al*., 1999; Darbyshire,1980; Snyder *et al*., 1983). In our experiments vaccinations reduced mean ciliostasis scores to approximately 2 to 3. However, in each of the vaccine groups also birds with complete ciliostasis (score 4) were present. Nevertheless, all birds in all vaccine

groups were completely protected against nasal discharge, indicating that the ciliostasis score does not, at least not in all cases, reflect protection of the respiratory tract as a whole.

In conclusion, it can be stated that spray vaccination at one-day-old against IB prevented broilers from clinical signs of IB and significantly reduced *E. coli*-airsacculitis after challenge with IBV-M41 and *E. coli* 506. Both the number of birds with airsacculitis and the severity of airsacculitis were reduced. However, even in a dose of $10^{7.4}$ EID_{50} of vaccine virus per bird, spray vaccination did not improve body weight uniformity and did not reduce systemic *E. coli* infections. In the latter respect, eye-drop vaccination might produce better results.

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Abbreviations

cfu = colony forming unit
D (v)= volume diameter
E.coli = *Escherichia coli*
EID_{50} = median embryo infective dose
GLM = Generalized Linear Model
HI = hemagglutination inhibition
IB = Infectious Bronchitis
IBV = Infectious Bronchitis Virus
i.t. = intratracheal
kg = kilogram
MDA = maternal derived antibodies
mg = milligram
MJ = megajoule
ml = millilitre
MLS = mean lesion score
PBS = phosphate-buffered saline
RIR = Rhode Island Red
s.d. = standard deviation
SPF = Specified Pathogen Free
SPF-WL = specified pathogen free white leghorn
µm = micrometer

Chapter 5

Progression of lesions in the respiratory tract of broilers after single infection with *Escherichia coli* compared to superinfection with *E. coli* after infection with infectious bronchitis virus

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Abstract

The progression of *Escherichia coli* lesions was studied in the respiratory tract of 4-week-old commercial broilers. Lesions were induced after a single intratracheal *E. coli* infection, and after an infection with *E. coli* preceded five days earlier by an oculo-nasal and intratracheal infectious bronchitis virus (IBV) infection of either the virulent M41 strain or the H120 vaccine strain. Trachea, lung and thoracic airsac lesions were examined macroscopically and microscopically. Tissue samples were taken at 3 hours post inoculation (hpi), and 1, 2, 4 and 7 days post inoculation (dpi) with *E. coli*. The location of both pathogens was assessed by immunohistochemistry. Single *E. coli* inoculation induced pneumonia and airsacculitis; in case it was preceded by IBV infection, the same macroscopical lesions and also viral tracheitis were found. The microscopical lesions induced by both pathogens in the trachea, lungs and airsacs were consistent with descriptions in literature. No clear difference existed between the single and dual infected birds with respect to inflammatory reactions in the lung, which had disappeared within 7 days, except for the presence of more follicles in dual infected birds. IBV antigen was detected in secondary bronchi and airsacs up to 2 dpi and in the trachea up to 4 dpi. *E. coli* bacteria were found in the tracheal lumen included in purulent material, the parabronchi and airsacs. In lung tissue *E. coli* antigen was found up to 4 dpi. No clear difference existed between single and dual inoculated birds regarding the presence of *E. coli* in the lung. In the airsacs, a few bacteria were found from 0.5 hpi up to 4 dpi in *E. coli* and IBV-*E. coli* inoculated birds. Although, both pathogens were cleared beyond detection at 7 dpi, lesions in the airsac in IBV-*E. coli* inoculated birds persisted, in contrast to broilers inoculated with *E. coli* only. In the present study it is shown that 4-week-old broilers are not resistant to intratracheal *E. coli* inoculation, however, these birds can overcome the induced *E. coli* infection within a short time span. Moreover, a preceding infection with vaccine or virulent IBV does not seem to impair the clearance of *E. coli* in the respiratory tract of broilers, but rather induces an exaggerated inflammatory response in the airsacs only.

Introduction

According to Barnes *et al. (*2003), Vaillancourt and Gross (2003), colibacillosis in chickens refers to any localized or systemic infection caused entirely or partly by *Escherichia coli*. This includes colisepticemia, coligranuloma, air sac disease, coliform cellulitis, swollen head syndrome, coliform peritonitis, coliform salpingitis, coliform osteomyelitis/synovitis, coliform panophtalmitis and coliform omphalitis/yolk sac infection. In broilers, colibacillosis mainly manifests itself as respiratory infection and peritonitis/pericarditis (Goren, 1978; Pourbakhsh *et al*., 1997; Matthijs *et al*., 2003). In practice, colibacillosis involving the respiratory tract in broilers is the most commonly encountered disease at slaughter, usually affecting birds from three to four weeks of age onwards. The infection is considered airborne, the air sacs probably being an important port of entry (Goren, 1978). In the present paper, colibacillosis in broilers refers to lesions induced by *E. coli* in the respiratory tract.

In the field, avian colibacillosis is characterised by low to moderate mortality, low to moderate incidence of septicemic lesions (mainly fibrinous polyserositis) and a high percentage of birds with airsacculitis (Cheville *et al*., 1978; Matthijs *et al*. 2003). Young chickens up to approximately the fourth week of life are susceptible to infection after intratracheal inoculation with *E. coli* as a single pathogen. In contrast, older chickens are considered resistant (Goren, 1978). Respiratory tract lesions, including air sac disease due to *E. coli*, are classified under systemic colibacillosis, and are frequently accompanied by septicaemia (Cheville *et al*., 1978; Pourbakhsh *et al*., 1997; Matthijs *et al*., 2003).

Most published research does not consider colibacillosis *per se*. It rather gives a description of the results of superinfection of *E. coli* after a preceding infection with a micro-organism which enhances the susceptibility such as infectious bronchitis virus (IBV) or Newcastle disease virus (NDV) or *Mycoplasma gallisepticum* (Peighambari *et al*., 2000; Gross, 1990). Experimentally, *E. coli* is usually applied by intratracheal or by supraconjunctival inoculation (Abdul-Rahman Al-Ankari *et al*., 2001), or by aerosol (Peighambari *et al*., 2000). In some occasions, air sac inoculation is applied if reactions of the air sac wall are studied (DeRosa *et al.,* 1992) or a higher efficacy of the infection model is desired (Pourbakhsh *et al.,* 1997).

Lesions and reactions induced by *E. coli* in the three compartments of the respiratory tract, i.e. the trachea and larynx, the lungs and the air sacs are well

described (Gross, 1957; Cheville *et al*., 1978; Pourbakhsh *et al.,* 1997). However, most of these descriptions refer to chickens that have been inoculated experimentally up to an age of two weeks and whose lesions were assessed only during a period of 48 hours. As colibacillosis in the field is most often seen after four weeks of age (Goren, 1978) and the course of the disease takes more than a few days, a window in time exists on which little information is available.

The aim of this study was to investigate the macroscopical and histopathological reactions induced upon intratracheal application of *E. coli* only and of a superinfection of *E. coli* after a preceding infection with vaccine or virulent IBV applied five days earlier in commercial broilers of four weeks of age. The location of the pathogens in the respiratory tract was investigated during a 7 days period after inoculation.

Material and methods

Experimental chickens

Eighteen-day-old embryonated eggs originating from a *M. gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the Department of Farm Animal Health (Utrecht University). Broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, the Netherlands) each with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^3 h^{-1}.

Broilers were fed a commercial ration containing 12.4 MJ of metabolizable energy per Kg and 19.5% crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75% of *ad libitum* intake on 'skip a day base' to prevent leg disorders and hydrops ascites. Tap water was always provided *ad libitum*. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35 ºC at day 1 to 20 ºC at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20 ºC.

All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, the Netherlands, in accordance with the Dutch regulation on experimental animals.

Inocula

IBV vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials, which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50%) per dose (Nobilis® IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, the Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50}/1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use, and contained at least $10^{3.0}$ EID_{50} /ml of H120 virus and $10^{4.6}$ EID_{50}/ml of IBV M41 virus.

The *E. coli* strain 506 (O78; K80) was isolated from a commercial broiler (Van Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs et al. (2003) and was used at a concentration of $10^{7.6}$ colony forming units (CFU)/ml.

Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age two groups were inoculated oculo-nasally (one droplet of 0.05 ml per bird in each eye and nostril) and intra-tracheally (1 ml per bird): group 1 and group 2 received distilled water, group 3 received IBV H120 vaccine and group 4 IBV M41. At 32 days of age, groups 2, 3 and 4 were intra-tracheally inoculated with 1 ml *E. coli* culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intra-tracheally per bird.

For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 vaccine and *E. coli* broth) as the H120 group, and group 4 (IBV M41 and *E. coli* broth) as M41 group.

Clinical and postmortem examination

Clinical signs of IBV infection were recorded 1, 2, 4 and 5 days after IBV inoculation and after *E. coli* inoculation and just before euthanizing. A bird was recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs *et al*., 2003).

From each group, 5 broilers were electrocuted and bled at 0.5 hpi (= hours post *E. coli* inoculation), 3 hpi, and at 1, 2, 4 and 7 dpi (days post *E. coli* inoculation). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (1991) in the left and right thoracic airsac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Van Eck and Goren, 1991).

Histology and immunohistochemistry

Sampling of tissues

After post-mortem examination, trachea and right lung were collected in 10% buffered formalin, fixed for 24 h and routine processed for histology. Also, samples of the trachea, the left lung and both thoracic airsacs, were collected and snap-frozen in liquid nitrogen for immunohistochemistry. Lung samples were taken from a transverse section where the bronchus enters the lung. The part of both thoracic airsacs, which was not attached firmly to the thoracic wall was sampled completely. Sections were cut in such a way to enable inspection of at least 20 mm of length of air sac wall.

Production of polyclonal antibodies against E. coli *506*

The bacterial inocula were prepared by submersing one frozen bead (-70 °C) of a batch containing the *E. coli* 506 strain in trypton soy broth (TSB) and by subsequent incubation for 20 h at 37 °C. The bacteria were washed with PBS and killed with methanol during 5 min. The suspension was centrifuged 10 min. at 1500g and the bacteria were washed twice with PBS and dissolved in 10 mg DDA (dimethyl dioctadecylammonium bromide)/ml PBS.

Two rabbits were injected 3 times, 21 days between each injection, subcutaneous with 1.5 ml of the PBS/DDA solution containing 10^9 *E. coli* bacteria per ml. Pre-immune and immune sera were tested for specific antibodies against *E. coli* 506 on dot blot, paraffin and cryostat sections. Both live and fixed bacteria were recognized.

Tissue staining procedure for IBV and E. coli

Trachea and lung were collected in 10% buffered formalin, fixed for 24 h and processed to paraffin. Sections were cut 5 µm thick, deparaffinised and placed in pure methanol with 0.75% H_2O_2 for 30 minutes to remove endogenous peroxidase activity. For IBV and *E. coli* staining in the airsac, cryostat sections (6 µm thick) were made from both thoracic airsacs. Cryosections were transferred to Superfrost Plus slides (Menzel-Glaser) and stored over silicagel for at least 24 h before use.

Sections were incubated with rabbit serum specific for *E. coli* (*E. coli* 506) or with mouse sera with mAbs 48.4 against IBV nucleoprotein (Koch et al.,1990) for 1 h. After washing in PBS, the slides were co-incubated for 1 h with rabbit anti-mouse serum (Dako, Denmark) or goat anti-rabbit serum (Dako, Denmark) conjugated to horseradish peroxidise. The slides were washed and stained with 0.5

mg 3, 3-diaminobenzidine-tetrahydrochloride (DAB, Sigma, USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.03% H_2O_2.
DAB-stained sections were counterstained with hematoxylin and mounted with aquamount (BDH, UK).

Statistical analysis
Between-group differences per time point were non-parametrically analyzed for mean lesion score (MLS) using the Mann-Whitney U test. Nasal discharge, macroscopic colibacillosis lesions (airsacculitis, perihepatitis and/or pericarditis) and microscopic lesions (tracheitis, pneumonia, airsacculitis) per time point were non-parametrically analyzed between groups with Fischer Exact Test with Bonferroni correction.

Results

Mortality
During the course of the experiment no mortality occurred.

Nasal discharge
Mucous nasal discharge was observed from 2 to 9 days after IBV inoculation. From 2 to 5 days following IBV inoculation the number of birds with nasal discharge in the M41 group was significantly higher in comparison with the H120 group (Table 1).

Table 1. Number of broilers with mucous nasal discharge at different time points after inoculation with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days.

	Days after IBV inoculation							
Group	1	2	4	5*	6	7	9	12
PBS[$]	0/30[A]	0/30[A]	0/30[A]	0/30[A]	0/5[A]	0/5[A]	0/5[A]	0/5[A]
E. coli[$$]	0/30[A]	0/30[A]	0/30[A]	0/30[A]	0/5[A]	0/5[A]	0/5[A]	0/5[A]
H120	0/30[A]	1/30[A]	2/30[A]	3/30[A]	1/5[A]	2/5[A]	3/5[A]	0/5[A]
M41	0/30[A]	15/30[B]	28/30[B]	30/30[B]	3/5[A]	3/5[A]	1/5[A]	0/5[A]

[AB]Groups with different letters within a column are significantly different ($P<0.05$).
[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.
*Day of inoculation with *E. coli*.

Table 2. Macroscopical colibacillosis lesions: number of broilers with airsacculitis and/or pericarditis/perihepatitis and the mean lesion score (MLS ± SD). At each timepoint and within each group five birds were analysed. Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age.

		Time after *E. coli* 506 strain inoculation					
Broilers with		0.5 h	3 h	1 d	2 d	4 d	7 d
Airsacculitis	PBS[$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli[$$]	0^{A}	0^{A}	5^{B}	2^{AB}	3^{AB}	0^{A}
	H120	4^{AB}	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}
	M41	5^{B}	5^{B}	5^{B}	4^{AB}	5^{B}	5^{B}
Perihepatitis	PBS[$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
and/or	*E. coli*[$$]	0^{A}	0^{A}	1^{A}	0^{A}	2^{A}	0^{A}
Pericarditis	H120	0^{A}	0^{A}	0^{A}	0^{A}	2^{A}	1^{A}
	M41	0^{A}	0^{A}	0^{A}	0^{A}	3^{A}	2^{A}
MLS	PBS[$]	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}
	E. coli[$$]	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	1.6 ± 1.6^{B}	1.6 ± 2.2^{AB}	3.4 ± 3.4^{AB}	0.0 ± 0.0^{A}
	H120	0.4 ± 0.2^{A}	0.5 ± 0.0^{A}	2.4 ± 1.5^{B}	3.4 ± 1.9^{B}	6.6 ± 3.5^{B}	5.0 ± 4.1^{B}
	M41	0.5 ± 0.0^{A}	0.5 ± 0.0^{A}	3.4 ± 0.9^{B}	2.0 ± 1.9^{B}	6.4 ± 2.5^{B}	4.5 ± 3.0^{B}

[AB]Groups with different letters within a column are significantly different ($P<0.05$).
[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

Macroscopical lesions

Broilers that received IBV **(**H120 and M41 group) showed veiled airsacs at 0.5 and 3 hpi. No lesions were found at that time in the PBS group and the *E. coli* group.

In all infected groups airsaccultis, pericarditis and perihepatitis was observed from 1 dpi. Pericarditis and perihepatis were only found in broilers with severe airsacculitis. Lesions were more pronounced in the H120 and M41 group than in the *E. coli* group, however the difference was not significant. Seven dpi no macroscopical lesions were found in the *E. coli* group, whereas airsacculitis was present in all and serositis in some birds of the H120 and M41 group. The number of broilers with airsacculitis, perihepatitis and/or pericarditis and mean macroscopic lesion score are presented in Table 2.

Microscopical lesions

Trachea

In the PBS group, at all time points, tracheas were lined with pseudostratified ciliated columnar respiratory epithelium with few goblet cells and varying numbers of mucoid glands. The lamina propria was one to three cells wide and no infiltrates were present (Figure 1a). A few single granulocytes per section were present. Plasma cells were present in low numbers varying from 5 to 30 plasma cells per section.

In the *E. coli* group, no lesions were found in the tracheas at any stage except for a slight increase of plasma cells in the lamina propria at 2 and 4 dpi.In the H120 and M41 group, from 3 hpi, the epithelium was not ciliated, grossly cuboid, and partially flat (Figures 1b and 1c). At 4 dpi the epithelium had regained the pseudostratified ciliated morphology in the tracheas of the birds in the M41 group; at 7 dpi in both IBV groups the epithelium resembled that of the PBS group (Figure 1d). The lamina propria in the H120 and M41 group was more than 10 cells wide due to mononuclear and mixed cell infiltration (mononuclear cells and granulocytes). The mononuclear infiltrate in the lamina propria of these groups declined slightly to 3 cells wide at 7 dpi, some larger mononuclear aggregates and several follicles remaining locally.

Granulocytes were present in the lamina propria in numbers varying from a few to more than hundred per field of view and also between epithelial cells resulting in thousands per trachea section at 1 dpi, decreasing at 2 and 4 dpi to only a few per section at 7 dpi. Plasma cells were found varying from 30 to 100 per section at 3 hpi. Plasma cell numbers increased at 2 and 4 dpi to 40 to 250 per section and diminishing to 30 to 50 per section at 7 dpi. At 1 dpi few follicles were found in some tracheas of the IBV groups; at 7 dpi 1 to 4 per section were found in tracheas of birds of the M41 group and 4 to 8 in H120 group.

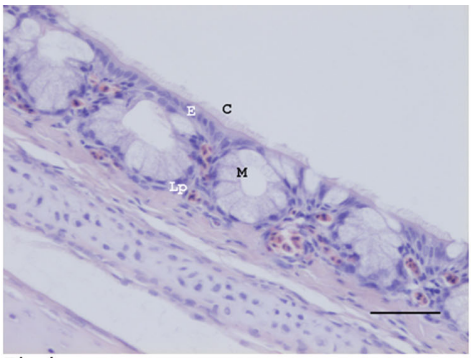

Fig 1a

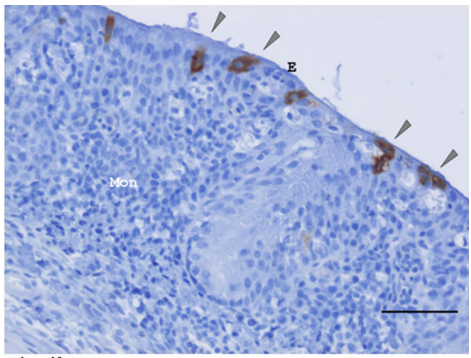

Fig 1b

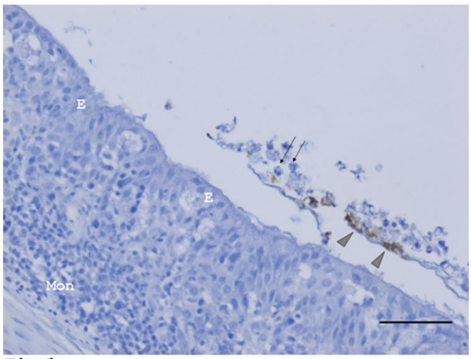

Fig 1c

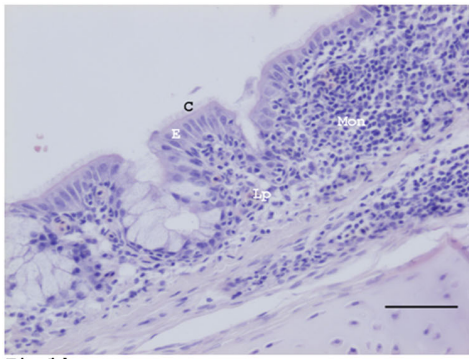

Fig 1d

Figure 1. Trachea of broilers inoculated oculonasally and intratracheally with either PBS (Fig 1a) or IBV H120 (Fig 1b, c, d) at 27 days of age and intratracheally with PBS-diluted glucose broth (Fig 1a) or *E. coli* (Fig 1b, c, d) at 32 days of age.
(a) Trachea sampled at one day after inoculation with PBS-diluted glucose broth. Haematoxylin and eosin stain, bar = 50 microns. Normal tracheal mucosa with pseudostratified columnar epithelium (E), bearing cilia (C), lamina propria (Lp) of only few cells thickness and mucous glands (M).
(b) Trachea sampled one day after inoculation with *E. coli*. Immunoperoxidase IBV – haematoxylin stain, bar = 50 microns. Epithelial cells (E) have irregular morphology and do not bear cilia, epithelial cells containing IBV are stained (arrowheads; note the negative cast of the nucleus); in the lamina propria mononuclear infiltrate (Mon) of several tens of cells in thickness is present.
(c) Trachea sampled at one day after inoculation with *E. coli*. Immunoperoxidase *E. coli* – haematoxylin stain, bar = 50 microns. Epithelial cells (E) do not bear cilia, in the lamina propria mononuclear infiltrate (Mon) more than ten cells in thickness is present. Stained *E. coli* bacteria (arrowheads) are present in purulent exudation containing heterophils with bilobar nuclei (arrows); the mucosal surface does not appear to be colonized by *E. coli*.
(d) Trachea sampled at seven days after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 50 microns. Mucosa consists of pseudostratified columnar respiratory epithelium (E) and bearing cilia (C); in the lamina propria (Lp) mononuclear infiltrate (Mon) is present.

