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C. Neubauer, M. De Souza-Pilz, Anders Miki Bojesen, Magne Bisgaard ...+1 more authors

Institutions: University of Veterinary Medicine Vienna, University of Copenhagen

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Tissue distribution of haemolytic Gallibacterium isolates in laying birds showing clinical signs of egg peritonitis

Claudia Neubauer, Magdalena de Souza-Pilz, Miki Bojesen, Magne Bisgaard, Michael Hess

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Tissue distribution of haemolytic *Gallibacterium anatis* isolates in laying birds with reproductive disorders

Neubauer, C. *[1], De Souza-Pilz, M. [1], Bojesen, A. M. [2], Bisgaard, M. [2] and Hess, M. [1]

[1]Department for Farm Animals and Veterinary Public Health, Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria, and [2]Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, DK, 1870 Frederiksberg C, Denmark

Short title: *Gallibacterium* in laying birds with egg peritonitis

*To whom correspondence should be addressed. Tel: +43 1 250775158. Fax: +43 1 250775192. E-mail: claudia.neubauer@vu-wien.ac.at

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Abstract

Gallibacterium anatis biovar *haemolytica* has been suggested to have a causal role in peritonitis and salpingitis in chickens. Therefore, the aim of this study was to investigate the occurrence of *G. anatis* biovar *haemolytica* in chickens with reproductive disorders. One hundred and 41 birds from 31 layer flocks were submitted for necropsy and the following organs were examined for bacteria: choana, trachea, lung, heart, liver, spleen, ovary, oviduct, duodenum and cloaca. Examination for *Escherichia coli* was included as it can induce reproductive disorders. *G. anatis* was isolated in pure culture from the reproductive tract of affected birds in six of the 31 flocks while *E. coli* was obtained in pure culture from 10 of them. Both *G. anatis* and *E. coli* were isolated from the reproductive tract of 14 of the 31 flocks. The genetic diversity of the *Gallibacterium* isolates was assessed by amplified fragment length polymorphism on a subset of 83 isolates. Generally, each flock was infected with a single clone, which could be isolated from various sites in the birds. However, in two flocks, the majority of birds yielded positive samples from the internal organs, indicating that these particular clones may be more invasive. The findings support previous suggestions that *G. anatis* biovar *haemolytica* is associated with infection of the reproductive tract of chickens making it a likely cause of lowered productivity and an animal welfare concern.

Introduction

The genus *Gallibacterium* was recently established within the family of *Pasteurellaceae* Pohl 1981 (Christensen *et al*., 2003). The genus contains avian bacteria formerly known as *Pasteurella haemolytica*, *Actinobacillus salpingitidis* or *Pasteurella anatis* and currently includes the species *Gallibacterium anatis* and *Gallibacterium* genomospecies 1 and 2. Two biovars are described within *G. anatis*, a haemolytic biovar *haemolytica* and a non-haemolytic, biovar *anatis*. *Gallibacterium* spp. can be isolated from a great variety of birds, such as chickens, turkeys, ducks, geese, psittacine birds, partridges and guinea fowl (Harbourne, 1962; Hacking & Pettit, 1974; Mushin *et al*., 1979; Addo & Mohan, 1984; Bisgaard, 1993).

Members of the genus *Gallibacterium* have been isolated from birds with various clinical conditions. In fact, *Gallibacterium* spp. can be isolated from healthy birds and it has been suggested that these bacteria may be part of the normal microbiota in the upper respiratory and the lower genital tracts (Bisgaard, 1977; Mushin *et al*., 1979). Contrary to this, *Gallibacterium* spp. have been obtained from layers with lesions affecting the reproductive organs including salpingitis and severe drops in egg production (Kohlert, 1968; Matthes *et al*., 1969; Hacking & Pettit, 1974; Gerlach, 1977; Bisgaard & Dam, 1981; Mirle *et al*., 1991). Furthermore, a mortality of 73% was noted in experimentally immunosuppressed 15-week-old brown layers after intravenous infection, emphasizing the importance of *Gallibacterium* spp. as a pathogen (Bojesen *et al*., 2004). In a field study performed in Denmark Bojesen *et al*. (2003b) demonstrated that the biosecurity level influenced the prevalence of *Gallibacterium* spp. with a lower level of biosecurity resulting in a more frequent detection of these pathogens.

In spite of all the experimental and epidemiological data reported so far, no data have been published on the presence of *G. anatis* in different organs of laying birds showing reproductive disorders. The present study was designed to investigate the prevalence of haemolytic *G. anatis* in individual birds kept in alternative husbandry systems and suffering from reproductive disorders.

