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Research paper

## 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

R. Marius Dwars<sup>a,b,1</sup>, Mieke G.R. Matthijs<sup>a,b,1</sup>, Angeline J.J.M. Daemen<sup>a,b</sup>, Jo H.H. van Eck<sup>a,b</sup>, Lonneke Vervelde<sup>a,c</sup>, Wil J.M. Landman<sup>d,\*</sup>

<sup>a</sup> Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3508 TD Utrecht, The Netherlands

<sup>b</sup> Department of Farm Animal Health, The Netherlands

<sup>c</sup> Department of Infectious Diseases and Immunology, The Netherlands

<sup>d</sup> Animal Health Service (GD), Arnsbergstraat 7, 7418 EZ, Deventer, The Netherlands

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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 5 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 h 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. 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, in IBV-*E. coli* inoculated birds lesions in the airsac 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, which seems to be the mechanism behind the pattern of airsacculitis in commercial poultry in the field.

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\* Corresponding author at: Animal Health Service (GD), Arnsbergstraat 7, 7418 EZ Deventer, The Netherlands. Tel.: +31 570 660386; fax: +31 570 660354.

E-mail address: [w.landman@gddeventer.com](mailto:w.landman@gddeventer.com) (Wil J.M. Landman).

<sup>1</sup> These authors contributed equally to this work.

### 1. Introduction

According to Barnes et al. (2003), colibacillosis in chickens refers to any local 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 panophthalmitis and coliform omphalitis/yolk sac infection. In broilers, colibacillosis mainly manifests itself as respiratory infection and peritonitis/pericarditis (Goren, 1978; Pourbakshsh 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 3 to 4 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 characterized by low to moderate mortality, low to moderate incidence of septicemic lesions (mainly fibrinous polyserositis) and a high percentage of birds with airsacculitis (Cheville and Arp, 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 and Arp, 1978; Pourbakshsh 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 (Al-Ankari et al., 2001), or by aerosol (Peighambari et al., 2000). In some occasions, airsac inoculation is applied if specifically reactions of the airsac wall are studied (DeRosa et al., 1992) or a higher efficacy of the infection model is desired (Pourbakshsh 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 airsacs, are well described (Gross, 1957; Cheville and Arp, 1978; Pourbakshsh et al., 1997). However, most of these descriptions refer to chickens that have been inoculated experimentally up to an age of 2 weeks and whose lesions were assessed only during a period of 48 h. As colibacillosis in the field is most often seen after 4 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 5 days earlier in commercial broilers of 4 weeks of age. The location of the pathogens in the respiratory tract was investigated during a 7 days period after inoculation.

## 2. Material and methods

### 2.1. 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<sup>3</sup> and fitted with a wire floor of 0.93 m<sup>2</sup>. Isolators were ventilated at a rate of approximately 40 m<sup>3</sup> h<sup>-1</sup>.

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 °C at day 1 to 20 °C at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20 °C.

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.

### 2.2. Inocula

IBV vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials, which contained at least 10<sup>3.0</sup> EID<sub>50</sub> (egg infective dose 50%) per dose (Nobilis<sup>®</sup> IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, the Netherlands, as freeze-dried vials containing 10<sup>8.3</sup> EID<sub>50</sub>/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<sup>3.0</sup> EID<sub>50</sub>/ml of H120 virus and 10<sup>4.6</sup> EID<sub>50</sub>/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<sup>7.6</sup> CFU/ml.

### 2.3. 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 intratracheally (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 inoculated intratracheally with 1 ml *E. coli* 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 vaccine and *E. coli* broth) as the H120 group, and group 4 (IBV M41 and *E. coli* broth) as M41 group.

#### 2.4. Clinical and postmortem examination

Clinical signs of IBV infection were recorded 1, 2, 4 and 5 days after IBV inoculation and immediately 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 applying mild pressure directed from the nasal bone region towards 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 airsacs, 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).

#### 2.5. Histology and immunohistochemistry

##### 2.5.1. Sampling of tissues

After postmortem examination (for time points see Table 1), trachea and right lung were collected in 10% buffered formalin, fixed for 24 h and routine processed