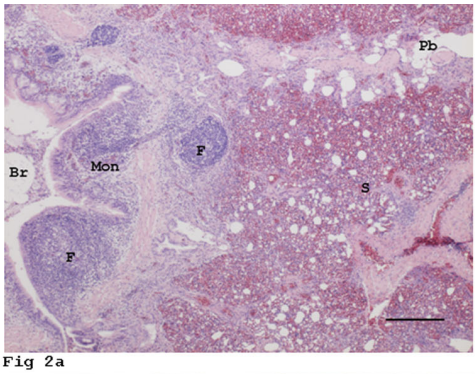

Fig 2a

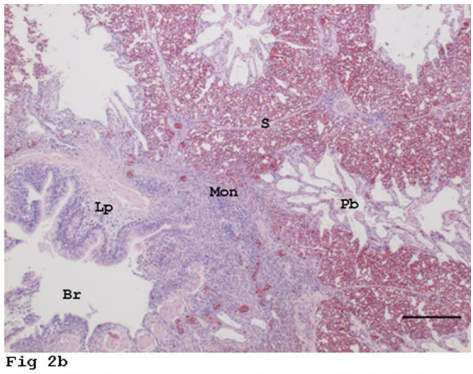

Fig 2b

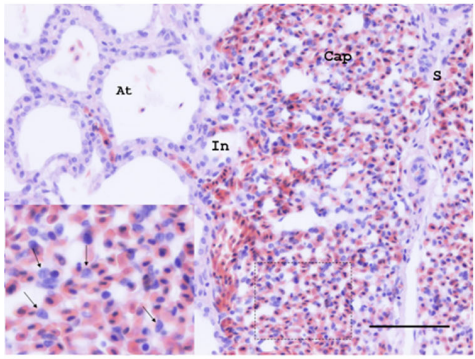

Fig 2c

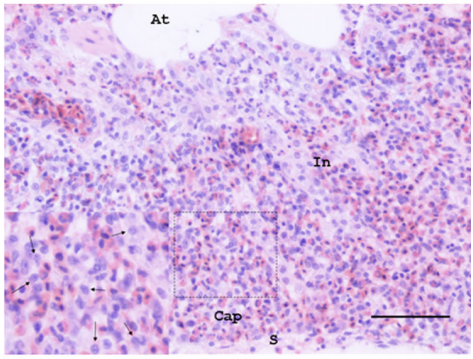

Fig 2d

Figure 2. Lung of broilers inoculated oculonasally and intratracheally with either PBS (Fig 2b, c, d) or IBV H120 (Fig 2a, e) or IBV M41 (Fig 2f, g) at 27 days of age and intratracheally with PBS-diluted glucose broth (Fig 2c) or *E. coli* (Fig 2a, b, d, e, f, g) at 32 days of age.
(a) Lung tissue sampled at seven days after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 200 microns. Parabronchi (Pb) appear aerated; the lamina propria of the bronchus is heavily infiltrated (Mon) and several follicles (F) are present in both the lamina propria of the bronchus (Br) and in the peribronchial area. Most interparabronchial septa (S) are without infiltrate.
(b) Lung tissue sampled at seven days after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 200 microns. Parabronchi (Pb) appear aerated; the lamina propria of the bronchus (Br) is only slightly infiltrated. Adjacent to the bronchus some mononuclear infiltrate (Mon) exists. Most interparabronchial septa (S) are thin and without infiltrate.
(c) Lung tissue sampled at one day after inoculation with PBS-diluted glucose broth. Haematoxylin and eosin stain, bar = 50 microns. Normal aerated aspect of atria (At), infundibulum (In), and capillary region (Cap) with open air capillaries as indicated by the position of the epithelial cells with two nucleoli visible (arrows), in insert. Interparabronchial septum (S) is thin and without oedema.
(d) Lung tissue sampled at one day after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 50 microns. Massive infiltration in capillary region (Cap) and apparent extensive loss of aeration in atria (At), infundibulum (In), and collapse of capillary region as indicated by the increase in density of respiratory epithelial cells with two nucleoli visible (arrows), in insert. Interparabronchial septum (S) shows oedema.

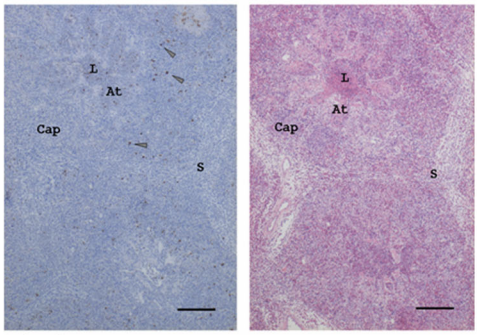

Fig 2e

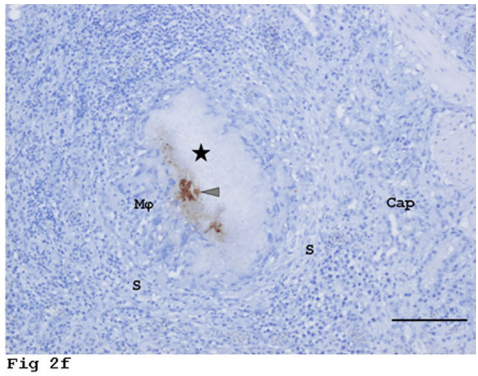

Fig 2f

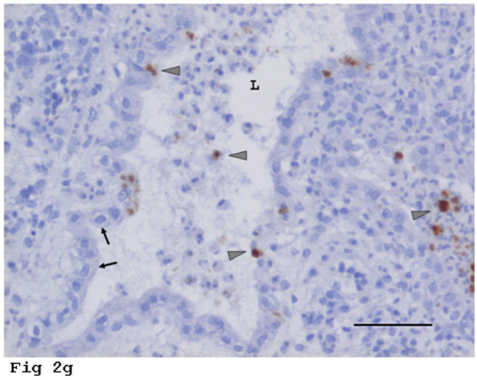

Fig 2g

(e) Lung tissue sampled at one day after inoculation with *E. coli*. Left and right represent serial sections of the same parabronchi taken at small distance. Bar = 200 microns.
Left: Immunoperoxidase *E. coli* – haematoxylin stain. *E. coli* bacteria (arrowheads) are present in the capillary region (Cap), in atria (At) and in the central lumen (L) as deeply staining aggregates of antigen representing many bacteria. In the interparabronchial septum (S) oedema is present.
Right: Haematoxylin and eosin stain. Parabronchi demonstrate infiltration of capillary region (Cap) and exudation in the atria (At) and in the central lumen (L). The capillary region does not appear aerated, only a few atria seem partially aerated. Massive oedema exists in the interparabronchial septum (S).
(f) Lung tissue sampled at seven days after inoculation with *E. coli*. Immunoperoxidase *E. coli* – haematoxylin stain, bar = 100 microns. Remnants of parabronchus with heavily infiltrated interparabronchial septum (S); from the capillary region and the lumen a necrotic core (asterisk) surrounded by macrophages (Mφ), partially multinucleated, remains. *E. coli* bacteria within the core are stained (arrowhead). Adjacent a parabronchus with apparently aerated air capillaries (Cap).
(g) Lung tissue sampled at three hours after inoculation with *E. coli*. Immunoperoxidase *E. coli* – haematoxylin stain, bar = 50 microns. Epithelium lining central parabronchial lumen shows increased thickness, many epithelial cells have vacuolated cytoplasm (arrows) and in several epithelial cells aggregates of stained *E. coli* bacteria (arrowheads) are visible. *E. coli* bacteria are also present as deeply staining aggregates in the atria and in the central lumen (L). Exudation is present containing heterophils with bilobar nuclei.

Lungs

Bronchi

The adventitia of the bronchi of broilers of the *E. coli*, H120 and M41 group showed oedema and cellular infiltrations consisting mainly of granulocytes and mononuclear cell types, which were present from 0.5 hpi and 2 dpi, respectively. In most birds of the *E. coli* group the lamina propria of bronchi were changed. Slight to moderate oedema and hyperaemia and granulocyte infiltrates were present; in some occasions granulocytes were present between epithelial cells. In the IBV groups, marked changes were present, consisting of lymphocyte infiltrates, granulocyte infiltrates extending into the epithelial layer, hyperaemia and oedema in varying but considerable amounts. Follicles were also present mainly in the lamina propria and some also in the adventitia surrounding the bronchi. The mean number of follicles per section of bronchus varied from 2 to more than 15 in both IBV groups from 1 dpi onwards, i.e. from 6 days after IBV inoculation (Figure 2a, lung at 7 dpi). In the PBS group and the *E. coli* group, few follicles were present, i.e. a mean of 1 follicle per section of bronchus (Figure 2b).

Parabronchi

In the PBS group, at all time points, the capillary area of parabronchi, infundibula and atria appeared open and aerated. The atrial septa consisted of a thin stroma lined with flat epithelium. In the capillary area predominantly erythrocytes, air capillary epithelium and endothelium were recognized, also a few mononuclear cells were present, mostly fibrocytes and macrophages (Figure 2c).

The histopathological changes found in the parabronchi of the *E. coli* group, the H120 group and the M41 group were similar. One bird of the *E. coli* group sampled at 1 dpi did not show any reaction.

Most altered parabronchi were located adjacent to and surrounding the primary bronchus. In some occasions also the parabronchi running at the periphery of the lung were affected. Heavily affected parabronchi were frequently situated next to intact parabronchi. Some lengthwise sectioned parabronchi were only affected partially, the healthy part abruptly transitioning into clearly inflamed tissue. In most affected parabronchi the central lumen was not clogged.

At 3 hpi several parabronchi appeared collapsed due to loss of air in the capillary area resulting in a reduction of the outer diameter of the parabronchus and an increase of the number of cells per field of view (Figure 2d, lung at 1 dpi). In several birds of all three infected groups, oedema and granulocyte infiltration of the

parabronchus was noticed at this time point. At 1 dpi collapse oedema and abundant cellular infiltration consisting mainly of granulocytes and to lesser extend of mononuclear cell types were observed in the capillary area of many parabronchi of almost all infected birds, which diminished from 2 to 4 dpi. The parabronchial septa were thickened due to oedema and cellular infiltration. In a few severely affected parabronchi, passage of air appeared impossible through oedema and cellular infiltration, collapse of air capillaries and accumulation of exudation in the central lumen (Figures 2e and 2f). Margination and diapedesis of granulocytes was observed in arteries including the branches of the *arteria pulmonalis* running at the triads where three parabronchi meet. In several parabronchi of all three infected groups serous fluid and inflammatory cells were present in the atria and the central lumen.

The epithelium lining the central lumen increased in thickness and changed to a nearly cuboid shape from 3 hpi onwards in all *E. coli* inoculated groups. In a few of these epithelial cells bacteria were found in immunohistochemically stained sections (Figure 2g).

In some instances, at 1 and 2 dpi, a segment of the parabronchus showed suppurative necrotizing parabronchitis. A core of debris with a faint representation of the preexistent architecture, surrounded by partially multinucleated phagocytes enclosed within the interparabronchial septum remained of these parabronchi. In this core of debris, small nests of bacteria were present. These structures had assumed the form of a granuloma and multinucleate macrophages were present from 2 dpi onwards (Figure 2f, lung at 7 dpi).

Oedema and granulocytes disappeared gradually from 2 dpi onwards. Subsequently, predominantly mononuclear infiltrates remained in affected parabronchi. At 7 dpi, most parabronchi seemed to have recovered completely and normal architecture was seen. They appeared inflated and had a diameter similar to that of the control birds (Figure 2a and 2b). At this time point, few parabronchi in several birds of all infected groups were only partially or not inflated and infiltrated or necrotized. Birds with these changes have been classified as having pneumonia in Table 3.

In several lungs of the *E. coli*, the H120 and the M41 group few isolated small focal mononuclear infiltrates were present in parabronchial/atrial septa (Figures 2a and 2b). The number of affected parabronchi and severity of parabronchial lesions are presented in Table 4.

Table 3. Microscopical lesions: number of broilers with tracheitis, pneumonia and airsacculitis. At each timepoint and within each group five broilers were analysed. Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age

		Time after *E. coli* inoculation					
Broilers with		0.5 h	3 h	1 d	2 d	4 d	7 d
Tracheitis	PBS[$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli[$$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	H120	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}
	M41	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}
Pneumonia	PBS[$]	n.d. [$$$]	n.d.	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli[$$]	n.d.	n.d.	5^{B}	5^{B}	3^{AB}	3^{A}
	H120	n.d.	4^{A}	3^{AB}	5^{B}	5^{B}	3^{A}
	M41	n.d.	5^{A}	5^{B}	4^{B}	2^{AB}	3^{A}
Airsacculitis	PBS[$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli[$$]	3^{AB}	4^{AB}	5^{B}	3^{AB}	3^{AB}	0^{A}
	H120	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}
	M41	5^{B}	5^{B}	5^{B}	4^{AB}	5^{B}	5^{B}

[AB]Groups with different letters within a column are significantly different ($P<0.05$).
[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.
[$$$]n.d. = not determined

Table 4. Percentage of damaged parabronchi in broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age. At each timepoint and within each group five birds were analysed. Percentage of damaged parabronchi per field section: - = 0%, +/- = 0-10%, + = 10-30%, ++ = >30%. Severity of damage of the parabronchi: - = no changes, +/- = minor cell infiltration/oedema in part of parabronchus, + = generalized cell infiltration in whole parabronchus, ++ = massive cell infiltration and/or necrosis in parabronchus.

	Damaged parabronchi					Severity of damage				
	Time after *E. coli* inoculation									
Group	3 h	1 d	2 d	4 d	7 d	3 h	1 d	2 d	4 d	7 d
PBS[$]	n.d.[$$$]	-	-	-	-	n.d.	-	-	-	-
E. coli[$$]	n.d.	++	++	+	+/-	n.d.	++	+	+/-	+/-
H120	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-
M41	+	++	+	+/-	+/-	+/-	+	+/-	+/-	+/-

[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.
[$$$]n.d. = not determined.

Airsacs

In the PBS group, at all time points, the wall of the cranial thoracic airsacs consisted of a thin layer of stroma covered with serosa of one cell thickness. On the respiratory side areas covered with columnar ciliated epithelium of the respiratory tract alternated with areas covered with flat epithelium.

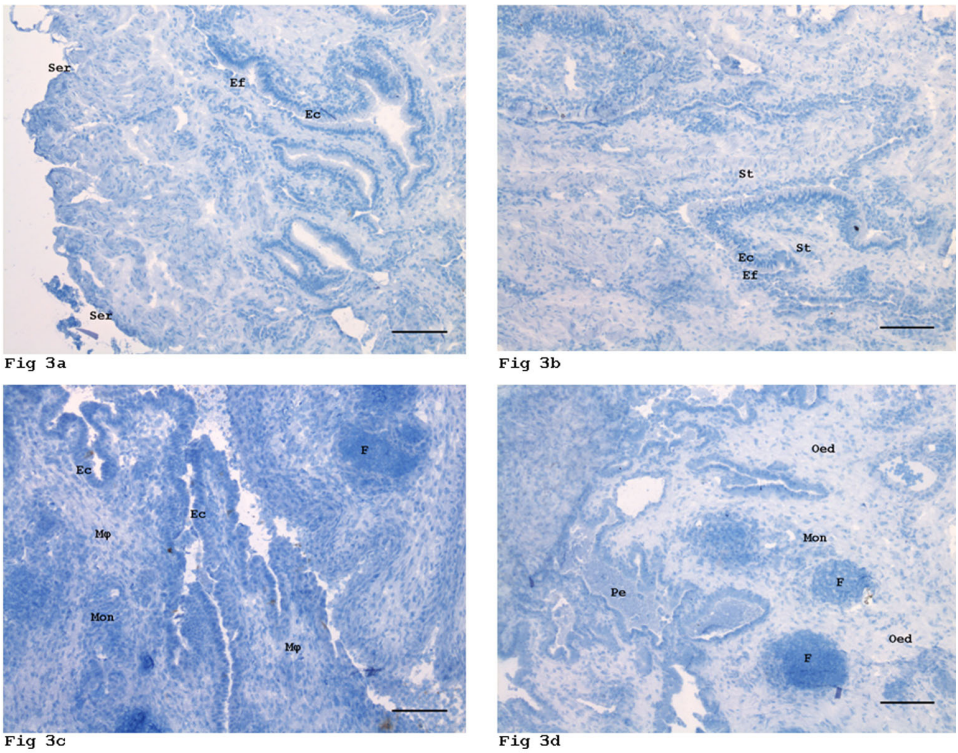

Figure 3. Airsac of broilers inoculated oculonasally and intratracheally with either PBS (Fig 3a,b) or IBV M 41 (Fig 3c,d) at 27 days of age and intratracheally with PBS-diluted glucose broth (Fig 1a) or *E. coli* (Fig 3b,c,d) at 32 days of age.
(a) Airsac samples at four days after inoculation with PBS-diluted glucose broth. Immunoperoxidase IBV – haematoxylin stain, bar = 100 microns. Columnar respiratory epithelium bearing cilia (Ec) alternated with flat epithelium (Ef). Serosa of one cell thickness (Ser).
(b) Airsac sampled at seven days after inoculation with *E. coli*. Immunoperoxidase IBV – haematoxylin stain, bar = 100 microns. Intact columnar respiratory epithelium bearing cilia (Ec) alternated with flat epithelium (Ef). No mononuclear infiltrate is present in the stroma (St).
(c) Airsac sampled at 0,5 hours after inoculation with *E. coli*. Immunoperoxidase IBV – haematoxylin stain, bar = 100 microns. Columnar epithelium (Ec) is intact, a mononuclear infiltrate (Mon) mostly consisting of macrophages (Mφ) as shown previously by immunohistochemistry (Matthijs *et al*., 2008) and a follicle (F) are present.
(d) Airsac sampled at seven days after inoculation with *E. coli*. Immunoperoxidase IBV – haematoxylin stain, bar = 100 microns. Oedema (Oed), mononuclear infiltrate (Mon) and several follicles (F) are present in the stroma as well as purulent exudation (Pe) in the airsac lumen.

Table 5. The extent of oedema and purulent exudation in the thoracic airsac in broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age. At each timepoint and within each group five birds were analysed.
Oedema and pus present per field section:- = no oedema or pus, +/- = localized oedema and pus, + = generalised oedema and pus, ++ = massive oedema and pus.

	Oedema						Purulent exudation					
	Time after *E. coli* inoculation						Time after *E. coli* inoculation					
Group	0.5 h	3 h	1 d	2 d	4 d	7 d	0.5 h	3 h	1 d	2 d	4 d	7 d
PBS[$]	-	-	-	-	-	-	-	-	-	-	+/-	-
E. coli[$$]	+/-	+/-	+/-	+	+	-	-	-	+/-	+/-	+/-	-
H120	++	++	+	+	++	+/-	+	+	++	++	++	++
M41	++	++	++	++	++	+/-	+	+	++	++	++	++

[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

A distinct lamina propria was not recognizable. No oedema, infiltrates and follicles were present (Figure 3a).
In the *E. coli* group, mononuclear infiltrates were present in some broilers at 1 dpi and had disappeared at 7 dpi. At 7 dpi, the epithelium was completely intact (Figure 3b).
In most broilers of both IBV groups, mononuclear infiltrate and several follicles were present in the stroma at 0.5 hpi (Figure 3c) and at 3 hpi up to 7 dpi. No noticeable difference existed between the number of follicles observed at 1 dpi and observed at 7 dpi.

