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Multi Locus Sequence Typing (MLST) and plasmid profile characterization of avian pathogenic *Escherichia coli* associated with increased mortality in free-range layer flocks

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Abstract

Avian pathogenic *Escherichia coli* (APEC) strains originating from ten free-range layer flocks were characterized by Multi Locus Sequence Typing (MLST) and plasmid profile analysis to investigate their phylogenetic relationship and diversity, respectively. In addition to colibacillosis, all flocks tested positive for antibodies against avian Metapneumovirus (aMPV) during production, and six of the flocks were concurrently affected by histomonosis. Accumulated average mortality for flocks concurrently affected by colibacillosis and histomonosis made up 17.4%, while the average mortality for *E. coli* infected flocks was 16.5%. A total of eight different sequence types (STs) and 47 different plasmid profiles were demonstrated among the *E. coli* isolates. Within each flock between one to four different STs and three to 13 different plasmid profiles were demonstrated. A statistical significant difference in STs and plasmid profile diversity of the population of *E. coli* was not demonstrated between flocks affected by histomonosis compared to histomonosis-free flocks.

Only minor clonal diversity was demonstrated for each flock, and in all, but one flock, colibacillosis started before antibodies against aMPV were detected.

All isolates, except two, carried plasmids greater than 100 kilobases, but only a single plasmid replicon type, IncFIB, was demonstrated, suggesting plasmids representing this type might represent a common pathogenicity factor for the different STs of *E. coli*. Within each flock a clonal tendency was observed, indicating that only certain clones of *E. coli* possess a significant pathogenic potential. These clones act as primary rather than secondary pathogens, resulting in colibacillosis without predisposing factors, including histomonosis and aMPV.

Introduction

Avian pathogenic *Escherichia coli* (APEC) associated with colibacillosis represents a major welfare problem in addition to imposing significant economic losses on the poultry industry worldwide (Barnes *et al.*, 2008). Most studies on the population of *E. coli* associated with colibacillosis have concerned broilers (Peighambari *et al.*, 2002; Ask *et al.*, 2006a; Ask *et al.*, 2006b; Gregersen *et al.*, 2010b), but an increased importance of colibacillosis in layers has been observed since the mid-1990s (Vandekerchove *et al.*, 2004a).

Traditionally, *E. coli* has been regarded as an opportunistic pathogen, and colibacillosis regarded a secondary disease following a primary infection (Dwars *et al.*, 2009) or lack of appropriate management (Vandekerchove *et al.*, 2004b). However, an increased significance, and a primary rather than secondary role of APEC in layers, has been reported both from the Netherlands and Denmark (Vandekerchove *et al.*, 2005; Gregersen *et al.*, 2010a). To provide knowledge on the role of *E. coli* as a primary or a secondary pathogen, genetic characterization is inevitable, representing a precondition for proper understanding and subsequently treatment of outbreaks (White *et al.*, 1993; Gregersen *et al.*, 2010a).

A high degree of genome plasticity has been demonstrated for *E. coli* (Liebchen *et al.*, 2008) which represents four phylogenetic groups (Herzer *et al.*, 1990). APEC, however, mainly belongs to group B2 and to a lesser extent group D (Mora *et al.*, 2009). According to the "mix and match" hypothesis (Mokady *et al.*, 2005) a successful clone of *E. coli* is likely to develop and spread horizontally and vertically. For the same reasons clonal outbreaks are expected to be dominated by a single sequence type (ST). Besides chromosomal contents, *E. coli* isolates frequently possess large plasmids and many of these plasmids are likely to contain virulence-related sequences (Johnson *et al.*, 2007; Tivendale *et al.*, 2009; Mellata *et al.*, 2010).