**Table 1**

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

Broilers with	Time after <i>E. coli</i> inoculation					
	0.5 h	3 h	1 day	2 days	4 days	7 days
<b>Tracheitis</b>						
PBS <sup>a</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>
<i>E. coli</i> <sup>b</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>
H120	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>
M41	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>
<b>Pneumonia</b>						
PBS <sup>a</sup>	n.d.	n.d.	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>
<i>E. coli</i> <sup>b</sup>	n.d.	n.d.	5 <sup>B</sup>	5 <sup>B</sup>	3 <sup>AB</sup>	3 <sup>A</sup>
H120	n.d.	4 <sup>A</sup>	3 <sup>AB</sup>	5 <sup>B</sup>	5 <sup>B</sup>	3 <sup>A</sup>
M41	n.d.	5 <sup>A</sup>	5 <sup>B</sup>	4 <sup>B</sup>	2 <sup>AB</sup>	3 <sup>A</sup>
<b>Airsacculitis</b>						
PBS <sup>a</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>	0 <sup>A</sup>
<i>E. coli</i> <sup>b</sup>	3 <sup>AB</sup>	4 <sup>AB</sup>	5 <sup>B</sup>	3 <sup>AB</sup>	3 <sup>AB</sup>	0 <sup>A</sup>
H120	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>
M41	5 <sup>B</sup>	5 <sup>B</sup>	5 <sup>B</sup>	4 <sup>AB</sup>	5 <sup>B</sup>	5 <sup>B</sup>

<sup>AB</sup>Groups with different letters within a column are significantly different ( $P < 0.05$ ).

n.d. = not determined.

<sup>a</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.

<sup>b</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

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 airsac wall.

##### 2.5.2. Production of polyclonal antibodies against *E. coli* 506

The bacterial inocula were prepared by submersing one frozen bead ( $-70^{\circ}\text{C}$ ) of a batch containing the *E. coli* 506 strain in TSB and incubation during 20 h at  $37^{\circ}\text{C}$ . The bacteria were washed with PBS and killed with methanol during 5 min. The suspension was centrifuged 10 min at  $1500 \times g$  and the bacteria were washed twice with PBS and dissolved in 10 mg DDA/ml PBS.

Two rabbits were injected 3 times, 21 days between each injection, subcutaneously 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.

##### 2.5.3. Tissue staining procedure for IBV and *E. coli*

Trachea and lung tissue samples were collected in 10% buffered formalin, fixed for 24 h and processed to paraffin. Sections were cut  $5\ \mu\text{m}$  thick, deparaffinised and placed in pure methanol with 0.75%  $\text{H}_2\text{O}_2$  for 30 min to remove endogenous peroxidase activity. For IBV and *E. coli* staining in the airsacs, cryostat sections ( $6\ \mu\text{m}$  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 (CIDC, Lelystad, The Netherlands) 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%  $\text{H}_2\text{O}_2$ .

DAB-stained sections were counterstained with hematoxylin and mounted with aquamount (BDH, UK).

#### 2.6. 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.

### 3. Results

#### 3.1. Mortality

During the course of the experiment no mortality occurred.

#### 3.2. 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 (for detailed information see Matthijs et al., 2008).

#### 3.3. 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 perihepatitis were only found in broilers with severe airsaccultis. Lesions were more pronounced in the H120 and M41 group than in the *E. coli* group, however the difference was not significant. At 7 dpi no macroscopical lesions were found in the *E. coli* group, whereas airsaccultis was present in all and serositis in some birds of the H120 and M41 group. Detailed information on the number of broilers with airsaccultis, perihepatitis and/or pericarditis and mean macroscopic lesion score has been given elsewhere (Matthijs et al., 2008).

#### 3.4. Microscopical lesions

##### 3.4.1. 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 (Fig. 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, at 3 hpi, the epithelium was not ciliated, grossly cuboid, and partially flat (Fig. 1b and c). 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 (Fig. 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 40–250 per section and diminishing to 30–50 per section at 7 dpi.

At 1 dpi few follicles were found in some tracheas of the IBV groups; at 7 dpi 1–4 per section were found in tracheas of birds of the M41 group and 4–8 in the H120 group.