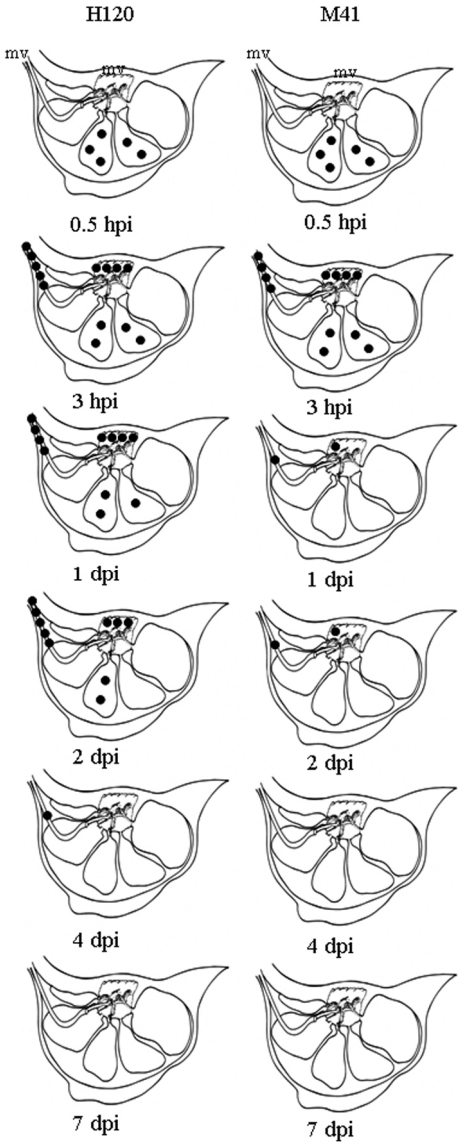

Figure 4. The localization of IBV antigen in the respiratory tract (trachea, lung and thoracic airsacs) of broilers (n = 5 per time point (hours post *E. coli* inoculation = hpi/days post *E. coli* inoculation = dpi) at 32 days of age, preceded by IBV vaccine H120 (H120 group) or virulent IBV M41 (M41 group) at 27 days of age).

At 7 dpi in both IBV groups oedema and mononuclear infiltrates and several follicles were present in the stroma as well as purulent exudations in the airsac lumen.

The extent of oedema and pus in the thoracic airsac is presented in Table 5. No difference in oedema and purulent exudation were found between the H120 and M41 groups (Figure 3d). In the *E. coli* group only localized oedema and pus were found.

The number of broilers with microscopical lesions in trachea, lung and airsacs are presented in Table 3.

Location of pathogens

IBV antigen

IBV antigen was found in respiratory epithelial cells present in the trachea (Figure 1b), the bronchi and the airsac of the H120 and M41 groups. IBV was not found in mucoid glands in the trachea, neither in the flat epithelium lining part of the airsac nor in the parabronchi.

The number of broilers with IBV antigen and its localisation at different time points after *E. coli* inoculation is presented in Figure 4.

E. coli *antigen*

The tracheas of all broilers inoculated with *E. coli* appeared free of bacteria from 2 dpi to 7 dpi except one bird of the H120 group that had *E. coli* bacteria in the trachea at 4 dpi. In case *E. coli* bacteria were found in trachea at 1 dpi this was never accompanied by lesions.

Adhesion of bacteria to the epithelium was not observed (Figure 1c). Moreover, bacteria were not encountered in the deeper layers of the tracheal wall.

E. coli was present in the capillary region and the larger lumina of the parabronchi in the lungs. In the affected parabronchi the amounts of bacteria in the capillary area, the atria and in the purulent debris within parabronchial and bronchial lumina were similar; between parabronchi the amount varied considerably. Most bacteria were found in the parabonchi adjacent to the bronchus. The bacteria detected with specific antiserum were present as free bacteria and as round agglomerates of several microns of diameter not recognisable as individual bacteria (Figures 2e and 2g). Few bacteria were found within individual epithelial cells from the lining of the parabronchial central lumen (Figure 2g). Positively stained material was found mainly as small round agglomerates and free bacteria

were not seen in the lung tissues at 2 and 4 dpi. These agglomerates of antigen were found in the capillary part and in the lumen of the parabronchi. No evidence for the presence of bacteria in endothelial cells of the capillary part of the parabronchus was found. The occurrence of bacteria in lung tissue was accompanied by inflammatory changes of the parabronchus.

In plugs of debris, resulting from complete purulent necrosis of parabronchi, small round colonies of bacteria were enclosed in cracks in the debris from 2 dpi onwards. Small nests of bacteria were still present in cracks in the solid necrotic material within the remains of necrotized parabronchi at 7dpi; in these lungs this was the only site where bacteria were found (Figure 2f).

In the airsacs, positively stained material was found predominantly in pus located at the surface. Individual bacteria were present in the *E. coli* group and only few individual bacteria and also small round agglomerates of antigen were present in both IBV groups. At 3 hpi the presence of bacteria was accompanied by oedema of the air sac wall in all broilers of the IBV groups, but not in the broilers of the *E. coli* group. At 2 and 4 dpi in the *E. coli* and IBV groups, the presence of bacteria was also accompanied by inflammatory changes i.e. cellular infiltrates in the stroma and/or the respiratory epithelium. These broilers also showed purulent exudation on the respiratory surface of the airsacs. At 7 dpi no bacteria were detected in the air sacs of any group.

Bacterial antigen was also found in association with pleuritis. It was found in the *E. coli* group at 1 dpi (4 broilers), 2 dpi (2 broilers) and 4 dpi (2 broilers). In the H120 group and the M41 group pleuritis was found at 4 dpi in only 1 bird per group. The number of broilers with *E. coli* antigen and its localisation at different time points after *E. coli* inoculation is presented in Figure 5.

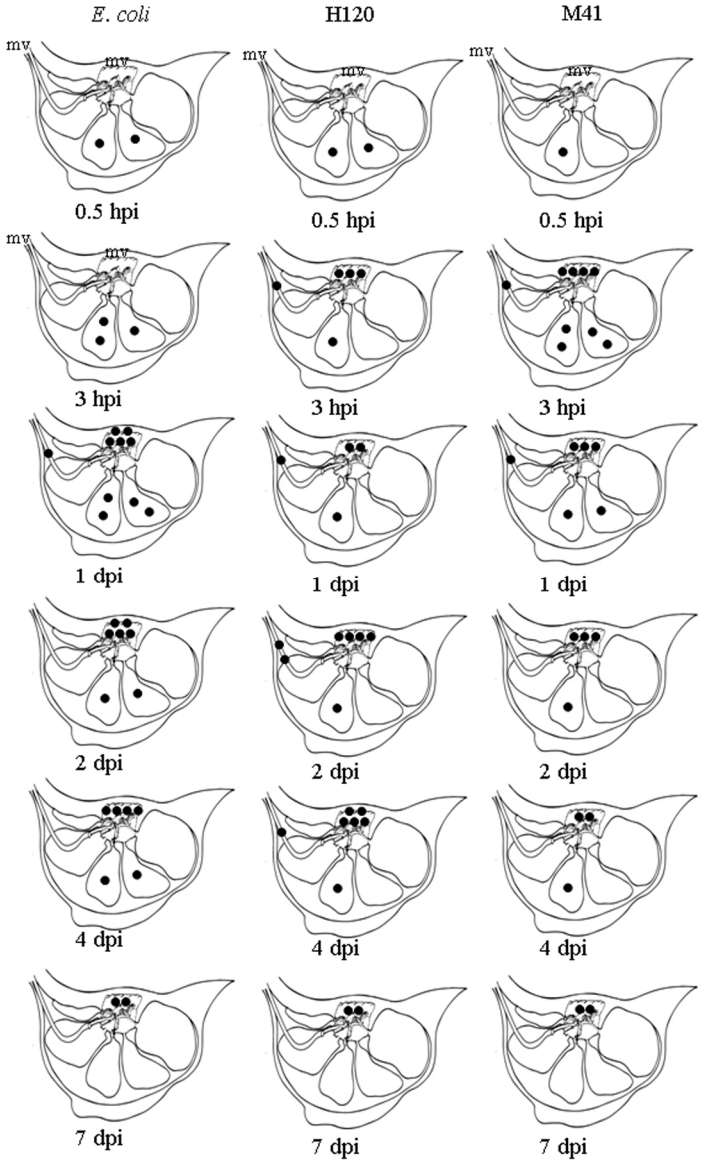

Figure 5. The localization of *E. coli* antigen in the respiratory tract (trachea, lung and thoracic airsacs) of broilers (n = 5 per time point (hours post *E. coli* inoculation = hpi/days post *E. coli* inoculation = dpi) at 32 days of age, preceded by inoculation with distilled water (*E. coli* group) or IBV vaccine H120 (H120 group) or virulent IBV M41 (M41 group) at 27 days of age).

Discussion

In the present study, broilers of four weeks of age were inoculated intratracheally with *E. coli* as a single agent or as a superinfection after a preceding infection with IBV of either virulent M41 strain or H120 vaccine strain. Both approaches resulted in pneumonia and airsacculitis.

An important difference was that broilers only infected with *E. coli* recuperated within a week after inoculation, whereas the dually infected birds still suffered from persisting airsacculitis and systemic infection despite the fact that they had cleared trachea and lungs from immunohistochemically detectable IBV and *E. coli*. Regarding the lesions in the lungs, only minor differences were found between the *E. coli* and IBV groups. In neither group the trachea appeared to be involved in the infection with *E. coli*. Only a few bacteria were occasionally seen in some tracheas; they appeared freely distributed in the lumen and not attached to epithelial cells. No signs of colonization by *E. coli* as mentioned by Nakamura *et al.* (1992) of tracheal epithelium were seen. Various other authors reported colonization of fowl tracheas by *E. coli* based on countings of CFU after grinding tracheal tissue (Marien *et al*., 2005; 2006). By doing so, exudation containing *E. coli* bacteria may have been included in these samples, adversely suggesting colonization of the tracheal mucosa. Another difference in the microscopic lesions was more pronounced lymph follicular reactions in the IBV groups compared to the *E. coli* group.

Our results indicate that 4-week-old broilers are not *in sensu stricto* naturally resistant against an experimental infection with a high dose of *E. coli,* but are capable to recover from an acquired infection within several days, showing only minimal recognisable damage in the respiratory tract. Indications for the clearance of bacteria from the respiratory tract were found after immunohistochemical study of the tracheas, the lungs and the airsacs. In case bacteria were still encountered at 7 dpi, they were always found within cores of necrotic material from severely affected parabronchi. These cores resembled fibriscesses (Huchzermeyer *et al*., 2000), which are considered the result of a defence mechanism to prevent bacteraemia in reptiles and birds, thus suggesting that clearance mechanisms were effectively operational. Moreover, some other authors (Peighambari *et al*., 1999; Yoder *et al*., 1989) mention a discrepancy between the occurrence of bacteria as determined by cultural methods and the presence of lesions in the respiratory tract after infection with *E. coli*, also suggesting that clearing of this bacterium might be

a common phenomenon. Apparently it occurs rapidly and effectively within days, and is therefore presumably based upon non-specific defence mechanisms.

One of the reactions in the lungs observed as early as 3 hpi is deinflation resulting in collapse of the capillary region of the parabronchus. The event resembles the collapse of the mammalian lung after disappearance of surfactant in inflammatory reactions in the lung by decreased production and increased reabsorption of surfactant by type-II pneumocytes mediated by e.g. surfactant protein (SP)-A (Griese, 1999). In this respect, the observed increase in size of parabronchial epithelium and the vacuolized aspect of the cytoplasm may be a consequence of the uptake of surfactant by pneumocytes during the early defence against bacterial infection and the preparation for reactive changes in the lung. It is noteworthy that the avian type-II pneumocyte is present in the parabronchial epithelium, which lines the central lumen (Lorz et al, 1997). However, it must be kept in mind that concerning the origin, the composition and structure and the metabolism major differences exist between mammalian and avian lung surfactants (Scheuermann et al, 1997) and surfactant proteins (Bernhard *et al*., 2001).

In contrast to the capillary region, the central lumen in most affected parabronchi remains passable for air. The affected parabronchi were situated around the primary bronchus: this distribution may explain that pneumonia in chicken lung is hard to recognize macroscopically.

The preceding infection with IBV did not seem to affect the clearance of *E. coli* from the lungs and the airsacs. This finding is in line with the work of Ariaans *et al*. (2008, accepted Veterinary Immunology and Immunopathology), who described that the effector function of phagocytic cells in peripheral blood and in the spleen is not affected by an infection with IBV.

IBV antigen was found in the airsacs, persisting five to seven days after inoculation with IBV. It appears that from the three compartments of the respiratory tract: trachea, lungs (bronchi and parabronchi) and the airsacs, the airsacs are the only site which may harbour both IBV and *E. coli* during a maximum of 2 dpi. A plausible explanation for the longer persistence of airsac lesions compared to trachea and lungs upon successive infection of broilers with both IBV and *E. coli* was given previously by Matthijs *et al*. (2008, accepted Veterinary Immunology and Immunopathology), who demonstrated the occurrence of a reduction in the number of macrophages a week after inoculation with *E. coli,* whereas these cells were still present in high numbers at this stage in the airsacs of IBV–*E. coli* infected broilers. Possibly, upon the influence of both pathogens the

high concentrations of macrophages will be prompted to overproduce pro-inflammatory cytokines, favouring the occurrence of the persistent airsacculitis. In this regard, Gross (1990) also considers the *E. coli*-complicated respiratory infection the result of an overspecialized cellular response.

Based on the presented data we conclude that four-week-old broilers are not resistant to intratracheal *E. coli* inoculation with high doses, however, these birds can within a short time span overcome the induced *E. coli* infection. Moreover, a preceding infection with IBV vaccine and field strain does not impair the clearance of *E. coli* in the respiratory tract of broilers, but rather induces an exaggerated inflammatory response only in the airsacs. Further research on superinfections with IBV and *E. coli* in broilers, should focus on the airsacs because it is the only site of the respiratory tract where both pathogens were demonstrated and the persistence of inflammatory changes was observed.

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Abbreviations

Infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg infective dose 50% (EID_{50}), specified pathogen free (SPF), colony forming units (CFU), hours post *E. coli* inoculation (hpi), days post *E. coli* inoculation (dpi), mean lesion score (MLS), trypton soy broth (TSB), dimethyl dioctadecylammonium bromide (DDA), 3,3-diaminobenzidine-tetrahydrochloride (DAB)

Chapter 6

Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*

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Abstract

Colibacillosis results from infection with avian pathogenic *Escherichia coli* bacteria. Healthy broilers are resistant to inhaled *E. coli*, but previous infection with vaccine or virulent strains of Infectious Bronchitis Virus (IBV) predisposes birds for severe colibacillosis. The aim of this study was to investigate how IBV affects the course of events upon infection with *E. coli*. Broilers were inoculated with IBV H120 vaccine virus or virulent M41 and challenged 5 days later with *E. coli* 506. A PBS and *E. coli* group without previous virus inoculation were included. Sections of trachea, lung and airsacs were stained for CD4, CD8, γδ - TCR, αβ1-TCR, and for macrophages (KUL-01) and both pathogens. Changes in the mucociliary barrier of trachea, lung and airsacs did not predispose for bacterial superinfection. The disease in the lungs of the *E. coli* group and both IBV/*E. coli* groups was similar. Lesions in the airsacs were more pronounced and of longer duration in the IBV/*E. coli* groups. The immunocytological changes differed substantially between the *E. coli* group and both IBV/*E. coli* groups. In trachea, lungs and airsacs the CD4+ and CD8+ populations were significantly larger than in the *E. coli* and PBS groups. In the lungs and the airsacs the macrophages were more numerous in the IBV/*E. coli* and the *E. coli* groups than in the PBS group. The presence of great numbers of T cells and macrophages in IBV infected birds most likely induced an altered immune response, which is responsible for the enhanced clinical signs of colibacillosis.

Introduction

In humans as well as in animals it is well known that viral infection of the respiratory tract can predispose for bacterial infections (reviewed by Heinzelmann *et al.,* 2002; Matthijs *et al.,* 2003). Two hypotheses for the underlying mechanisms have emerged. One hypothesis is that bacterial superinfection emerges from viral damage to the respiratory tissue, characterized by loss of cilia and ciliated cells (Bakaletz, 1995), decreased ciliary activity and mucociliary clearance (Wilson *et al.,* 1996), and/or that damage to epithelium may provide more attachment sites to bacteria (El Ahmer *et al.,* 1999). A second hypothesis is that the immune responses after viral infection may increase the susceptibility for bacterial infections. Phagocytosis of bacteria by macrophages (Debets-Ossenkopp *et al.,* 1982) or neutrophils (Engelich *et al.,* 2001; Navarini *et al.,* 2006) has shown to be hampered due to a previous viral infection. Moreover, the innate anti-viral responses, especially type I IFN, may have a severe granulotoxic effect that increases susceptibility to bacterial superinfection (Navarini *et al.,* 2006). The cell populations in tissues of superinfected animals differ significantly from cell populations in tissues of animals infected with only one pathogen. In addition, superinfections may result in an overproduction of inflammatory cytokines, which may contribute to immunopathology due to exacerbated immune responses (Beadling and Slifka, 2004; Sluijs *et al.,* 2006; Speshock *et al.,* 2007).

Two hypotheses to explain the enhanced susceptibility have been suggested but the underlying mechanisms are still not fully understood (Navarini *et al.,* 2006). The results are not conclusive and further experimental studies are needed to elucidate this phenomenon. Most experiments concerning superinfections of the respiratory tract have been performed in laboratory animals (Beadling and Slifka, 2004; Sluijs *et al.,* 2006). These experimental models have several advantages, but an important disadvantage is that the animals used are usually not natural host for the infectious agents used.

The use of chickens as experimental animal in combination with avian pathogens could overcome this disadvantage. A superinfection model with Infectious Bronchitis Virus (IBV) and *E. coli* in chickens has been developed (Goren, 1978). Matthijs *et al*. (2003) used this model and showed increased susceptibility for *E. coli* after previous infection with virulent IBV. Remarkably, this phenomenon was also observed after infection with a mild IBV vaccine virus. This suggested that

mechanical tissue damage was not the cause of the enhanced susceptibility to *E. coli*.

This infection model offers the opportunity to investigate how a preceding infection with either a virulent or a mild IBV strain may affect the course of a subsequent *E. coli* infection in the respiratory tract (trachea, lung and airsac) of broilers. The aim of this study was therefore to investigate two possible mechanisms of enhanced susceptibility: tissue damage and alteration of the immune response. The immunocytological changes were observed over a time course from 0.5 hours to 7 days after application of *E. coli* in the trachea. During that period dynamics of IBV, *E. coli*, lymphocytes (CD4, CD8, γδ-TCR and αβ1-TCR) and macrophages (KUL-01) in the trachea, lung and airsacs of broilers were studied.

Materials and Methods

Experimental chickens

Eighteen-day-incubated eggs originating from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) each with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^3 h^{-1}.

Broilers were fed a commercial ration containing 12.4 MJ of metabolically energy per Kg and 19.5% crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75% of *ad libitum* intake on 'skip a day base' to diminish leg disorders and hydrops ascites. Tap water was provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35ºC at day 1 to 20ºC at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20ºC.

All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

Inocula

IBV vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials, which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50%) per dose (Nobilis® IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, The Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50} /1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use, and contained at least $10^{3.0}$ EID_{50} /ml of H120 virus and $10^{4.6}$ EID_{50}/ml of IBV M41 virus.

The *Escherichia coli* strain 506 (O78; K80) was isolated from a commercial broiler (Van Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs et al. (2003) and was used at a concentration of $10^{7.6}$ CFU/ml.

Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age all groups were inoculated oculo-nasally (one droplet of 0.05 ml per bird in each eye and nostril) and intra-tracheally (1 ml per bird): groups 1 and 2 received distilled water, group 3 received IBV H120 vaccine and group 4 IBV strain M41. At 32 days of age, groups 2, 3 and 4 were intra-tracheally inoculated with 1 ml *E. coli* culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intra-tracheally per bird.

For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 vaccine and *E. coli* broth) as the H120 group, and group 4 (IBV strain M41and *E. coli* broth) as M41 group.

Clinical and post mortem examination

Clinical signs of IBV infection were recorded 1, 2, 4 and 5 days after IBV inoculation and after *E. coli* inoculation and just before euthanizing. A bird was recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs et al., 2003).

From each group, 5 broilers were electrocuted and bled at 0.5 hour, 3 hours, and at days 1, 2, 4 and 7 after *E. coli* inoculation (hpi/dpi). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (1991) in the left and right thoracic airsac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Van Eck and Goren, 1991).

