A survey of *Enterococcus cecorum* isolates from a kinky-back affected broiler farm.

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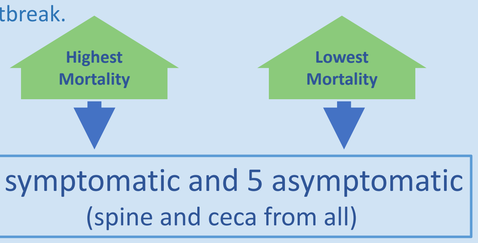
Abstract

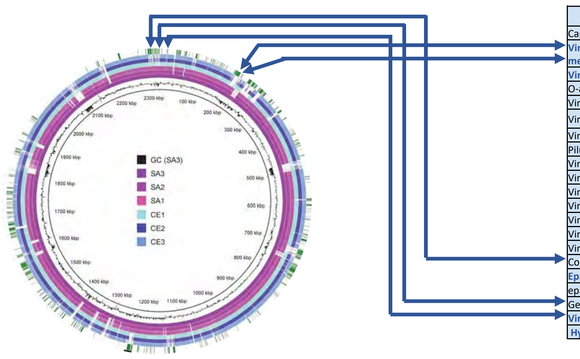
Enterococcal spondylitis (ES), commonly known as kinky-back, is an inflammatory disease in poultry caused by infection and inflammation of the avian caudal thoracic vertebrae. ES has garnered growing attention in the industry due to high mortality and loss associated with the disease in recent years. *Enterococcus cecorum* has been isolated from the spines of ES affected birds and has been identified as the causative bacterial agent. *E. cecorum* is also a predominant commensal bacterium in the avian gastrointestinal tract. Previous research has indicated that virulent strains of *E. cecorum* have emerged in commercial poultry settings, which may explain the rapid increase of ES cases observed in broiler systems. To test this hypothesis, we collected *E. cecorum* isolates from the cecal contents and spines of 10 ES affected and 10 non-ES affected 42 day old birds from a southern US broiler farm suffering high ES mortality. The birds were sourced from two houses on the farm; 5 affected and 5 unaffected from the house experiencing the highest ES mortality (25.6%) and the same from the house experiencing the lowest mortality (12.8%). All *E. cecorum* isolates were screened by PCR for a set of five presumptive virulence factors and characterized using RAPD-PCR fingerprinting. Virulence screening results show that, of the factors tested, there is no single virulence factor or combination of virulence factors that correlates with *E. cecorum* isolates collected from ES affected birds or spines. Moreover, RAPD fingerprinting shows high diversity among all isolates, indicating a lack of genetic similarity. Clusters identified with greater than 95% similarity contain isolates from affected and unaffected birds and from both spine and cecal samples, preventing any distinctions to be made between virulent and commensal isolates. These results suggest that infection by *E. cecorum* may be host-associated (i.e. gut barrier integrity, immune status) and related to *E. cecorum* concentration in the gut rather than exclusively contingent on emergence of distinct virulence factors.

Design

Sample Collection

Ceca and spines were collected from 5 recently symptomatic broilers (birds exhibiting lameness, but not showing signs of prolonged symptoms, such as dehydration or blindness) and 5 asymptomatic broilers from both the highest enterococcal spondylitis (ES) mortality house (25.6%) and the lowest ES mortality house (12.8%) from a southern US broiler farm experiencing an ES outbreak.





