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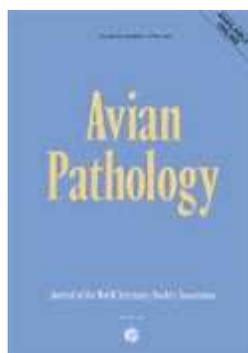
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***Enterococcus cecorum* infections in broiler breeders and their offspring: molecular epidemiology**

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Enterococcus cecorum infections in broiler breeders and their offspring:
molecular epidemiology

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Abstract

Increased mortality and problems with lameness were reported in Dutch broiler flocks from the year 2008 onwards. Therefore, a field inventory, including 10 affected broiler flocks, nine corresponding broiler breeder flocks and five hatcheries, was carried out. The onset of clinical signs (lameness and increased mortality) started at about two weeks of age. The flock mortality varied from 3.1 to 8.1% at slaughter. Postmortem lesions of broiler flocks were characterized by the occurrence of pericarditis/hydropericardium, arthritis and femoral head necrosis. *E. cecorum* was isolated from approximately 30% of the lesions. In the broiler breeders, *E. cecorum* was not isolated from any lesions. However, it was isolated from 31 out of 65 (47%) cloacal swabs, from 2 out of 65 (3%) oviduct samples, from 1 out of 65 (1.5%) bone marrow samples and 2 out of 25 (8%) blood samples. *E. cecorum* was not isolated from the air samples or dead-in-shell originating from the hatcheries involved. In total, 78 isolates were subjected to further typing by means of tRNA Intergenic Spacer PCR and confirmed as *E. cecorum*. The genetic relatedness of these cocci was subsequently studied using pulsed-field gel electrophoresis. The banding patterns of approximately 68% of *E. cecorum* isolates originating from parent stock flocks were clonal to one or more isolates of the same or other parent flocks. In contrast, isolates originating from their diseased offspring showed much greater genetic variation. Therefore, the vertical transmission of *E. cecorum* could not be demonstrated.

Introduction

Enterococci are normal inhabitants (commensals) or transient residents of the intestinal flora of birds and mammals, which explains their worldwide occurrence (Devriese *et al.*, 1992a; Devriese *et al.*, 1994; Devriese *et al.*, 1991b; Devriese *et al.*, 1992b; Kaukas *et al.*, 1986). Disease outbreaks caused by enterococci are therefore considered opportunistic, while predisposing factors (e.g. deficiencies, other infections, immunosuppression, antibiotic treatment, vaccinations) are considered crucial for outcome of the pathology induced (Landman *et al.*, 2000; Lewis & Zervos, 1990; Linden & Miller, 1999; Steentjes *et al.*, 2002). In poultry, enterococci are frequently isolated from dead-in-shell and day-old chicks, often affecting the yolk sac (Cortes *et al.*, 2004; Deeming, 2005; Gross & Domermuth, 1962). In many cases multiple species of bacteria can be isolated and soiled eggs are regarded as an important source of contamination (Carter *et al.*, 1973; De Reu *et al.*, 2006).

Enterococcus faecalis has been associated most frequently with poultry diseases amongst which endocarditis (Gross & Domermuth, 1962; Jortner & Helmboldt, 1971), hepatic granulomas in turkeys (Hernandez *et al.*, 1972) and, arthritis and amyloidosis in both brown layers (Landman *et al.*, 1994) and broiler breeders (Landman *et al.*, 1998; Steentjes *et al.*, 2002) have been most frequently reported. It has also been isolated from arthritic joints of ducks (Bisgaard, 1981), while *E. faecium* has been incriminated in acute septicaemia of white Peking ducklings (Sandhu, 1988). Furthermore, *E. faecalis* has been associated with ascites in hens and pulmonary hypertension in broilers (Tankson *et al.*, 2001, 2002). Other enterococci also associated with valvular endocarditis are *E. faecium* and *E. durans* (Domermuth & Gross, 1969). *E. durans* infection in young chickens has been associated with bacteraemia and encephalomalacia (Cardona *et al.*, 1993). Another enterococcus associated with brain lesions (focal necrosis) in the young chicks is *E. hirae* (Devriese *et al.*, 1991a; Randall *et al.*, 1993).

E. hirae has also been found in cases of endocarditis in broilers (McNamee & King, 1996) and diarrhoea in 1 to 7 day old layer chicks (Kondo *et al.*, 1997). In chickens with spontaneous and induced *E. faecalis* endocarditis, central nervous system lesions related to bacterial emboli have been seen (Jortner & Helmboldt, 1971).