##### 3.4.2. Lungs

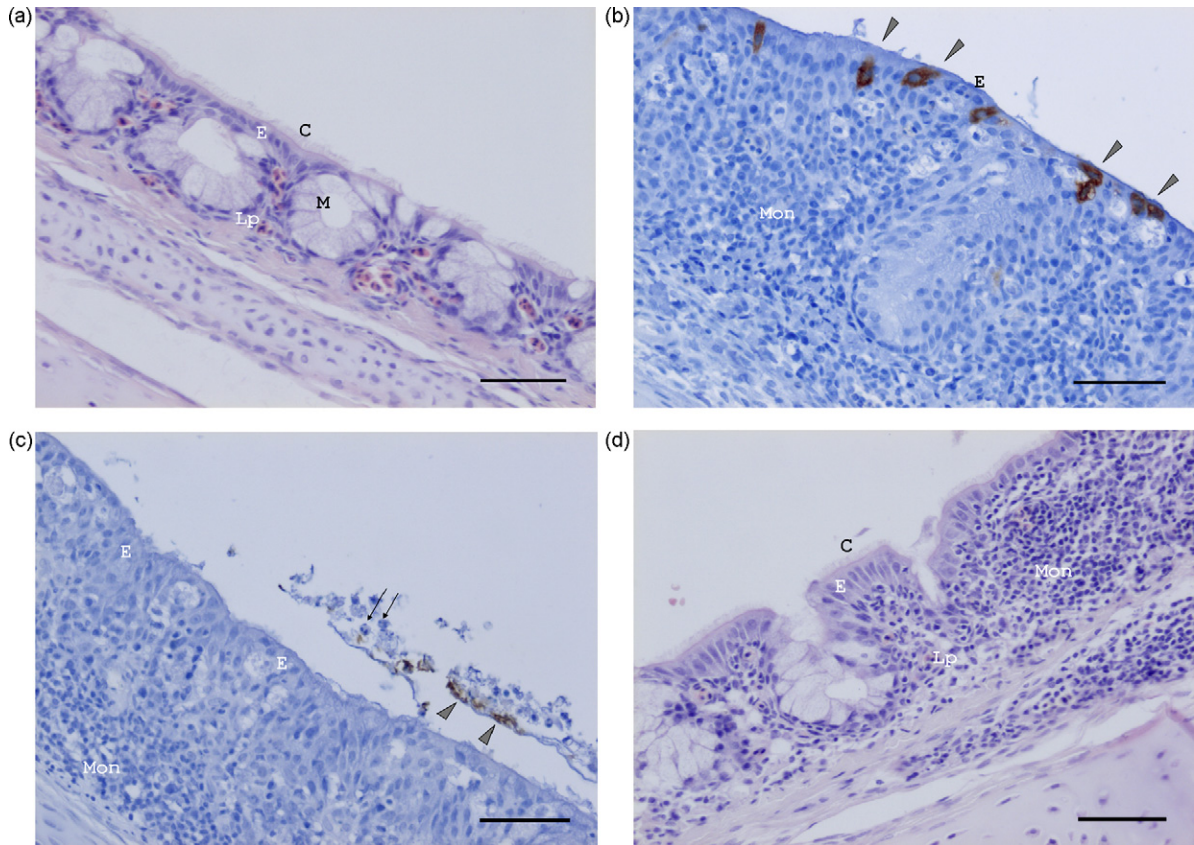
**3.4.2.1. Bronchi.** The adventitia of the bronchi of broilers of the *E. coli*, H120 and M41 group showed oedema and cellular infiltrations consisting mainly of granulocytes and mononuclear cell types, which were present from 0.5 hpi and 2 dpi, respectively.

In most birds of the *E. coli* group the lamina propria of bronchi was 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, consisting of lymphocyte infiltrates, granulocyte infiltrates extending into the epithelial layer, hyperaemia and oedema in varying but considerable amounts, were present. 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 (Fig. 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 (Fig. 2b).

**3.4.2.2. 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 (Fig. 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



