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Core genome multilocus sequence typing (cgMLST) confirms systemic spread of avian pathogenic *Escherichia coli* (APEC) in broilers with cellulitis

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ABSTRACT

Broiler cellulitis has emerged as an important cause of economic losses for farmers and slaughter plants from carcass condemnation at processing. Avian pathogenic *Escherichia coli* (APEC) has been identified as the main causative agent. The aim was to characterize *E. coli* isolated from cellulitis and organs in broilers at slaughter by whole genome sequencing analysis to study if systemic spread could be confirmed. Isolates were collected post-mortem from 101 carcasses condemned due to dermatitis/cellulitis from five commercial farms and six flocks. Forty-six isolates were characterised to determine serotypes, sequence types and virulence-associated genes. Analysis by cgMLST was performed to study the genetic similarity between isolates from the same broiler, among birds from the same flock and between flocks. *Escherichia coli* was isolated from 90% of birds from subcutaneous samples. In 20 broilers, *E. coli* was isolated from organs in pure culture or mixed with sparse growth of other bacteria. In eight of these, there were post-mortem findings suggestive of systemic bacterial spread. The majority of the isolates from the same bird and flock belonged to the same serotype and sequence type and were genetically indistinguishable, but differed when compared between flocks. Common APEC virulence genes, i.e. *chuA, fyuA, hlyF, iroN, irp2, iss, ompT, sitA, TerC, TraT*, were present in > 87% of the isolates. We conclude that evidence of systemic spread of *E. coli* from cellulitis was present in some birds at time of slaughter but cannot be reliably detected at meat inspection.

1. Introduction

Cellulitis in broiler chickens was first described in 1984 (Randall et al., 1984) and has become economically important as it leads to complete condemnation or down-grading of affected carcasses at slaughter plants. The definition of cellulitis in broilers includes a subcutaneous serosanguineous to caseated fibrinous plaque covered by discoloured and thickened skin, often located on the abdomen (Randall et al., 1984; Nolan et al., 2020). Avian pathogenic *Escherichia coli* (APEC) is believed to be the main causative agent. As of today, there is no common *E. coli* serotype or virulence gene profile defined in association with cellulitis in broilers. The pathogenesis is believed to involve bacterial infection of the subcutaneous tissue introduced through skin scratches or other skin lesions often caused by other broilers (Messier et al., 1993; Peighambari et al., 1995). The portal of entry and timing of

events are however difficult to study as chickens with cellulitis display few clinical signs during grow-out and are therefore first identified at meat inspection. The disease development has been associated with managemental risk factors such as large flock size and certain types of litter (Elfadil et al., 1996; Schulze Bernd et al., 2020).

In some cases, concurrent systemic manifestations of *E. coli* infection can be observed, such as pericarditis, airsacculitis and perihepatitis suggesting systemic bacterial spread from the cellulitis lesion as the primary site (Gomis et al., 1997). In a study by Gomis et al. (1997), *E. coli* that belonged to the same O serogroup was isolated from the cellulitis lesion and organs from the same broiler, suggesting systemic spread of bacteria. In addition, *E. coli* was isolated from organs even in the absence of gross findings (Gomis et al., 1997). More recently, Poulsen et al. (2018) found the same pulsed-field gel electrophoresis (PFGE) profile in *E. coli* isolates from cellulitis and spleen from the same

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broiler. Further, Silva et al. (2021) demonstrated that *E. coli* isolates from cellulitis and liver lesions from the same broilers possessed the virulence-associated genes *iss* and *iutA* as determined by PCR. However, more definite proof of systemic spread to organs is needed. This can be done through whole genome sequencing (WGS) and core genome multilocus sequence typing (cgMLST) of isolates from the same carcass. A similar approach based on core genome single nucleotide polymorphism analysis of *E. coli* isolates collected from broiler flocks with increased first week mortality was recently published (Kravik et al., 2022). The analysis of isolates from multiple sample sites of the same bird showed no sequence diversity, suggesting identical isolates in all organs. The question whether systemic spread is present at slaughter in carcasses with cellulitis is important in terms of production economics. Partial instead of complete condemnation of carcasses with cellulitis could possibly mitigate economic losses if applied when signs of systemic manifestations of *E. coli* infection are absent. This aspect has not yet been thoroughly studied with WGS sequencing, however several authors have suggested that full condemnation of birds with cellulitis should be implemented at slaughter (Poulsen et al., 2018; Silva et al., 2021).