Immunocytochemical staining

After post-mortem examination, trachea, left lung and both thoracic airsacs were collected and snap-frozen in liquid nitrogen for immunocytological examination. Samples for immunocytological staining were taken from the middle of trachea, in the area of the entrance of the mediodorsal secondary bronchi in the lung and of both thoracic airsacs in total. Cryostat sections (6 µm) were transferred to Superfrost Plus slides (Menzel-Glaser) and stored over silicagel for at least 24 h before use. Slides were fixed in pure acetone, air-dried and incubated with mAbs in appropriate concentrations for 1 h; mouse anti-chicken CD4, CD8β, γδ-TCR, αβ1-TCR and KUL-01 (Southern Biotechnology, Birmingham, USA). All mAbs were diluted in PBS containing 1% BSA (Sigma) and 0.1% sodium azide (PBS/BSA). Slides were washed in PBS and incubated for 1 h with biotinylated horse anti-mouse IgG (Vectastain®). Slides were washed with PBS and incubated for 1 h with avidin: biotinylated enzyme complex (Vectastain® Elite ABC kit) diluted in PBS. Then the slides were incubated with 1 mg 3, 3-diaminobenzidine-tetrahydrochloride (DAB, Sigma, USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.06% H_2O_2.

To ensure no over-or under staining, slides were monitored during the reaction under the microscope. Slides were washed with PBS, counterstained with haematoxylin and mounted in Glycergel mounting medium (Dako, USA). All incubations were performed at room temperature in a humidified box. Control slides were incubated as described above, except that mAbs were omitted.

Immunocytological analysis of the tissue sections

Trachea

Three sections of the trachea per mAbs (CD4, CD8, γδ-TCR, αβ1-TCR and KUL-01) were examined by light microscopy magnification. The score given per animal was the cell count of the 3 trachea rings in total divided by three. Cell populations were scored using the following system for CD4, CD8, KUL-01, γδ-TCR and αβ1-TCR cells: 0 = 0-3 cells, 1 = 4-10 cells, 2 = 11 cells or 1 cluster of cells, 3 = multiple clusters. A cluster is a group of tens of cells located close together.

Left Lung

Of each left lung and for each mAbs (CD4, CD8, γδ-TCR, αβ1-TCR and KUL-01) three parabronchi in the proximity of the Bronchial Associated Lymphoid Tissue (BALT), but not next to the BALT, were analysed. This area was chosen to examine the same well-defined area in every lung consistently. The score given per animal was the total cell count of three parabronchi divided by three. Cell populations were scored using the following system for CD4: 1 = 0-20 cells, 2 = more than 20 cells and/or 1 cluster, 3 = multiple cell clusters; for CD8: 1 = 0-10 cells, 2 = 11-20 cells, 3 = more than 20 cells; for KUL-01: 1 = 20-50 cells, 2 = 51-200 cells, 3 = more than 200 cells; for γδ-TCR and αβ1-TCR: 1 = less than 10 cells, 2 = 11-40 cells, 3 = more than 40 cells.

Left en right thoracic airsacs

Because of lack of a well-defined morphological unit in the airsacs the complete section of the airsacs was examined. Four levels of relative cell density were distinguished for all cell subpopulations: 0 = no cell influx, 1 = few scattered cells throughout the stroma, 2 = many cells scattered throughout the stroma, 3 = complete cell infiltration of the stroma of the airsac wall.

Detection of IBV and E. coli

Production of polyclonal antibodies against E. coli 506

The bacterial inocula were prepared by submersing one frozen bead (-70 °C) of a batch containing the *E. coli* 506 strain in trypton soya broth and by subsequent incubation for 20 h at 37 °C. The bacteria were washed with PBS and killed with methanol during 5 min. The solution was centrifuged 10 min. at 1500g and the bacteria were washed twice with PBS and dissolved in 10 mg DDA (dimethyl dioctadecylammonium bromide)/ml PBS. Two rabbits were injected 3 times, 21

days between each injection, subcutaneous with 1.5 ml of the PBS/DDA solution containing 10^9 *E. coli* bacteria per ml. Pre-immune and immune sera were tested for specific antibodies against *E. coli* 506 on dot blot, paraffin and cryostat sections. Both live and fixed bacteria were recognized.

Tissue staining procedure for IBV and E. coli

Trachea and lung were collected in 10% buffered formalin, fixed for 24 h and processed to paraffin. Sections were cut 5 µm thick, deparaffinised and placed in pure methanol with 0.75% H_2O_2 for 30 minutes to remove endogenous peroxidase activity. Sections were incubated with rabbit serum specific for *E. coli* (*E. coli* 506) or with mouse sera with mAbs 48.4 against IBV nucleoprotein (Koch et al., 1990) for 1 h. After washing in PBS, the slides were co-incubated for 1 h with rabbit anti-mouse serum (Dako, Denmark) or goat anti-rabbit serum (Dako, Denmark) conjugated to horseradish peroxidase. The slides were washed and stained with 0.5 mg 3, 3-diaminobenzidine-tetrahydrochloride (DAB, Sigma USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.03% H_2O_2.

For IBV and *E. coli* staining in the airsac, cryostat sections (6 µm thick) were made from both thoracic airsacs in total. The procedure was the same as used in the immunohistochemical staining. The complete section of each organ was examined for general pathology and the location of both pathogens. The number of *E. coli* bacteria in different parts of the respiratory tract were recorded and classified. Four classes were distinguished: 0, 1, 2, and 3. For trachea and bronchi, the following classes were distinguished: in class 0 no bacteria were found; in class 1 1-5 bacteria; in class 2 6-10, and in class 3 more than 10 bacteria were counted. For the air sacs the classes were: class 0 when no bacteria were found; class 1 for 1-20 bacteria; class 2 20-100 bacteria and class 3 when more than 100 were counted.

Statistical analysis

Between-group differences per time point were non-parametrically analyzed for mean lesion score (MLS) using the Mann-Whitney test. Nasal discharge, airsacculitis and pericarditis/perihepatitis per time point were non-parametrically analyzed between groups with Fischer Exact Test with Bonferroni correction. Lymphocyte subpopulations were analyzed with Fischer Exact Test with the Bonferroni correction.

Examination of birds has not taken place for all organs at the same time points. For the statistical analysis of the airsac all time points were used, but for the other

organs the time points of 0.5 and 3 hpi are missing. Both airsacs were examined separately. The classification of birds with respect to the number of *E. coli* was 87% identical; in 6% of the birds a difference of one class was found. Therefore only the right airsac was analysed statistically. Because of the small number of birds per group and time point, no distinction was made between the various classes for number of *E. coli* found. So, for the statistical analysis the result per bird was recorded as 'with (at least one) *E. coli'* or 'no *E. coli*' found (Table 3).

A generalized linear model was performed on the number of birds without or with *E. coli* with a poisson distribution. The explanatory variables were treatment group (*E. coli*, H120 or M41), time and class (with / without) of *E. coli.* The interaction between time and class and group and class represent the relation between the distributions of the number of birds in the classes with *E. coli*. The best model was based on the (lowest) Akaike's Information Criterion (R development core team, 2007; Pawitan, 2001) but the larger model should have at least a difference of two in AIC to be selected as the best model. For the generalized linear model the statistical program R version 2.5.1 (R Development Core Team, 2007) was used.

Results

After inoculation with IBV, the birds were clinically examined, and mucous nasal discharge was observed from 2 to 9 days p.i. (Table 1). After inoculation with *E. coli*, the birds were examined for clinical signs of colibacillosis, and post mortem examination was performed to quantify colibacillosis lesions. The number of birds with airsacculitis, perihepatitis and/or pericarditis and mean lesion score (macroscopical lesions) are presented in Table 2.

Trachea

Location of pathogens

H120 virus was detected for 4 days after *E. coli* inoculation in 15 broilers, and M41 virus was detected for 2 days in 6 broilers. Virus was found in the columnar ciliated cells, and in the lamina propria. *E. coli* bacteria were not found either attached to the epithelium or in the underlying tissue of the trachea (Table 3). No time effect was found in number of birds with *E. coli*. In birds in the H120 group, *E. coli* was more often found than in birds of the *E. coli* group and the M41 group.

No difference was found in number of birds with *E. coli* between the *E. coli* and M41 groups.

Table 1. Number of broilers with mucous nasal discharge at different time points after inoculation with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age.

	Days after IBV inoculation							
Group	1	2	4	5*	6	7	9	12
PBS[$]	0/30 [A]	0/30[A]	0/30[A]	0/30 [A]	0/5 [A]	0/5 [A]	0/5 [A]	0/5 [A]
E. coli[$$]	0/30 [A]	0/30 [A]	0/30[A]	0/30 [A]	0/5 [A]	0/5 [A]	0/5 [A]	0/5 [A]
H120	0/30 [A]	1/30 [A]	2/30[A]	3/30 [A]	1/5 [A]	2/5 [A]	3/5 [A]	0/5 [A]
M41	0/30 [A]	15/30 [B]	28/30[B]	30/30[B]	3/5 [A]	3/5 [A]	1/5 [A]	0/5 [A]

[AB]Groups with different letters within a column are significantly different ($P<0.05$).
[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.
*Day of inoculation with *E. coli*.

Immunocytological changes

In the PBS and the *E. coli* group no influx of lymphocytes was found. The few lymphoid cells found in the lamina propria were CD4+ cells and macrophages. In contrast, in the H120 and the M41 group massive lymphocyte infiltrations were found at all time points after *E. coli* challenge. These infiltrates consisted of CD4+ and CD8+ lymphocytes, and macrophages (Figure 1A). The CD4+ cells expressed the αβ1-TCR+, whereas the CD8+ cells expressed either the γδ-TCR+ or αβ1-TCR+. No significant differences in number of CD4+ and CD8+ cells were found between the H120 and the M41 groups. From 2 dpi onwards the expression of the T cell receptors, both γδ-TCR and αβ1-TCR, reduced drastically. The expression of CD4 and CD8 in tissues of birds in these groups remained high (data not shown).
All cell subpopulations were found scattered throughout the tissue, but CD4+ and CD8+ cells were also found in clusters. The CD8+ cells were found around B cell follicles, and CD4+ cells were located within B cell follicles.

Table 2. Macroscopical colibacillosis lesions: number of broilers with airsacculitis and/or pericarditis/perihepatitis and the mean lesion score (MLS ± SD). At each timepoint and within each group five birds were analysed. Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age.

		Time after *E. coli* 506 strain inoculation					
Broilers with	Group	0.5 h	3 h	1 d	2 d	4 d	7 d
Airsacculitis	PBS[$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli[$$]	0^{A}	0^{A}	5^{B}	2^{AB}	3^{AB}	0^{A}
	H120	4^{AB}	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}
	M41	5^{B}	5^{B}	5^{B}	4^{AB}	5^{B}	5^{B}
Perihepatitis	PBS[$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
and/or	*E. coli*[$$]	0^{A}	0^{A}	1^{A}	0^{A}	2^{A}	0^{A}
Pericarditis	H120	0^{A}	0^{A}	0^{A}	0^{A}	2^{A}	1^{A}
	M41	0^{A}	0^{A}	0^{A}	0^{A}	3^{A}	2^{A}
MLS	PBS[$]	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}
	E. coli[$$]	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	1.6 ± 1.6^{B}	1.6 ± 2.2^{AB}	3.4 ± 3.4^{AB}	0.0 ± 0.0^{A}
	H120	0.4 ± 0.2^{A}	0.5 ± 0.0^{A}	2.4 ± 1.5^{B}	3.4 ± 1.9^{B}	6.6 ± 3.5^{B}	5.0 ± 4.1^{B}
	M41	0.5 ± 0.0^{A}	0.5 ± 0.0	3.4 ± 0.9^{B}	2.0 ± 1.9^{B}	6.4 ± 2.5^{B}	4.5 ± 3.0^{B}

[AB]Groups with different letters within a column are significantly different ($P<0.05$).
[$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$]Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

Table 3. Number of birds with or without *E. coli* bacteria in different parts of the respiratory tract detected by immunostaining. Birds were inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age. At each time point and within each group five birds were analysed.

		Time after *E. coli* infection					
Tissue	Group	0.5h	3h	1d	2d	4d	7d
Right airsac	PBS	0/5[$]	0/5	0/5	0/5	0/5	0/5
	E. coli	2/3	2/3	3/2	2/3	2/3	0/5
	H120	2/3	1/4	1/4	1/4	0/5	0/5
	M41	1/4	1/4	1/4	1/4	0/5	0/5
Trachea	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	E. coli	n.d.	n.d.	1/4	0/5	0/5	0/5
	H120	n.d.	1/4	1/3	2/2	1/4	0/5
	M41	n.d.	1/4	1/4	0/5	0/4	0/5
Parabronchi	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	E. coli	n.d.	n.d.	5/0	5/0	4/1	2/3
	H120	n.d.	3/2	2/3	4/1	5/0	2/3
	M41	n.d.	4/1	3/1	3/1	2/2	2/3
Bronchi	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	E. coli	n.d.	n.d.	5/0	4/1	1/3	0/5
	H120	n.d.	4/1	1/3	2/2	2/3	0/5
	M41	n.d.	3/2	2/2	0/5	1/3	0/4

[$] 5/0 : the number of birds with at least one *E. coli* detected / the number of birds without *E. coli* in that part of the respiratory tract.
n.d. = not determined; samples were missing

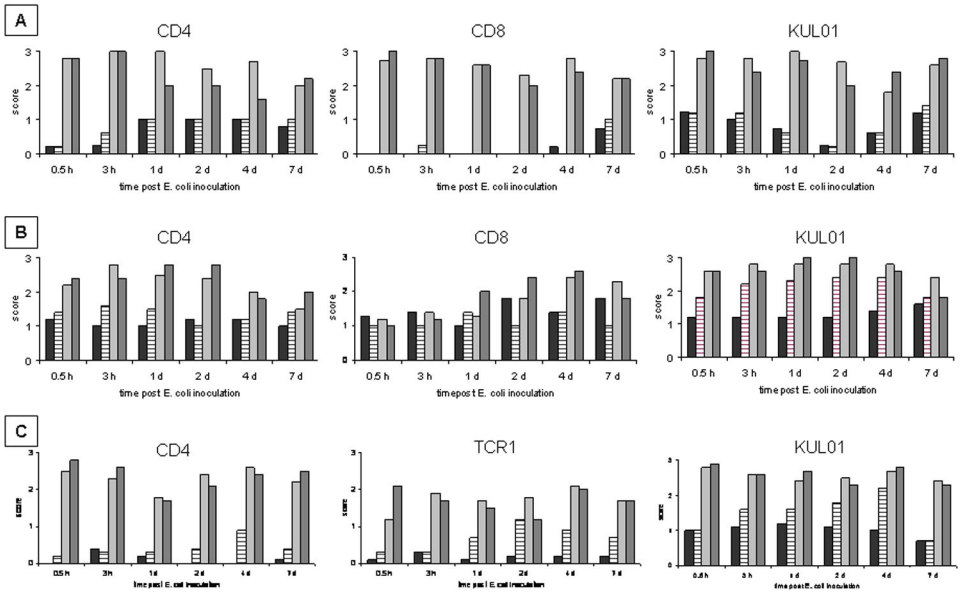

Figure 1 Number of CD4+, CD8+ and KUL-01+ cells in different parts of the respiratory tract of broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41). All birds except those in the PBS group were inoculated with *E. coli* at day 5 after IBV inoculation. At each time point and within each group five birds were analysed. Black bars: PBS group; hatched bars: *E. coli* group; grey bars: H120 group; dark bars: M41 group.
A: trachea: Mean cell count per three trachea rings. CD4+, CD8+, KUL-01+ cells: 0: 0-3 cells, 1: 4-10 cells, 2: 11 cells or 1 cluster of cells, 3: more than 1 cell cluster. A cluster is a group of tens of cells located close together.
B: lungs: Mean cell count per three parabronchi. CD4+ cells: 1: 0-20 cells, 2: more than 20 cells and 1 cluster, 3: multiple cell clusters, for CD8+ cells: 1: 0-10 cells, 2: 11-20 cells, 3: more than 20 cells, for KUL-01: 1: 20-50, 2: 51-200, 3: more than 200 cells.
C: airsacs: The complete section of the airsac was examined. CD4, CD8, KUL-01: 0 = no cell influx, 1 = few scattered cells throughout stroma, 2 = many cells scattered throughout stroma, 3 = massive cell infiltration of stroma of airsac wall.

Lung

Location of pathogens

IBV (H120 and M41) was found in the columnar ciliated cells of the epithelium in the bronchi, but not in the parabronchus. *E. coli* was found as free bacteria and agglomerates in the capillary area, atria, and pus within parabronchial and bronchial lumina (Table 3). At 2 dpi and 4 dpi *E. coli* was mainly found as small

agglomerates in the capillary part and lumen of the parabronchus. The presence of bacteria was accompanied by inflammatory changes of the parabronchi (data not shown). In both the parabronchi and secondary bronchi no time or group effect was found in the number of broilers with *E. coli*.

Immunocytological changes

The number of affected parabronchi and the severity of affection of parabronchi are presented in Table 4.

Table 4. Percentage of damaged parabronchi in broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with *E. coli* at 32 days of age. At each timepoint and within each group five birds were analysed. Percentage of damaged parabronchi per field section: -: 0%, +/-: 0-10%, +: 10-30%, ++: more than 30%. Severity of damage of the parabronchi: -: no changes, +/-: minor cell infiltration/oedema in part of parabronchus, +: generalized cell infiltration in whole parabronchus, ++: massive cell infiltration and/or necrosis in parabronchus.

	Damaged parabronchi					Severity of damage				
	Time after *E. coli* inoculation									
Group	3 h	1 d	2 d	4 d	7 d	3 h	1 d	2 d	4 d	7 d
PBS[$]	n.d.[$$$]	-	-	-	-	n.d.	-	-	-	-
E. coli[$$]	n.d.	++	++	+	+/-	n.d.	++	+	+/-	+/-
H120	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-
M41	+	++	+	+/-	+/-	+/-	+	+/-	+/-	+/-

[$] Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.
[$$] Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.
[$$$]n.d. = not determined.

At 0.5 hpi many CD4+ cells and macrophages were present in lungs of IBV infected birds. At that time no difference in number of CD8+ cells was found between the different groups (Figure 1B). From 1 to 7 d after *E. coli* infection, an

increase in CD8+ cells was detected in the M41 group and at 4 and 7 dpi in the H120 group. The numbers of CD4+, CD8+ and KUL-01+ cells in H120 and M41 groups significantly exceeded the numbers of these cells in the *E. coli* group.

In H120 and M41 groups many macrophages were present until 4 dpi. In the *E. coli* group, the number of macrophages increased after inoculation, but did not equal the levels in the H120 and M41 groups. At 7 dpi the number of macrophages was decreased in the *E. coli* and M41 groups to the level found in the PBS group, but it remained high in the H120 group. Up to 2 dpi most macrophages were elongated, whereas from 4 dpi most macrophages were enlarged and rounded up suggesting activation of these cells.

No significant differences in the number of γδ-TCR+ cells in the lung were found between the four groups. In the H120 and M41 groups, the number of αβ1-TCR+ cells increased 4 dpi coinciding with the increase of CD8+ cells (TCR data not shown; Figure 1B). No significant difference between H120 and M41 groups were found in numbers of CD4+, CD8+, KUL-01+, γδ-TCR+ and αβ1-TCR+ cells.

Airsac

Location of pathogens

IBV was found in the columnar ciliated cells of respiratory epithelium in the airsacs of birds in the H120 and M41 groups; H120 virus was detected for 2 days after *E. coli* inoculation in 14 birds, and M41 virus was detected for 3 hours after *E. coli* inoculation in 9 birds. *E. coli* was found predominantly in pus located at the respiratory surface in the airsacs. For the right airsac no group effect was found on number of birds with *E. coli* (Table 3).

Immunocytological changes

In the PBS and *E. coli* groups only few CD4+ cells were observed. In contrast, massive infiltrations of CD4+ and CD8+ lymphocytes were found in birds in the H120 and M41 groups from 0.5 hpi till 7 dpi (Figure 1C). Macrophages were present in H120 and M41 groups at 0.5 hpi, and their number remained high up to 7 dpi (Figure 1C). In the PBS and *E. coli* groups, few macrophages were present at 0.5 hpi. Only in the *E. coli* group a substantial increase was found which was highest at 4 dpi. Nevertheless, the number of macrophages in this group remained lower than the number of macrophages in the H120 and M41 groups. Seven dpi the number of macrophages in the *E. coli* group decreased to a level comparable to the level of the birds in the PBS group (Figure 1C). The numbers of CD4+, CD8+ and

KUL-01+ cells in H120 and M41 groups exceeded significantly the numbers of these cells in the *E. coli* group.