Material and Methods

Flocks investigated. In total 31 layer flocks were investigated: 14 organic free range flocks (54 birds), six conventional free range flocks (32 birds) and 11 conventional deep litter flocks (55 birds). Data on the size and the age of these flocks are shown in Table 1. The flocks were selected on the following criteria: a) an increase of mortality reported by the farmer (compared with the normal baseline mortality of the flock), b) a drop in egg production (up to 10% within one week), c) apathy of birds, d) pasting around the vent and e) pathological signs of salpingitis/peritonitis noted during necropsy performed on site. During the site visit the clinical status of the flocks was recorded.

All flocks were vaccinated according to a standard vaccination schedule including vaccinations against coccidia, *Salmonella* Enteritidis, avian metapneumovirus, infectious bronchitis virus, infectious bursal disease virus and Newcastle disease virus. In addition some flocks were vaccinated against *E. coli* (eight with a commercially available vaccine, Nobilis® E. coli inac., and two with an autogenous vaccine), *Pasteurella multocida* (one flock with an autogenous vaccine) and *Mycoplasma gallisepticum* (one flock with a commercially available vaccine, Poulvac® MG).

Prior to any therapeutic treatment three to eight birds per flock were brought to the clinic and killed for necropsy followed by a very detailed sampling regime. The only exception was a group of birds from a single conventional deep litter flock which were treated with neomycin directly before sampling.

Necropsy findings. The necropsy findings of the birds were recorded according to the following protocol: trachea: haemorrhages; lungs: airsacculitis and oedema/hyperaemia; heart: haemorrhages, hydropericardium and pericarditis; liver: hepatomegaly, haemorrhages, necrosis and perihepatitis; ovary: haemorrhages, atrophy, deformed follicles, broken follicles, regression; oviduct: haemorrhages, non-functional; kidney: renomegaly and haemorrhages; spleen: splenomegaly; abdominal cavity: peritonitis and ascites.

Bacteriological investigations. Bacteriological investigations were carried out from the following ten sites: choana, trachea, lung, heart, liver, spleen, ovary, oviduct, duodenum and cloaca. Each sample was plated out directly on a blood agar plate (Columbia agar with 5% sheep blood; BioMeriéux, Vienna, Austria) and a McConkey agar plate (BioMeriéux, Vienna, Austria) for isolation of *Gallibacterium* spp. and *E. coli*, respectively. The plates were incubated aerobically at 37°C for 24 h.

Haemolytic *Gallibacterium* isolates were identified by their growth on blood agar within 24 h which is characterized as follows: circular, raised colonies with an entire margin, shiny and semi-transparent with beta-haemolytic zone. Such colonies were regarded as suspicious of *Gallibacterium*. Suspect colonies were subcultured on blood agar to obtain pure cultures. Gram-staining and cytochrome oxidase tests were performed for all isolates.

Up to five *E. coli* colonies per plate were tested for avian pathogenic serovars O1:K1, O2:K2 and O78:K80 using slide agglutination (Veterinary Laboratories Agency, Surrey, UK). Briefly, one drop of the agglutination sera was mixed with one single *E. coli* colony on an object slide and swirled for 2 min. The absence/appearance of agglutination with each serovar test sera was recorded.

Extraction of DNA and PCR. The QIAGEN DNEasy Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction following the manufacturer's protocol. All isolates suggestive of *Gallibacterium* spp. were investigated according to Bojesen *et al*. (2007). Using this PCR, two fragments of approximately 790 bp and 1080 bp were expected for strains of the genus *Gallibacterium*. All PCR products were separated by electrophoresis on 2% agarose gels following a standard procedure (Sambrook & Russel, 2001).

Amplified Fragment Length Polymorphisms (AFLP). The genetic diversity of a subset of 83 isolates from 18 flocks was evaluated by AFLP as reported previously (Christensen *et al.,* 2003). From each flock at least one isolate from the respiratory tract and one from liver, heart, spleen, ovary, oviduct or intestinal tract was chosen. Briefly, the non-selective *Bgl*II primer (FAM- 5´GAGTACACTGTCGATCT 3´) and the non-selective *Bsp*DI primer (5´ GTGTACTCTAGTCCGAT 3´) were used to amplify the fragments subsequent to restriction digestion and ligation to their corresponding adaptors. All AFLP reactions were repeated at least twice to allow evaluation of the reproducibility of the method.