E. cecorum, which was previously classified as a streptococcus (Williams *et al.*, 1989), was first described as a new enterococcus species from the intestines of chickens (Devriese *et al.*, 1983). Later on, this bacterial species was also reported to form part of the intestinal flora of pigeons (Baele *et al.*, 2002) and turkeys (Scupham *et al.*, 2008). Further, *E. cecorum* has been found to be associated with bone lesions in broiler chickens characterized by chondronecrosis and osteomyelitis (Wood *et al.*, 2002). *E. cecorum* has also been reported as the cause of one (Devriese *et al.*, 2002) and subsequently several (De Herdt *et al.*, 2008) outbreaks of disease in broilers characterized by sepsis, pericarditis, local myositis and, most prominently, bone and joint lesions (purulent arthritis of the hock, femoral head necrosis and osteomyelitis of vertebra T6). More recently, outbreaks of disease with similar clinical signs and lesions from which *E. cecorum* was also isolated, were reported in both a broiler and a broiler breeder flock in Canada (Stalker *et al.*, 2010).

Spontaneous cases of *E. cecorum* infections matching previous descriptions (i.e. characterized by increased mortality, pericarditis, femoral head necrosis and arthritis) were increasingly seen amongst Dutch broiler flocks from 2008 onwards. In some cases the source of the *E. cecorum* problems was tentatively attributed to the broiler breeder flock of origin by veterinarians and/or hatcheries. Both, the clinical problems in broilers and a possible link to parent stock formed the rationale to conduct a field study on the occurrence of *E. cecorum* and lesions associated with it in both types of poultry as well as in dead-in-shell and hatcheries. The clonality of the *E. cecorum* obtained from the broiler flocks and the corresponding parent

stock was studied using pulsed-field gel electrophoresis (PFGE) in order to assess the possible vertical transmission of this micro-organism.

Materials and methods

Broilers and broiler breeders. Ten broiler farms with increased mortality and lameness problems were reported to the Animal Health Service (GD, Deventer, the Netherlands) by practitioners. The flock size varied from approximately 19000 to 58000 birds. The age at sampling ranged from 13 to 36 days (Table 1). All flocks were vaccinated against Newcastle disease (mandatory), and most cases also against Gumboro disease and infectious bronchitis.

A requirement to participate in the study was that the problem flock should originate from a single breeder flock and that the parent birds should be alive at the time of sampling the broilers in order to be able to include the breeder flocks and dead-in-shell in the study. Moreover, participating broiler flocks were required to be free of antibiotics during at least one week. In total 10 broiler flocks originating from 9 broiler breeder flocks were studied. Two of the broiler flocks originated from the same breeder flock. Only live birds were submitted for postmortem. The selection of birds was done by the farmers or their veterinarians, who were instructed to collect lame and/or sick birds. In case no clinical signs occurred, as in broiler breeder flocks, chickens were selected randomly.

Postmortem. The birds were stunned using a mixture of CO₂ and O₂ and exsanguinated by incision of the jugular vein. A thorough general routine postmortem examination was performed. All macroscopic abnormalities were recorded and samples were taken for bacteriology and histopathology.

Bacteriology. Bone marrow, pericardium and joints were sampled from broilers for bacteriology, while from the broiler breeders bone marrow, cloaca swabs, oviduct and blood samples were taken. The isolation of *E. cecorum* was done using sheep blood agar (K204, BioTrading Benelux B.V., Mijdrecht, the Netherlands) and kanamycin aesculin azide agar (CM0591, Oxoid B.V., Badhoevedorp, the Netherlands), which were incubated for up to 48 hours at 37°C under aerobic and micro-aerophilic conditions. Identification of the genus *Enterococcus* was done by means of agglutination using Streptex® (Remel Europe Ltd., Dartford, Kent, UK) and growth in trypton phosphate broth with 6.5% NaCl. Further identification to the species level was performed using the Rapid ID32 STREP from bioMérieux (bioMérieux Benelux b.v., Zaltbommel, the Netherlands). *E. cecorum* isolates were stored at -70°C for further molecular analysis.

Histopathology. At necropsy, tissue samples from the two most frequently found lesions (i.e. femoral head necrosis and pericarditis) were collected for histological examination. Proximal femur and heart tissue were fixed in 10% neutral buffered formalin and routinely processed for histology. Bone samples were decalcified in 36.8% formic acid and 6.8% sodium formiate (1/1 v/v) for 14 days. Paraffin embedded tissue sections 4-6 µm thick were stained with haematoxylin and eosin (H&E) before light microscopy.

Hatcheries. The ten broiler flocks that participated in this study originated from 5 different hatcheries, which were all sampled.

Air samples. The occurrence of *E. cecorum* in hatchery air was studied by analyzing air samples taken with the Airport MD8 (Sartorius AG, Göttingen, Germany) coupled to gelatine

filters with a 3 µm pore size and a diameter of 80 mm (type 17528-80-ACD; Sartorius AG). Between 30 and 90 l of air were sampled from the hatcher cabinets during the hatch of offspring from the 5 first broiler breeder flocks (i.e. 5 different hatcheries) participating in the field inventory. The gelatine filters were dissolved in 50 ml of buffered peptone water (BPW) at 37°C for bacteriology. A 0.1 ml sample of the solution was pipetted onto sheep blood agar and incubated for 24 hours under micro-aerophilic and aerobic conditions. Identification of enterococci was performed as described under bacteriology. Detection limits were 10^{4.2} cfu/m³ hatchery air when 30 l of air was sampled and 10^{3.7} cfu/m³ when 90 l was sampled.