Another reason to study *E. coli* from broilers with cellulitis is the possible influence on food safety. Concerns regarding genetic similarity among *E. coli* isolated from cellulitis in broiler chickens and human extraintestinal pathogenic *E. coli* (ExPEC) were raised early (Kumor et al., 1998). It has been shown that APEC strains can share certain traits with uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) based on phylogenetic cluster analysis, presence of virulence genes and plasmid replicon types (Jorgensen et al., 2019). Johnson et al. (2008) reported a common phylogenetic cluster for APEC, NMEC and UPEC isolates, which could represent isolates having zoonotic potential, although most of the characterized APEC, NMEC and UPEC clustered separately in subgroups and could be considered as separate subpathotypes.

The aim of the present study was to characterise *E. coli* isolates from broiler chickens condemned due to cellulitis and to investigate if systemic spread could be confirmed from the cellulitis lesion as the assumed primary portal of entry for *E. coli*. To this end, *E. coli* isolates were collected from broiler chickens at a slaughter plant and isolates were studied by WGS and cgMLST analysis.

2. Materials and methods

2.1. Study population

The study population consisted of Ross 308 broiler chickens (target body weight 2200 g) from large conventional farms that were approved by an official veterinarian at ante-mortem inspection at a Swedish slaughter plant. Flocks included in the study were those that were available for sampling on six weekdays during five consecutive weeks in May and June 2021.

In total, 101 broilers slaughtered at 35–38 days of age were examined post-mortem ($N = 6$ flocks, $N = 5$ farms). The collection of carcasses was performed by slaughter plant staff based on the condemnation code for dermatitis/cellulitis (Swedish Food Agency, 2021). One flock was included in the study per day and was represented by 12–20 carcasses each. The carcasses were retrieved at the first meat inspection position after passing through the scalding tank, defeathering and removal of head and feet, but before evisceration. During the first three study days, carcasses with gross evidence of a subcutaneous plaque were specifically targeted for sampling. Carcasses were collected sequentially from the slaughter line in batches of up to six birds. They were individually placed in sealed plastic bags, transferred to a separate building, sampled and examined by two poultry veterinarians within approximately 60 min.

2.2. Sampling and post-mortem examination

Sampling for bacterial culture was performed before post-mortem examination. All sampling was performed with aseptic techniques using clean instruments and Amies swabs (Copan, USA), with minimal handling of tissues and organs to avoid cross-contamination. First, the site of cellulitis was sampled as far away as possible from meat inspection incisions, if present. Then, after incision of the body cavity with sterile instruments, the pericardium and spleen were sampled from all carcasses. The pericardium was swabbed and the spleen was incised with a sterile scalpel after surface searing before sampling of the parenchyma. In addition, a maximum of two additional organs or tissues per carcass were sampled if gross signs of bacterial infection were observed. Following sampling, post-mortem examinations were performed according to a routine but simplified avian necropsy protocol (National Veterinary Institute SVA, Sweden). Information on sex and body condition (thin, normal, obese) and gross findings were documented. The skin and subcutaneous tissues were examined for all types of abnormalities such as discoloration, thickening, congestion, oedema, presence of exudate, scratches and other skin defects. Organs and tissues were examined with special attention to signs of bacterial infection such as enlargement, presence of exudate and parenchymatous lesions. The femoral head and the proximal tibiotarsus were incised and assessed for osteomyelitis, and tendon sheaths, hock, knee and hip joints were opened and examined for signs of infection.

2.3. Bacterial culture and MALDI-TOF MS

The swabs were streaked immediately after sampling on 5% bovine blood agar and Cysteine Lactose Electrolyte Deficient (CLED) agar (SVA, Sweden). CLED agar plates were used to prevent swarming of *Proteus* spp. The agar plates were placed in bags on ice packs and transported to the laboratory in the evening on the same day and incubated at 37 °C for 24 h. One presumptive *E. coli* colony (i.e. 2–3 mm in diameter, opaque, grey on blood agar or yellow on CLED) from each tissue or organ was randomly selected for confirmation by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), using a Maldi Biotyper Microflex LT System (Bruker Daltonik GmbH) with the MBT BDAL 8468 MSP library. Upon species confirmation, the same colony was re-cultured on 5% bovine blood agar at 37 °C for 24 h, followed by storage in 1.5 ml Brain Heart Infusion broth supplemented with 15% glycerol (SVA, Sweden) at − 80 °C pending further analyses.