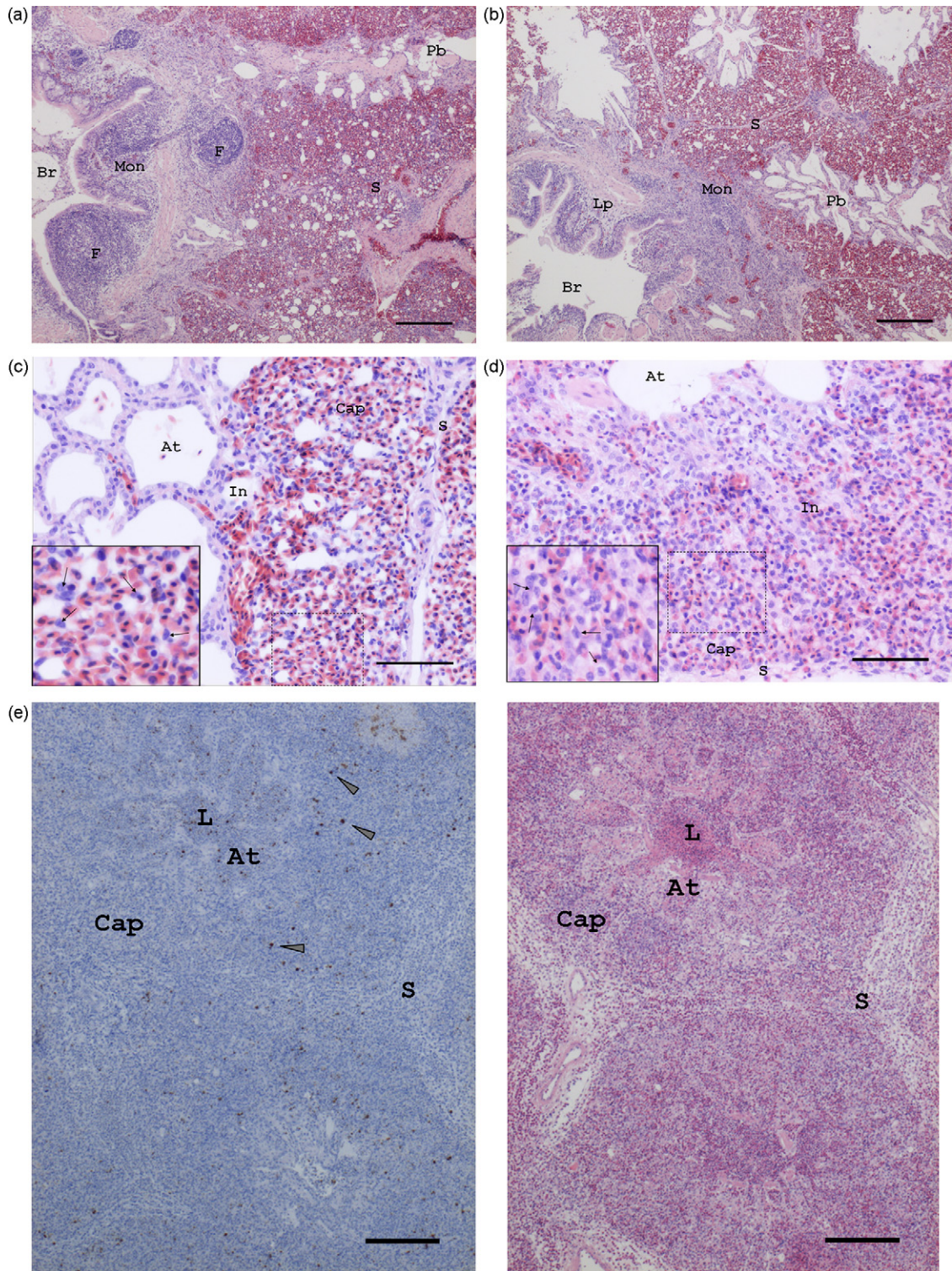
**Fig. 1.** Trachea of broilers inoculated oculonasally and intratracheally with either PBS (a) or IBV H120 (b, c, and d) at 27 days of age and intratracheally with PBS-diluted glucose broth (a) or *E. coli* (b, c, and d) at 32 days of age. (a) Trachea sampled at 1 day after inoculation with PBS-diluted glucose broth. Haematoxylin and eosin stain, bar = 50  $\mu\text{m}$ . 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 1 day after inoculation with *E. coli*. Immunoperoxidase IBV-haematoxylin stain, bar = 50  $\mu\text{m}$ . 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 1 day after inoculation with *E. coli*. Immunoperoxidase *E. coli*-haematoxylin stain, bar = 50  $\mu\text{m}$ . 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 7 days after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 50  $\mu\text{m}$ . Mucosa consists of pseudostratified columnar respiratory epithelium (E) and bearing cilia (C); in the lamina propria (Lp) mononuclear infiltrate (Mon) is present.

(Fig. 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 extent 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 (Fig. 2e and f). 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 (Fig. 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 (Fig. 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



**Fig. 2.** Lung of broilers inoculated oculonasally and intratracheally with PBS (b, c, and d) or IBV H120 (a and e) or IBV M41 (f and g) at 27 days of age and intratracheally with PBS-diluted glucose broth (c) or *E. coli* (a, b, d, e, f, and g) at 32 days of age. (a) Lung tissue sampled at 7 days after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 200 µm. 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 7 days after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 200 µm. 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 1 day after inoculation with PBS-diluted glucose broth. Haematoxylin and eosin stain,

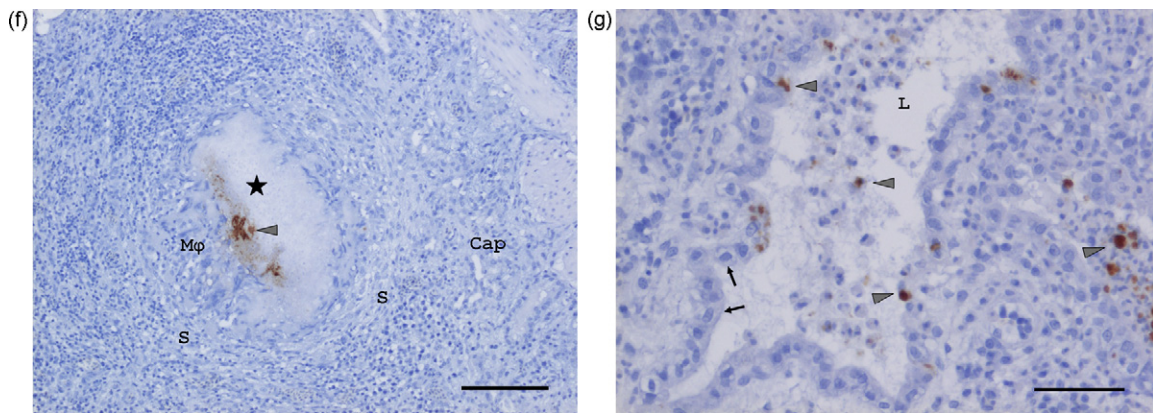


Fig. 2. (Continued).