Dead-in-shell embryos. Sixty non-pipped eggs per broiler breeder flock (n = 9) were submitted for analysis. Subsequently, 10 fresh dead-in-shell chicks per submission were selected for bacteriological analysis of bone marrow. Additionally, bacteriological analysis of the yolk sac was performed as described under bacteriology from dead-in-shell originating from the last 4 breeder flocks included in the study (flocks 6 through 9).

Molecular identification and epidemiology. *tRNA Intergenic Spacer PCR.* *E. cecorum* isolates originating from the broiler flocks (n = 42) and the broiler breeders (n = 36) were subjected to confirmatory molecular identification using a tRNA Intergenic spacer PCR (Baele *et al.*, 2000).

PFGE. Genetic relatedness of *E. cecorum* isolates originating from the broiler and broiler breeder flocks was studied using PFGE analysis. It was performed on isolates from the pericardium (n=23), bone marrow (n=16) and joint (n=3) from 10 different broiler flocks and, in addition, on isolates from the cloaca (n=31), blood (n=2), bone marrow (n=1) and oviduct

(n=2) from 9 different broiler breeder flocks, following a modification of a procedure described previously (Kuzucu *et al.*, 2005).

Briefly, colonies were collected from blood agar plates and resuspended in EET buffer (100 mM Na₂EDTA, 10 mM EGTA and 10 mM Tris-HCl) to an OD₆₀₀ between 1.2 and 1.4. Agarose gel blocks of genomic DNA were prepared by mixing the cell suspension with an equal volume of 1% low-melting point agarose. The plugs were incubated for 4 hours at 37°C with 1 ml (10 mg/ml) lysozyme. After removing the lysozyme, 1 ml of Depro mixture (0.9 ml EET buffer, 0.5 ml proteinase K and 0.5 ml 20% SDS) was added to the plugs and incubated for 16 hours at 37°C. The Depro mixture was removed and the plugs were washed four times in TE buffer (10 mM Tris-HCl and 1 mM Na₂EDTA). Next, the blocks were washed (for 30 minutes on a rocker platform) with 200 µl of the restriction buffer. Subsequently, the DNA was digested by incubating a quarter of the agarose plugs in 200 µl restriction buffer containing 20 units *Sma*I for 16 hours at 25°C. Before electrophoresis, the plugs were rinsed and loaded on a 1% (w/v) PFGE certified agarose (Bio-Rad Laboratories, Hercules, USA). Electrophoresis was performed using the CHEF-DR III system (Bio-Rad Laboratories). Agarose gels were run in 0.5 X TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) at 14°C and 6 V/cm with an occluded angle of 120°. Pulse times varied from 5 to 15 sec over 10 hours. Agarose gels were stained with ethidium bromide, destained in distilled water and photographed.

Analysis and clustering of PFGE. DNA fingerprints were analyzed using the BioNumerics software package (Applied Maths NV, Sint-Martens-Latem, Belgium). Active zones on fingerprints ranged from 0% to 90%. Band matching was performed using the 5% relative to max filtering criteria. Uncertain bands were excluded from the analysis. The similarity coefficients were calculated using the band-based DICE algorithm with a 1% band position

tolerance window and 1% optimization. The unweighted pair-group method with arithmetic means (UPGMA) was used for clustering. Isolates with $\geq 95\%$ similarity were considered clonal.

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Results

Broilers. *Clinical signs.* The onset of clinical signs (lameness and increased mortality) started at about 2 weeks of age according to the farmers and their veterinarians. Later, decreased growth and a subsequent decrease in flock uniformity became apparent. The flock mortality varied from 3.1 to 8.1% at slaughter, while the percentage standard mortality of other well performing flocks raised earlier on the same farms varied between 2.5 and 3% according to the farmers (Table 1).

Nine out of ten affected flocks included in the study were administered one or more antibiotic treatments. In two cases up to five antibiotic treatments were given. The active compounds used were doxycycline hyclate, enrofloxacin, flumequine, amoxicillin, sulfametoxazol, potassium phenoxymethylpenicillin and trimethoprim sulphachlorpyridazine sodium (Table 1).

Macroscopic lesions. The major macroscopic lesions found at postmortem were pericarditis/hydropericardium (Figure 1), arthritis and femoral head necrosis (Figure 2 and Table 2), which were found in 53, 10 and 50% of clinically affected birds submitted for postmortem, respectively.

Sporadically, other lesions, such as hepatomegaly, necrotic myositis of the breast muscles, twisted legs, slipped tendon, chronic yolk sac inflammation and peritonitis were observed.