The consumer demand for organic eggs in the EU has increased from almost zero in the early nineties to 12 percent in 2006 (Hegelund *et al.*, 2006). Organic production, however, represents new challenges as to controlling biosecurity and pathogens. During free-range production poultry flocks are exposed to the caecal worm *Heterakis gallinarum*, the intermediate host of *Histomonas meleagridis* (Gibbs, 1962; Lee, 1971; McDougald, 2005). As chickens constitute an optimal host for the caecal worm (Lund *et al.*, 1974) this underlines the risk of outbreak of histomonosis in free-range layer flocks. Combined with the ban on nitroimidazoles for treatment this has paved the way for the re-emergence of major problems with histomonosis (Permin, 2003; McDougald, 2005). For unknown reasons many of these flocks are also affected by colibacillosis (Stokholm *et al.*, 2010). However, the relationship between *E. coli* and *H. meleagridis* in chickens remains presently unclear (Springer *et al.*, 1970). The interaction between *E. coli* and avian Metapneumovirus (aMPV) has been studied in more detail. Results obtained are, however, ambiguous (Majo *et al.*, 1997; Parreira *et al.*, 1998; Al-Ankari *et al.*, 2001; Vandekerehove *et al.*, 2004).

The aims of the present study included investigation of the genomic diversity of APEC associated with increased mortality in free-layers flock by MLST and plasmid profile analysis. In addition, the impact of aMPV and histomonosis on clonal diversity of *E. coli* and mortality rate were investigated.

Material and methods

Flock data. A longitudinal study was carried out on causes of mortality in ten flocks on seven farms with organic table egg production in Denmark. None of the layer farms were geographically

or epidemiologically related, and none of the included flocks were sister flocks. Neither were the rearing farms providing the flocks investigated geographically related. Animals from flocks A₁, A₂, E₁ and E₂ originated from the same rearing farm, but flocks at farm E were four months older than the two flocks at farm A. Animals from B₁ and B₂ also originated from the same rearing farm, but were reared in separate houses. Only one age group was present on each farm and each flock was contained in a separate house with access to separate outdoor pens. Although the flocks at farms A, B and E were housed separately they shared equipment and a common service room for egg collection. All the farms were operated according to an all-in all-out system. Flock sizes varied between 3,000 and 11,993 birds (Table 1). The flocks were observed from the day they were placed at the layer farms as 15 to 17 weeks old pullets until the end of the production at the age of 65 to 79 weeks. Layers dead on their own were randomly selected and submitted for *post mortem* examination during the whole production period, except layers from flock G which were only submitted once for *post mortem*.

Bacteriology. A total of 3044 layers were subjected to *post mortem* examinations, 2465 of which originated from farms affected by histomonosis. Organs demonstrating lesions typical of bacterial infection were decontaminated on the surface by searing before a sterile cotton swab was taken through the sterile surface and rotated within the lesions. Subsequently, material on the swab was plated onto blood agar (Blood Agar Base, CM55, Oxoid, Basingstoke, UK) containing 5% sterile bovine blood and incubated in sealed plastic bags at 37 °C overnight. From each blood agar plate demonstrating abundant growth in pure culture, a single colony was subcultured in Heart Infusion Broth (Difco, Brøndby, Denmark) and stored in 30% glycerol at -80 °C until used for identification and genetic characterization. Standard biochemical and physiological tests for identification were performed (Barrow & Feltham, 1993).

Plasmid characterisation of *E. coli*. A total of 180 randomly selected isolates, representing 180 chickens suffering from colisepticaemia (92 isolates) and chickens co-infected with *H. meleagridis* and *E. coli* (88 isolates) were characterized. Isolates characterized represented all dually infected farms while only two farms affected by *E. coli* alone were included. Isolates characterized covered the entire production period. Isolates were plasmid profiled using the method of Kado *et al.* (1981). Gels were stained in 2µg/ml ethidium bromide (Sigma), illuminated with ultraviolet light at 254nm using an UV transilluminator (UVP; model TS-40) and photographed with a Polaroid Land Camera (Polaroid; MP-4). Plasmid mobilities were calculated relative to the reference plasmids in *Escherichia coli* strains V517 (Kolodner & Macrina, 1987) and 39R861 (Threlfall *et al.*, 1986) and sizes estimated in accordance with the methods of Rochelle *et al.* (1986). Coiled covalently closed (ccc) and open circular (oc) DNA molecules were differentiated according to Hintermann *et al.* (1981). Differences in *E. coli* diversity between isolates obtained from flocks concurrently infected with histomonosis and histomonosis negative flocks as demonstrated by numbers of plasmid profiles were evaluated statistically by the unpaired t-test using GraphPad version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