2.4. Whole-genome sequencing and analyses

Forty-six *E. coli* isolates from 26 carcasses were selected for whole genome sequencing (WGS) analysis independently of gross findings. *Escherichia coli* isolates ($n = 36$) from subcutaneous tissue i.e. cellulitis and one to two organs from the same broilers were included if isolated in pure culture of with minimal growth of other bacteria upon primary culture. Isolates that met these criteria originated from four out of the six flocks (flock 1, 3, 4 and 5). In addition, to increase the number of characterized isolates, ten isolates with pure *E. coli* growth from cellulitis on primary plates were analysed from two randomly selected study flocks ($n = 10$ birds, flocks 3 and 5). Cultures from organ swabs from these ten birds showed no growth or sparse growth in mixed culture of no specific pathogenic bacteria. The isolates were named according to their flock of origin, carcass number and origin of sample, e.g. *3:5:Sc* (flock number 3: carcass number 5: subcutis). For DNA preparation, the selected isolates were retrieved from the frozen stock, passaged twice on 5% bovine blood agar, slurried in 500 μL nuclease-free water (Sigma-Aldrich, Germany) and lysed by heating at 100 °C for 5 min. DNA was extracted with the IndiMag Pathogen kit (Indical) on a TANBead Maelstrom-9600 system (TANBead). Library preparation was performed using Nextera chemistry and sequencing was done producing 2×150 bp reads on an Illumina NovaSeq instrument at SciLifeLab Clinical

Genomics, Solna, Sweden. Reads were checked for quality with FastQC (Andrews, 2010), trimmed with Trimmomatic (Bolger et al., 2014) and assembled with Unicycler (Wick et al., 2017). Serotype was determined with SerotypeFinder (Joensen et al., 2015) and multi-locus sequence typing (MLST) was done with mlst (Seemann, 2015) using the Achtman 7-locus MLST scheme available in the PubMLST database (Jolley et al., 2018). Assemblies were screened for virulence factors with Virulence-Finder (Camacho et al., 2009; Joensen et al., 2014; Tetzschner et al., 2020). The Enterobase cgMLST V1 scheme was used for the cgMLST analysis (Zhou et al., 2020) and clustering and visualization was done with Ridom SeqSphere+ (Junemann et al., 2013). The Enterobase database was also searched for isolates of human origin within the same HC50 HierCC V1 clusters as the isolates from the broiler chickens. A subset of the isolates of human origin were screened for virulence genes. Program versions and parameters are listed in Supplementary material T1. All raw sequencing data have been submitted to the European Nucleotide Archive and Enterobase and are available under accession number PRJEB52616.

2.5. Statistical analyses

All data were entered into Microsoft Excel 2016 (Microsoft Corporation, USA) where descriptive statistics (percentage, number of events and mean value) were calculated. Minor gross findings at post-mortem examination that could not be linked to a specific diagnosis were removed from the dataset.

3. Results

3.1. Slaughter data and post-mortem findings

The mean body weight of the sampled broiler flocks ranged from 2214 to 2312 g and the condemnation rate due to dermatitis/cellulitis ranged from 0.18% to 0.53%.

Among the 101 examined carcasses, 52% were males and 48% females. The mean carcass weight without head and feet was 1969 ± 274 g (out of 98 records). The majority of the carcasses were in normal body condition (89%) while 11% were thin (out of 94 records). A majority ($n = 73$, 94%) had an incision at the location of the skin lesion made at meat inspection (of 78 records).

A variety of different skin conditions were present in all 101 broilers. Gross findings in agreement with the definition of cellulitis (see introduction) or presence of a subcutaneous exudate considered as an early stage of plaque formation were observed in 79 out of 101 carcasses (78%). Of these, a majority had a subcutaneous plaque, intradermal green or yellow discoloration, subcutaneous oedema, subcutaneous congestion and inflammatory exudate/fibrin. In 35% of the carcasses with cellulitis, a localized lesion was observed on the abdomen, in the vent region or in dorsal or lateral position to the tail. Fewer carcasses (8%) showed a local lesion on the thigh or proximal trunk. In another 57%, the cellulitis lesion involved several areas of the body. The size of the primary cellulitis lesion ranged from 10 to 80 mm (recorded in 67 carcasses). Ninety-six percent of the carcasses with cellulitis displayed other localised skin lesions. These included single to multiple linear abrasions or lacerations up to 80 mm long in 84%, of which the majority were superficial and located on the abdomen (presumed scratches from claws). There were also single to multifocal dark discoloured 1–2 mm superficial circular erosions located on the abdomen in 68% of the carcasses and other skin defects in 33%. Twenty-two carcasses (22%) had gross findings not in agreement with the definition of cellulitis. Ten of these carcasses had a 5–40 mm lesion overlying the craniolateral breast muscle extending through the dermis with haemorrhage and inflammatory exudate on the muscle fascia. The other 12 carcasses had minor skin lesions on the abdomen, thigh and/or in lateral position to the tail. Other gross findings are summarised in Table 1. There was no sign of breast blisters or burns in any of the 101 carcasses. Signs of

Table 1
Summary of gross findings in addition to cellulitis and skin lesions at post-mortem examination of 101 broiler chickens from six flocks condemned on account of dermatitis/cellulitis at meat inspection at a Swedish slaughter plant.