Table 2

Percentage of damaged parabronchi in experimental broilers. Birds were inoculated with IBV vaccine H120 (H120 group) or virulent IBV strain M41 (M41 group) 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.

Group	Damaged parabronchi					Severity of damage				
	Time after <i>E. coli</i> inoculation									
	3 h	1 day	2 days	4 days	7 days	3 h	1 day	2 days	4 days	7 days
PBS <sup>a</sup>	n.d.	–	–	–	–	n.d.	–	–	–	–
<i>E. coli</i> <sup>b</sup>	n.d.	++	++	+	+/-	n.d.	++	+	+/-	+/-
H120	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-
M41	+	++	+	+/-	+/-	+/-	+	+/-	+/-	+/-

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.

n.d. = not determined.

<sup>a</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.

<sup>b</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

normal architecture was seen. They appeared inflated and had a diameter similar to that of the control birds (Fig. 2a and b). 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 1.

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 (Fig. 2a and b).

The number of affected parabronchi and severity of parabronchial lesions are presented in Table 2.

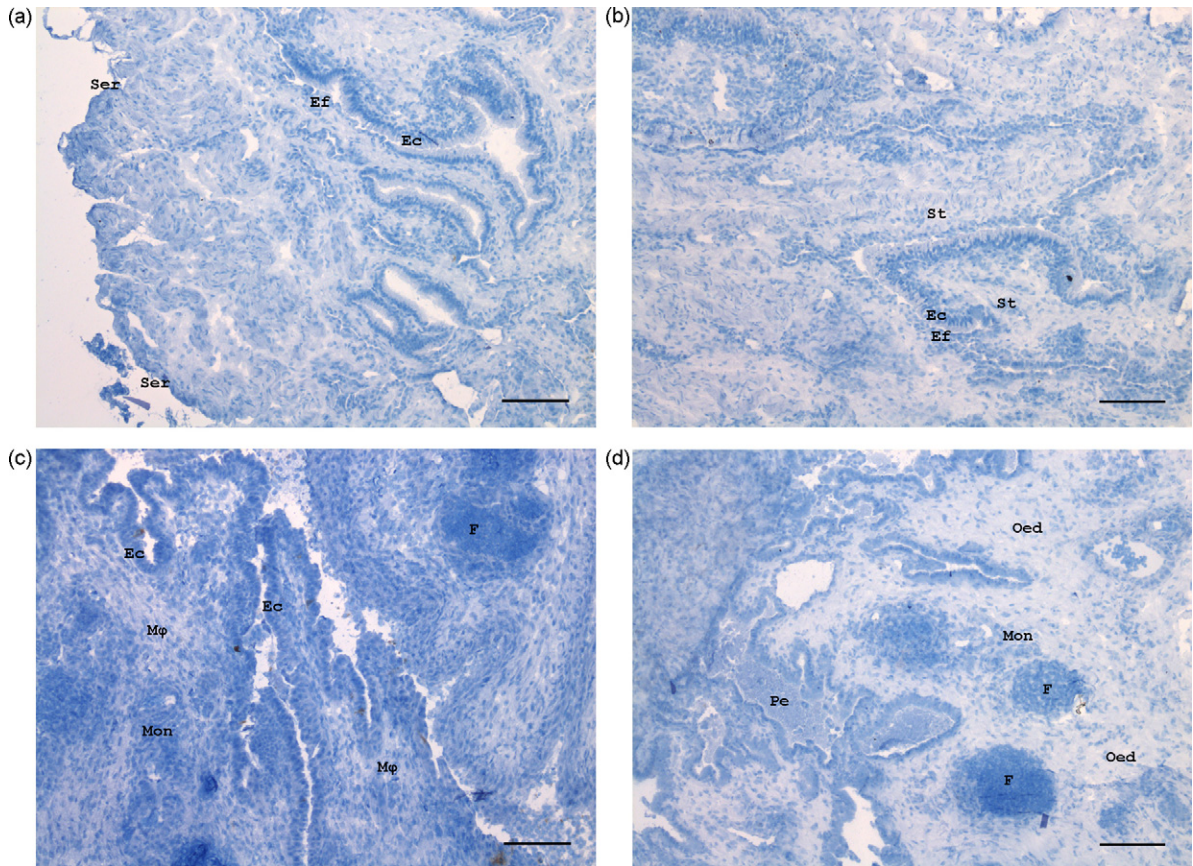
### 3.4.3. Airsacs

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. A distinct lamina propria was not recognizable. No oedema, infiltrates or follicles were present (Fig. 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 (Fig. 3b).