Multilocus sequence typing (MLST). A total of 102 isolates were characterized by MLST including at least six isolates of *E. coli* from each flock. Isolates were selected according to time of isolation in order to cover the entire period of each outbreak and by plasmid profile. Only a single isolate from each layer was characterized by MLST. The 102 isolates characterized represented a total of 38 different plasmid profiles. MLST was carried out according to Gregersen *et al.* (2010b). Briefly, seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified using 1 µl template DNA, 0.5 µl of each primer (20 pmol), 0.5 µl of the dNTPs (25 mM solution), 5

µl of 10×PCR buffer (Fermentas, Helsingborg, Sweden), 5 units of DreamTaq polymerase (Fermentas, Helsingborg, Sweden) and Millipore quality water to 50 µl. The PCR reaction conditions included a 2 min denaturation at 95°C, 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 58 °C, extension 2 min 72°C, and a final additional 5 min at 72°C.

PCR products were purified using 10 µl PCR product, 1 µl calf intestine alkaline phosphatase and 1 µl Exonuclease I (Fermentas, Helsingborg, Sweden). The mixture was incubated for 15 min at 37 °C and subsequently 15 min at 85°C.

Multilocus sequence analysis. Sequencing was performed at Macrogen Inc., Seoul, Korea, using the PCR primers. Sequences were assigned sequence types based upon the scheme described at the *E. coli* MLST database (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli>), which assigns allele designations to unique variants based on a 500-bp internal region of each gene. A phylogenetic tree was constructed based upon the concatenated sequences of the housekeeping genes by the neighbour-joining method using MEGA 4.0.2 software (Tamura *et al.*, 2007). Difference in *E. coli* diversity between isolates obtained from flocks concurrently infected with histomonosis and histomonosis negative flocks as demonstrated by the number of different STs were evaluated statistically by the unpaired t-test using GraphPad version 5.00 for Windows (GraphPad Software, San Diego, California, USA). The diversity of STs based on the maximum and minimum numbers of different nucleotides of the STs revealed by MLST was calculated using Basic Alignment Search Tool (BLAST) (Altschul *et al.*, 1997).

Plasmid replicon typing. Thirty-four isolates of *E. coli* representing 12 different plasmid profiles were selected for plasmid replicon typing. A minimum of two strains of *E. coli* per flock were included, except no isolates were included from flock F and G. Replicon typing was

carried out according to Carattoli *et al.* (2005), except boiling lysates were used instead of purified DNA.

Serological investigations. aMPV has been demonstrated to increase the risk of subsequent infection with *E. coli* (Al-Ankari *et al.*, 2001). To investigate the prevalence of aMPV, serum samples were randomly collected from 20 chickens from flocks A₂, B₁, C, D and E₁ at the age of 16 and 68 weeks, in addition to at peak of lay. Serum samples were tested for antibodies against aMPV using a commercial enzyme-linked immunosorbent assay (ELISA Kit (IDEXX), Schiphol-Rijk, The Netherlands) as recommended by IDEXX. All tests were performed on sera kept frozen until used.

Results

Post mortem lesions. A total of 290 layers out of 3044 submitted for *post mortem* (Table 1) were affected by histomonosis or showed coinfections by *H. meleagridis* and *E. coli*. Typical lesions of histomonosis in the liver and the caeca varied among flocks affected by histomonosis (Table 2). These lesions dominated in flock A₂ (100%) while only 18% demonstrated these lesions in flock D. On average, 46% showed typical lesions in both the liver and the caeca. On average, 51% of the layers suffering from histomonosis only demonstrated typical lesions within the caeca. These lesions dominated in flock B₂ (70%), but were absent in both flocks at farm A. Only 2% of the layers had typical lesions in the liver without lesions in the caeca. These lesions were only observed in flocks A₁ and B₁ (Table 2).

Gross lesions associated with histomonosis were in accordance with those previously observed in turkeys (McDougald, 2005). Lesions associated with colisepticaemia were also in

accordance with previous reports (Barnes *et al.*, 2008). Layers suffering from both colisepticemia and histomonosis showed general vascular disturbances, including congestion, discoloration of subperitoneal fat tissue and splenomegaly in addition to lesions typical of histomonosis in the caeca. Furthermore, a fibrinopurulent or purulent peritonitis with or without a salpingitis was found in 74.2% of these layers.