Number of carcasses / Finding	Cellulitis $n = 79$	Other skin lesions $n = 22$
Splenomegaly	22	2
Hepatomegaly	18	-
Perihepatitis	18	-
Miliary white spots in liver	12	-
Airsacculitis	11	-
Pericarditis and/or epicarditis	10	-
Coelomitis	8	-
Salpingitis	2	-
Focal serositis spleen	1	-
Other		
Ascites syndrome	5	2
Retained yolk sac	3	-
Dehydration	1	-
Right Müllerian duct cyst	-	1

suspected systemic bacterial infection were observed in 19% ($n = 19$) of the examined carcasses and only in broilers diagnosed with cellulitis. The most common gross findings indicative of systemic spread were splenomegaly and perihepatitis, followed by air sacculitis, pericarditis, hepatomegaly, other liver pathology and coelomitis.

3.2. Bacteriology

A total of 327 samples were cultured and *E. coli* was confirmed by MALDI-TOF MS in 44% of the samples and in 90% of the carcasses (Table 2). As seen in Table 2 there was variable growth in samples from subcutaneous tissue and organs. In samples originating from organs, pure growth of *E. coli* was found in 11%. In some of these, there was sparse growth of *E. coli* (1–10 colonies). Table 3 summarises culture results based on carcasses with and without gross findings suggestive of systemic bacterial infection.

Twenty out of the 101 carcasses had one or more sampled organ with growth of *E. coli* in pure culture or with sparse growth of other bacteria, of which 19 were diagnosed with cellulitis. Further, eight of these 20 carcasses (40%) had gross findings suggestive of systemic bacterial infection.

Table 2
Bacterial growth from cellulitis and organs samples ($N = 327$) obtained at post-mortem examination of broiler carcasses condemned because of dermatitis/cellulitis ($N = 101$). Numbers in brackets refer to bacterial growth from cellulitis and organ samples from the subgroup of carcasses with gross findings indicative of cellulitis ($n = 79$).

Number of sampled birds / Origin of sample	No growth	*E. coli*[1]	*E. coli* in mixed culture	Other species[1]	Mixed growth[2]
Subcutis $n = 101$	2 (1)	54 (54)	37 (22)	- (-)	8 (2)
Pericardium $n = 101$	42 (29)	9 (8)	15 (14)	7 (7)	28 (21)
Spleen $n = 101$	47 (31)	11 (11)	10 (9)	18 (15)	15 (13)
Other organ 1[3] $n = 21$	3 (3)	3 (3)	4 (3)	- (-)	11 (10)
Other organ 2[3] $n = 3$	- (-)	1 (1)	- (-)	- (-)	2 (2)

[1]Pure growth or growth in mixed culture with sparse growth of other bacteria
[2] ≥ 2 colonies with different morphology
[3]any 4th and 5th sample taken per carcass in one of the following location: liver, joint, air sac, tendon sheath, skeleton, coelomic cavity, pancreas or oviduct; liver being the most common sampled organ (n = 12).

Table 3
Distribution of bacterial growth or no growth, acquired at post-mortem examination from cellulitis and organs in broiler carcasses with gross findings indicating systemic spread of *E. coli* (S+, $n = 19$) and no systemic spread (S-, $n = 82$).

Origin of sample	Group	No growth	*E. coli*[1]	*E. coli* in mixed culture	Other species[1]	Mixed growth[2]
Subcutis	S+	-	13	5	-	1
$n = 101$	S-	2	41	32	-	7
Pericardium	S+	3	3	4	1	8
$n = 101$	S-	39	6	11	6	20
Spleen	S+	7	5	2	3	2
$n = 101$	S-	40	6	8	15	13
Other organ 1[3]	S+	2	2	2	-	6
$n = 21$	S-	1	1	2	-	5
Other organ 2[3]	S+	-	1	-	-	2
$n = 3$	S-	-	-	-	-	-

[1] Pure growth or growth in mixed culture with sparse growth of other bacteria
[2] ≥ 2 colonies with different morphology
[3] any 4th and 5th sample taken per carcass in one of the following location: liver, joint, airsac, tendon sheath, skeleton, coelomic cavity, pancreas or oviduct; liver being the most common sampled organ ($n = 12$).