bar = 50  $\mu$ m. 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 1 day after inoculation with *E. coli*. Haematoxylin and eosin stain, bar = 50  $\mu$ m. 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. (e) Lung tissue sampled at 1 day after inoculation with *E. coli*. Left and right represent serial sections of the same parabronchi taken at small distance, bar = 200  $\mu$ m. 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 7 days after inoculation with *E. coli*. Immunoperoxidase *E. coli*-haematoxylin stain, bar = 100  $\mu$ m. Remnants of parabronchus with heavily infiltrated interparabronchial septum (S); from the capillary region and the lumen a necrotic core (asterisk) surrounded by macrophages (M $\phi$ ), 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 3 h after inoculation with *E. coli*. Immunoperoxidase *E. coli*-haematoxylin stain, bar = 50  $\mu$ m. 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.





**Fig. 3.** Airsac of broilers inoculated oculonasally and intratracheally with either PBS (a and b) or IBV M 41 (c and d) at 27 days of age and intratracheally with PBS-diluted glucose broth (a) or *E. coli* (b, c, and d) at 32 days of age. (a) Airsac sampled at 4 days after inoculation with PBS-diluted glucose broth. Immunoperoxidase IBV–haematoxylin stain, bar = 100  $\mu$ m. Columnar respiratory epithelium bearing cilia (Ec) alternated with flat epithelium (Ef). Serosa of one cell thickness (Ser). (b) Airsac sampled at 7 days after inoculation with *E. coli*. Immunoperoxidase IBV–haematoxylin stain, bar = 100  $\mu$ m. 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 h after inoculation with *E. coli*. Immunoperoxidase IBV–haematoxylin stain, bar = 100  $\mu$ m. Columnar epithelium (Ec) is intact, a mononuclear infiltrate (Mon) mostly consisting of macrophages (M $\phi$ ) as shown previously by immunohistochemistry (Matthijs et al., 2008 in press) and a follicle (F) are present. (d) Airsac sampled at 7 days after inoculation with *E. coli*. Immunoperoxidase IBV–haematoxylin stain, bar = 100  $\mu$ m. Oedema (Oed), mononuclear infiltrate (Mon) and several follicles (F) are present in the stroma as well as purulent exudation (Pe) in the airsac lumen.

In most broilers of both IBV groups, mononuclear infiltrate and several follicles were present in the stroma at 0.5 hpi (Fig. 3c) and at 3 hpi up to 7 dpi. No noticeable difference existed between the number of follicles observed at 1 and at 7 dpi.

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 3. No difference in oedema and

**Table 3**

The extent of oedema and purulent exudation in the thoracic airsac of experimental broilers. Birds were inoculated with IBV vaccine H120 (H120 group) or virulent IBV strain M41 (M41 group) 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.

Group	Oedema						Purulent exudation					
	Time after <i>E. coli</i> inoculation						Time after <i>E. coli</i> inoculation					
	0.5 h	3 h	1 day	2 days	4 days	7 days	0.5 h	3 h	1 day	2 days	4 days	7 days
PBS <sup>a</sup>	–	–	–	–	–	–	–	–	–	–	+/-	–
<i>E. coli</i> <sup>b</sup>	+/-	+/-	+/-	+	+	–	–	–	+/-	+/-	+/-	–
H120	++	++	+	+	++	+/-	+	+	++	++	++	++
M41	++	++	++	++	++	+/-	+	+	++	++	++	++

Oedema and pus present per field section: – = no oedema or pus, +/- = local oedema and pus, + = generalised oedema and pus, ++ = massive oedema and pus.

<sup>a</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with PBS broth.

<sup>b</sup> Broilers inoculated at 27 days of age with distilled water and at 32 days of age with *E. coli* broth.

purulent exudation were found between the H120 and M41 groups (Fig. 3d). In the *E. coli* group only local oedema and pus were found.

The number of broilers with microscopical lesions in trachea, lung and airsacs are presented in Table 1.

### 3.5. Location of pathogens

#### 3.5.1. IBV antigen

IBV antigen was found in respiratory epithelial cells present in the trachea (Fig. 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 location at different time points after *E. coli* inoculation is presented in Fig. 4.