Bacteriology. Pure cultures of *E. coli* were obtained from 786 chickens out of 2465 birds received for *post mortem* examination from histomonosis affected flocks. One hundred and ninety of these birds also showed lesions typical of histomonosis. Out of 579 birds received for *post mortem* from four flocks only affected by colibacillosis pure cultures of *E. coli* were obtained from 272 layers showing lesions typical of *E. coli* septicemia. Isolates of *E. coli* included the whole outbreak period for all flocks.

Plasmid analysis. A total of 47 different plasmid profiles of *E. coli* were demonstrated among the 180 isolates investigated (Table 3). In flocks B₁, B₂, C and D dually infected with *H. meleagridis* and *E. coli* seven to 13 different profiles were demonstrated. In total, 26 profiles were demonstrated from layers only affected by colibacillosis compared to 30 profiles associated with flocks affected by histomonosis and *E. coli*. The slightly lower diversity of plasmid profiles obtained from single infections compared to dually infected flocks was not statistically significant ($p=0.3028$). In total, seventy different plasmid sizes were demonstrated, each isolate containing from 2 to 8 plasmids. All isolates contained large plasmids of a size varying between 80 – 300 Kb (a complete list stating the different plasmid profiles in detail is available as supplementary material 1).

MLST. Eight different STs were demonstrated by MLST (ST23, ST57, ST93, ST95, ST117, ST140, ST141 and ST369) (Table 4). Three of these STs (ST23, ST117 and ST140) were demonstrated among isolates from both histomonosis affected and histomonosis free flocks, whereas the remaining STs were unique for isolates from histomonosis affected flocks. ST117 and ST140 together made up 70 and 89% of the isolates characterized from histomonosis affected and nonaffected flocks, respectively. A least one of these two STs **was** present in all 10 flocks. ST140 and ST117 differ in 46 nucleotides, including all seven genes used for MLST characterization, and are phylogenetically only distantly related as shown by the phylogenetic tree of the concatenated sequences (Figure 1). No differences were found in the number of different STs demonstrated in flocks concurrently infected with *H. meleagridis* and *E. coli* compared with flocks only affected by *E. coli* ($p=0.8247$). The differences between STs based upon differences in nucleotides of the concatenated sequences varied between a single nucleotide difference observed between the phylogenetically closest related STs (ST369 and ST23) and 55 nucleotides observed between the distantly related ST140 and ST369.

A total of 15, 12, 5, 3 and 2 different plasmid profiles were demonstrated for ST140, ST117, ST23, ST57 and ST93, respectively

Plasmid replicon typing. All 34 isolates characterized by plasmid replicon typing revealed the same type, IncFIB, regardless of ST and plasmid profile (Table 5). Although plasmid profile typing demonstrated between two and eight plasmids for each *E. coli* isolate, only plasmids with replicon type IncFIB were typable with the method applied.

Serology. All flocks except C tested negative for antibodies against APV upon arrival (16 weeks). However, all flocks had started seroconversion at peaking. At peak of lay the percentages of

positive serum samples ranged from 5% to 45%. These figures ranged from 10% to 100% when the flocks were 68 weeks old (Table 1). Clinical signs, such as swollen head syndrome or respiratory disease, drop in egg production or other indications of aMPV infection were not observed in any of the flocks.

Discussion

According to Stockholm *et al.* (2010) major causes of mortality in commercial free-range layer flocks in Denmark include feather pecking, cannibalism, bacterial diseases including colibacillosis, parasitism with *Ascaridia galli*, and *H. meleagridis*, in addition to flock management faults impacting welfare and adding to production cost.

Several studies have indicated that only a limited number clones are associated with outbreaks of colibacillosis (White *et al.*, 1993; Blanco *et al.*, 1997; da Silveira *et al.*, 2003), whereas *E. coli* associated with “normal” mortality constitutes a multitude of clones (Gregersen *et al.*, 2010b). Characterization of the large number of strains (102) in this study demonstrated only limited diversity. Only a single clone dominated in each outbreak with the exception of flocks B2 and D. For those flocks two different clones were associated with each outbreak. These findings suggest a primary rather than a secondary nature of *E. coli* in certain outbreaks of colibacillosis.