Bacteriology. *E. cecorum* was isolated from 29 out of 84 (35%) swabs taken from the pericardium, from 3 out of 11 (27%) joints and 18 out of 62 (29%) bone marrow samples from the femur. Occasionally, other bacteria such as *Escherichia coli*, *E. hirae* and

Staphylococcus aureus were isolated. In total, 42 out of the 50 *E. cecorum* isolates were stored at -70°C for further molecular analysis (Table 2).

Histopathology. *Femur head.* Histopathological examination of the femoral head revealed a bacterial osteomyelitis within the physis and the proximal metaphysis, morphological consistent with enterococcal osteomyelitis.

Lesions were characterized by severe affection and effacement of the bone marrow between primary bone trabeculae, replaced by dense inflammatory infiltrates. The infiltrates consisted mainly of mixed populations of intact and degenerate heterophils, macrophages and few lymphocytes. Interspersed there were foci of necrotic debris, areas with hemorrhage and deposition of fibrin. Scattered vessels contained individual and small colonies of coccoid bacteria, both free in the vascular lumen and intracellular in macrophages.

Focally, few physeal vessels contained large colonies of coccoid bacteria that extended into the physeal cartilage (Figure 3). Within the adjacent area, many physeal vessels were either occluded by thrombi of fibrin and dense mixed inflammatory infiltrates or were effaced by necrotic debris.

Within the proximal metaphysis, there were randomly scattered areas where primary bone trabeculae retain central cartilage. There was also presence of microfractures characterized by fragmented trabeculae that were surrounded by several large active osteoclasts forming Howship's lacunae, indicative of active resorption. These areas also contained loosely arranged and highly vascularized fibrous tissue mixed with mild infiltrates of macrophages and heterophils .

Pericardium. Histopathological examination of the heart and pericardium revealed a chronic pericarditis and epicarditis. Both the pericardium and the epicardium were lined by

hyperplastic and hypertrophic mesothelium and their thickness is diffusely severely increased by deposition of abundant amounts of edematous and highly vascularized fibrous tissue, consistent with granulation tissue. Multifocal scattered fibrin depositions were present on the epi- and pericardial surface as well within the fibrous tissue.

Moderate to marked infiltrates of intact and degenerated heterophils, macrophages, lymphocytes and lesser plasma cells were present throughout the pericardium, epicardium and extending into the subepicardial myocardium. Focally within the pericardial cavity marked infiltrates of intact and degenerate heterophils and macrophages with protein rich and necrotic debris (purulent debris) was present (Figure 4). Small and medium sized blood vessels and lymphatic vessels within the pericardium and epicardium contained hypertrophic endothelium.

Broiler breeders. None of the farmers or hatcheries reported problems with disease, fertility or hatchability in any of the nine broiler breeder flocks analyzed. The age at postmortem ranged from 40 to 62 weeks of age, while the flock size varied from about 8000 to 44000 birds (Table 3). The macroscopic lesions found at postmortem were salpingitis, arthritis, peritonitis, fatty liver, pododermatitis, a cystic ovary and a liver tumor in a limited number of birds. *E. cecorum* was not isolated from any of these lesions. In a few birds in flocks 1, 2 and 8, intestinal worms were found (*Ascaridia*, *Heterakis* and/or *Raillietina*).

Bacteriology. *E. cecorum* was not isolated from any lesions in the broiler breeders. *E. cecorum* was nevertheless isolated from 31 out of 65 (47%) cloacal swabs, from 2 out of 65 (3%) oviduct samples, from 1 out 65 (1.5%) bone marrow samples and 2 out of 25 (8%) blood samples (Table 3).

Hatcheries. Bacteriology.

Air samples. *E. faecalis* was isolated in large amounts from all air samples, while *E. cecorum* was not detected.

Dead-in-shell embryos. Bacteriology of dead-in-shell embryos mainly yielded *E. faecalis* and/or *E. coli*. In the dead-in-shell of one breeder flock *Enterobacter cloacae* was isolated from the bone marrow and yolk sacs.

No bacteria were isolated from the dead-in-shells of two breeder flocks belonging to the same hatchery.

Molecular identification and epidemiology. *tRNA Intergenic Spacer PCR.* All stored isolates (n = 78) were confirmed as *E. cecorum* by means of tRNA Intergenic Spacer PCR.

PFGE. Approximately 50% of isolates per broiler breeder flock had identical banding patterns and belonged to the same clone. Three cloacal isolates from flock 3, one bone marrow isolate from flock 4 and four cloacal isolates from flock 6 showed the same PFGE pattern. Similarly, one oviduct isolate from flock 1, four cloacal isolates from flock 6, one oviduct and two cloacal isolates from flock 7 and five cloacal isolates from flock 8 were clonal. Finally, five cloacal isolates from flock 9 were clonal. In contrast, isolates originating from their diseased offspring showed a much greater genetic variation. However, a pericard isolate from broiler flock 1 and a bone marrow isolate from flock 3 showed the same PFGE pattern. Also two pericard isolates from flock 7 were clonal. Finally, five bone marrow isolates from flock 9 were clonal. The banding patterns of approximately 68% of *E. cecorum* isolates originating from parent stock flocks were clonal to one or more isolates of the same

or other parent flocks. In one broiler flock (nr. 9) five isolates from bone marrow had the same banding pattern as that of two oviduct and various cloacal isolates from three different parent flocks (nrs. 6, 7 and 8), but the offspring flock and the parent birds were not related (Figure 5).