3.3. Whole-genome sequencing and bioinformatics

Among the 46 sequenced isolates, 34 originated from organs and cellulitis lesions from 15 individual birds. Another ten isolates represented broilers with cellulitis without coliform growth in organs (single samples from cellulitis) and two isolates came from a carcass with a lesion on the craniolateral breast muscle (not diagnosed with cellulitis) (Table 4).

There were 11 serotype/sequence type (ST) combinations represented among the sequenced isolates (Table 4; Supplementary material T2). The serotype-sequence types differed between flocks, except for O2/O50:H5-ST95 and O120:H4-ST428 that was present in both flocks 4 and 5. One to five serotype-sequence type combinations were represented within flocks, while the majority of isolates from the same bird had the same serotype-sequence type.

All 46 isolates harboured the virulence genes *iss* (increased serum survival) and *TerC* (tellurium ion resistance protein), while 45 isolates (98%) harboured genes *hlyF* (hemolysin F), *iroN* (enterobactin siderophore receptor protein) and *ompT* (outer membrane protease) (Supplementary information T2). Another five genes were present in 40–43 isolates (87–94%) (*chuA*: outer membrane hemin receptor; *fyuA*: siderophore receptor; *irp2*: high molecular weight protein 2 non-ribosomal peptide synthetase; *sitA*: iron transport protein and *traT*: outer membrane protein complement resistance).

The minimum spanning tree (MST) showed that the majority of isolates from the same flock clustered with up to two allele differences between isolates and had the same sequence type (Table 5, Fig. 1). Isolates originating from the same carcass but from different locations showed an identical allelic profile or differed in one allele, with the exception of the two carcasses 3:13 and 4:1, in which the isolates from cellulitis and organ differed in the majority of alleles and had different serotypes and sequence types (Table 4; Fig. 1). In addition, the isolates from carcass 4:1 (isolates 4:1:Sc and 4:1:Sp) fell into two different clusters of isolates from flock 4. In two cases, isolates from different flocks were more similar than isolates from the same flock. Four isolates from flocks 4 and 5 of sequence type 428 were located in a shared cluster, with up to four allele differences between isolates and one isolate from flock 5 (5:9 Sc) was located 27 alleles from isolates from flock 4.

In Enterobase, 26 *E. coli* isolates of human origin were identified within 50 allelic differences from the current study isolates forming five clusters (Fig. S1). One to five isolates of human origin were present in

Table 4
Identification, serotype and sequence type for *E.coli* isolates ($n = 46$) acquired at post-mortem examination from subcutaneous tissue and organs in broilers condemned for dermatitis/cellulitis at a slaughter plant. The isolates were named according to flock, carcass number and location of sampling (Sc=subcutaneous tissue, Pc=pericardium, Sp=spleen, Li=liver, LA=liver and air sac). Isolates marked with * belonged to the carcass group not diagnosed with cellulitis.

Flock: Carcass no: Location	Serotype	Sequence type
1:6:Sc	O103:H21	101
1:6:Pc	O103:H21	101
3:3:Sc	O25:H4	131
3:5:Sc	O25:H4	131
3:5:Pc	O25:H4	131
3:6:Sc	O25:H4	131
3:6:Sp	O25:H4	131
3:7:Sc	O25:H4	131
3:7:Pc	O25:H4	131
3:7:Sp	O25:H4	131
3:8:Sc	O25:H4	131
3:11:Sc	O17/O44/O77:H18	69
3:12:Sc	O25:H4	131
3:13:Sc*	O132:H28	13199
3:13:Sp*	O23:H6	69
3:15:Sc	O25:H4	131
3:18:Sc	O78:H4	23
3:18:Sp	O78:H4	23
3:18:Li	O78:H4	23
4:1:Sc	O161:H4	117
4:1:Sp	O2/O50:H5	95
4:2:Sc	O161:H4	117
4:2:Pc	O161:H4	117
4:7:Sc	O120:H4	428
4:7:Pc	O120:H4	428
4:9:Sc	O161:H4	117
4:9:Sp	O161:H4	117
4:9:LA	O161:H4	117
4:12:Sc	O2/O50:H5	95
4:12:Pc	O2/O50:H5	95
5:1:Sc	O2/O50:H1	135
5:2:Sc	O2/O50:H1	135
5:2:Pc	O2/O50:H1	135
5:2:Sp	O2/O50:H1	135
5:3:Sc	O2/O50:H1	135
5:5:Sc	O2/O50:H1	135
5:5:Sp	O2/O50:H1	135
5:6:Sc	O2/O50:H1	135
5:7:Sc	O2/O50:H1	135
5:7:Pc	O2/O50:H1	135
5:9:Sc	O2/O50:H5	95
5:11:Sc	O2/O50:H1	135
5:13:Sc	O120:H4	428
5:13:Pc	O120:H4	428
5:19:Sc	O11:H25	457
5:19:Sp	O11:H25	457