In the H120 and M41 groups the number of γδ-TCR+ cells and αβ1-TCR+ cells was high from 0.5 hpi till the end of the experimental period. In the *E. coli* group an increase of γδ-TCR+ cells occurred from 0.5 hpi to 4 dpi, which was decreased at 7 dpi. Few γδ-TCR+ cells were found in the PBS group and hardly any αβ1-TCR+ cells were found in the PBS and *E. coli* groups. No significant differences in number of CD4+, CD8+, KUL-01+, γδ-TCR+ and αβ1-TCR+ cells were detected between the H120 and M41 groups.

Discussion

In this study the course of an *E. coli* infection after a previous infection with a virulent (M41) or vaccine (H120) strain of IBV in trachea, lungs and airsacs of broilers was examined. The aim of this study was to investigate two possible mechanisms of enhanced susceptibility: tissue damage and alteration of the immune response.

Despite significant differences in number of birds with nasal discharge 2 to 5 days after inoculation with either IBV strain, comparable clinical, macroscopical and microscopical changes were observed after infection with *E. coli* in both groups. Remarkably, 4 dpi mean lesion scores were comparable in both the *E. coli* and IBV groups, whereas at 7 dpi birds in the *E. coli* group were fully recovered but birds in both IBV groups still showed signs of colibacillosis. These findings suggest that due to a previous infection with either IBV strain birds were less capable of conquer the damage of the *E. coli* infection.

In the trachea, epithelial damage and mononuclear cell infiltration due to infection with IBV was found, however no additional effect of the subsequent infection with *E. coli* was noticed indicating that the apparent changes did not result in predisposition of the trachea for bacterial superinfection. In the lungs of both the *E. coli* group and the IBV groups acute purulent pneumonia of similar severity was observed, suggesting no additional effect of IBV on acute pneumonia. Moreover, the course of the disease in the lungs in both the *E. coli* group and the IBV groups was similar and birds of these groups were nearly completely recovered from pneumonia at 7 dpi. Although apoptosis of granulocytes and subsequent leukopenia is described after viral infection (McCullers, 2006; Navarini

et al., 2006) the massive granulocyte infiltration in the lungs and the presence of pus in the airsacs in both *E. coli* and the IBV- *E. coli* infected birds did not clearly suggest a reduction in the participation of granulocytes in the process. These findings suggest that neither trachea nor lungs (as suggested by DeRosa *et al.,* 1992; Smith *et al.,* 1985) were predilection sites for bacterial superinfection.
Lesions in the airsacs of both IBV groups were more pronounced and of longer duration than in the *E. coli* group. In contrast to the lungs, the airsacs from IBV infected birds were not recovered at 7 dpi whereas the airsacs of only *E. coli* infected birds were. The airsacs were the only location where both pathogens were found at the same time. Our results indicate that the difference in reaction upon superinfection with *E. coli* in IBV infected broilers compared to an *E. coli* infection without preceding virus infection is not caused by damage to the mucociliary barrier facilitating bacterial adhesion or penetration or by reduced clearance of bacteria.
The second hypothesis was that alteration of the immune response by a virus infection could make birds more susceptible to subsequent bacterial infections. At time of infection with *E. coli*, great numbers of CD4 cells, CD8 cells and macrophages were found in trachea and airsacs of birds of the IBV groups, whereas great numbers of CD4 cells and macrophages were found in the lung. The pronounced presence of macrophages at time of challenge with *E. coli* most likely increased the clearance of the bacteria, since fewer bacteria were found in lungs and airsacs of IBV infected groups. This finding suggests a reduced susceptibility for a superinfection with *E. coli*.

However, macrophages produce type I IFNs (IFN-α/β) after viral infection which have potent effects on viral replication, including that of IBV, but these cytokines can inhibit host defence against both Gram-positive and Gram-negative bacteria such as *Listeria monocytogenes* (O'Connell *et al.,* 2004), *Mycobacterium tuberculosis* in the lungs (Manca *et al.,* 1002), and *Salmonella typhimurium* (Navarini *et al.,* 2006), and possibly also against *E. coli* in broilers. The CD4 cells and CD8 cells also present in large numbers in IBV groups can produce type II IFN (IFN-γ) which is known to have a protective role in both viral and bacterial infections through the induction of a Th1 cell immune response (Decker *et al.,* 2002). However, its role in the activation of macrophages and airway epithelial cells can lead to an exaggerated inflammatory response (Konno *et al.,* 2002). Both T cells and macrophages present at time of *E. coli* inoculation might therefore be

responsible for the enhanced colibacillosis, due to overproduction of inflammatory cytokines.

In the airsacs, the numbers of macrophages remained high in the IBV groups, whereas the number of macrophages in the *E. coli* group decreased at 7 dpi to the level of the PBS group. Although both IBV and *E. coli* were cleared in all groups, the macrophages were still present in the airsacs at 7 dpi in the IBV groups. It is unclear whether the macrophages and T cells in the trachea, lungs and airsacs are functionally altered by the IBV infection, but the clear difference in the presence of these cells in IBV infected birds both at time of challenge and in the airsacs at 7 dpi might suggests that an altered microenvironment resulted in altered immune responses, as for example demonstrated in mice where lymphocytes were detrimental during the early innate immune responses against *Listeria monocytogenes* due to increased apoptosis inhibiting effector reactions (Carrero *et al.,* 2006). Both T cells and macrophages present at the time of *E. coli* inoculation might therefore be responsible for the increase of both the severity and the duration of the inflammatory reactions found in the airsacs in both IBV-*E. coli* infected birds, due to overproduction of inflammatory cytokines, as demonstrated in a study with turkeys (Rautenschlein *et al.,* 1998)

Ariaans *et al.* (in press) found that the phagocytic capacity and NO production of peripheral blood mononuclear cells and splenocytes was not affected by prior exposure to IBV, but that the proinflammatory response in the spleen of IBV infected birds seemed severely impaired compared to the only *E. coli* infected birds, and suggested that the virus modulated the innate immunity of the birds. Our study only showed immunocytological changes, as no functional study was performed.

In this study, only a limited number of samples were examined. However, the samples were considered representative, because they were taken in a uniform and reproducible manner and the lesions within each compartment were distributed evenly.

It is concluded that a preceding infection of the respiratory tract with IBV does not predispose for bacterial superinfection with *E. coli* by altering the mucociliary barrier but likely modulates the immune response.

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Chapter 7

The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection

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Abstract

Colibacillosis results from infection with avian pathogenic *Escherichia coli* bacteria. Healthy broilers are resistant to inhaled *E. coli*, but previous infection with vaccine or virulent strains of Infectious Bronchitis Virus (IBV) predisposes birds for severe colibacillosis. We investigated whether IBV affects recruitment and function of phagocytic cells and examined NO production, phagocytic and bactericidal activity, and kinetics of peripheral blood mononuclear cells (PBMC) and splenocytes. Moreover, we measured cytokine mRNA expression in lung and spleen samples. Broilers were inoculated with IBV H120 vaccine or virulent M41 and challenged 5 days later with *E. coli* 506. A PBS control and *E. coli* group without previous virus inoculation were also included. Birds were sacrificed at various time points after inoculation with *E. coli* (h/dpi). Inoculation with IBV induced extended and more severe colibacillosis than with *E. coli* alone. At 4 dpi, the number of PBMC in all *E. coli*-inoculated groups was significantly higher than in PBS-inoculated birds, which correlated with lesion scores. From 1 to 4 dpi, NO production by PBMC from all *E. coli*-inoculated animals was elevated compared to PBS birds. Bactericidal activity of PBMC in IBV-inoculated animals at 7 dpi was lower than in PBS- and *E. coli*-inoculated birds, but phagocytic capacity and recruitment were not severely impaired. In spleen samples of IBV-infected animals reduced expression of IL-1β, IL-6, IL-8, IL-10, IL-18 and IFN-γ mRNA was found 1 dpi. Our results suggest that enhanced colibacillosis after IBV infection or vaccination is caused at least by altered innate immunity and less by impairment of phagocytic cell function.

Introduction

Predisposition for bacterial infections in the course of respiratory viral infections is found in various species, both avian and mammalian. In chickens, turkeys and ducks colibacillosis is often observed secondary to infection with respiratory agents e.g. Infectious Bronchitis Virus (IBV), Newcastle Disease Virus, and *Mycoplasma gallisepticum* (Igbokwe *et al.,* 1996; Nakamura *et al.,* 1994). In mice, influenza virus increases susceptibility to *Streptococcus pneumoniae* infection (Seki *et al.,* 2004; Speshock *et al.*, 2007) and measles virus predisposes for a range of bacterial infections such as *Listeria monocytogenes* (Slifka *et al.,* 2003). In cattle, susceptibility for pneumonic pasteurellosis is greatly enhanced by a variety of respiratory viruses including bovine respiratory syncytial virus (Liu *et al.,* 1999), bovine herpes virus-1 (Leite *et al.*, 2002) and bovine coronavirus (Storz *et al.*, 2000) . In man, pneumonia has been a leading cause of death during influenza pandemics, supporting the widely held view that influenza virus predisposes for streptococcal infections (McCullers, 2006).

The mechanisms behind enhanced susceptibility to bacterial superinfection after viral infection have been studied extensively (Beadling and Slifka, 2004; Hament *et al.,* 1999) but are still not well understood. A first set of hypotheses suggests increased susceptibility due to tissue damage in the respiratory tract resulting in functional impairment. Three possible causes have been described as mechanisms for functional damage. Viral replication in the upper respiratory tract causes loss of cilia and ciliated cells (Bakaletz, 1995), decreased ciliary activity impairs mucociliary clearance (Wilson *et al.,* 1996) and finally, damage to epithelium may provide more attachment sites for bacteria (Ahmer *et al.,* 1999).

A second set of hypotheses suggests altered innate immune responses. Impairment of innate effector functions, i.e. adhesion and entry, phagocytosis, killing, nitric oxide (NO) and superoxide production have been suggested previously (Ficken *et al.,* 1987; Naqi *et al.,* 2001; Read *et al.,* 1999) . Changes in recruitment or function of macrophages and neutrophils after bacterial superinfections have been described in mice (Navarini *et al.,* 2006; Slifka *et al.,* 2003).

Modulation of other innate functions affecting induction and / or control of adaptive responses might have a long term effect upon the host-pathogen interaction. Type I interferons (IFNs I) induced after viral infection have an antiviral effect, but can be detrimental for induction of anti-bacterial responses

(Navarini *et al.,* 2006; O'Connell *et al.,* 2004), whereas type II IFNs play an important role in bacterial infections (Shtrichman *et al.,* 2001).

Goren (1978) developed an experimental model in broilers that demonstrated enhanced susceptibility to *Escherichia coli* after inoculation with IBV. Moreover, not only a virulent strain (IBV M41) enhanced inflammatory reactions on superinfection with *E. coli*, but the mild IBV vaccine strain (IBV H120) widely used in the field also enhanced susceptibility in a very similar way (Matthijs *et al.,* 2003).

In our study to investigate which mechanisms underlie enhanced susceptibility to colibacillosis after IBV infection or vaccination, we examined the dynamics of pathogens and of immunopathological changes (M. G. R. Matthijs *et al.*, submitted for publication).

The aim of this study was to detect whether innate effector functions such as the recruitment of effector cells and function of phagocytic cells, measured by NO production and bactericidal capacity had been affected after IBV infection. As an alternative explanation for the enhanced colibacillosis we also examined altered expression of cytokine mRNA in spleen and lung samples.

Materials and methods

Experimental chickens

Eighteen-day-incubated eggs originating from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) each with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^3 h^{-1}.

Broilers were fed a commercial ration containing 12.4 MJ of metabolically energy per Kg and 19.5 % crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75 % of *ad libitum* intake to diminish leg disorders and hydrops ascites. Tap water was provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35 ºC at day 1 to 20 ºC at day

31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20 ºC. All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

Inocula

The IB vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50 %) per dose (Nobilis® IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, the Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50} / 1.2 ml / vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use and contained at least $10^{3.0}$ EID_{50} / ml of H120 virus and $10^{4.6}$ EID_{50} / ml of M41 virus. The *Escherichia coli* strain 506 (O78; K80) was isolated from a commercial broiler (Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs *et al.* (Matthijs *et al.,* 2003) and was used at a concentration of $10^{7.6}$ CFU / ml.

Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age, all groups were inoculated oculo-nasally (one droplet of 0.05 ml per bird in each eye and nostril) and intratracheally (1 ml per bird): groups 1 and 2 received distilled water, group 3 received IBV H120 virus and group 4 IBV M41 virus. At 32 days of age, groups 2, 3 and 4 were intratracheally inoculated with 1 ml *E. coli* 506 culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intratracheally per bird. For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 virus and *E. coli* broth) as the H120 group, and group 4 (IBV M41 virus and *E. coli* broth) as M41 group.

Clinical and post-mortem examination

Clinical signs of IBV infection were determined 1, 2, 4 and 5 days after IBV inoculation and after *E. coli* inoculation just before euthanizing. A bird was

recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs *et al.,* 2003).

From each group, 5 broilers were electrocuted and blood collected at 0.5 hour, 3 hours and at days 1, 2, 4 and 7 after *E. coli* inoculation (hpi / dpi). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (Eck and Goren, 1991) in the left and right thoracic air sac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Matthijs *et al.,* 2005).

Flow cytometric analysis of cell composition

Peripheral blood, spleen and lung were collected from sacrificed birds. Spleen tissue was squeezed through a 70 μm mesh to prepare single cell suspensions. Splenocytes and PBMC were isolated by density gradient centrifugation for 20 min at 850 g using Ficoll-Hypaque (density 1.078), washed twice with PBS (Cambrex) and adjusted to $5x10^7$ cells / ml in RPMI1640 medium supplemented with 10 % FBS, 2 mM glutamax-I (Gibco) and 100 U / ml penicillin and streptomycin (Gibco).

Cell suspensions were fluorescently labeled for 30 minutes on ice with mAb against thrombocytes (mouse anti-chicken CD41/CD61:FITC, Serotec), monocytes (mouse anti-chicken monocyte/macrophage KUL-01:FITC, Southern Biotechnology Associates) and $CD8^+$ cells (mouse anti-chicken CD8α:FITC, mouse anti-chicken CD8β:RPE, Southern Biotechnology Associates). Subsequently, cell-associated fluorescence was analysed by flow cytometry using Cell Quest software (Becton Dickinson). Results are expressed as percentages of total viable cells.

Nitric oxide production assay

Cells were seeded in flatbottom 96-well plates at $2.5x10^6$ cells in 50 μl culture medium (RPMI1640 + 2 % chicken serum + 2 mM glutamax-I (Gibco) and 100 U / ml penicillin and streptomycin (Gibco). Cells were incubated for 48 h at 41 °C (5 % CO_2) with 100 μl of either culture medium to determine background production of nitric oxide (NO), medium supplemented with 10 μg / ml heat-killed *E. coli* 506, or medium with 10 μg / ml *E. coli* LPS (from *E. coli* O55:B5, Sigma) to measure maximum NO production. After incubation, 50 μl / well of culture supernatant was transferred to sterile flatbottom 96-well plates. An NO dilution series (200–3.13 μM) was included on each plate as a standard curve to determine the amount of NO

produced. To each well, 50 µl of Griess reagent (1 % sulfanilamide and 0.3 % naphtylenediamine, 1:1 in 2.5 % phosphoric acid) was added, plates were incubated for 10 minutes at room temperature on a plate shaker and absorbance was recorded at 550 nm. For each chicken, NO secretion was calculated as total amount of NO produced by the cells after stimulation with heat-killed *E. coli* or *E. coli* LPS, minus the background NO production by unstimulated cells.

Phagocytosis and killing assay

Splenocytes and PBMC were incubated for 30 minutes at 10^8 cells in 1 ml RPMI 1640, 5 % FBS containing 10^9 *E. coli* bacteria, in roundbottom 12 ml polypropylene tubes (Greiner Bio-One) at 37 °C, 5 % CO_2, to allow bacterial adhesion and entrance. At this point, gentamycin (200 µg / ml; Gibco) was added to each tube to kill extracellular bacteria and cells were incubated for an additional 30 minutes at 37 °C. Cells were washed once with PBS to remove the gentamycin, and either lysed directly with 1 % saponin (T = 0.5 h) to release intracellular bacteria, or incubated for 14 h or 24 h in RPMI 1640 medium supplemented with 5 % FBS and 20 µg / ml gentamycin before cell lysis.

The cells were cultured in the presence of gentamycin to prevent re-infection and unlimited growth in the medium of *E. coli* released from dead cells. After saponin treatment, cell lysates were centrifuged and pellets were resuspended in 200 µl PBS and plated in duplicate (100 µl lysate per plate) on McConky Agar (MCA) plates (Biotrading) to determine the number of viable bacteria. The MCA plates were incubated overnight at 37 °C and colonies were counted blindly. Results were calculated as the sum of colonies on the duplicate plates. If the sum of colony counts on the duplicate plates was below 100 colonies, cells were considered successful in clearing the *E. coli*, whereas cells were considered unable to clear the bacteria when counts were over 300 colonies.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples isolated from spleen and lung were screened for mRNA encoding IL-1β, IL-4, IL-6, IL-8, IL-10, IL-18, IFNα, IFNβ, and IFNγ. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Purified RNA was eluted in 30 µl RNase-free water and stored at -80 °C. Reverse transcription was performed using iScript cDNA Synthesis Kit (Biorad). Primers and probes (Applied Biosystems) were designed according to

Kaiser *et al.* (Kaiser et al., 2003) and Rothwell *et al.* (Rothwell et al., 2004) and are listed in Table 1.

Table 1. Real-time quantitative RT-PCR primers and probes.

RNA target		Probe/primer sequence (5' – 3')	Accession Number
28S	probe	(FAM) - AGGACCGCTACGGACCTCCACCA - (TAMRA)	X59733
	F primer	GGCGAAGCCAGAGGAAACT	
	R primer	GACGACCGATTTGCACGTC	
IL-1β	probe	(FAM) - CCACACTGCAGCTGGAGGAAGCC - (TAMRA)	AJ245728
	F primer	GCTCTACATGTCGTGTGTGATGAG	
	R primer	TGTCGATGTCCCGCATGA	
IL-4	probe	(FAM) - AGCAGCACCTCCCTCAAGGCACC - (TAMRA)	AJ621735
	F primer	AACATGCGTCAGCTCCTGAAT	
	R primer	TCTGCTAGGAACTTCTCCATTGAA	
IL-6	probe	(FAM) - AGGAGAAATGCCTGACGAAGCTCTCCA - (TAMRA)	AJ309540
	F primer	GCTCGCCGGCTTCGA	
	R primer	GGTAGGTCTGAAAGGCGAACAG	
IL-8	probe	(FAM) - TCTTTACCAGCGTCCTACCTTGCGACA - (TAMRA)	AJ009800
	F primer	GCCCTCCTCCTGGTTTCAG	
	R primer	TGGCACCGCAGCTCATT	
IL-10	probe	(FAM) - CGACGATGCGGCGCTGTCA - (TAMRA)	AJ621614
	F primer	CATGCTGCTGGGCCTGAA	
	R primer	CGTCTCCTTGATCTGCTTGATG	
IL-18	probe	(FAM) - CCGCGCCTTCAGCAGGGATG - (TAMRA)	AJ276026
	F primer	AGGTGAAATCTGGCAGTGGAAT	
	R primer	ACCTGGACGCTGAATGCAA	
IFNα	probe	(FAM) - CTCAACCGGATCCACCGCTACACC - (TAMRA)	U07868
	F primer	GACAGCCAACGCCAAAGC	
	R primer	GTCGCTGCTGTCCAAGCATT	
IFNβ	probe	(FAM) - TTAGCAGCCCACACACTCCAAAACACTG - (TAMRA)	X92479
	F primer	CCTCCAACACCTCTTCAACATG	
	R primer	TGGCGTGCGGTCAAT	
IFNγ	probe	(FAM) - TGGCCAAGCTCCCGATGAACGA - (TAMRA)	Y07922
	F primer	GTGAAGAAGGTGAAAGATATCATGGA	
	R primer	GCTTTGCGCTGGATTCTCA	

For the quantitative RT-PCR assay TaqMan Universal PCR Master Mix (Applied Biosystems) was used. Primers were used at 300 nM and probes at 100 nM concentration. Amplification and detection of specific products was achieved with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) with the following cycle profile: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 10 sec and 59 °C for 1 min.