Amplification products were detected on an ABI 377 automated sequencer (PE Biosystems). Each lane included internal-lane size standards labelled with ROX dye (Applied Biosystems). GeneScan 3.1 fragment analysis software (Applied Biosystems) was used for fragment size determination and pattern analysis. AFLP profiles comprising fragments in the size range 50-500 bp were considered for numerical analysis with the software GelCompar II (Applied Maths, Kortrijk, Belgium). Normalised AFLP fingerprints were compared using the Dice similarity coefficient and clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA).

Results

Flocks investigated. In addition to the criteria applied for selecting flocks the most frequent additional findings were lack of uniformity (17/31), cannibalism (13/31), shaking heads (10/31) and nasal discharge (5/31).

Necropsy findings. Three to eight birds per farm were investigated, totalling 141 birds. In addition to peritonitis (21%) the main pathological findings were lesions in the reproductive tract as summarized in Table 2. A high percentage of birds demonstrated regression of the ovaries (40%) and a non-functional oviduct (31%). Furthermore, 28% of the birds had deformed follicles and in 10 birds, severe egg concretions were found within the oviduct.

Fibrinous perihepatitis and pericarditis were found in only three and two birds, respectively. During necropsy gastrointestinal helminths (*Ascaridia* spp.) were found in the small intestine of birds from three organic free-range flocks. Many birds (36/141) showed signs of fatty liver syndrome. Severe pecking wounds due to cannibalism, mainly localized around the cloaca were observed in 63 birds. No lesions were found in the respiratory tract.

Bacteriological investigations. A total of 310 bacterial isolates suggestive of haemolytic *G. anatis* were obtained. All these demonstrated a wide beta-haemolytic zone (1 to 2 mm) as reported by Bisgaard (1982) and Christensen *et al.* (2003). All were Gram-negative rods that were sometimes pleomorphic, and were cytochrome oxidase-positive.

Data for bacteriological isolation are summarized in Table 3. Most of the isolates (161/310) were obtained from the respiratory tract. From heart, liver, spleen, intestine and reproductive tract 16, 14, 19, 63 and 37 isolates were found, respectively. In addition to the intestinal tract (244/847) *E. coli* was obtained mainly from the respiratory tract (274/844) and the reproductive tract (167/844). From heart, liver and spleen 55, 53, and 54 isolates were recovered, respectively. Almost half of the isolates belonged to avian pathogenic *E. coli* (405/847). In nine flocks *E. coli* strains with the antigenic profile O1:K1 were observed while O2:K1 was found in flock numbers 14 and 24 and O78:K80 was found only once in flock number 10. Furthermore, mixed infections with *E. coli* O1:K1 and O2:K1 were noted in two flocks and mixed infection of O1:K1 and O78:K80 and one further flock.

In reproductive tract co-infections of haemolytic *G. anatis* and *E. coli* were found in 18 birds from 14 flocks. Haemolytic *G. anatis* isolates were obtained from eight birds of six flocks and infection of the reproductive tract with only *E. coli* was seen in 39 birds from 10 flocks. Both *E. coli* and haemolytic *G. anatis* were isolated from birds expressing lesions in either the ovaries or the oviduct. However, these microorganisms were also found in birds without lesions in the reproductive tract.

Identification of *Gallibacterium* by PCR. All 310 isolates that were tentatively identified as *Gallibacterium* were tested with a *Gallibacterium* specific PCR, and from all isolates amplicons of approximately 790 bp and 1080 bp were obtained as exemplified in Figure 1.

AFLP. Eighty-three isolates from 18 flocks were investigated. Using a cut-off level of 90% to define a genotype (clone), 34 individual clones were identified. Generally, a high genetic similarity was demonstrated both between and within flocks. In 11 flocks, only one clone was present, this includes the three flocks (6, 7, 8), where only one isolate was obtained. In four flocks two clones were demonstrated. One flock (flock number 4) had three and two flocks (flock numbers 16 and 19) had six clones, respectively (Figure 2). One clone was present in four different flocks.

Discussion

The present study aimed to investigate the association between the presence of haemolytic *G. anatis* and reproductive disorders in laying birds kept in alternative husbandry systems as birds kept in these systems are more likely to be infected with *G. anatis* (Bojesen *et al*., 2003b).

E. coli is considered to be the most common bacterium associated with reproductive disorders in chickens (Bisgaard & Dam, 1981; Jones & Owen, 1981; Jordan *et al*., 2005). However, other reports suggest that *Gallibacterium* spp. may cause these disorders and their importance is likely to be underestimated due to overgrowth by other bacterial species and the difficulties of identification (Mirle *et al*., 1991; Bojesen *et al*., 2003a).