Gene	USA Broiler Pathogen	USA Broiler Pathogen	USA Broiler Pathogen	EU Breeder Pathogen	EU Layer Pathogen	EU Broiler Pathogen	USA Broiler Commensal	USA Broiler Commensal	USA Broiler Commensal	EU Layer Commensal	EU Layer Commensal
Capsular region	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Virulence Region 1 membrane	Present	Present	Present	Present	Present	Partial	Partial	Partial	Partial	Partial	Partial
Virulence Region 1 hyp. prot.	Present	Present	Present	Present	Present	Partial	Partial	Partial	Partial	Partial	Partial
O-antigen	Present	Present	Present	Present	Present	Present	Absent	Absent	Absent	Absent	Absent
Virulence Region 2	Present	Present	Present	Present	Present	Present	Absent	Absent	Absent	Partial	Partial
Virulence Region 3	Present	Present	Present	Present	Present	Present	Absent	Absent	Absent	Absent	Absent
Virulence Region 4/CRISPR	Present	Present	Present	Present	Absent	Present	Present	Present	Absent	Present	Present
Pilus Region	Present	Present	Present	Present	Present	Partial	Partial	Partial	Present	Present	Present
Virulence Region 5	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Virulence Region 6	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Virulence Region 7	Absent	Absent	Present				Absent	Absent	Absent		
Virulence Region 8		Absent	Present							Absent	Absent
Virulence Region 9	Present	Present	Present	Present	Present	Present	Absent	Absent	Absent	Present	Present
Virulence Region 10	Present	Present	Present	Absent	Present	Absent	Absent	Absent	Absent	Present	Present
Virulence Region 11		Absent	Present							Absent	Absent
Collagen region	Present	Present	Present	Present	Present	Present	Absent	Absent	Absent	Present	Present
Epa Locus	Present	Present	Present	Present	Present	Partial	Partial	Partial	Partial	Partial	Partial
epaL-P	Present	Present	Present	Present	Present	epaL-O	Absent	Absent	Absent	Absent	Absent
Genes between epaH and L	Present	Present	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent
Virulence Region 12	Present	Present	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent
Hypothetical prot. 2298	Partial	Partial	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent

Figure 1: A comparative analysis of 6 *Enterococcus ceorum* genomes (three virulent strains [SA1, SA2, SA3] and three commensal strains [CE1, CE2, CE3]) from Borst, et al. (2015).

Table 1: A comparison of the presence or absence of several candidate virulence factors among full genomes of various *Encercoccus cecorum* strains isolated from poultry, both from the United States (US) and the European Union (EU).

Methods

E. cecorum Isolation

Cecal samples were plated onto de Man, Rogosa and Sharpe (MRS) agar and incubated at 35 °C. Multiple isolates were picked from each birds, grown up in MRS broth and gDNA was isolated.

Spinal samples were opened to reveal the caudal thorasic vertebral column and swabbed. Swabs were plated on phenylethyl alcohol (PEA) blood agar and incubated at 35 °C. Presumptive *Enterococococcus* isolates were picked and grown up in MRS broth and gDNA was isolated.

Cecal and spinal *Enterococcus cecorum* isolates were identified by 16S rRNA gene sequencing. Non-*E. cecorum* isolates were not included in downstream analysis.

E. cecorum Virulence mPCR and RAPD-type

All confirmed *E. cecorum* isolates were subjected to a multiplex PCR (mPCR) designed to detect 5 candidate virulence factors. mPCR products were analyzed on a capillary gel electrophoresis system (Fragment Analyzer™, Advanced Analytical Technologies, Inc.). A pairwise comparison of the results was conducted using the binary Dice correlation (BioNumerics, Applied Maths)

All isolates were also characterized by random amplification of polymorphic DNA (RAPD) PCR fingerprinting (RAPD primer 2). RAPD PCR products were run on a capillary gel electrophoresis system (Fragment AnalyzerTM, Advanced Analytical Technologies, Inc.) and a RAPD fingerprint dendrogram was constructed using the Dice method with the unweighted pair group method with arithmetic mean (UPGMA) algorithm for pairwise analysis (BioNumerics, Applied Maths).

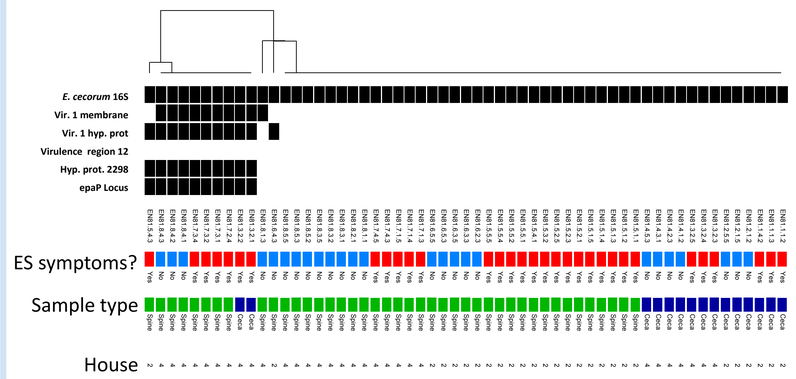


Figure 2: Binary (presence/absence) pairwise dendrogram of the results of the mPCR designed to detect the 5 candidate *E. cecorum* virulence factors (Virulence region [VR] 1 membrane protein, VR 1 hypothetical protein, VR 12, hypothetical protein 2298, and the epaP locus) of individual isolates collected from spines and ceca of affected and unaffected birds. This analysis also includes the *E. cecorum* 16S rRNA gene, which they all possess.

