

Understanding *Gallibacterium*-Associated Peritonitis in the Commercial Egg-Laying Industry

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Introduction

Peritonitis is an important disease of laying hens in commercial table egg operations. Despite its importance, the etiology and pathogenesis of this disease have not been completely clarified. Two bacterial species are routinely isolated from cases of avian peritonitis: avian pathogenic *Escherichia coli* (APEC) and *Gallibacterium anatis*. Although APEC's contributions to peritonitis have been well documented (7), the exact role played by *G. anatis* in the pathogenesis of this disease has only recently been recognized. Danish researchers have reported that *G. anatis* is frequently associated with peritonitis lesions (1-6), with or without concurrent APEC isolation. While current wisdom suggests that APEC and *G. anatis* infections are opportunistic in nature, co-infection studies involving these two species inoculated into the oviduct of laying hens were highly lethal towards chickens. Such research suggests that the pathogenesis of peritonitis might be quite complex, and could involve interplay between multiple microbial species (i.e., polymicrobial infection). A better understanding of APEC and *G. anatis* is needed in order to elucidate their pathogenesis.

Although much effort has been devoted to understanding APEC's virulence mechanisms, little effort has been directed towards the study of the virulence mechanisms of *G. anatis*. Since epidemiological and *in vivo* studies suggest that *G. anatis* plays an important role in avian peritonitis, lack of understanding of *G. anatis* virulence renders control of peritonitis problematic. In this study, we performed draft genome sequencing on two *G. anatis* strains, one classified as highly virulent and another classified as avirulent. We compared these two genomes to identify putative *G. anatis* virulence genes. We then determined the prevalence of some of these genes among *G. anatis* isolates from healthy hens and from peritonitis lesions, and used these data in an effort to examine possible correlations between clonality and virulence genotype.

Materials and Methods

Objective 1. Sequence the genomes of a virulent and an avirulent strain of *G. anatis*. Through our collaborators in Denmark, we obtained the most well-characterized set of *G. anatis* isolates in the world. These isolates have been extensively characterized for their ability to cause disease, phenotypic characteristics, and phylogeny using a variety of molecular genetic techniques (1-6). This collection includes strains isolated from the lesion sites of diseased birds as well as those isolated from the upper respiratory tracts of healthy birds. From this collection of *G. anatis* isolates, we selected the avirulent *G. anatis* type strain ATCC 43329 for draft genome sequencing (6). This strain was isolated from the flora of a healthy bird in Europe. Additionally, we collaborated with Dr. Anders M. Bojesen at the University of Copenhagen to compare this sequence with the highly virulent type strain 12656/12, recently sequenced in his laboratory (6). We then performed draft sequencing on a 3rd *G. anatis* strain isolated from the peritonitis lesion of a laying hen from a commercial operation in Iowa. *Draft Sequencing.* The three strains selected for sequencing were grown overnight in BHI broth. Cultures were then pelleted, and the genomic DNA from each pellet was extracted using a commercial kit and size fractionated into 300-500 bp fragments. A ligated library was prepared at the University of Minnesota's Biomedical Genomics Facility. Pyrosequencing was performed on the GS FLX instrument involving 2 full 70 X 75 mm plates.

Finishing / Gap Closure. Software associated with the 454 Sequencer acquired an .sff image read for each fragment sequenced. These reads were processed and assembled using the Newbler Assembler. The initial assembly was then converted into an .ace file and imported into SeqMan software from DNASTAR (Madison, WI). Finishing was performed on these strains the pooled primer technique previously described and successfully applied to other projects. Primers were designed outside of repeat regions to ensure accuracy, and PCR products were obtained using a high fidelity, proofreading *taq* polymerase. Products were then sequenced and added to the assembly to aid in gap closure.

Objective 2. Identify putative virulence genes of *G. anatis* using comparative genomics. The finished sequences were analyzed for ORFs using GeneQuest from DNASTAR (Madison, WI) in cooperation with Manatee from The Institute for Genomic Research (Rockville, MD), followed by manual inspection. Translated ORFs were compared to known protein sequences using BLAST. The sequences and annotations of these strains are currently being finished and inspected, and will be deposited in GenBank upon completion. Using a local BLAST tool, the three *G. anatis* sequences were compared to each another for nucleotide and protein similarities. From these analyses, we identified a subset of DNA regions present in the virulent *G. anatis* strains, but absent from the avirulent commensal *G. anatis* strain. These pathogen-specific regions will be the focus of future studies involving the virulence mechanisms of *G. anatis* that cause peritonitis in laying hens.