four of the five clusters harbouring two to six genetically identical isolates from the current study in each cluster. The fifth cluster harboured 18 isolates of human origin and one isolate from the current study. Out of the 26 isolates from humans, 11 were isolated from urine, one from liver and blood respectively, and 13 lacked this information. When comparing the presence of virulence genes in one to two of the isolates of human origin within each cluster with the current study isolates in the same cluster, 14–22 shared virulence genes were identified in both groups. The most commonly occurring genes were *lpfA* (long polar fimbriae), *ompT* (outer membrane protease) and *terC* (tellurium ion resistance protein) that were present in all analysed isolates of human origin and isolates from the current study in the five clusters. The genes *cvaC* (microcin C), *fyuA* (siderphore receptor), *gad* (glutamate decarboxylase), *hlyF* (hemolysin F), *iroN* (enterobactine siderophore receptor protein), *irp2* (iron regulatory protein 2), *iss* (increased serum survival), *mchF* (ABC transporter protein) and *sitA* (iron transport protein)
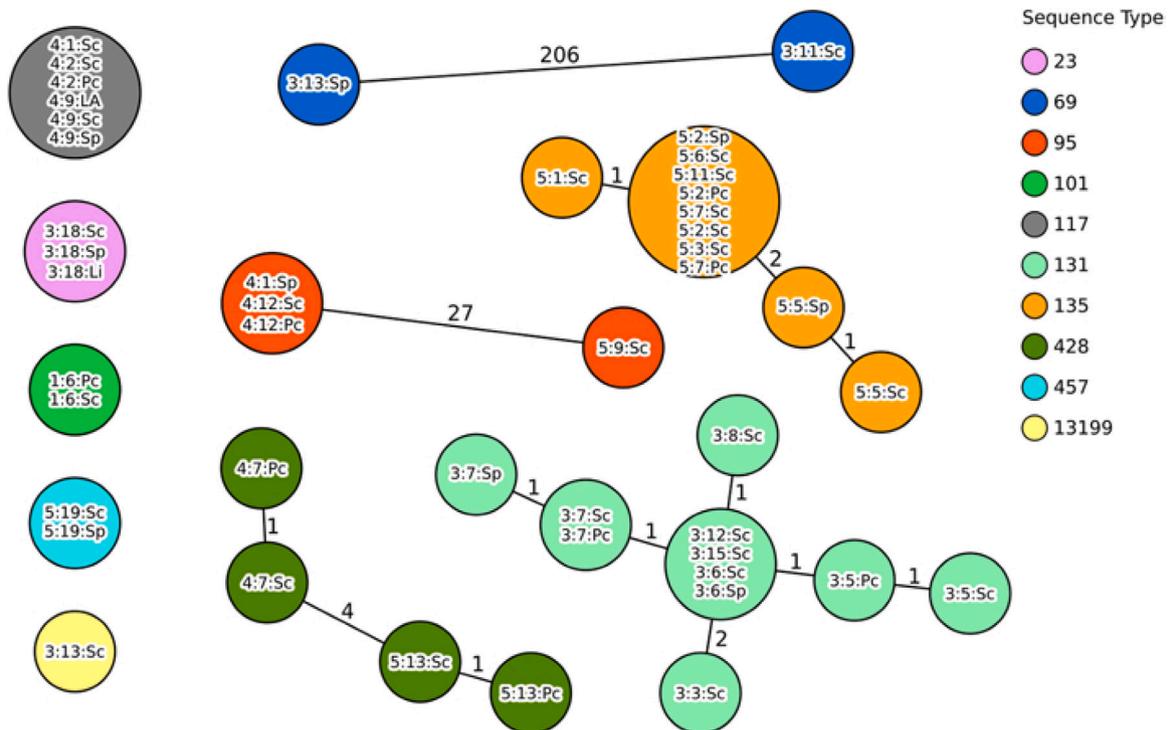
Fig. 1. Minimum spanning tree based on the cgMLST data from *E. coli* isolates (n = 46) from carcasses condemned for cellulitis at a Swedish slaughter plant. Colours represent different sequence types. Each circle represents an allele profile with lines that connects closely related isolates forming clusters. The number on the line denotes how many alleles that differed between the isolates. No line implies that the isolates differs in > 1870 alleles.

occurred in both isolates of human origin and current study isolates in four out of five clusters. The gene *lpfA* (long polar fimbriae) was present only in the 14 isolates from the current study that clustered with those of humans origin, as well as in a single cellulitis isolate (3:11:Sc) that differed by 206 alleles from the isolates from the current study that clustered with isolates of human origin.