Comparing the STs of *E. coli* obtained from layers with those previously reported from Danish broiler breeders (Gregersen *et al.*, 2010b), only two STs were shared (ST23 and ST117). This probably reflects that table egg production and broiler production are completely separated in Denmark. Although all flocks had access to outdoor areas and contact with the avifauna two STs, ST117 and ST140, made up more than two thirds of the isolates characterized, questioning the risk

of the avifauna as a direct source of infection, as a much broader diversity of clones is expected from this source (Souza *et al.*, 1999)

The carriage of large plasmids is considered characteristic of APEC isolates (Doetkott *et al.*, 1996) and with the exception of two isolates, all *E. coli* carried plasmids greater than 100 kilobases. A large number of different plasmid profiles was demonstrated, and isolates each carried up to eight different plasmids, underlining the highly developed ability of *E. coli* to acquire and lose plasmids (Stehling *et al.*, 2007). This statement is underlined by each ST demonstrated was associated with a number of different plasmid profiles (e.g the 33 strains assigned ST140 were associated with 15 different plasmid profiles).

Isolates characterized by plasmid replicon typing all revealed the same replicon type, IncFIB, irrespective of plasmid profile and STs. This particular plasmid replicon type has previously been associated with virulence in poultry (Johnson *et al.*, 2007; Johnson *et al.*, 2008) and horizontal transfer of plasmids of this replicon type might represent a risk factor for changing a commensal *E. coli* into a more virulent form (Cusumano *et al.*, 2010)

It has been suggested that aMPV infection may pave the way for a subsequent *E. coli* infection via the respiratory tract (Jones, 2008). If so, a significantly higher prevalence of birds were expected to have seroconverted at peak of lay (Table 1) since the *E. coli* infections started at approximately 20 weeks of lay. However, in all flocks except flock C the outbreaks started before seroconversion for aMPV was observed making it unlikely that the virus should have acted as the “door-opener” for the *E. coli* infections. Moreover, as *E. coli* were selected to cover the entire period of colibacillosis for all flocks, including the period before and after the flocks had sampled positive for aMPV, and the same clone(s) dominated throughout each outbreak, it is more likely that an intrinsic ability of the particular clones cause disease rather than the way was paved by aMPV.

These findings are in agreement with those of Vanderchoves *et al.* (2004) who failed to detect any consistent interaction between *E. coli* and aMPV.

With the trend towards free-range and organic poultry production, the need for control of parasites is becoming more important as they may have a negative impact on production. Worldwide there has been an increase in reports of histomonosis in free-range poultry (Ganapathy *et al.*, 2000; Esquenet *et al.*, 2003; Permin, 2003; McDougald, 2005). A longitudinal study of 15 Danish organic egg producing flocks demonstrated outbreaks of histomonosis in six of these flocks, the mortality reaching 23% in a single flock (Stockholm *et al.*, 2010). In contrast to lesions observed in turkeys (Karaman *et al.*, 2009), previous studies in chickens have concluded that liver lesions often are absent in free-range layers (Esquenet *et al.*, 2003). These observations were confirmed in the present study in which more than half (51%) of the layers only demonstrated typical lesions within the caeca. Under experimental conditions the pathogenicity of *H. meleagridis* seems to depend on the presence of bacteria as demonstrated in the cecal ligation model (Kemp, 1974). However, if the hypothesis, that typhlitis due to *H. meleagridis* / *H. gallinarium* represents a predisposing factor for subsequent infection with *E. coli* is correct, a multitude of STs are expected to be associated with subsequent colibacillosis due to the diversity of potential pathogenic *E. coli* in the intestine (Ewers *et al.*, 2009). However, it can not be excluded that specific clones might develop in accordance with the “mix and match” theory (Mokady *et al.*, 2005). In the present study, differences in diversity of STs of *E. coli* from histomonosis affected and histomonosis negative flocks were not observed. In addition, plasmids of the same replicon type were found in both groups, probably adding to the virulence potential of the *E. coli* associated with the outbreaks. These results do not provide evidence for histomonosis as a predisposing factor for *E. coli* infection. However, additional studies are needed to fully elucidate the hypothesis.