Based on specific signs in the flocks, birds were selected for further investigations in accordance with Vanderkerchove *et al*. (2004). Drop in egg production, apathy of birds, pasting around the vent and increased mortality were all signs used by the farmer to initiate

further investigations as reported in the present study. The results of the *post mortem* investigations performed on the farms and the notification of salpingitis/peritonitis were additional criteria to include the farm in the study. In total, 141 birds were killed and the samples were taken from fresh carcasses. Except the five birds from flock no. 15, all birds (136/141) were investigated prior to any antibiotic treatment to increase the recovery of bacteria from the different organs.

In the present study most of the haemolytic *G. anatis* isolates were obtained from the respiratory tract, especially from the choana and trachea. This is not surprising considering previous reports by Bisgaard (1977), Mushin *et al*. (1979) and Bojesen *et al*. (2003b) indicating that *Gallibacterium* spp. constitute part of the normal tracheal microbiota in chickens and that under certain conditions they might act as opportunists and cause respiratory disease. In the case of *E. coli*, the natural route of infection is generally thought to be via the respiratory tract (Gross, 1961; Carlson & Whenham, 1968) and a similar route might exist for *Gallibacterium*. The present investigation supports this assumption for both bacteria, as 52% of the *G. anatis* and 32% of the *E. coli* isolates were recovered either from the choana, trachea or the lungs of the birds. Penetration of *Gallibacterium* organisms from the mucosa of the respiratory tract into the systemic circulation may be due to the influence of an impaired host immunity, co-infections and environmental factors (Gerlach, 1977; Matthes & Löliger, 1976; Nagaraja *et al*., 1984; Shaw *et al*., 1990; Leitner & Heller, 1992).

From the case history of the flocks it was known that cannibalism occurred in nearly half of the flocks (13/31) and that numerous birds showed extensive pecking wounds around the cloaca. This is in contrast to the findings reported by Jones & Owen (1981) and Jordan *et al*. (2005) who observed only a few cases of vent cannibalism in birds suffering from salpingitis/peritonitis. However, Cumming (2001) attributed considerable importance to such injuries from which the bacteria may spread into the blood stream resulting in bacteraemia with bacteria to be isolated from the heart, liver, spleen and the reproductive tract. In the present investigation ascending infections may have contributed to the finding of *G. anatis* and *E. coli* in the reproductive tract, in addition to the heart, liver and spleen. Bojesen *et al*. (2004) used fluorescence *in situ* hybridisation to demonstrate bacteria in the spleen and liver following intraperitoneal infection of chickens with *Gallibacterium*, indicating that ascending infections might be of relevance.

The gross pathological lesions of reproductive disorders due to *Gallibacterium* infection cannot be distinguished from infections with *E.* coli and for this reason all samples were also investigated for *E. coli*. With an increase of birds/flock showing pathological signs

in the ovaries or the oviduct *E. coli* was isolated more frequently. This might have been because *G. anatis* was overgrown in such cases as already mentioned above. *E. coli* are present in the normal microbiota of the intestinal tract in poultry, but certain strains of avian pathogenic *E. coli* (APEC) possess specific virulence factors and are able to cause severe infections. The APEC serogroups O1, O2 and O78 are most frequently recovered from infected chickens (Sojka & Carnaghan, 1961; Glantz *et al*., 1962; Cloud *et al*., 1985; Dozois *et al*., 1992). In the present investigation *E. coli* was isolated from birds of each flock and APECs (O1:K1, O2:K1 and O78:K80) were found in more than 50% of the flocks. The bacteria were isolated from the respiratory tract (274/844) and the reproductive tract (166/844). Bisgaard & Dam (1981) investigated 150 salpinx samples from layer carcasses condemned at slaughterhouses due to salpingitis and found that *E. coli* was isolated most frequently. Their findings were confirmed by Jones & Owen (1981) investigating 292 birds in which salpingitis/peritonitis was diagnosed over a period of two years. Furthermore, these authors also found mixed infections of *E. coli* and haemolytic *Gallibacterium* spp., as shown in nearly half of the cases in the present study.