Results were expressed as fold changes between samples, relative to the PBS group (Philbin *et al.,* 2005). To correct for variation in RNA preparation and sampling, Ct values for cytokine-specific product for each sample were standardized using the Ct value of 28S-specific product for the same sample. To normalize RNA levels between samples within an experiment, the mean Ct value for 28S-specific product was calculated by pooling Ct values of all samples in that experiment.

Well-to-well variations in Ct value of 28S-specific product about the experimental mean were calculated. The slope of the 28S dilution series regression lines was used to calculate differences in input of total RNA. Fold differences in cytokine expression between treatment groups were calculated relative to the reference ribosomal RNA, using a method adapted from Philbin *et al.* (Philbin *et al.,* 2005). Fold differences were calculated from the C_T values C (for the cytokine) and C' (for ribosomal RNA) using the equation $^{10}\log R_{(A/B)} = [(C_A - C_B)/S] - [(C'_A - C'_B)/S']$, where S and S' are, respectively, the slopes of plots of C_T value against the ^{10}logarithm of concentration for serial dilutions of cytokine DNA and ribosomal RNA, assayed on the same plate. This calculation avoids assumptions about the efficiency of the PCR amplifications and reduces to the common $\Delta\Delta C_T$ method in the case that both have perfect efficiency.

Statistical analysis

Between-group differences per time point were non-parametrically analyzed for each assay using the Mann-Whitney test. Partial correlations between different assays were determined per time point, with treatment as a control variable. Analysis was performed using the SPSS program and the probability level for significance was taken as $P < 0.05$.

Results

Clinical and macroscopical observations

Five days after IBV inoculation, all broilers in the M41 group and 10 % of the broilers in the H120 group showed nasal discharge. Birds were examined macroscopical for colibacillosis lesions (Fig. 1).

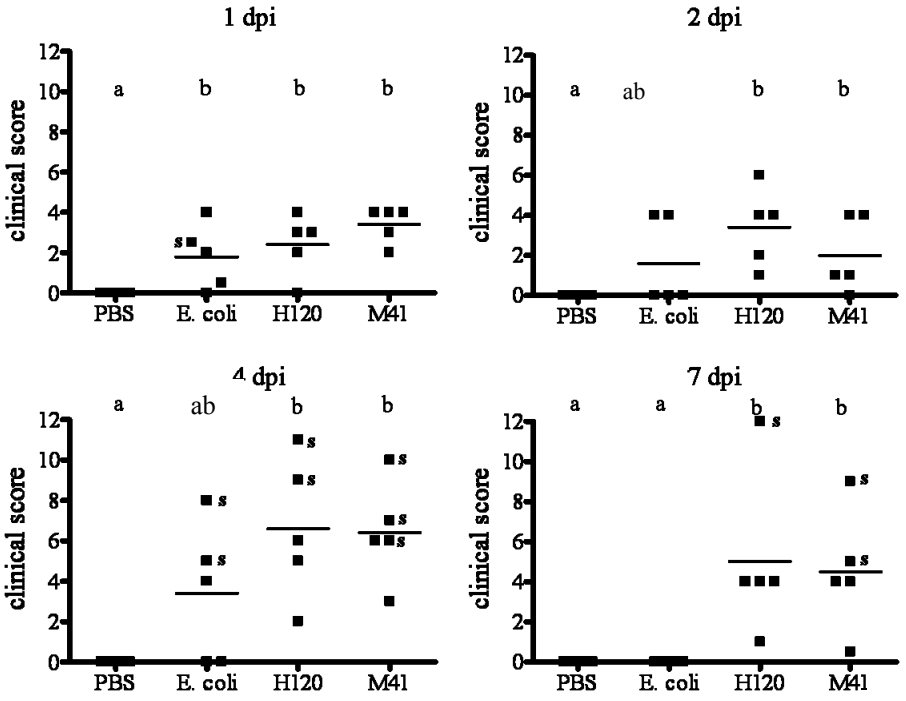

Figure 1. Colibacillosis lesion scores at different time points after *E. coli* inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before *E. coli* inoculation, whereas the PBS group received no IBV or *E. coli*. Per treatment group, 5 birds were sampled at each time point. Each dot represents the total lesion score of an individual bird with a maximum score of 12. The horizontal lines indicate the mean scores of each treatment group. Birds with systemic signs of colibacillosis, characterized by lesions in liver and/or pericardium, are designated with 's'. Groups with different letters are significally different ($P<0.05$).

No lesions were observed in birds of the *E. coli* group at 0.5 h and 3 h after *E. coli* inoculation, at these time points all broilers of the H120 and the M41 groups, except one in the H120 group, had airsacculitis. Airsacculitis (colibacillosis lesions in the thoracic air sacs) was observed in chickens of all *E. coli*-inoculated groups between 1 and 4 dpi. At 7 dpi, no signs of colibacillosis were observed anymore in the *E. coli* group whereas all birds of the H120 and the M41 groups had airsaccullitis. Systemic lesions (perihepatitis / pericarditis) were observed in 1 bird of the H120 group and 2 birds of the M41 groups at 7 dpi. No macroscopical signs of colibacillosis were observed in birds of the PBS group throughout the course of the experiment. More detailed clinical data can be found in Matthijs *et al*. (submitted for publication).

Flow cytometric analysis of blood mononuclear cells and splenocytes

The frequencies of thrombocytes, monocytes / macrophages, $CD8\beta^+$ and $CD8\alpha^+$ cells were analyzed as percentages of total viable cells in PBMC and splenocyte cell suspensions by flow cytometry at 1, 2, 4 and 7 dpi. Thrombocytes accounted for 60-75 % of PBMC and 2-4 % of splenocytes, with no significant differences between the treatment groups or fluctuations in time (data not shown). The percentage monocytes in peripheral blood of all *E. coli*-inoculated birds increased up to 4 dpi, and at 7 dpi, dropped to levels found in the PBS group (Fig. 2).

A significant positive correlation ($P < 0.01$) between monocyte percentages and colibacillosis lesion scores was found at 4 dpi. Whereas an effect of the *E. coli* inoculation on monocyte frequencies was apparent, monocyte frequencies were not significantly decreased nor increased in IBV-inoculated birds when compared to birds that received *E. coli* only. In the spleen at 1 dpi, a significantly higher percentage of macrophages was found in IBV-infected birds, and only in the H120 group, a higher percentage of macrophages was found at all time points. These changes were not significantly correlated to lesion scores. Significant variations in the percentages of $CD8\beta^+$, PBMC and splenocytes between the treatment groups were observed at different time points, but these variations were not related to an effect of either *E. coli* or IBV (data not shown). Similar fluctuations in the percentages of $CD8\alpha^+\beta^-$ cells were found, apart from 1 dpi, where the M41 group showed higher percentages of $CD8\alpha^+\beta^-$ cells than the other groups, but no

significant correlation was found with the *E. coli* or IBV inoculation (data not shown).

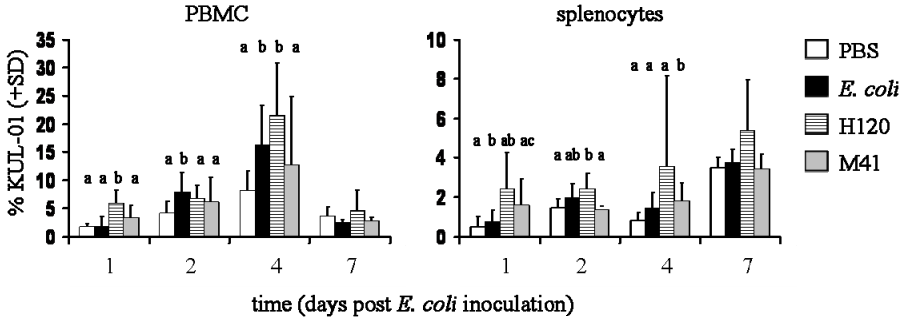

Figure 2. Percentage ± SD of monocytes / macrophages (KUL-01) in PBMC and splenocytes at different time points after *E. coli* inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before *E. coli* inoculation, whereas the PBS group received no IBV or *E. coli*. Bars represent the average frequency of labeled cells per treatment group (n=5), as a percentage of total live cells. Groups with different letters are significally different ($P<0.05$).

Nitric oxide production

The Griess reaction assay was used to determine whether IBV inoculation had an effect on the production of NO by splenocytes and PBMC after subsequent stimulation with heat-killed *E. coli* bacteria or *E. coli* LPS. NO production by PBMC of the *E. coli*-inoculated groups at 1 dpi was significantly higher than of the PBS group (fig. 3). Furthermore the IBV-inoculated groups, most notably the H120 group, showed higher NO production than the *E. coli* group, but at 2 dpi, NO production of the IBV-inoculated groups almost halved. At 7 dpi, NO production of the *E. coli* group had dropped to the level of the PBS group, whereas NO production by both the H120 and M41 groups was elevated compared to the PBS and *E. coli* groups.

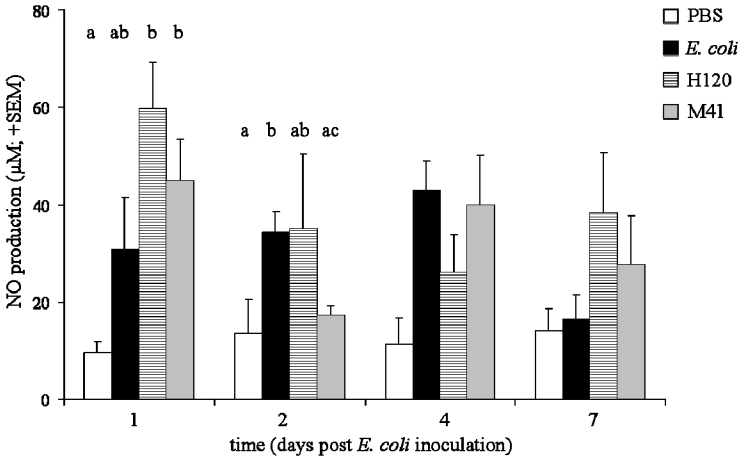

Figure 3. Means ± SEM of *E. coli*-induced NO production in response to heat-killed *E. coli 506* by PBMC per group, at various time points after *E. coli* inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before *E. coli* inoculation, whereas the PBS group received no IBV or *E. coli*. NO production by cells of individual chickens is calculated as the total NO production after stimulation with heat-killed *E. coli* or LPS, minus the background NO production of unstimulated cells. Groups with different letters are significantly different ($P<0.05$).

NO production by PBMC and splenocytes after *E. coli* LPS-stimulation showed a pattern comparable to stimulation with heat-killed *E. coli*, although the amount of NO produced was approximately twice as high (data not shown).

Phagocytosis and killing assay

The ability of PBMC and splenocytes to kill *E. coli* bacteria after internalization was determined at 4 and 7 dpi, based on the number of colonies found after 24 h incubation. Uptake of bacteria was confirmed after 14 h incubation as the number of *E. coli* colonies retrieved from the PBMC and splenocytes and was higher than 300 in all chickens (data not shown). At 7 dpi, in the H120 and M41 groups the PBMC of 3 respectively 4 out of 5 chickens did not kill engulfed bacteria based on high colony counts, whereas only 1 chicken in the PBS group and 1 chicken in the *E. coli* group did not kill intracellular bacteria (table 2). At 4 dpi, splenocytes from all groups successfully killed *E. coli* bacteria. At 7 dpi however, splenocytes from 2

chickens in the H120 group were not able to kill the bacteria within 24 h incubation (data not shown).

Table 2. Phagocytosis and killing of bacteria by PBMC of chickens killed 4 and 7 days after *E. coli* inoculation (dpi). Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine strain H120 (group: H120), virulent IBV strain M41 (group: M41) or PBS (groups: PBS and *E. coli*) and 5 days later inoculated with *E. coli*, the PBS group with PBS. Each group consisted of five broilers. Numbers represent the number of chickens in each group displaying the specified killing efficiency. For each chicken, samples were run in duplicate.

	Group	colony counts 4 dpi*			colony counts 7 dpi*		
		< 100	100-300	> 300	< 100	100-300	> 300
PBMC	PBS**	3	0	2	0	4	1
	E. coli	0	3	2	2	2	1
	H120	0	2	3	1	1	3
	M41	1	1	3	0	1	4

* The number of colonies retrieved from phagocytic cells after lysis was used as a measurement for killing efficiency; retrieval of less than 100 colonies was considered successful clearance, whereas retrieval of more than 300 colonies was considered as a failure to clear *E. coli*.
** Inoculated at 32 days with glucose broth instead of *E. coli* broth.

Real-time quantitative RT-PCR

To examine the effects of IBV and *E. coli* inoculation on systemic immune functions, cytokine mRNA expression of spleen and lung samples were measured by real-time quantitative RT-PCR. No significant differences between the groups were found at any time point for IL-4, IFNα and IFNβ in either spleen or lung samples (data not shown). At 1 dpi, spleen mRNA levels for IFNγ and the pro-inflammatory cytokines IL-1β and IL-6 (data not shown) as well as IL-8, IL-18 and IFNγ (figure 4A) were significantly upregulated in the *E. coli*-inoculated group in comparison to the IBV-inoculated groups. Similarly, mRNA levels for the anti-inflammatory cytokine IL-10 were increased significantly in *E. coli*-inoculated birds compared to the IBV-inoculated birds.

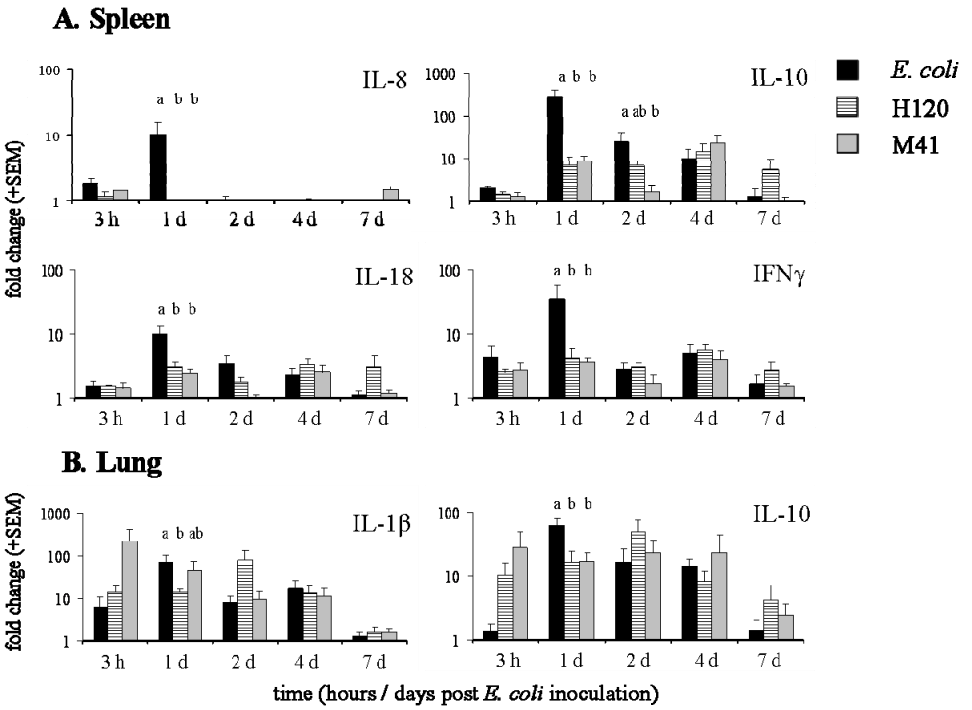

Figure 4. Real-time quantification of cytokine mRNA expression by cells isolated from A) splenocytes and B) lung tissue samples of PBS chickens (white bars), chickens inoculated with *E. coli* only (black bars), IBV H120 + *E. coli* (hatched bars) and IBV M41 + *E. coli* (grey bars). Data are expressed as mean relative fold increase at different time points after *E. coli* inoculation, compared to samples of PBS birds. Error bars show SEM for triplicate samples of 5 birds per treatment group. Groups with different letters are significally different ($P<0.05$).

The mRNA levels of these cytokines dropped sharply from 2 dpi onward. Interestingly, at 7 dpi, only in the H120 group, IL-6, IL-10 and IFNγ mRNA expression was still higher than the *E. coli* group. Tests were carried out to find whether altered expression of cytokine mRNA in IBV-inoculated birds was also found in lung samples (figure 4B). In lung samples, no significant differences between the groups were found at any time point for IL-18, IFNβ and IFNγ (data not shown). In contrast to our findings in the spleen, at 3 hpi, mRNA levels for IL-

6, IL-8, IFNγ (data not shown), IL-10 and IL-1β (figure 4B) in lung samples of birds of the H120 and especially the M41groups were higher than those of the *E. coli* group. However, at 1 dpi, mRNA expression of these cytokines in the *E. coli* group had increased similar to, or higher than that of the birds in the IBV infected groups. Levels remained elevated at 2 dpi, with highest expression in the IBV H120 group. At 7 dpi, mRNA expression levels of all three *E. coli*-inoculated groups had dropped to the level of the birds of the PBS group.

Discussion

Enhanced susceptibility for bacterial infections as a consequence of viral infection is reported in varying combinations in various species (Hament *et al.,* 1999). In poultry, severe colibacillosis after infections with Infectious Bronchitis Virus is well documented (Peighambari *et al.,* 2000; Vandekerchove *et al.,* 2004) and is a serious problem in commercial chickens.

Interestingly, not only the virulent IBV M41 field strain induces enhanced susceptibility to subsequent *E. coli* infection, but the mild H120 vaccine strain also induces this enhanced colibacillosis to about the same level (Matthijs *et al.,* 2003). A model based on these findings was used to study the phenomenon and test the contribution of different possible factors causing the phenomenon. In a separate paper, we addressed the question whether local changes at the surfaces of the trachea, lung and airsacs might facilitate local bacterial replication and subsequent entry of bacteria. The results of that study showed that the clearance of the bacteria from the lung was comparable for all the groups inoculated with *E. coli*, irrespective of previous exposure to virus (Matthijs *et al*., submitted).

In this paper we examined whether prior exposure to IBV virus (M41 or H120) affected the effector functions of mononuclear cells resulting in the phenomenon of enhanced susceptibility, a hypothesis supported by several papers (Ficken *et al.,* 1987; Naqi *et al.,* 2001; Slifka *et al.,* 2003). For this question, NO production by mononuclear cells isolated from the spleen and PBMC were tested. After *E. coli* inoculation, the NO production in samples upon stimulation with heat-killed *E. coli* bacteria or *E. coli* LPS (data not shown) was strongly increased in all groups, irrespective of prior exposure to IBV, although at 1 dpi the increase was highest in IBV-inoculated birds. Another difference was observed at 7 dpi, where birds exposed to *E. coli* alone showed diminished NO production compared to birds also

exposed to either IBV strain (M41 or H120). This is in line with the clinical picture of bacterial persistence in broilers previously exposed to IBV.

Subsequently, the internalization and intracellular killing of bacteria was tested in splenocytes and PBMC of all groups. Again all groups exposed to *E. coli*, irrespective of earlier exposure to IBV, responded similarly in the assay at 4 dpi, in that the cells had a diminished killing capacity. At a later time point (7 dpi), bactericidal capacity in the groups exposed to IBV was still lower than the bactericidal capacity of the group exposed to *E. coli* alone, suggesting a slower recovery. This once more was in line with the clinical recovery observed in the *E. coli* alone group.

The results of the NO production and of the internalization and intracellular killing of bacteria by the individual splenocytes or PBMCs suggest that the small changes in effector functions of those cells cannot be the only reason for the phenomenon of enhanced colibacillosis in animals previously exposed to virus. However, changes in the number of cells and / or their recruitment could still influence the outcome of the secondary infection. We therefore evaluated the percentage of effector cells by FACS analysis. Major differences that could explain the enhanced colibacillosis in the samples of the dual infected groups were not found.

Because no major changes in the effector functions of the mononuclear cells could be found to explain the phenomenon of enhanced colibacillosis, other mechanisms to explain the findings were sought. Supported by the observation that clinical colibacillosis seemed mainly the result of a systemic effect, and not directly of a local lung condition, we set out to find other explanations and looked at whether cytokine profiles would clarify the clinical picture. This might lead to insights into how the innate capacity of the animals was modulated at the time of *E. coli* exposure. Therefore, we examined mRNA expression in splenocytes and in samples of the lung.