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Discussion

Increased mortality and problems with lameness were reported in Dutch broiler flocks from the year 2008 onwards. Although isolated cases of *E. cecorum*-associated disease were reported earlier (Devriese *et al.*, 2002; Wood *et al.*, 2002), the recent increase in disease outbreaks was striking. This may have been the consequence of the spread of isolates with disease inducing potential or an increased susceptibility of broilers due environmental factors, changes in genetic make-up, the occurrence of underlying infections, etc. Alternatively, a combination of these factors may have been at stake.

Diseased flocks were often treated with antibiotics; however, the effect of antibiotic therapy was temporary despite the fact that named treatments were regularly given based on antibiotic sensitivity testing and repeatedly. This has been speculatively attributed to the fact that *E. cecorum* invades organs such as bone tissue and pericardium and/or remains secluded in cells, where likely insufficient concentrations of antibiotic are obtained. Other possible explanations are reinfections from the environment and predisposing factors (increased susceptibility) as discussed above, which might be present after cessation of the antibiotic treatment.

At postmortem affected broiler flocks were characterized by the occurrence of pericarditis, arthritis and femoral head necrosis in varying degrees, which were found in 53, 10 and 50% of clinically diseased birds submitted for postmortem, respectively. *E. cecorum* was isolated from 35% of samples from the pericardium, 27% of joint samples and 29% of bone marrow samples from femur (Table 2). The relatively low number of positive *E. cecorum* isolations from affected organs at bacteriology could have been the consequence, at least in some cases, of the chronic character of the lesions and/or previous treatments with

antibiotics, despite the fact that participating flocks were required to be free of antibiotics for at least one week.

In broiler flock number 8 characteristic lesions associated with *E. cecorum* infections were hardly found. This was explained by the fact that clinical symptoms associated with *E. cecorum* infections are not specific, therefore occasionally birds may be selected for analysis that are not good representatives for the disease outbreak. Also the frequent antibiotic treatments given may have influenced the occurrence and severity of *E. cecorum* lesions. Alternatively, a combination of both may have occurred explaining the low occurrence of *E. cecorum*-associated lesions in this flock.

Broilers flocks with above average mortality (including culling), signs of lameness and decreased growth and uniformity were reported to the Animal Health Service. The onset of clinical signs i.e. increased mortality and lameness were observed from approximately 2 weeks of age onwards. The mortality of the affected flocks varied from 3.1 to 8.1% at slaughter, while the reference mortality rate including culling of other unaffected flocks on the same farms varied between 2.5 and 3%. In two flocks (4 and 5) the mortality at slaughter appeared low but this was explained by the fact that culling had been performed on a very limited scale (Table 1).

The course of disease, the clinical signs, the macroscopic lesions and the results of bacteriology are in agreement with a field study reported previously (De Herdt *et al.*, 2008). A major difference in the current study was the fact that osteomyelitis of thoracic vertebrae, which has also been described by (Wood *et al.*, 2002), was not observed in the birds, despite the fact that the spinal column was carefully examined for deformities by palpation and by visual inspection after cutting the vertebral column sagittally. This may have been the result of the relatively small number of birds examined or due to the relatively young age of the birds at sampling. A more likely explanation is the fact that veterinarians were asked to submit

lame, not paralyzed, broilers. This is in agreement with the fact that in subsequent pathogenesis research, *E. cecorum* isolates originating from the present flocks were able to induce osteomyelitis of the vertebra T6 in a number of experimental broilers and breeders (unpublished results).

On some farms *E. cecorum*-associated clinical signs and pathology reoccurred between successive flocks, suggesting persistence at the farm, which has been suggested previously (De Herdt *et al.*, 2008). In some cases the source of *E. cecorum* problems found in the broiler flocks was tentatively attributed to the broiler breeder of origin by the practitioners and/or hatcheries, therefore the parent stock and the hatcheries concerned were included in the present field study. *E. cecorum* could not be detected in air samples from the hatcheries, in contrast to *E. faecalis*, which was isolated in large amounts similar to in previous research (Landman *et al.*, 2000). An explanation for this is that *E. faecalis* is the dominant enterococcal species in the gut flora of young chickens during the first few days of life. Approximately 64 to 99% of enterococci isolated at day 1 (Devriese *et al.*, 1991b; Kaukas *et al.*, 1986, 1987) are *E. faecalis*. Consequently, the high number of airborne *E. faecalis* found during air sampling at the hatchery, with highest concentration being present in the hatcher and chicken processing room, was not unexpected.