According to the existing literature detection of members of *Pasteurellaceae* by traditional methods often results in difficulties at isolation, cultivation and identification. The weak and unreliable reactions observed for some of the phenotypic identification tests add to these uncertainties (Christensen *et al*., 2003). Diagnostic PCR assays were therefore developed to complement these diagnostic methods (Bojesen *et al*., 2007) and were found very useful in confirming the classification of the isolates. The genetic diversity assessed by AFLP analysis of 83 isolates confirmed previous indications of high genetic relatedness among isolates from the same flock (Bojesen et al., 2003a). Furthermore, the fact that the same clone could be isolated from the mucosal lining of the upper respiratory tract and from lesions in the reproductive tract, heart, liver and spleen, suggests that, given the right opportunity, *Gallibacterium* isolates residing in the upper respiratory or lower genital tract may gain access to the systemic circulation and/or the upper reproductive tract and cause disease,.

In conclusion, we have shown that haemolytic *G. anatis* isolates are prevalent in layers with reproductive disorders. The results from the current study provide strong support for the theory that *Gallibacterium* plays a role in reproductive disorders. Furthermore, we have demonstrated that *Gallibacterium* isolates from different locations i.e. heart, spleen, upper respiratory and lower genital tract were highly similar, indicating that isolates residing in their

natural habitat may cause reproductive disorders and/or systemic disease under certain conditions.

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Figure legends

Figure 1. *Agarose gel electrophoresis of amplification products from haemolytic* Gallibacterium anatis *field isolates. Lane M, 100 bp DNA ladder (Invitrogen); lanes 1 and 10, isolates from cloaca; lanes 2, 6, 11 and 12, isolates from choana; lanes 3 and 8, isolates from intestine; lanes 4 and 7, isolates from trachea; lane 5, isolate from ovary; lanes 9 and 13, isolates from lungs; lanes 14 and 16, negative controls; lane 15, positive control.*

Figure 2. *Dendrogram (unweighted pair group method using arithmetic averages) of AFLP similarities of 83* Gallibacterium *isolates. S_D, band-based Dice similarity coefficient (%). Flocks, the flock number from which the isolates originate. Isolates grouping at a 90% similarity level or higher were pooled as indicated by grey triangles. The dotted line indicates the clonal cut-off level at 90% similarity.*

Table 1. *Size and age of the flocks investigated*

Size	<1000	1000-2000	2000-3000	>3000								
Age (weeks)	<30	30-40	>40	<30	30-40	>40	<30	30-40	>40	<30	30-40	>40
Organic/free-range	1		1	1	1	3	1	1	3	1	1	
Conventional/free-range		2				2		1			1	
Conventional/deep litter			1			1	1	1	1	1	4	1

Table 2. *Main necropsy findings*

Lesion	Organic/free-range (54 birds/14 flocks)	Conventional/free-range (32 birds/6 flocks)	Conventional/deep litter (55 birds/11 flocks)	N=141
Haemorrhages in ovary	10	13	3	26 (18%)
Atrophy of ovary	25	12	21	58 (40%)
Deformed follicles	16	13	10	39 (28%)
Broken follicles	10	8	4	22 (16%)
Haemorrhages in oviduct	11	5	1	17 (12%)
Non-functional oviduct	17	8	19	44 (31%)
Peritonitis	15	9	6	30 (21%)

Table 3. *Isolation of haemolytic* G. anatis *and* E. coli *from different organs displayed in connection with the housing system and the number of birds expressing lesions in either the ovaries and/or the oviduct*