In conclusion, only a limited diversity was demonstrated for the *E. coli* population associated with affected flocks, indicating a primary rather than secondary nature of *E. coli* associated with outbreak of colibacillosis. A large diversity of plasmids profiles was observed among the isolates and all, except two isolates, carried plasmids larger than 100kb. All plasmids investigated contained the same plasmid replicon type, suggesting that this replicon might represent a common pathogenicity factor among avian pathogenic *E. coli*. Neither histomonosis nor aMPV were shown to represent a predisposing factor for subsequent colibacillosis; however, further studies addressing these issues are needed to fully elucidate the importance of these factors.

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Figure 1. Phylogenetic relationship between concatenated sequences of seven housekeeping genes of sequence types (ST) determined for *E. coli* isolates based on the neighbour-joining analysis. Support for phylogenetic groups analysis is indicated as numbers out of 100.

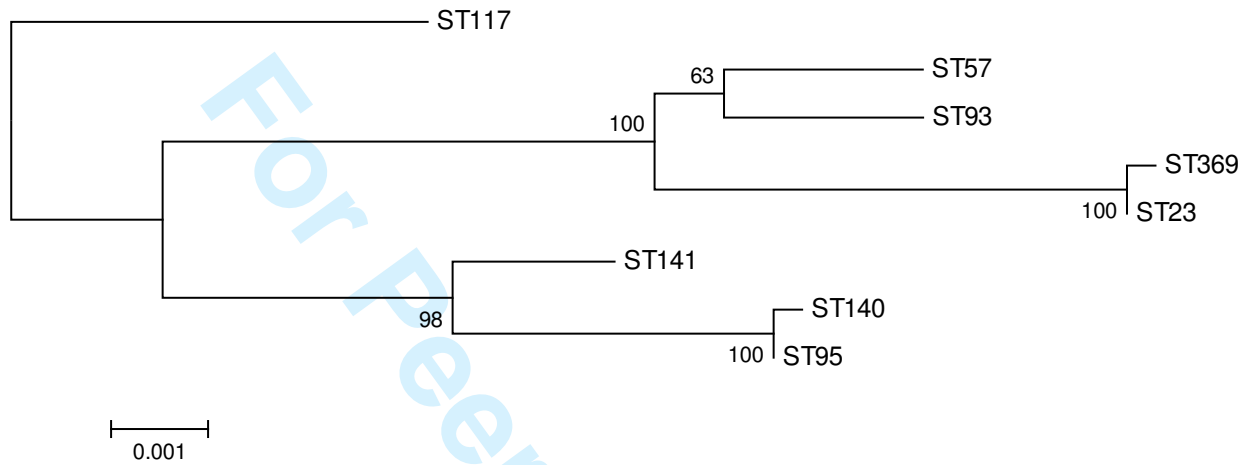


Table 1. Production data including flock size, accumulated mortality, numbers of birds received for post mortem examination, post mortem findings as to histomonosis and colisepticaemia, duration of outbreaks and demonstration of antibodies against avian Metapneumovirus (aMPV).

Farm	Flock	Flock size	Accumulated mortality (%) based on farmers registrations	Total number of layers submitted for post mortem out of total number of dead layers	Post mortem findings in birds received for examination			Duration of outbreaks			% positive samples tested for antibodies against aMPV		
					Histomonosis (%)	Colisepticaemia (%)	Histomonosis & colisepticaemia (%)	Histomonosis (weeks)	Colisepticaemia (weeks)	Histomonosis & colisepticaemia (weeks)	16 weeks	Peak of lay	68 weeks
A	A ₁ ¹⁾	3000	14.7	99/441	15.15%	17.17%	4.04%	+ (20-32)	+ (20-57)	+ (20-41)	-	-	-
	A ₂ ¹⁾	3000	15.1	64/454	4.69%	21.88%	4.69%	+ (20-23)	+ (20-63)	+ (20-24)	0	5	100
B	B ₁ ²⁾	4500	18.2	684/821	5.99%	16.23%	8.77%	+ (22-36)	+ (21-72)	+ (21-32)	0	20	10
	B ₂ ²⁾	3958	16.4	441/649	4.08%	22.68%	19.27%	+ (21-35)	+ (21-70)	+ (21-26)	-	-	-
C	C	4522	6.3	219/285	10.05%	16.89%	10.05%	+ (22-30)	+ (20-61)	+ (20-29)	35	45	30
D	D	3000	33.4	958/1003	0.12%	36.95%	1.86%	+ (25)	+ (25-79)	+ (25-30)	0	25	10
E	E ₁ ¹⁾	3600	15.7	215/566	0.00%	43.72%	0.00%	- -	+ (18-65)	- -	0	10	30
	E ₂ ¹⁾	3600	13.8	268/497	0.00%	34.33%	0.00%	- -	+ (18-65)	- -	-	-	-
F	F ₁ ³⁾ F ₂ ³⁾	4000 4000	} 15.0	66/1198	0.00%	89.39%	0.00%	- -	+ (27-66)	- -	NA	NA	NA
G	G	11993	21.5	30/2578	0.00%	90%	0.00%	- -	+ (23-47)	- -	NA	NA	NA