PFGE was performed in order to address two questions: firstly, to assess whether vertical transmission of *E. cecorum* could have occurred and, secondly, to study whether *E. cecorum* isolates originating from broilers were clonal within and between flocks. The banding patterns of approximately 68% of *E. cecorum* isolates originating from parent stock flocks were clonal to one or more isolates of the same or other parent flocks. In the isolates originating from the broiler flocks this was only the case in about 20% of the isolates. Why *E. cecorum* populations at the broiler farm level show more genetic diversity compared to the breeder farm level is currently unknown. It should be noted that isolates from broilers

originated from lesions, while those from broiler breeders were mainly obtained from cloacal swabs.

Although the isolates from parent stock for the greater part originated from cloacal swabs and not from lesions, it was postulated that they could have been transmitted to the offspring through eggshell contamination during and after lay or internal contamination in the oviduct similar to arthropathic and amyloidogenic *E. faecalis* (Landman *et al.*, 2001; Landman *et al.*, 1999). However, vertical transmission of *E. cecorum* could not be demonstrated by PFGE despite the fact that this bacterium was isolated twice from an oviduct and blood samples, which initially suggested that vertical transmission, might be possible. PFGE is a whole-genome fingerprinting technique well suited for examining whether greater genomic variations between isolates/strains occur, but, it will likely not detect small mutations such as point mutations. Although PFGE does not yield information on genomic changes with time (i.e. isolates showing the biggest difference in banding pattern are not necessarily phylogenetically the most distant), it is unlikely that large genomic changes will arise after one passage (van Belkum *et al.*, 2007). Therefore, the *E. cecorum* outbreaks in broilers within and/or between broiler flocks can not be attributed to a single clone, which either suggests multiple sources of infection and/or the polyclonal nature of *E. cecorum* populations at the farm resulting in opportunistic infections in broilers.

The results presented here indicate that the vertical transmission of *E. cecorum* is not likely to occur in broilers. Therefore, the question on how these birds become infected with this bacterium remains unanswered. Ongoing pathogenesis studies focusing on a variety of infection routes may provide an adequate answer.

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Figure Legends

Figure 1. Heart of a broiler showing macroscopical signs of pericarditis. Note the opacity of the pericardial membrane.

Figure 2. A/ Illustration of early stage of femoral head necrosis. Note the purulent necrotic focus (large arrows) and the fissure (small arrows), likely leading to fracture of the femoral head. B/ Femoral head necrosis observed after dislocation of the hip joint during postmortem.

Figure 3. Overview of a femur physis with osteomyelitis: the bone marrow between primary bone trabeculae is replaced by dense mixed inflammatory infiltrates. Large colonies of coccoid bacteria are present in two vessels, extending into the physeal cartilage (arrows). Adjacent the physeal vessels are effaced by necrotic debris, mixed with fibrin and a mixed infiltrate of heterophils and macrophages. Haematoxylin and eosin, bar=100 μ m.

Figure 4. Pericarditis, epicarditis: multifocal scattered fibrin depositions were present on the epi- and pericardial surface as well within the fibrous tissue. Focally within the pericardial cavity, marked infiltrates of intact and degenerated heterophils and macrophages with protein rich and necrotic debris (purulent debris) were present (arrow). Haematoxylin and eosin, bar=200 μ m.

Figure 5. Restriction endonuclease digestion patterns obtained by PFGE of 42 *E. cecorum* isolates originating from lesions of broilers and 35 isolates from broiler breeders. Numbers 1 to 9 identify the breeder flocks with their corresponding offspring. Orange labels designate isolates from breeder flocks, green labels designate those of broilers and blue labels

designate isolates from the second broiler flock of breeder flock number 8. Identification of the tissue/body fluid of origin of E. cecorum isolates was as follows: B = bone marrow, Blo = blood, C = cloaca, O = oviduct, P = pericardium, S = synovial fluid. Isolates were considered clonal if similarity percentages were ≥ 95 . Clonal groups are indicated with red branches. Broiler isolates showed great genetic variability, while isolates from broiler breeders appeared to be clonal in about 68% of the cases. None of the isolates of the broiler breeders showed a clonal relationship to isolates of their offspring.

Table 1. *Data of broiler flocks*

Flock number	Flock size (n)	Age of broilers at sampling (days)	Age of broiler breeder		% mortality broilers at slaughter ^c	Slaughter age	Antibiotic treatment ^d (n)
			n	flock ^b (weeks)			
1	58000	18	6	32	8.1	45	Yes (4)
2	36990	21	13	38	5.7	38	Yes (2)
3	31390	30	10	31	5.7	45	Yes (5)
4	18750	17	12	36	3.6	37	No
5	29200	16	11	56	3.1	41	Yes (1)
6	24300	25	14	54	4.7	41	Yes (5)
7	33000	14 + 18	6 + 11	50	5.8	41	Yes (1)
8 ^a	24200	36	15	31	6.5	42	Yes (4)
9 ^a	30000	17	12	35	5.4	37	Yes (1)
10	48000	13	11	33	4.9	35	Yes (2)

^aBoth flocks originated from one broiler breeder flock.