Flock no.	Housing system[a]	N[b]	Sero-typing	Heart		Liver		Spleen		Choana		Trachea		Lung		Duodenum		Cloaca		Ovary		Oviduct	
				G. a.[c]	E. c.[d]	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.	G. a.	E. c.
1	cfr	5/8	O1:K1	0	0	1	0	0	0	3	3	8	6	3	2	3	5	1	7	1	0	0	0
2	ofr	2/3	O1:K1	0	0	0	0	0	0	0	1	3	2	0	1	0	3	0	3	0	0	0	0
3	cfr	2/4	O1:K1	1	0	0	0	0	0	1	3	4	0	2	0	1	2	0	4	3	0	3	0
4	ofr	1/3	O2:K1	1	0	2	0	1	0	2	3	3	1	0	0	2	2	2	3	1	2	1	2
5	ofr	1/3	*E. coli*	0	0	0	0	0	0	1	0	1	1	0	0	0	3	1	2	1	1	2	1
6	ofr	3/3	O1:K1	0	0	0	0	0	2	0	2	0	3	1	3	0	3	0	3	0	3	0	3
7	ofr	1/3	O1:K1	0	3	0	1	0	0	1	1	0	3	0	3	0	3	0	3	0	3	0	3
8	ofr	0/3	O1:K1	0	0	0	0	0	0	1	3	0	0	0	3	0	3	0	3	0	3	0	0
9	ofr	3/3	*E. coli*	0	0	0	0	2	2	1	2	1	0	0	3	1	3	2	3	0	3	0	2
10	ofr	3/3	O78:K80	0	0	0	0	0	0	2	1	1	1	0	3	0	3	0	3	0	3	0	3
11	cdl	2/5	*E. coli*	0	0	0	0	0	0	4	5	2	0	2	0	2	5	2	5	1	0	0	0
12	cfr	5/5	*E. coli*	0	0	0	0	0	0	1	5	1	0	0	0	0	4	1	5	0	3	1	3
13	cfr	1/5	O1:K1+ O2:K1	0	0	0	0	0	0	2	4	2	4	1	0	2	5	1	5	1	0	0	0
14	cdl	0/5	O1:K1+ O2:K1	0	0	0	0	0	0	4	1	2	3	3	0	0	2	3	4	1	1	2	1
15	cdl	4/5	O1:K1	0	1	0	1	0	1	1	5	0	2	1	4	0	2	0	5	0	5	0	0
16	cfr	2/5	*E. coli*	0	0	0	0	0	0	5	5	4	2	2	3	1	5	4	5	1	3	0	2
17	ofr	1/5	O1:K1	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	2	0	0	1	0
18	cdl	4/5	*E. coli*	0	2	0	4	0	3	0	5	0	5	0	5	0	5	0	5	0	5	0	5
19	cdl	1/5	*E. coli*	3	4	1	3	1	4	0	5	1	5	0	4	1	5	2	5	0	5	1	4
20	ofr	3/5	*E. coli*	0	5	2	5	3	4	1	5	1	5	4	5	0	5	0	5	0	5	2	4
21	ofr	4/5	*E. coli*	0	5	1	5	1	5	0	5	2	5	2	5	1	5	1	5	0	5	2	5
22	ofr	5/5	O1:K1 + O78:K80	0	2	0	2	1	5	4	5	5	5	1	5	1	5	2	5	2	5	1	5
23	ofr	5/5	*E. coli*	0	5	0	5	0	5	0	5	2	5	0	5	0	5	0	5	0	5	0	5
24	cfr	5/5	O2:K1	0	5	0	5	0	5	2	5	3	5	1	5	0	5	1	5	0	5	1	5
25	cdl	4/5	*E. coli*	0	5	1	5	0	3	1	4	1	5	0	4	0	5	1	5	0	4	0	4
26	cdl	1/5	O1:K1	0	5	0	5	0	5	4	5	4	5	1	5	1	5	5	5	0	5	0	5
27	cdl	5/5	*E. coli*	1	5	0	5	1	5	5	5	1	5	0	5	0	5	1	5	1	5	0	5
28	ofr	0/5	*E. coli*	2	5	1	5	0	4	3	5	2	5	2	5	0	5	1	5	1	5	1	5
29	cdl	0/5	*E. coli*	3	2	3	2	4	0	4	4	2	3	0	5	2	5	3	5	1	4	0	5
30	cdl	0/5	*E. coli*	2	0	1	0	1	0	5	0	4	0	3	0	2	0	5	3	1	0	1	0
31	cdl	1/5	*E. coli*	3	1	1	0	4	1	3	1	4	1	4	1	2	1	2	2	0	1	2	1
Total number of isolates				16	55	14	53	19	54	63	103	64	87	34	84	22	114	41	130	16	89	21	78