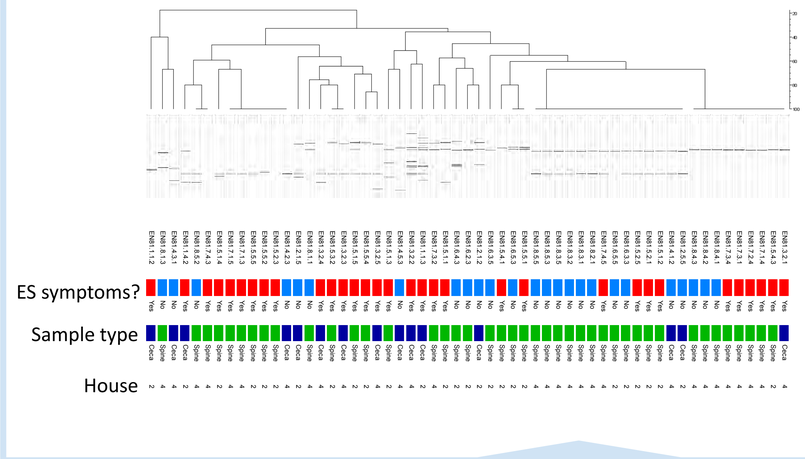


Figure 3: A pairwise dendrogram of the RAPD fingerprints of individual *E. cecorum* isolates collected from spines and ceca of affected and unaffected birds.

Citations

Borst, L.B., et al. (2015). Comparative genomic analysis identifies divergent genomic features of pathogenic *Enterococcus cecorum* including a type IC CRISPR Cas system, a capsule locus, an epa-like locus, and putative host tissue binding proteins. PloS One *10*, e0121294.

Dolka, B., et al. (2015a). Draft Genome Sequences of Five Clinical *Enterococcus cecorum* Strains Isolated from Different Poultry Species in Poland: TABLE 1. Genome Announc. *3*, e01082-15

Dolka, B., et al. (2015b). Draft Genome Sequences of Two Commensal *Enterococcus cecorum* Strains Isolated from Chickens in Belgium. Genome Announc. *3*.

Borst, L.B., et al. (2012). Molecular epidemiology of Enterococcus cecorum isolates recovered from enterococcal spondylitis otbreaks in the southeastern United States, Avian Pathology,41:5, 479-485

Results

-Cecal *Enterococcus cecorum* was detected in 30% of birds exhibiting symptoms of ES, while E. cecorum was detected in 50% of ceca from asymptomatic birds. Of the total isolates collected from all ceca, 16% were determined to be *E. cecorum.*

-At least one *E. cecorum* isolate was detected in all spinal samples except one sample from an asymptomatic bird and one symptomatic bird.

-Results of multiplex PCR of candidate virulence factors (Fig. 2) did not correlate with ES symptoms or anatomical location (spine/ceca). However, isolates that possess any of the candidate virulence factors tend to possess others. Virulence region 12 was not detected in any isolates.

- RAPD-typing of the *E. cecorum* isolates (Fig. 3) revealed a genetically diverse group of isolates. Clustering did not correlate with either ES symptoms or anatomical location.

Discussion

Enterococcus cecorum was detected in the spines of both symptomatic and asymptomatic birds. The presence of *E. cecorum* in the spines of asymptomatic birds may be due to the progression of the disease wherein the infection is established, but symptoms have not yet manifested. Individuals may have varying degrees of resistance to ES, possibly due to host immune or gut integrity phenotypes or a protective microbiome.

The lack of data correlating spinal *E. cecorum* isolates to virulence factors (fig. 2) or genetic relatedness (Fig. 3), provides evidence that the emergence of ES in broilers may not be exclusively contingent on clonal, pathogenic strains. Previous studies have shown *E. cecorum* isolates obtained from clinical ES lesions to be genetically identical by pulse field gel electrophoresis, while isolates collected from the GI tract (considered commensal) are both distinct from ES lesion isolates and genetically diverse (Borst, 2012). This data indicates that the majority of spinal isolates belong to many genetically distinct groups, with all major clusters also containing cecal isolates, implicating the GI tract as a reservoir for ES agents. This suggests that ES may be host-associated (i.e. gut barrier integrity, immune status) and related to enteric *E. cecorum* concentration rather than exclusively contingent on distinctive virulence factors.