^bAge of breeders at the time that eggs yielding the offspring flock were produced (breeder birds were sampled about 7 to 14 weeks later).

^cStandard mortality of other unaffected flocks on these farms was approximately 2.5- 3%.

^dDoxycycline hyclate, enrofloxacin, flumequine, amoxicillin, sulfametoxazol, potassium phenoxymethylpenicillin, trimethoprim sulphachlorpyridazine sodium.

Table 2. *Macroscopic lesions and isolation of E. cecorum from broilers with clinical signs*

Flock number	Bird examined (n)	Macroscopic lesions			Isolation of <i>E. cecorum</i>		
		Pericarditis/hydro-pericardium	Arthritis	Femoral head necrosis	Pericardium	Joint	Bone marrow
1	6	5	0	3	5/5	n.d.	2/3
2	13	10	0	0	2/5	n.d.	n.d.
3	10	7	1	9	1/5	n.d.	1/4
4	12	0	5	4	1/5	0/3	1/4
5	11	5	1	3	0/4	1/1	n.d.
6	14	9	3	12	3/12	2/3	3/10
7	6 + 11	11	1	11	9/17	0/1	4/11
8	15	0	1	0	1/12	0/1	1/12
9	12	8	0	11	3/10	0/2	4/11

10	11	9	0	7	4/9	n.d.	2/7
Total	121	64	12	60	29/84	3/11	18/62
Percentage		53	10	50	35	27	29

n.d.= not done

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Table 3. *Data of broiler breeder flocks*

Flock number	Bird examined (n)	Flock size ^a (n)	Age at postmortem (weeks)	Isolation of <i>E. cecorum</i>			
				Cloaca	Oviduct	Bone marrow	Blood
1	6	20270	40	0/5	1/5	0/5	0/5
2	10	43610	48	1/5	0/5	0/5	2/5
3	8	24860	41	5/5	0/5	0/5	0/5
4	5	18100	44	0/5	0/5	1/5	0/5
5	6	7840	63	0/5	0/5	0/5	0/5
6	10	20930	62	8/10	0/10	0/10	n.d.
7	12	12310	59	3/10	1/10	0/10	n.d.
8	10	17645	45	6/10	0/10	0/10	n.d.
9	12	24870	42	8/10	0/10	0/10	n.d.
Total	79			31/65	2/65	1/65	2/25

Percentage	47%	3%	1,5%	8%
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^aFlocks were kept in several houses if the flock size was >10000 birds, but always originated from one grand parent flock .

n.d. = not done.