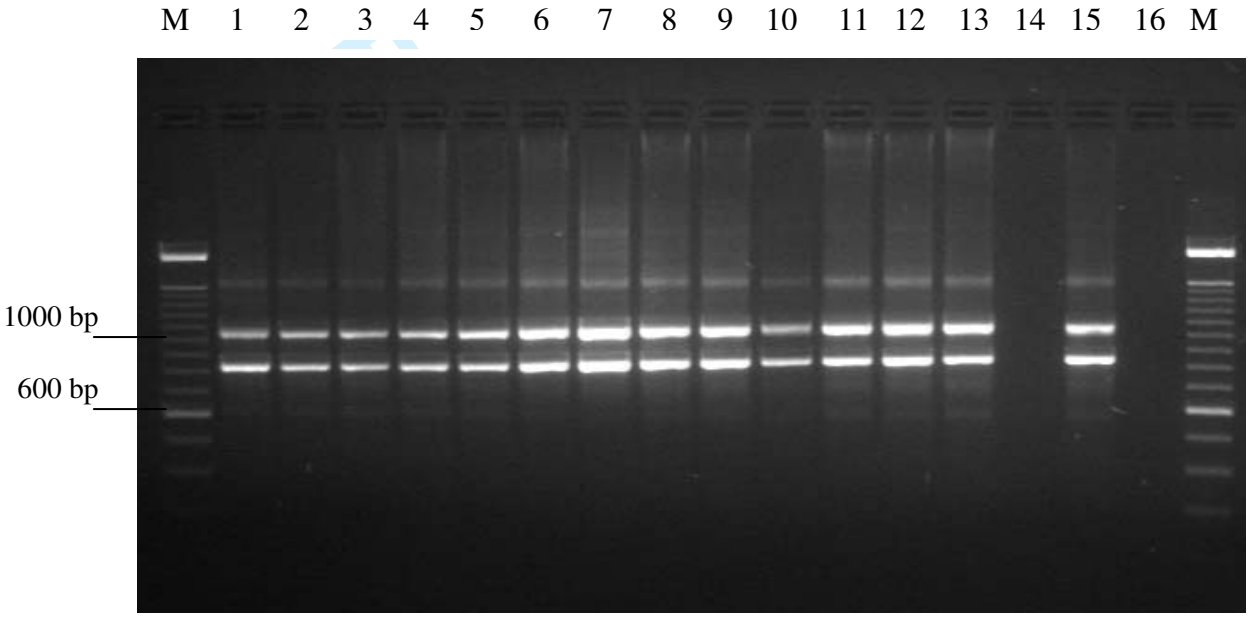
[a]Housing system according to Table 1; cfr: conventional free range; ofr: organic free range; cdl: conventional deep litter, [b]Number of birds with lesions in the ovaries and/or the oviduct according to Table 2/number of birds investigated, [c]Number of birds from which haemolytic *G. anatis* was isolated, [d]Number of birds from which *E. coli* was isolated.

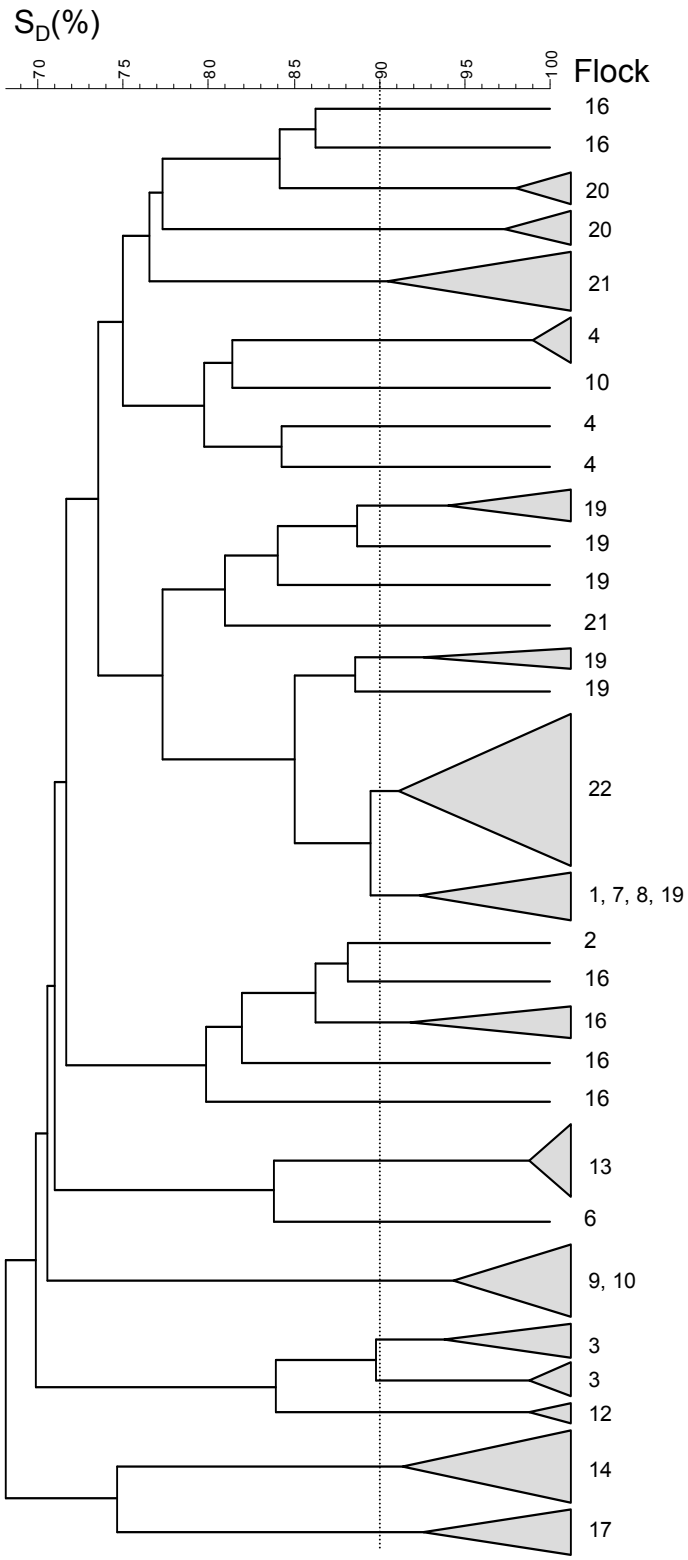
Table 4: Escherichia coli *obtained from different organs*