4. Discussion

In the present study, *E. coli* in pure culture was isolated from organs both with and without gross findings of suspected bacterial infection beyond the assumed primary site of cellulitis in broiler chickens. The isolates originated from six different healthy flocks as suggested by ante-mortem inspection and mean body weight. The condemnation rate of the flocks due to dermatitis/cellulitis was similar to figures reported in previous studies in Europe (Poulsen et al., 2018; Schulze Bernd et al., 2020). The majority of *E. coli* isolates from cellulitis and organs in the same carcass were genetically indistinguishable. Our results thus confirm earlier studies (Gomis et al., 2001; Poulsen et al., 2018; Silva et al., 2021) that *E. coli* may have spread systemically in broiler carcasses with cellulitis sampled at slaughter. The present study also suggests that gross findings of suspected systemic bacterial infection is not a reliable tool for identification of this subset of birds at meat inspection.

As cellulitis is difficult to diagnose in live broilers on farms, the present study and most earlier studies have relied on sampling at slaughter plants. The commercial slaughter of broilers results in various species of bacteria being present in the slaughter plant environment and on the broiler skin (Moazzami et al., 2021). A likely cause is that water is used during the slaughter process, e.g. during scalding and defeathering, which could introduce contaminating bacteria on and under the skin and possibly within the body cavity. Moreover, many carcasses are incised at meat inspection to confirm cellulitis, which could introduce contaminants to the subcutaneous sampling location. Therefore, it was an expected finding that some samples in the present study showed growth of bacteria in mixed culture, with or without *E. coli* (Table 2).

This phenomenon occurred at a similar rate on all six sampling days despite an aseptic sampling technique, sampling in a separate building within a short time of death and prior to post-mortem examination. In accordance with our results, Messier et al. (1993) reported growth of bacteria in mixed culture in samples from cellulitis when sampling was performed at a slaughter plant. Another study described that *E. coli* was isolated from cellulitis lesions and organs but no results were included regarding the level of mixed bacterial growth (Onderka et al., 1997). Other researchers have reported only pure cultures (Gomis et al., 1997; Poulsen et al., 2018). The present study clearly illustrates the difficulties in obtaining pure primary cultures from broilers at slaughter plants, despite a careful sampling strategy designed to obtain representative and good quality samples.

Several previous studies have suggested that *E. coli* can spread systemically from the primary cellulitis lesion to organs (Gomis et al., 2001; Poulsen et al., 2018; Silva et al., 2021). In contrast to these earlier studies, which were based on serotyping, virulence gene detection by PCR and fingerprinting (PFGE), our study is the first to report evidence of systemic spread based on cgMLST. Core genome-MLST analysis is a genome-wide genotyping method representing the core genome and it thus provides a solid and reliable way to identify identical or closely related *E. coli* isolates. Ideally, signs of systemic spread should be detected at the slaughter plant during meat inspection. However, in the present study we confirmed findings of other researchers, that *E. coli* can be isolated from organs in carcasses even when no gross findings of systemic spread were observed (Gomis et al., 1997; Onderka et al., 1997). Interestingly, in the present study only a few *E. coli* colonies were cultured from some organ samples. This could be a sign of early or low-grade systemic infection, possibly bacteraemia or sample contamination. In contrast, there were also carcasses with gross findings of systemic infection from which cultures revealed no bacterial growth. This could indicate bacterial clearance as discussed in a previous paper by Gomis et al. (2001). Thus, it is most likely difficult to reliably identify birds with systemic spread at slaughter, especially taking in consideration the short time available to inspect each carcass and the mild nature

of gross lesions sometimes present in early infection stage. It should be mentioned that although it is assumed in the current study that cellulitis is the primary lesion from where the systemic spread occurred, the sequence of events in coliform cellulitis have not been fully studied and determined.