#### 3.5.2. *E. coli* antigen

The tracheas of all broilers inoculated with *E. coli* appeared free of bacteria from 2 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 (Fig. 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 parabronchi 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 (Fig. 2e and g). Few bacteria were found within individual epithelial cells from the lining of the parabronchial central lumen (Fig. 2g). Positively stained material was found mainly as small round agglomerates and free bacteria were not seen in the lung tissue 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 7 dpi; in these lungs this was the only site where bacteria were found (Fig. 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

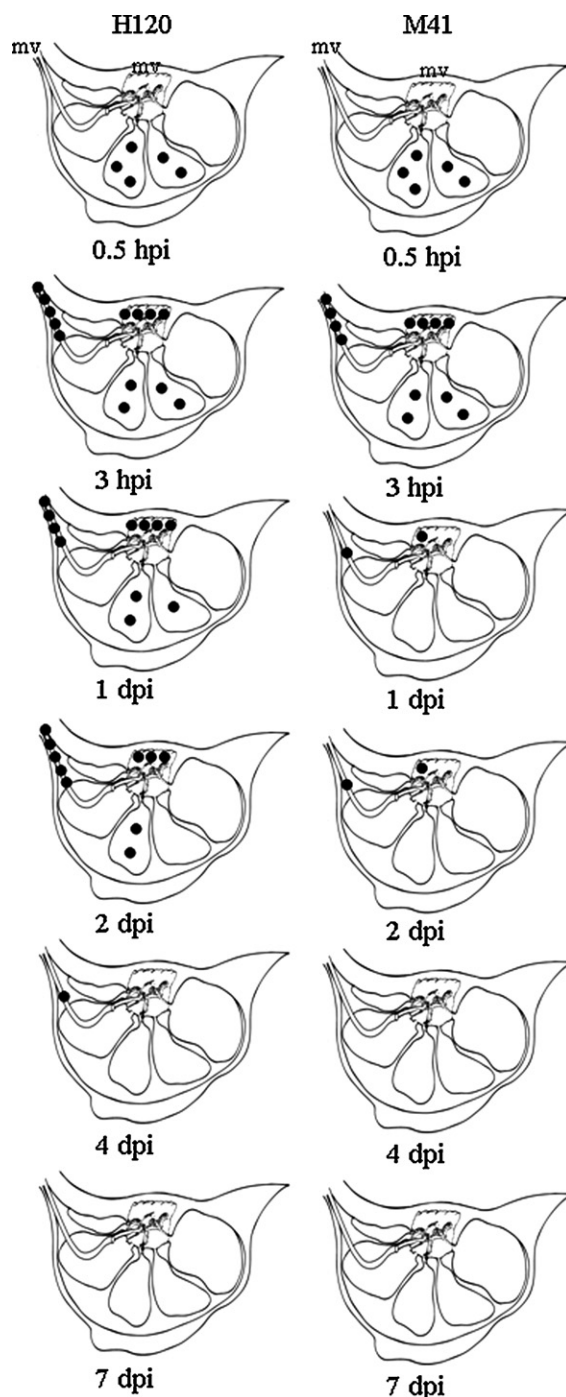


Fig. 4. The location of IBV antigen in the respiratory tract (trachea, lung and thoracic airsacs) of broilers ( $n = 5$  per time point; hours post-*E. coli* inoculation = hpi/days post-*E. coli* inoculation = dpi) at 32 days of age, preceded by IBV vaccine H120 (H120 group) or virulent IBV M41 (M41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