No.	*E. coli* Typing	Heart	Liver	Spleen	Choana	Trachea	Lung	Duod.	Cloaca	Ovary	Oviduct
1	O1:K1	0[a)]/8[b)]	0/8	0/8	3/8	6/8	2/8	5/8	7/8	0/8	0/8
2	O1:K1	0/3	0/3	0/3	1/3	2/3	1/3	3/3	3/3	0/3	0/3
3	O1:K1	0/4	0/4	0/4	3/4	0/4	0/4	2/4	4/4	0/4	0/4
4	O2:K1	0/3	0/3	0/3	3/3	1/3	0/3	2/3	3/3	2/3	2/3
5	*E. coli*	0/3	0/3	0/3	0/3	1/3	0/3	3/3	2/3	1/3	1/3
6	O1:K1	0/3	0/3	2/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3
7	O1:K1	3/3	1/3	0/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3
8	O1:K1	0/3	0/3	0/3	3/3	0/3	3/3	3/3	3/3	3/3	0/3
9	*E. coli*	0/3	0/3	2/3	2/3	0/3	3/3	3/3	3/3	3/3	2/3
10	O78:K80	0/3	0/3	0/3	1/3	1/3	3/3	3/3	3/3	3/3	3/3
11	*E. coli*	0/5	0/5	0/5	5/5	0/5	0/5	5/5	5/5	0/5	0/5
12	*E. coli*	0/5	0/5	0/5	5/5	0/5	0/5	4/5	5/5	3/5	3/5
13	O1:K1 O2:K1	0/5	0/5	0/5	4/5	4/5	0/5	5/5	5/5	0/5	0/5
14	O1:K1+ O2:K1	0/5	0/5	0/5	1/5	3/5	0/5	2/5	4/5	1/5	1/5
15	O1:K1	1/5	1/5	1/5	5/5	2/5	4/5	2/5	5/5	5/5	0/5
16	*E. coli*	0/5	0/5	0/5	5/5	2/5	3/5	5/5	5/5	3/5	2/5
17	O1:K1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5
18	*E. coli*	2/5	4/5	3/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
19	*E. coli*	4/5	3/5	4/5	5/5	5/5	4/5	5/5	5/5	5/5	4/5
20	*E. coli*	5/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5
21	*E. coli*	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
22	O1:K1 + O78:K80	2/5	2/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
23	*E. coli*	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
24	O2:K1	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
25	*E. coli*	5/5	5/5	3/5	4/5	5/5	4/5	5/5	5/5	4/5	4/5
26	O1:K1	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
27	*E. coli*	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
28	*E. coli*	5/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
29	*E. coli*	2/5	2/5	0/5	4/5	3/5	5/5	5/5	5/5	4/5	5/5
30	*E. coli*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	0/5	0/5
31	*E. coli*	1/5	0/5	1/5	1/5	1/5	1/5	1/5	2/5	1/5	1/5
Total		55	53	54	103	87	84	114	130	89	78

[a)] Number of birds from which *E. coli* was isolated
[b)] Number of birds investigated

Figure 1: Agarose gel electrophoresis of amplification products from haemolytic *Gallibacterium anatis* field isolates
Lane M: 100 bp DNA ladder (Invitrogen); lanes 1,10: isolates from cloaca; lanes 2,6,11,12: isolates from choana; lanes 3,8: isolates from intestine; lanes 4,7: isolates from trachea; lane 5: isolate from ovary; lanes 9,13: isolates from lungs; lanes 14,16: negative control; lanes 15: positive control




S_D(%)
70
75
80
85
90
95
100
Flock
16
16
20
20
21
4
10
4
4
19
19
19
21
19
19
22
1, 7, 8, 19
2
16
16
16
16
13
6
9, 10
3
3
12
14
17