After *E. coli* inoculation alone, the pro-inflammatory cytokines IL-1β and IL-6 were strongly increased in both splenocytes and lung tissue. This was expected, because both cytokines play an important role in initiating an acute-phase immune response against invading pathogens and activating a wide range of immune cells such as macrophages and T cells (Wigley and Kaiser, 2003).

The pro-inflammatory chemokine IL-8 was found to be upregulated in lung samples as early as 3 hpi, and in splenocytes at 1 dpi. The findings in the lung are in agreement with its function as a chemotractant, produced at the infection site in

order to recruit heterophils and initiate a rapid local inflammatory response (Withanage *et al.,* 2004). The upregulation in spleen samples was only found in the *E. coli* group and is likely due to the fact that the bacteria are not retained in the respiratory tract but spread systemically. Surprisingly, in the IBV groups no IL-8 mRNA is found in the spleen, which might suggest that a lack of response in the spleen can contribute to prolonged colibacillosis. The mRNA expression of pro-inflammatory mediators IL-1β and IL-6 in the spleen at 1 dpi in IBV groups also lagged behind that of the *E. coli* group.

Not only the cytokines IFNγ and IL-18, but also IL-10 were found to be upregulated in splenocytes and lung samples of birds inoculated with *E.coli* alone. IFNγ is a potent macrophage-activating factor and IL-18 is an important inducer of IFNγ production (Wigley and Kaiser, 2003). Both IFNγ and IL-18 are strongly linked to a cell-mediated Th1-like immune response (Staeheli *et al.,* 2001). IL-10 on the other hand is a promotor of Th2-like immune responses, predominantly by inhibiting pro-inflammatory and Th1 cytokines (Rothwell *et al.,* 2004), which can increase host susceptibility to bacterial diseases through its anti-inflammatory effects, including suppression of macrophage function. Previous studies in chickens showed that IL-10 production could be accompanied by either downregulation of IFNγ production (Abdul-Careem *et al.,* 2007; Rothwell *et al.,* 2004), but also by concurrent upregulation of IL-10 and IFNγ (Hong *et al.,* 2006). Moreover, it should be noted that IL-10 is known to have immunostimulatory effects in certain species, inducing MHC class II upregulation and cytotoxic T-cell stimulation (Groux and Cottrez, 2003).

In our chicken model, high mRNA expression of pro- and anti-inflammatory cytokines at 1 dpi in spleen samples was not found in birds inoculated with IBV and *E. coli*, but only in *E. coli* inoculated birds, and thus seems to be associated with recovery of colibacillosis. The differences in mRNA expression are consistent with the finding that virus-induced modulation of the immune response plays an important role in the susceptibility to subsequent bacterial infection.

In conclusion, we observed that both vaccine and virulent IBV caused enhanced colibacillosis, but IBV did not significantly affect phagocytic capacity and NO production of peripheral mononuclear cells and splenocytes. However, IBV did alter the systemic cytokine mRNA expression patterns after *E. coli* inoculation in commercial broilers likely resulting in enhanced colibacillosis.

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Abbreviations

dpi = days post *Escherichia coli* inoculation; EID_{50} = median embryo infective dose; IBV = Infectious Bronchitis Virus; i.t. = intratracheal; RPE = R-phycoerythrin

Chapter 8

Summarizing discussion

Introduction

Infectious bronchitis (IB), caused by a corona virus, is a respiratory disease of poultry with a world-wide distribution. Although clinical signs in broilers are rather mild, vaccination is common practice in the poultry industry. The vaccines used are modified live vaccines, which have been passaged several times in embryonated eggs to reduce their virulence. Vaccine virus is able to replicate in respiratory epithelium and to induce an immune response in chicken that provides clinical protection after infection with a virulent strain of the same serotype (Cavanagh, 2005; 2007). Besides causing respiratory signs, infectious bronchitis virus (IBV) can predispose broilers for colibacillosis, a disease caused by a respiratory infection with *E. coli* strains (Cook *et al*., 1986; Glisson, 1998; Vandekerchove *et al*., 2004). Colibacillosis in broiler flocks is characterised by increased mortality, loss of uniformity, reduced body weight gain, increased feed conversion and high condemnation rate at slaughter, and is therefore an economically important disease (Goren, 1991; Elfadil *et al.,* 1996; Barnes *et al.,* 2003). Moreover, the disease severely reduces welfare.

Although the vaccine industry does not claim any effect of IB vaccines related to colibacillosis, large scale use of these vaccines in broilers in the Netherlands, predominantly at one or 15 days of age, is partly based upon the supposed effect on IBV induced colibacillosis. In a study in the Netherlands of 10 not IB vaccinated broiler flocks, Lambers *et al.* (2000) observed the following: all flocks became seropositive to IBV, nasal discharge (a prominent sign of virulent IB) was not observed and at slaughter great numbers of birds were condemnated due to colibacillosis. It was hypothesized that these observations might be explained by assuming that the broiler flocks had become infected with IB vaccine virus. This hypothesis is partly supported by findings of Meulemans *et al.* (2001) in Belgium and Farsang *et al.* (2002) in Sweden, who isolated IBV vaccine strains from flocks that had not been vaccinated against IB. The question that arose was whether IBV vaccine strains were able to increase susceptibility for colibacillosis in broilers substantially.

The first aim of this thesis was, therefore, to assess the ability of vaccine virus (H120 and H52) to enhance the susceptibility for colibacillosis in comparison with the ability of virulent IBV (M41 and D387). Clinical signs and pathological findings after the superinfection were recorded and compared (Chapter 2). Only if vaccine viruses can spread efficiently, they could be a potential risk factor for the

occurrence of colibacillosis. So, the next question to be investigated was whether an IBV vaccine virus (H120) was able to spread in a flock (Chapter 3). Furthermore, it was investigated whether vaccination with IBV vaccine was able to protect broilers against an *E. coli* superinfection after infection with a virulent IBV (Chapter 4).

In the second part of the thesis (Chapters 5, 6 and 7) the pathological changes and immunological responses after an *E. coli* superinfection in IBV inoculated broilers were studied. A better understanding of the processes involved in this phenomenon might contribute to develop other types of vaccines, or to develop intervention measures with respect to prevention of colibacillosis.

Increase of susceptibility to colibacillosis by IBV vaccines

It was demonstrated experimentally that the ability of the IBV vaccines H120 and H52 (further referred to as H120 and H52) to increase colibacillosis susceptibility in four-week-old commercial broilers did not differ significantly from that of the virulent IBV strains M41 and D387 (further referred to as M41 and D387) (Chapter 2). This finding contrasted with the observation that after inoculation with these vaccines strains (H120 and H52) significant fewer birds developed nasal discharge than after inoculation with the virulent strains (M41 and D387). Apparently, the presence or absence of nasal discharge does not seem to predict whether an IBV strain might or might not increase the broilers' susceptibility for colibacillosis. The results in Chapter 2 indicated that IBV vaccines could play a role in the occurrence of colibacillosis in commercial broilers in the field.

In Chapter 4 the protective effect of vaccination of day-old broilers with H120 against colibacillosis after M41 challenge at four weeks of age was examined. Vaccination (spray and eye-drop) not only prevented the occurrence of clinical signs of IB (nasal discharge) as expected, it also reduced both the number of birds with *E. coli*-induced airsacculitis and the severity of airsacculitis. Spray vaccination, however, even with a high dose per bird ($10^{7.4}EID_{50}$/bird), did not reduce the number of birds with fibrinous pericarditis and perihepatitis nor the severity of it and had, as a consequence, no beneficial effect on body-weight uniformity. The results after eye-drop vaccination did not allow a straight forward interpretation as discussed in Chapter 4.

Although the results of Chapter 4 indicate that vaccination with H120 does not prevent broilers from increased susceptibility to colibacillosis upon M41 challenge, it is questionable whether this will also happen after a natural infection in the field. In the study described in Chapter 4, birds were individually inoculated with M41. De Wit *et al.* (1998), however, showed that vaccination with H120 effectively stopped transmission of M41. This implies that after introduction of M41 in a H120 vaccinated flock, the number of birds that become infected will remain limited, and, as a result, IBV-related colibacillosis will also occur at a limited scale only. However, it should be realized that De Wit *et al.* (1998) used SPF layer type chickens, which were vaccinated twice, in the absence of maternally derived antibodies, whereas the birds used in the experiment in Chapter 4 were commercial broilers vaccinated only once.

Implications for the broiler industry

The question that arises from the results described in Chapters 2 and 3 is whether mass application of IBV vaccines in the field might pose a risk not only for vaccinated flocks but also for unvaccinated flocks. In case H120 is administered to broilers at day of hatch or at fifteen days of age, which is common practice in Dutch broiler industry, the vaccine is able to spread rapidly among birds as demonstrated in a transmission experiment described in Chapter 3.

It was also shown that birds that became vaccinated by contact with inoculated ones were also protected against signs of IB after challenge with M41. Even if not all broilers have been effectively in contact with vaccine after mass application, it was demonstrated (Chapter 3) that nearly all birds, contact-exposed to vaccinated ones, became infected with the vaccine virus within 15 days, and subsequently were also protected against signs of IB after challenge with M41.

Consequently, it is not likely that vaccination with H120 at day of hatch or at fifteen days of age will cause increased susceptibility for colibacillosis in the second half of the growing period of broilers, the period in which the *E. coli* concentration in the air of broiler houses might be high (Harry and Hemsley, 1965; Carslon and Whenham, 1968), because H120 spreads rapidly in the experimental groups, as demonstrated from the virus excretion data in Chapter 3.

It should be realised, however, that IBV can establish a persistent infection in chicken (Cook *et al.,* 1968; Alexander *et al.,* 1978; Toro *et al*., 1996). It has also

been demonstrated (Naqi *et al.*, 2003) that IBV vaccine exposure of one day old chicks can lead to intermittent shedding of the virus. Whether persistence and subsequent reactivation of the IB infection also plays a role in the increased susceptibility to colibacillosis in the field is yet unknown, and could be subject of future research.

The vaccine virus H120 might only be a risk for unvaccinated flocks if H120 is able to spread between flocks. Introduction of IBV vaccine during the second half of the growing period might result in an increased risk for colibacillosis. The estimated reproduction ratio and the 95% CI of IBV H120 following inoculation at day of hatch was [2.1-∞] and [1.8-∞] after inoculation at 15 days of age, which implies that H120 might spread between flocks. Another indication for between-flock spread can be found in studies of Meulemans *et al.* (2001) and Farsang *et al.* (2002), who demonstrated the presence of IBV vaccine in unvaccinated flocks in Belgium and Sweden, respectively.

An abundance of publications on the beneficial effect of IB vaccination in chickens, i.e. prevention of clinical signs of IB, is available (amongst others: Davelaar and Kouwenhoven, 1980; Andrade *et al.,* 1982; Darbyshire and Peters, 1985; Cook *et al.,* 1999; Bijlenga *et al.,* 2004). Also in experiments described in Chapter 4 this beneficial effect was obtained. On the other hand, IBV vaccine may increase the susceptibility to colibacillosis by spreading of virus from vaccinated flocks to unvaccinated broiler flocks. However, the quantitative contribution of IBV in enhancement of susceptibility to colibacillosis under field conditions has not been demonstrated in this thesis. Besides IBV, other viruses, like Avian Pneumo virus and Newcastle Disease (vaccine) virus and even factors like dust and ammonia (NH_3) may play a role (Goren, 1991; Barnes *et al*., 2003; Vandekerchove *et al.,* 2004). Nevertheless, because of this uncertainty, at least for the time being, it is advisable to vaccinate all broilers flocks against IB in the first part of the growing period (in this period the *E. coli* concentration in the air of broiler houses is low as a rule). By doing so, clinical IB will be prevented and *E. coli* airsacculitis induced by virulent IBV or IB vaccine virus will at least be reduced.

Underlying mechanisms of an E. *coli* superinfection in IBV-infected broilers

The clinical signs of colibacillosis can be reduced by application of antibiotics. This might, however, increase resistance of *E. coli* against these therapeutics, and, moreover, broilers cannot be treated at the end of the production period, because of the withdrawal period. More insight in the pathogenesis of a superinfection of *E. coli* in IBV-infected broilers and in the underlying mechanisms of the increased susceptibility to colibacillosis might lead to new methods in prevention or reduction of colibacillosis. Chapters 5, 6 and 7 report studies into the pathogenesis and immunological response after *E. coli* infection in IBV-infected broilers. Two possible pathways for superinfections which have been proposed in literature, tissue damage and interaction with the immune system (Heinzelmann *et al.*, 2002; McCullers, 2006), were studied.

Single *E. coli* infection

It has been known for a long time (Goren, 1978; Goren, 1991) that an infection with *E. coli* alone does not induce colibacillosis in broilers from three weeks of age onwards, as they do not develop clinical signs or macroscopical pathological lesions after challenge with *E. coli*. The explanation given was that broilers were either not colonised at all by the bacteria, or that the bacteria were cleared rapidly. In the studies described in Chapters 2 and 4, the evaluation of the course of *E. coli* infection in the various groups was based on macroscopical examination carried out seven days after intratracheal *E. coli* inoculation.

The findings were consistent with those of Goren (1978): broilers inoculated with *E. coli* alone did not develop colibacillosis. In Chapters 5, 6 and 7 various tissues and organs were examined not only seven days after *E. coli* inoculation but also at different time points after *E. coli* inoculation. In these studies, it was shown that four-week-old broilers develop colibacillosis almost to the same extent as superinfected (*E. coli*–IBV) broilers, but in contrast to the superinfected ones they are capable to recover and clear their organs within seven days, demonstrating a properly working immunological defence mechanism. So, results described in Chapter 5 indicate that four-week-old broilers are not *in sensu stricto* "naturally resistant" against an experimental infection with *E. coli.*

***E. coli* superinfection in IBV-infected broilers**

Infectious bronchitis virus and *E. coli* were detected in different parts of the respiratory tract. Virus was found in the trachea, bacteria in the lungs, and both pathogens were found in the airsacs (Chapter 5, 6, 7).

Because IBV causes severe damage to the ciliated tracheal epithelium, investigators (Nakamura *et al.,* 1992; 1996; Arné *et al.*, 2000; Vandemaele *et al.,* 2002) suggested the destruction of trachea tissue by IBV as the cause of the increased susceptibility to *E. coli* superinfections. In the study presented in Chapter 6, *E. coli* bacteria were not detected adhered to or invaded in the tracheal tissue of H120 and M41 inoculated broilers. This indicates that tracheal tissue damage due to replication of IBV is not likely the mechanism for increased susceptibility to colibacillosis.

Macrophages and granulocytes are responsible for clearance of bacteria, and it is known that viral infections can hamper the functioning of these cells (Ficken *et al.,* 1987; Read *et al.*, 1999). Several human, mammalian and avian viruses are known to cause dysfunction of macrophages and granulocytes (Navarini *et al.*, 2006; Slifka *et al.*, 2003). Naqi *et al*. (2001) suggested that IBV had an effect on the macrophages. Nevertheless, in the studies described in chapter 6 and 7 of this thesis, no effect of a preceding IBV infection was found on the recruitment of macrophages in trachea, lungs and airsacs or on the function of phagocytic cells in blood and spleen. Moreover, the macrophages seemed able to clear lungs and airsacs from *E. coli*.

A remarkable finding was that the number of macrophages in the airsacs decreased at seven days after *E. coli* inoculation to basic levels in the *E. coli* group, whereas they remained high in the IBV - *E. coli* groups (Chapter 6). Macrophages not only have a phagocytic function, but they also produce cytokines which modulate the immune response. An overproduction of cytokines in broilers previously infected with IBV might be responsible for the increase of the severity and the duration of the inflammatory reactions (Beadling and Slifka, 2004). However, the cytokine production measured in the lungs was not influenced by the preceding IBV infection (Chapter 7).

In contrast to the lungs the innate antibacterial and anti-inflammatory cytokine production (IL-1β, IL-6, IL-8, IL-18) and the regulatory cytokine production (IL-10) in the spleen was affected (no up-regulation) by the preceding IBV infection. Broilers inoculated with *E. coli* alone had a significant augmentation of these cytokines, which seemed beneficial since these birds had recovered at seven days

after *E. coli* inoculation, as shown in chapter 7. Also IFNγ in the spleen did not seem to be up-regulated in broilers infected with IBV and *E. coli*, whereas in the spleen of *E. coli* inoculated broilers it was. Down-regulation of IFNγ presumably acts as a negative feedback signal for virus replication and spread, and may therefore be involved in the establishment of the latent infection (Adair, 1996).

Utility of the model used in this thesis to study superinfections

A lot of models have been used to study the mechanisms behind superinfections (amongst others: Bakaletz, 1995; Wilson *et al.,* 1996; Hament *et al.,* 1999; Beadling and Slifka, 2004; McCullers, 2006). The infection model described in this thesis allows studying *E. coli* superinfection in IBV–infected broilers. This model has the advantage that the animals used in the experiments are also the animals of interest, contrary to human research where mice are most frequently used to study pathogenesis of human viruses and bacteria. A question is whether this model could be a more generic model to study bacterial superinfections following viral infections.

Examples of superinfection models suggest that each virus-bacterium superinfection has its own pathogenesis. In a study where mice were infected with measles virus and *Listeria monocytogenes*, the function and the recruitment of macrophages and granulocytes was affected (Slifka *et al.*, 2003). In a study of Navarini *et al.* (2006), in which mice were infected with Lymphocytic choriomeningitis virus and *L. monocytogenes* or *Staphylococcus aureus*, the infiltration of granulocytes at the site of bacterial superinfection was drastically reduced. In this thesis no altered function or recruitment of the macrophages or altered infiltration of granulocytes were found. Van der Sluijs *et al.* (2006) found in a mouse superinfection model that post-influenza pneumococcal pneumonia was associated with uncontrolled inflammatory responses in the lungs in which excessive IL-10 production was involved in enhanced bacterial overgrowth. In this thesis, the regulatory cytokine production (IL-10) in the spleen was affected (no up-regulation) by the preceding IBV infection.

Future research

Colibacillosis in the second half of the growing period of broilers, as a rule, occurs as secondary infection. Besides IBV infection, also infections with other respiratory viruses such as ND vaccine virus and APV, as well as *Mycoplasma gallisepticum* infection and non-infectious factors like high NH_3 and dust levels in the air of broiler houses, might increase the susceptibility to colibacillosis in broilers (Gross, 1991; Goren, 1991; Barnes *et al.,* 2003; Vandekerchove *et al.,* 2004)

- First of all it is important to establish the proportion of colibacillosis cases in the field associated with IB vaccine virus in order to determine if further research in this field has priority. For this purpose, monitoring of broiler flocks for the presence of vaccine virus and investigating its association with colibacillosis is needed.

For a good and more complete understanding of the role of IB vaccine virus in the occurrence of colibacillosis in broilers, following studies are needed as well.

- Experimental study on the role of persistence and the intermitting virus shedding of IB vaccine virus in broilers related to colibacillosis.
- A study into the transmissibility of virulent IB virus and IB vaccine virus in commercial broilers vaccinated once against IB.

Some pro-inflammatory cytokines do not seem to be up-regulated in the spleen of broilers infected with IBV and *E. coli*, while this up-regulation does take place in only *E. coli* inoculated broilers. This difference suggests an IB virus-induced modulation of the immune response resulting in an inability to conquer the bacterial superinfection. But further research is needed.

- An experimental study on the IBV induced modulation of the cytokine response. Starting with a repetition and later on an extension of the study presented in Chapter 7.

Final conclusions

- Although a significant difference in clinical signs (nasal discharge) was observed between broilers infected with IB vaccines (H120 and H52) and virulent IBV (M41 and D387), no significant differences were demonstrated between these vaccines and virulent IBV strains in their ability to increase susceptibility to colibacillisosis.
- Under experimental conditions, IB vaccine H120 is able to spread efficiently among broilers.
- Based on the former final conclusions, IB vaccine virus might induce colibacillosis under field conditions in unvaccinated broiler flocks in the second half of the growing period.
- Vaccination with IB vaccine H120 protects broilers against clinical IB, reduces the proportion of birds with airsacculitis after challenge with virulent IBV M41 and *E. coli*, but does not protect the birds against pericarditis, perihepatitis and loss of body weight uniformity after such a challenge.
- A preceding viral IBV infection does not impair the clearance of *E. coli* in the respiratory tract of broilers, but rather induces an exaggerated inflammatory response in the airsacs only.
- Due to a preceding IBV infection the innate antibacterial and anti-inflammatory cytokine response in the spleen was not up-regulated following *E. coli* inoculation, as it was in broilers inoculated with *E. coli* alone.