Moreover, the cgMLST analysis in our study confirmed that most *E. coli* isolates from the same flock of origin belonged to the same genetic cluster, while isolates from different flocks belonged to separate clusters. This suggests that each flock has its own predominant cellulitis-associated *E. coli* populations. This has previously been suggested by Singer et al. (1999) based on PFGE analysis. In addition, in our study, one cluster consisted of isolates with close genetic similarity, which originated from two different farms. This indicates that in addition to endemic *E. coli* populations, the same genotypes associated with cellulitis can be found on several farms, which also is in accordance with previous results by de Brito et al. (2003) using extragenic palindromic PCR (REP-PCR) fingerprint technique. Together, these results point to the possibility of bacterial transmission of APEC within the broiler industry network.

Avian pathogenic *E. coli* is a heterogeneous group of bacteria that is not well defined when it comes to the presence of virulence genes (Mehat et al., 2021). While cellulitis-associated *E. coli*, including the isolates in the present study, fits in the general APEC definition, there is no shared set of virulence genes defining this group of bacteria (Jeffrey et al., 2002; de Brito et al., 2003; Barbieri et al., 2013; Poulsen et al., 2018). The genes *kpsMTII*, *ibeA* and *gimB* have been suggested as clinically important virulence genes in association with cellulitis (Barbieri et al., 2013). In our study, the *ibeA* gene was identified in a majority of the sequenced isolates. On the other hand, the *kpsM* gene was present only in a subset of isolates. In another study, the cytotoxin *E. coli* vacuolating Factor (ECVF) was shown to induce microscopic skin lesions in an animal experimental setting (Quel et al., 2013). The author suggested that ECVF contributes to the pathogenesis of cellulitis. As no ECVF sequence yet has been published, further analysis of our study isolates was not possible.

Our study showed that cellulitis could be associated with multiple serotypes and sequence types, virtually all of which have previously been associated with the broader APEC pathotype. Our findings included STs 23, 95, 117, and 428, which commonly cause systemic disease in poultry (Mehat et al., 2021). Our results strengthen the proposed pathogenesis that cellulitis development is dependent on both an infecting strain specialised in causing extraintestinal infection and the condition of the host together with management factors (Jeffrey et al., 2002; Schulze Bernd et al., 2020). Poulsen et al. (2018) identified a predominant *E. coli* sequence type (ST117) and PFGE profile in broilers with cellulitis in Denmark. They studied *E. coli* isolates from 34-days-old broilers with cellulitis and carcasses condemned at slaughter due to cellulitis. A subset of eight study isolates with ST117 were subjected to WGS and six of the isolates differed by only 7–36 SNPs. In another study by Ronco et al. (2017), closely related isolates of sequence type ST117 were reported as a predominant pathogen in several Nordic countries. Ronco et al. (2017) studied 114 isolates from broilers and broiler parents, and ST117 was identified in a clade consisting of 62 isolates. Moreover, 47 of these isolates belonged to serotype O78:H4. In both studies, it was suggested that the O78:H4-ST117 could have originated from a common breeding stock source in Sweden (Ronco et al., 2017; Poulsen et al., 2018). In our study, there were only six ST117 isolates, all originating from the same flock and cluster, but with a different serotype (O161:H4) than the one presented by Ronco et al. (2017). Serotype O78:H4 was however present in three isolates from another flock but in combination with ST23. It should be added that there is no study published on APEC isolates from Sweden confirming the presence of O78:H4-ST117 during the same time period as in previous reports (Ronco et al., 2017; Poulsen et al., 2018). Thus, our study could not confirm the presence of O78:H4-ST117 as the predominant *E. coli* lineage in Sweden in 2021. Although speculative, there could have been another unknown

common source, or other strains could have since replaced this particular serotype-sequence type in Sweden.

In our study, a similar set of virulence genes were detected among the genetically related isolates of human origin and the study isolates. There was limited information on the source and disease conditions of the isolates of human origin, but at least 11 of 29 isolates were collected from urine indicating that the isolates belong to the UPEC group. These results support previous research that APEC and human pathogenic *E. coli* are similar in terms of both phylogeny and virulence factors, although it is not clear if APEC cause infections in humans (Manges, 2016; Jorgensen et al., 2019). There is no routine genomic characterization of ExPEC isolates collected in the Swedish healthcare system, and therefore no direct comparison on the national level was possible in the present study.

5. Conclusion

Based on genomic comparison we conclude that *E. coli* isolated from cellulitis in broiler chickens frequently have characteristics in agreement with other APEC and can spread systemically. Identifying broilers with systemic spread of *E. coli* based on gross findings is however not a reliable method that can be used at slaughter plants, as *E. coli* isolates that are genetically indistinguishable from the cellulitis isolates can be found in organs without gross findings.

We also conclude that sampling for bacterial isolation from broiler carcasses in the slaughter plant is challenging, due to the bacterial flora in the slaughter process environment as well as on the carcasses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109755.

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