¹⁾ Flocks A₁, A₂, E₁ and E₂ originated from same rearing farm, but were separated according to time since flocks on farm E were four months older than those flocks on farm A. None of the flocks were sister flocks. ²⁾ Flocks B₁ and B₂ originated from the same rearing farm but were reared in separate houses. ³⁾ Only the accumulated data from flock F₁+F₂ were available, therefore F₁+F₂ were considered one flock in analysis of data. NA: Not available.

Table 2. *Distribution of lesions associated with histomonosis in layers only affected by histomonosis.*

Farm	Flock	Liver lesions only	Caecal lesions only	Liver & caecal lesions
A	A ₁	16%	0%	84%
	A ₂	0%	0%	100%
B	B ₁	4%	55%	41%
	B ₂	0%	70%	30%
C	C	0%	14%	86%
D	D	0%	82%	18%
All flocks		2%	51%	46%

Tabel 3. Summary of plasmid profiles demonstrated.

Farm	Flock	No. of isolates plasmid profiled*/Total No. of isolates	No. of plasmids profiled	Plasmid profile followed by no. of each plasmid profile, single infection/dual infection of <i>Histomonas meleagridis</i> and <i>E. coli</i> infection, respectively
A	A ₁	8/21	3	1(6, 4/2), 2(1, 0/1), 3(1, 0/1)
	A ₂	8/19	2	1(6, 5/1), 2(2, 0/2)
B	B ₁	26/179	10	4(8, 1/7), 5(2, 0/2), 6(1, 0/1), 7(7, 1/6), 8(2, 1/1), 9(2, 0/2), 10(1, 1/0), 11(1, 0/1), 12(1, 0/1), 13(1, 0/1)
	B ₂	36/209	7	7(24, 3/21), 14(1, 1/0), 15(3, 3/0), 16(3, 3/0), 17(3, 0/3), 18(1, 1/0), 19(1, 0/1)
C	C	26/68	13	20(8, 0/8), 21(2, 0/2), 22(1, 0/1), 23(3, 1/2), 24(3, 2/1), 25(2, 1/1), 26(1, 0/1), 27(1, 1/0), 28(1, 0/1), 29(1, 0/1), 30(1, 0/1), 31(1, 0/1), 32(1, 1/0)
D	D	37/374	10	33(18, 10/8), 34(8, 5/3), 35(4, 4/0), 36(1, 1/0), 37(1, 0/1), 38(1, 0/1), 39(1, 0/1), 40(1, 1/0), 41(1, 1/0), 42(1, 1/0)
E	E ₁	18/105	3	43(14, 14/-), 44(3, 3/-), 45(1, 1/-)
	E ₂	21/106	4	44(2, 2/-), 45(15, 15/-), 46(2, 2/-), 47(2, 2/-)
	8	180/1081		

* The single plasmid profiles are stated in detail in supplementary material 1.

Table 4. *Distribution of sequence types (ST) of E. coli in ten different free-range layer flocks.*

Flock	Histo- monosis affected	No. of isolates characterized by MLST	ST23	ST57	ST93	ST95	ST117	ST140	ST141	ST369
A1	+	8		1			7			
A2	+	6		1			5			
B1	+	15			1		1	12		1
B2	+	11				5	2	4		
C	+	13		3			9		1	
D	+	14	6		1			7		
		67	6	5	2	5	24	23	1	1
E1	-	7					2	5		
E2	-	8					2	6		
F	-	6	4				1	1		
G	-	14						14		
		35	4				5	26		
Total		102	10/102 (9.8)	5/102 (4.9)	2/102 (1.9)	5/102 (4.9)	29/102 (28.4)	49/102 (48.3)	1/102 (1.0)	1/102 (1.0)