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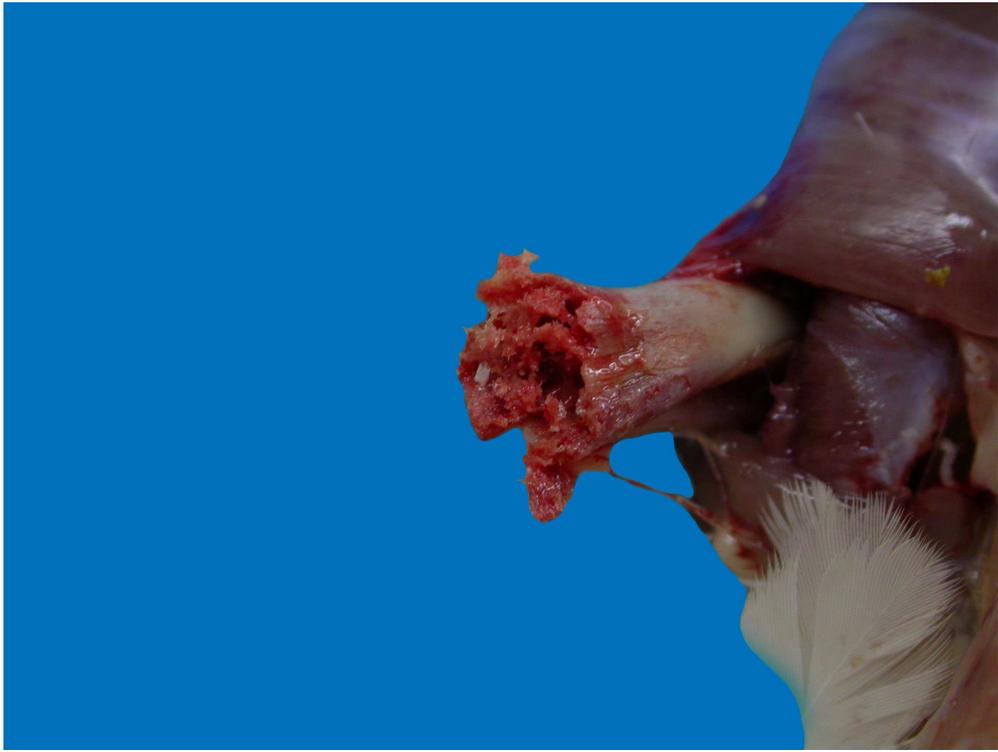
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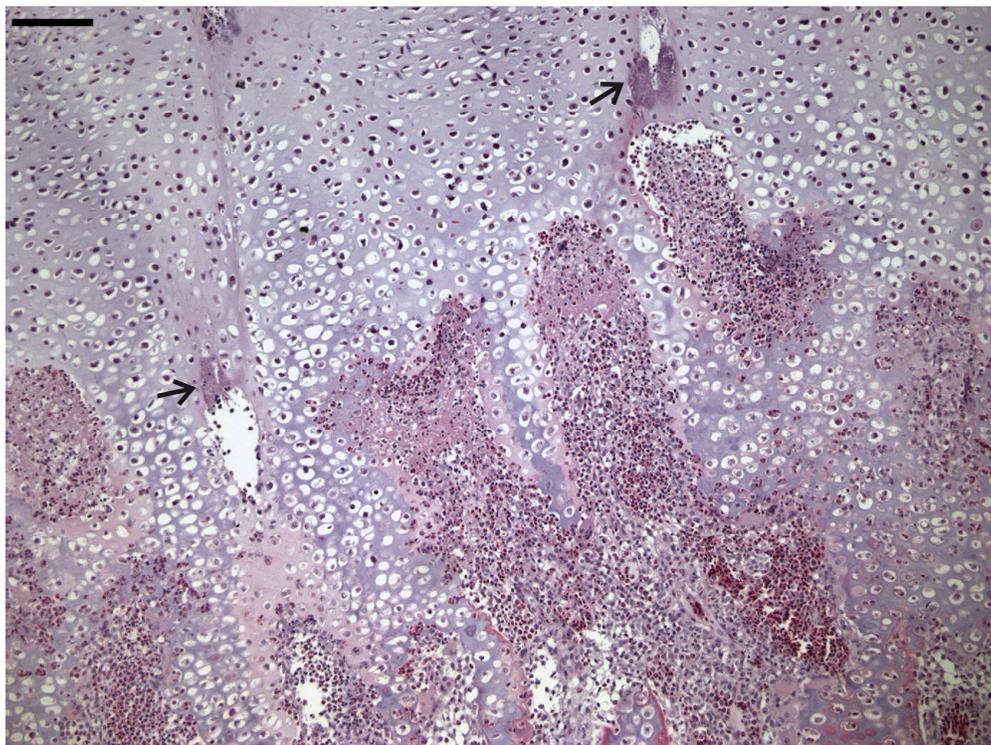
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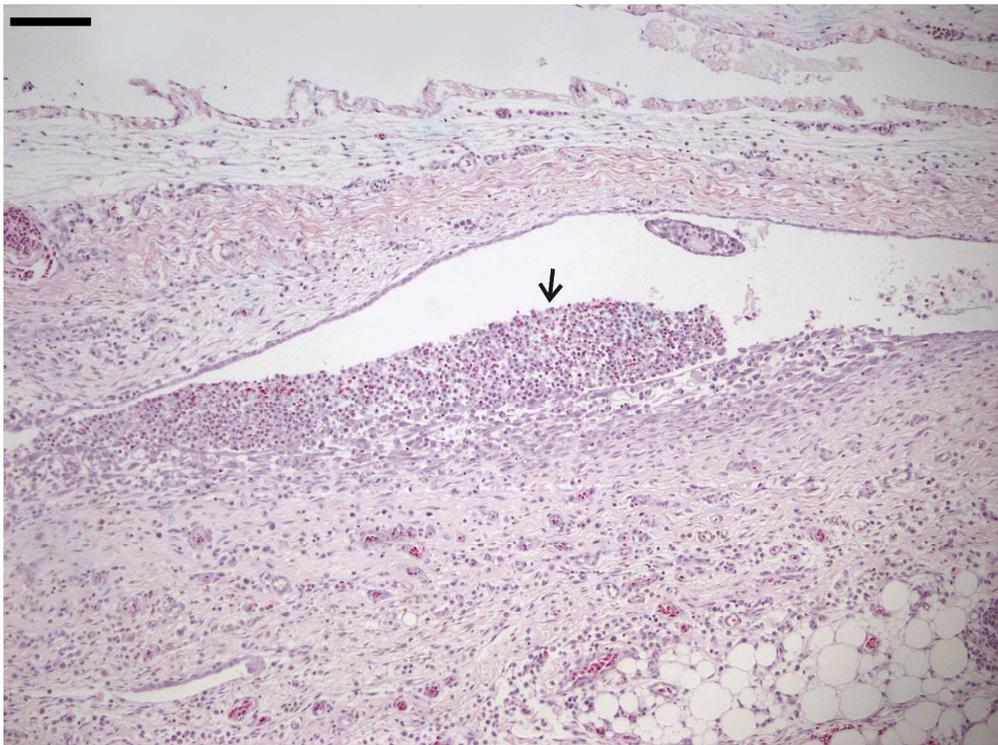
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