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Samenvatting

Inleiding

Infectieuze bronchitis (IB), veroorzaakt door een coronavirus, is een ademhalingsziekte bij kippen die in de hele wereld voorkomt. Hoewel de klinische verschijnselen bij vleeskuikens mild zijn, wordt IB vaccinatie in de vleeskuikenhouderij algemeen toegepast. De gebruikte vaccins zijn meestal geattenueerde levende vaccins, die om hun virulentie te verminderen meermaals gepasseerd zijn in geëmbryoneerde eieren. Een levend vaccinvirus is in staat zich te vermeerderen in het epitheel van het ademhalingsapparaat en induceert zodoende een immuunreactie zodat vleeskuikens klinisch beschermd zijn tegen een infectie met een virulent infectieuze bronchitis virus (IBV) behorend tot hetzelfde serotype als het IBV vaccin. Naast het veroorzaken van afwijkende ademhalings-verschijnselen kan het IBV vleeskuikens triggeren voor secundaire bacteriële infecties, meestal *Escherichia coli (E. coli)* infecties waardoor het vleeskuiken colibacillose ontwikkelt. In koppels vleeskuikens die lijden aan colibacillose wordt verhoogde uitval, verlies van uniformiteit, verminderde groei, verhoogde voerconversie en een verhoogd afkeuringpercentage aan de slachtlijn gezien. Om die redenen is colibacillose bij vleeskuikens economisch gezien een belangrijke ziekte. Ook belangrijk is dat bij een uitbraak van colibacillose in een koppel vleeskuikens het welzijn van deze dieren ernstig wordt aangetast.

Hoewel de vaccinindustrie geen enkele aanspraak maakt op enig effect van IB vaccins op IBV geïnduceerde colibacillose, is het gebruik van deze vaccins in de vleeskuikenhouderij in Nederland voor een groot deel gebaseerd op dit veronderstelde effect. In een door de schijfster van dit proefschrift in Nederland uitgevoerde studie van 10 niet tegen IB gevaccineerde koppels vleeskuikens, werd het volgende geconstateerd: snot (een belangrijk symptoom van de infectie met virulent IBV) werd niet waargenomen, terwijl wel alle koppels op het einde van de groeiperiode antilichamen tegen IBV bezaten. Bovendien was het afkeuringpercentage aan de slachtlijn door colibacillose hoog. Deze waarnemingen leiden tot de hypothese dat infectie met IB-vaccinvirus (mede) oorzaak zou kunnen zijn van de verschijnselen zoals die werden waargenomen in de niet tegen IB gevaccineerde koppels vleeskuikens. Deze hypothese wordt deels gesteund door

buitenlandse bevindingen waarbij IBV vaccinstammen werden geïsoleerd uit niet tegen IB gevaccineerde koppels. De vraag rees dan ook of IBV vaccinstammen in staat waren de gevoeligheid voor colibacillose van vleeskuikens te verhogen.

In dit proefschrift werd daarom allereerst het colibacillose inducerend effect van de IB vaccinvirussen H120 en H52 vergeleken met dat van de virulente IBV stammen M41 en D384 (Hoofdstuk 2).
Bovendien, alleen indien IBV vaccinvirussen kunnen spreiden, kunnen ze een mogelijke risicofactor vormen voor het optreden van colibacillose. Daarom werd vervolgens onderzocht of het IBV vaccinvirus in staat was te spreiden in koppels vleeskuikens (Hoofdstuk 3). Verder werd onderzocht of het mogelijk was door IB vaccinatie vleeskuikens te beschermen tegen een *E. coli* superinfectie na infectie met virulent IBV van hetzelfde serotype als het vaccinvirus (Hoofdstuk 4).

In het tweede deel van het proefschrift (Hoofdstukken 5, 6 en 7) werden bij vleeskuikens de pathologische veranderingen en de immuunreacties na een IBV - *E. coli*-superinfectie bestudeerd. Een beter inzicht in de processen die betrokken zijn bij dit fenomeen zou kunnen bijdragen tot de ontwikkeling van andere typen vaccins of tot de ontwikkeling van nieuwe interventiemethoden ter preventie van colibacillose.

De door IBV-vaccins geïnduceerde verhoogde gevoeligheid voor colibacillose

Experimenteel werd aangetoond dat er geen of nauwelijks verschil is tussen de IBV-vaccins H120 en H52 (verder aangeduid als H120 en H52) en de virulente IBV-stammen M41 en D387 (verder aangeduid als M41 en D387) met betrekking tot het colibacillose inducerend vermogen bij vleeskuikens (Hoofdstuk 2). Deze bevinding contrasteerde met de bevinding dat het aantal vleeskuikens met snot na inoculatie met de vaccinstammen (H120 en H52) significant geringer was dan na inoculatie met de virulente stammen (M41 en D387). Snot is derhalve geen goede parameter voor de colibacillose gevoeligheid na een IB infectie.

In Hoofdstuk 4 werd onderzocht of vaccinatie van eendagsvleeskuikens met H120, op vier weken leeftijd bescherming gaf tegen colibacillose na challenge met M41. Vaccinatie (spray- en oogneusdruppelenting) voorkwam niet alleen het optreden van klinische verschijnselen van IB (snot), maar verminderde ook significant het aantal vleeskuikens met door *E. coli* veroorzaakte luchtzakontsteking en verminderde bovendien significant de ernst van de

luchtzakontsteking. Sprayvaccinatie echter, zelfs indien het vaccin toegediend werd in een hoge dosering ($10^{7.4}$ EID_{50}/kuiken), verminderde het aantal kuikens met fibrineuze pericarditis en perihepatitis niet, noch de ernst van deze laesies. De resultaten, verkregen met de oogdruppelmethode, lieten geen eenduidige interpretatie toe, zoals weergegeven in Hoofdstuk 4.
Hoewel de resultaten van Hoofdstuk 4 erop wijzen dat vaccinatie met H120 de vleeskuikens niet volledig beschermt tegen verhoogde gevoeligheid voor colibacillose na blootstelling aan M41, is het zeer de vraag of dit ook zal gebeuren in de veldsituatie na een natuurlijke blootstelling aan het veldvirus. In de studie beschreven in Hoofdstuk 4 werden de kuikens individueel besmet met het M41 virus, terwijl door andere onderzoekers is aangetoond dat vaccinatie met H120, spreiding van het M41 virus voorkomt. Dit impliceert dat na introductie van M41 in een gevaccineerd koppel het aantal kuikens dat geïnfecteerd wordt, beperkt zal blijven en dat bijgevolg IBV-gerelateerde colibacillose slechts op beperkte schaal zal optreden. Vermeld dient evenwel dat deze andere onderzoekers SPF- legkippen (zonder maternale antilichamen) gebruikten, die bovendien tweemaal werden gevaccineerd, terwijl de kippen die gebruikt werden in het experiment beschreven in Hoofdstuk 4 commerciële vleeskuikens waren, die slechts eenmaal werden gevaccineerd.

Implicaties voor de vleeskuikenhouderij

Uit de resultaten beschreven in de Hoofdstukken 2 en 3 rijst de vraag of massa-applicatie van IBV-vaccins in het veld een risico zou kunnen vormen voor het ontstaan van colibacillose, niet alleen voor IB gevaccineerde maar ook voor niet IB gevaccineerde koppels vleeskuikens. Als H120 wordt toegediend aan vleeskuikens op een leeftijd van 1 dag of 15 dagen, wat gebruikelijk is in de Nederlandse vleeskuikenhouderij, is het vaccin in staat snel onder de kuikens te spreiden, zoals aangetoond in het transmissie-experiment beschreven in Hoofdstuk 3. In dat hoofdstuk werd tevens aangetoond dat kuikens die besmet raakten met het vaccinvirus door contact met de gevaccineerde kuikens, na challenge met M41 ook beschermd waren tegen klinische verschijnselen van IB. Zoals gesteld, spreidt H120 toegediend aan vleeskuikens op de leeftijd van 1 dag of 15 dagen, onder experimentele omstandigheden snel (Hoofdstuk 3). Om die reden is het onwaarschijnlijk dat H120 in de tweede helft van de groeiperiode, de periode

waarin de *E. coli* concentratie in de stallucht hoog kan zijn, verhoogde gevoeligheid voor colibacillose induceert. Opgemerkt dient echter dat IBV een persisterende infectie kan veroorzaken, waarbij langdurige intermitterende virusuitscheiding kan optreden. Of persistentie en reactivatie van het IBV een rol spelen bij het induceren van verhoogde gevoeligheid voor colibacillose bij vleeskuikens in het veld is onbekend en kan onderwerp worden van toekomstig onderzoek.

Het vaccinvirus H120 kan alleen een risico zijn voor niet IB gevaccineerde koppels, als H120 ook in staat is te spreiden tussen koppels. De introductie van het IBV vaccin in niet IB gevaccineerde koppels gedurende de tweede helft van de groeiperiode kan resulteren in een verhoogd risico op het ontstaan van colibacillose. Het 95% betrouwbaarheidsinterval van de reproductieratio van H120 was bij vleeskuikens geinoculeerd op 1 dag leeftijd [2.1-∞] en [1.8-∞] bij kuikens geinoculeerd op 15 dagen leeftijd. Dit betekent dat H120 mogelijk ook kan spreiden tussen koppels. Een andere aanwijzing voor de spreiding tussen koppels werd verkregen in buitenlands onderzoek, waarbij het IB vaccinvirus werd geïsoleerd uit niet tegen IB gevaccineeerde koppels.

Er is een groot aantal publicaties dat het positieve effect van IB vaccinatie bij kippen beschrijft met betrekking tot preventie van klinische verschijnselen van IB. Ook in de experimenten beschreven in Hoofdstuk 4 is dit effect verkregen. Aan de andere kant kan het IBV-vaccin de gevoeligheid voor colibacillose verhogen door spreiding van het virus van gevaccineerde koppels vleeskuikens naar niet-IBV gevaccineerde koppels. De kwantitatieve bijdrage van het IBV aan de verhoging van de gevoeligheid voor colibacillose in het veld is in dit proefschrift echter niet onderzocht. Een dergelijk onderzoek is van belang omdat naast het IBV ook andere virussen, zoals het Aviaire Pneumovirus en het Newcastle Disease (vaccin) virus en ook factoren zoals stof en ammoniak (NH_3) een rol spelen in de verhoging van de gevoeligheid van vleeskuikens voor colibacillose.

Gelet op het voorgaande is het aan te raden alle koppels vleeskuikens vooralsnog te vaccineren tegen IB in het eerste deel van de groeiperiode (in deze periode is de *E. coli*-concentratie in vleeskuikenstallen, als regel, laag). Daardoor zal klinische IB worden voorkomen en *E. coli*-luchtzakontsteking geïnduceerd door virulente IBV of IB vaccinvirus worden verminderd.

De onderliggende mechanismen van een *E. coli* superinfectie in vleeskuikens besmet met IBV

De klinische verschijnselen veroorzaakt door colibacillose kunnen bij vleeskuikens worden verminderd door toediening van antibiotica. Toediening van antibiotica kan de resistentie van *E. coli* tegen antibiotica echter doen toenemen. Bovendien kunnen vleeskuikens door de wachttijd voor antibiotica niet tot het einde van de groeiperiode worden behandeld. Meer inzicht in de pathogenese van een superinfectie van *E. coli* in vleeskuikens besmet met IBV en in de onderliggende mechanismen van verhoogde gevoeligheid voor colibacillose kan leiden tot nieuwe methoden in de preventie of vermindering van colibacillose. Hoofdstukken 5, 6 en 7 bevatten onderzoek naar de pathogenese en de immuunrespons na een *E. coli* infectie bij vleeskuikens geïnfecteerd met IBV. In de literatuur worden twee mogelijkheden beschreven voor de verhoogde gevoeligheid voor bacteriële infecties na een virale infectie: als eerste, weefselbeschadiging door de virale infectie waardoor de bacterie beter kan aanslaan en, ten tweede, verandering van de immuunreactie door de virale infectie.

Enkelvoudige *E. coli*-besmetting

Het is al langer bekend dat bij vleeskuikens ouder dan circa drie weken inoculatie van *E. coli* alleen, geen klinische verschijnselen en macroscopische afwijkingen veroorzaakt. De verklaring hiervoor is, dat de kuikens ofwel helemaal niet gekoloniseerd worden door de *E. coli* bacteriën ofwel dat de bacteriën snel door het kuiken worden geëlimineerd. In het onderzoek beschreven in Hoofdstukken 2 en 4 werd het verloop van een *E. coli* infectie in 4 weken oude vleeskuikens gebaseerd op macroscopisch postmortaal onderzoek dat werd uitgevoerd 7 dagen nadat de kuikens intratracheaal waren geïnoculeerd met *E. coli*. De resultaten van dit onderzoek kwamen overeen met die uit het verleden, namelijk vleeskuikens geïnoculeerd met *E. coli* alleen, ontwikkelden geen colibacillose. In de Hoofdstukken 5, 6 en 7 werden verschillende weefsels en organen niet alleen onderzocht op 7 dagen na *E. coli* inoculatie maar ook op eerdere tijdstippen. In dit onderzoek werd aangetoond dat kuikens van 4 weken oud, na *E. coli* inoculatie, in bijna dezelfde mate colibacillose ontwikkelden als supergeïnfecteerde (IBV- *E. coli*) kuikens. In tegenstelling tot de supergeïnfecteerde kuikens bleken kuikens die

geinoculeerd waren met *E. coli,* echter in staat om binnen 7 dagen volledig te herstellen en binnen dit tijdsbestek *E. coli* te verwijderen uit alle onderzochte organen. Hieruit blijkt dat vleeskuikens van 4 weken oud een goed werkend immuunapparaat bezitten.. Bovendien tonen de resultaten in Hoofdstuk 5 aan dat 4 weken oude vleeskuikens *in sensu stricto* niet "natuurlijk resistent" zijn tegen een experimentele infectie met *E. coli*.

E. coli-superinfectie bij vleeskuikens besmet met IBV

Het IBV en de *E. coli* bacterie bleken te verschillen met betrekking tot de delen van het ademhalingsapparraat die werden gekoloniseerd. Het virus werd aangetroffen in de trachea, bacteriën in de longen en beide pathogenen in de luchtzakken (Hoofdstukken 5, 6, 7).
Omdat replicatie van het IBV het gecilieerd trachea epitheel ernstig beschadigt, suggereerden andere onderzoekers dat daardoor de *E. coli* bacterie gemakkelijker kan koloniseren. In het onderzoek uitgevoerd in Hoofdstuk 6 werden echter in de trachea van H120 en M41 geinoculeerde kuikens, geen *E. coli* bacteriën aangetoond die kleefden aan of binnendrongen in het tracheaweefsel. Dit wijst erop dat de verhoogde gevoeligheid voor colibacillose na IBV-infectie niet veroorzaakt wordt door beschadiging van tracheaweefsel door IBV.

Het is bekend dat na virale infecties de functies van macrofagen en granulocyten, verantwoordelijk voor het opruimen van bacteriën, aangetast kunnen zijn. Zowel bij mens als dier zijn verschillende virussen bekend die de functie van macrofagen en granulocyten verstoren. Ook is gesuggereerd dat IBV invloed heeft op de functie van macrofagen. In de Hoofdstukken 6 en 7 van dit proefschrift zijn echter noch op de rekrutering noch op de functies van macrofagen in trachea, longen en luchtzakken net zo min als op de werking van de fagocyten in bloed en lever, effecten van IBV aangetoond.

Opmerkelijk was dat het aantal macrofagen in de luchtzakken van de kuikens die alleen geinoculeerd waren met *E. coli*, 7 dagen na *E. coli* inoculatie weer op het oorspronkelijke niveau zat, terwijl het aantal macrofagen in de luchtzakken van kuikens die zowel met IBV als met *E. coli* waren geinoculeerd, 7 dagen na *E. coli* inoculatie nog steeds erg hoog was (Hoofdstuk 6). Macrofagen hebben niet alleen een fagocyterende functie, maar ze kunnen tevens cytokines produceren die de immuunrespons moduleren. Overproductie van cytokines in vleeskuikens die

zowel geinoculeerd werden met IBV als *E. coli* is mogelijkerwijs verantwoordelijk voor de ernst en de duur van de ontstekingsreactie in de luchtzakken van deze dieren.

De cytokineproductie in de longen van *E. coli* geïnfecteerde vleeskuikens werd echter niet beïnvloed door een voorafgaande infectie met IBV (Hoofdstuk 7). Daarentegen werd in de lever de productie van enkele cytokines (IL-1β, IL-6, IL-8, IL-18, IL-10) wel beïnvloed door de voorafgaande IBV infectie; er vond namelijk geen up-regulatie van deze cytokines plaats in de IBV-*E.coli* geinoculeerde kuikens terwijl dit wel het geval was in vleeskuikens die alleen met *E. coli* waren geinoculeerd. Deze up-regulatie leek een gunstig effect te hebben, aangezien deze dieren 7 dagen na *E. coli* inoculatie volledig hersteld waren (Hoofstuk 7). In levers van vleeskuikens geinoculeerd met IBV en *E. coli* was ook INFγ niet ge-up-reguleerd terwijl dit wel het geval was in levers van vleeskuikens die alleen geinoculeerd waren met *E. coli*. INFγ heeft een remmend effect op replicatie en verspreiding van virussen. Het zou kunnen zijn dat IBV om "die reden" de productie van IFNγ remt, zodat IBV kan spreiden en latent in de kip aanwezig kan blijven.

Eindconclusies

- Hoewel een significant verschil is vastgesteld in kliniek (snotverschijnselen) tussen vleeskuikens geinoculeerd met IB vaccinvirussen (H120 en H52) en vleeskuikens geinoculeerd met virulente IB virussen (M41 en D387), zijn geen significante verschillen aangetoond tussen deze vaccins en virulente IBV-stammen in hun vermogen om de gevoeligheid voor colibacillose te verhogen.
- Onder experimentele omstandigheden is het IB vaccinvirus in staat snel en efficiënt te spreiden onder vleeskuikens.
- Uitgaande van de vorige conclusies, zou het IB vaccinvirus in het veld colibacillose kunnen induceren bij vleeskuikens in de tweede helft van de groeiperiode.
- Vaccinatie met het IB vaccin H120 beschermt vleeskuikens tegen de klinische verschijnselen van IB, vermindert het aantal kuikens met *E. coli* luchtzakontsteking nadat de kuikens zijn blootgesteld aan het virulente IBV M41 en *E. coli*, maar beschermt de vleeskuikens niet tegen IBV geïnduceerde *E. coli* pericarditis en perihepatitis. Deze laatste aandoening beinvloedt uniformiteit en lichaamsgewicht negatief.
- Een voorafgaande IBV infectie verhindert de opruiming van *E. coli* in het ademhalingsstelsel van vleeskuikens niet, maar veroorzaakt veeleer een "overdreven" ontstekingsreactie in de luchtzakken.
- Door een voorafgaande IBV infectie wordt de aangeboren antibacteriële en anti-inflammatoire cytokinerespons in de lever niet ge-up-gereguleerd na *E. coli* inoculatie, zoals wel het geval is bij vleeskuikens die alleen met *E. coli* werden geinoculeerd.

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Curriculum Vitae

Mieke, **Geertrui, Raymonda Matthijs** werd geboren op 31 augustus 1973 in Gent (België). In 1987 behaalde zij het diploma LSO, afdeling Latijn-Wiskunde aan het Sint-Maartencollege te Lede, in 1990 het diploma HSO, afdeling Latijn-Wetenschappen in het Sint-Gertrudisinstituut in Wetteren.
Zij studeerde Diergeneeskunde aan de Universiteit Gent, waar zij in 1995 het diploma van kandidaat in de Diergeneeskundige Wetenschappen behaalde en in 1998 het diploma van Dierenarts, met onderscheiding.
In maart 1999 trad zij in dienst als junior docent/junior onderzoeker bij de Hoofdafdeling Landbouwhuisdieren van de Faculteit Diergeneeskunde van de Universiteit Utrecht. In 2001 startte zij dit promotieonderzoek dat in 2008 werd voltooid.

Nunc est bibendum!

Nostrorum sanitas

Prosit senior, prosit corona, ad fundum