Table 5. Sequence type (ST), plasmid profile and plasmid replicon type of *E. coli* isolates

Farm	Flock	ST	Plasmid profile	Plasmid replicon type	
A	A ₁	117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		57	2	IncFIB	
	A ₂	117	3	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		117	1	IncFIB	
		57	2	IncFIB	
		B	B ₁	140	4
B ₂	95		7	IncFIB	
C	C	117	17	IncFIB	
		117	22	IncFIB	
D	D	140	30	IncFIB	
		23	17	IncFIB	
E	E ₁	140	40	IncFIB	
		140	40	IncFIB	
		140	40	IncFIB	
		117	40	IncFIB	
		140	40	IncFIB	
		117	41	IncFIB	
		140	42	IncFIB	
		E ₂	117	41	IncFIB
			140	42	IncFIB
			140	42	IncFIB
	140		42	IncFIB	
	140		42	IncFIB	
			140	42	IncFIB
			117	43	IncFIB

Supplementary material 1. Outlining of different size plasmids (kilobases) for each plasmid profile.

Farm	Flock	No. of isolates plasmid profiled/Total No. of isolates	Plasmid profile	No. of each plasmid profile	Size (kilobases) of plasmids for each plasmid profile															
					Isolates obtained from															
					singel infec.	dual infec.														
A	A ₁	8/21	1	6	4	2	175			39	7		3.1	2.6	2.2					
			2	1		1	147	80						2.2						
			3	1		1	147	122				7		3.1	2.6	2.2				
A	A ₂	8/19	1	6	5	1	175			39	7		3.1	2.6	2.2					
			2	2		2	147	80							2.2					
B	B ₁	26/179	4	8	1	7	175						5.4	5.1						
			5	2		2	175							5.4	5.1	4	2.7			
			6	1		1	175								5.1	3	2.5			
			7	7	1	6	118										2.8			
			8	2	1	1	175			98	41			5.4	5.1					
			9	2		2	175				39	21	7			3.1	2.6	2.2	1.7	
			10	1	1		250	147	94				6	5.2		3.1	2.6			
			11	1		1	118	105		41								2.1	1.7	
			12	1		1				98		23	6.3	5.4	5.1					
			13	1		1	175			98				5.4	5.1			2.2	1.7	
			B	B ₂	36/209	7	24	3	21	118									2.8	
						14	1	1		185			47	8						2.4
						15	3	3		240	147		54	6		5.2				
16	3	3					185									4.1				
17	3					3	200				35	7				4.3	2.8	2.4		
18	1	1					200													
19	1		1	200		94					5.4	4.9								
C	C ₁	26/68	20	8		8	147		82						6.9	6.1	2.7	2.45		
			21	2		2		125										2.45		
			22	1		1	147					38		7				2.45		
			23	3	1	2			115		76									
			24	3	2	1													No plasmid contents	
			25	2	1	1	147									6.9	6.1	2.7	2.45	
			26	1		1	147					63						2.4	1.9	
			27	1	1		160			94	66			7.4	6.4			2.4		
			28	1		1						105								
			29	1		1		130											2.1	
			30	1		1		125												
			31	1		1	147												2.7	2.45
			32	1	1					115		76							2.7	
D	D ₁	37/374	33	18	10	8	185	100												
			34	8	5	3	115	54	1.9	1.8										
			35	4	4		115	63	5.4	1.9										
			36	1	1		94	48	1.65											
			37	1		1	115	13.5	5.4	4.2	2.1	1.7								
			38	1		1	147	100	2.8											
			39	1		1	185	100	2.8	2.6										
			40	1	1		115	54	4.8	16	12	8.8	5.8							
			41	1	1		54													
			42	1	1		115	63	5.4				8	5.8	3.5					
E	E ₁	18/105	43	14	14		300	52		15.5	5.1									
			44	3	3		300	52			7	3.4	2.8	2.4						
			45	1	1		300													
E	E ₂	21/106	44	2	2		300	52			7	3.4	2.8	2.4						
			45	15	15		300													
			46	2	2		300				7	3.4	2.8	2.6	2.4					
			47	2	2		300	147												
					92	88														