Objective 3. Determine the prevalence and putative virulence genotypes of *G. anatis* isolates in healthy hens and in hens with peritonitis. Chickens with peritonitis lesions were collected from three commercial egg-laying operations. Additionally, commensal *G. anatis* isolates were taken from the same three commercial egg-laying operations. *G. anatis* isolates were also collected from houses in which no disease was present. Sites sampled included the respiratory tract (trachea), intestinal tract (crop, small intestine, cloaca), reproductive tract (oviduct), and peritoneal cavity (surface of the ovary). A total of 100 samples were collected. Swabs were streaked on sheep blood agar and incubated overnight at 37° C. For *G. anatis* isolation, both hemolytic and non-hemolytic colonies exhibiting biochemical traits consistent with *G. anatis* (indole⁻, urea⁻, trehalose⁺, maltose⁻, xylose⁺, arabinose⁻, mannitol⁺, and sorbitol⁻) were picked into BHI broth overnight and frozen in glycerol. PCR products specific for the 16S rRNA gene were used to verify *G. anatis*. In an effort to determine if the isolates from sick and healthy birds differed in their genetic content, multiplex PCR panels were designed to identify pathogen-specific genes. Two-way cluster analysis was used to identify correlations between the isolates and genes studied.

Results and Discussion

Three *G. anatis* genomes were subjected to draft sequencing using the GS FLX technology. The initial assemblies yielded from 75-125 contiguous sequences per genome, at an average fold-coverage of 25X-30X, with total estimated genome sizes ranging from 2.5-2.8 Mbp. Gap closure has reduced the number of contigs to less than 20 per genome. These contigs have been annotated and analyzed for pathogen-specific genes. From this analysis, 24 genes were selected for further study (Fig. 1). Multiplex PCR and Southern hybridizations were used to examine the prevalence of these 24 genes among 100 *G. anatis* isolates collected from commercial layer operations in Iowa over a 2-year-period. The genes identified as highly prevalent among these isolates included genes of two different ABC transporter systems, possibly involved in iron transport; capsular biosynthesis genes; heme utilization genes; and a putative outer membrane protease (Fig. 1). These results provide targets for future work involving study of the virulence mechanisms of *G. anatis* or vaccine development. Multilocus sequence analysis (MLST) of 94 isolates, combined with the genotyping data, revealed that certain clonal types appear to dominate intra-flock, and intra-farm (Fig. 2). Also, genotype and clonal type do correlate with one another to at least some degree, since certain MLST clusters tended to be more likely than other to possess a gene or system.

Overall, this project produced the first genome sequences of *G. anatis* implicated in peritonitis in commercial layers. These sequences are powerful tools for future work targeting vaccine candidates with which to control peritonitis in laying hens. The genotyping and MLST schemas resulting from this work is publicly available (<http://www.umn.edu/~joh04207/>), and will be highly useful from a diagnostic perspective. Their refinement will result in the ability to effectively predict the

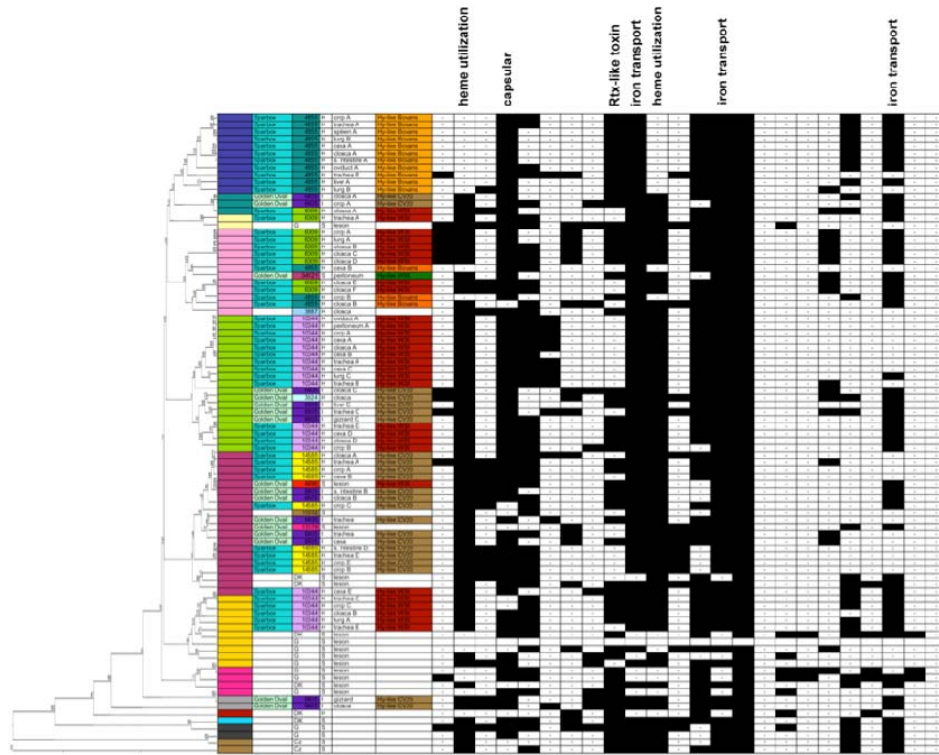


Figure 2. MLST analysis of 94 *G. anatis* isolates combined with genotyping results. Column 1 colors depict MLST cluster, column 2 colors depict company, column 3 colors depict farm, column 4 depicts swabbing site, and column 5 depicts bird type. The remaining 24 columns depict the presence (black) or absence (white) of a gene among each isolate.

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