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

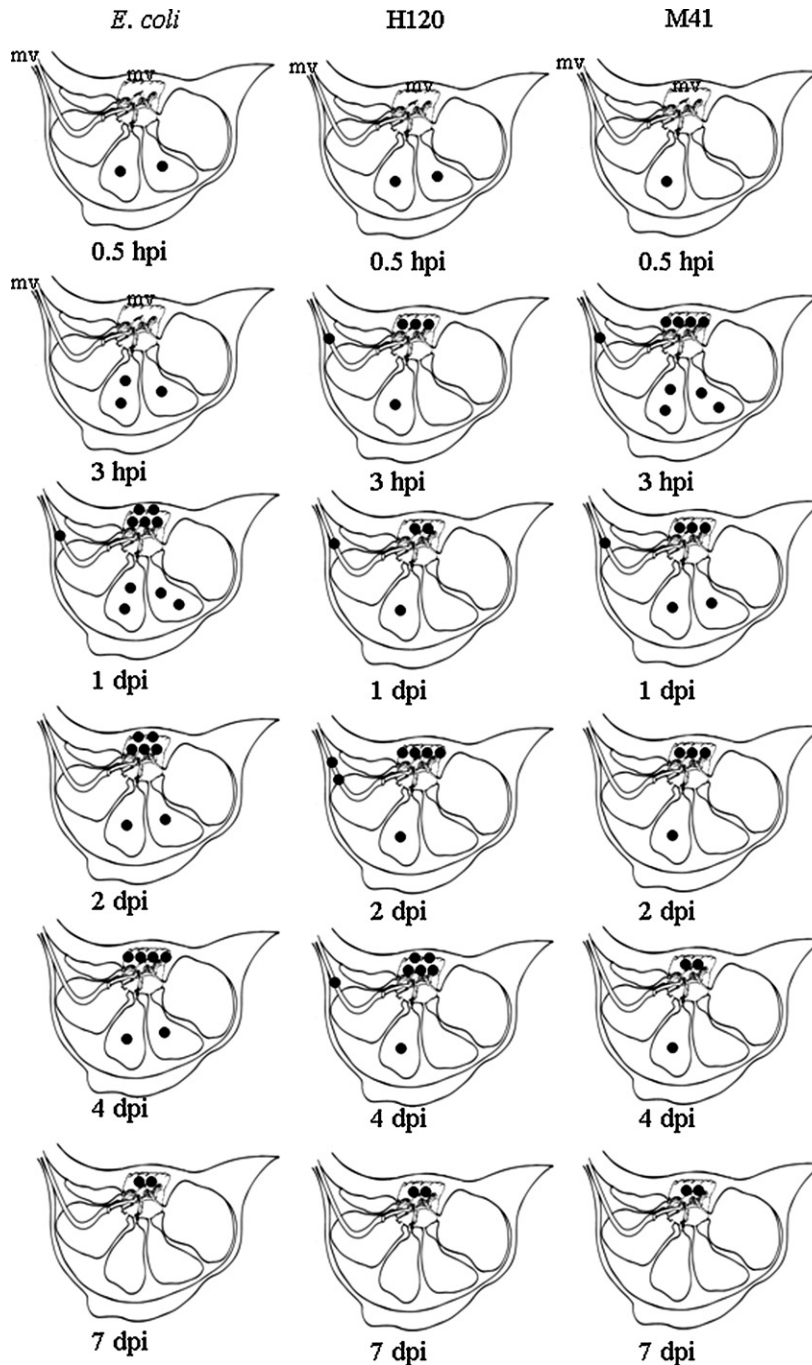


Fig. 5. The location of *E. coli* antigen in the respiratory tract (trachea, lung and thoracic airsacs) of broilers ( $n=5$  per time point; hours post-*E. coli* inoculation = hpi/days post-*E. coli* inoculation = dpi) at 32 days of age, preceded by inoculation with distilled water (*E. coli* group)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

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 location at different time points after *E. coli* inoculation is presented in Fig. 5.

#### 4. Discussion

The aim of our experiments was to study the possible (synergistic) effect of IBV on the course of a subsequently

induced *E. coli* infection. Control groups infected with IBV alone were not included because it was not the aim to study IBV-induced histopathology, which has already been extensively described in literature (Dhinakar and Jones, 1997; Di Matteo et al., 2000; Fulton et al., 1993; Cavanagh and Naqi, 2003; Geilhausen et al., 1973; Janse et al., 1994; Kotani et al., 2000; Nakamura et al., 1992; Ratanasethakul and Cumming, 1983; Riddell, 1987), but to assess the increase in macro- and microscopical lesions of the respiratory tract in broilers infected with IBV and infected with *E. coli* 5 days later in comparison with broilers infected with *E. coli* alone. In mixed infection models it is very difficult, if not impossible, to quantify and qualify exactly which lesions can be ascribed to either pathogen due to the possible occurrence of synergism. In other words: it is very likely not a matter of 'simple addition or subtraction' of lesions.

Based on what has been mentioned previously, it can be concluded that control groups infected with IBV alone would not have given surplus value to the presented research.

The time interval between IBV and *E. coli* infection was 5 days based on earlier research described by Goren (1978) and Peighambari et al. (2000), who found the greatest effect of IBV on the course of a subsequent infection with *E. coli* at a time interval of 4 and 6 days, respectively.

Both approaches, i.e. *E. coli* as a single agent or as superinfection, 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 determination of the number of CFUs 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 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 fibriscences (Huchzermeyer and Cooper, 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., 2000; 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 was disinflation 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 and Lopez, 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 remained 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), 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 5–7 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. (Matthijs et al., 2008), 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 considered the *E. coli*-complicated respiratory infection the result of an overspecialized cellular response.

Based on the presented data we conclude that 4-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. Our findings may contribute to the elucidation of the mechanism behind the pattern of airsacculitis observed in commercial poultry.

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