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Microbiological Investigations of Bacteria Infecting Yolks of Broiler and Broiler Breeder Embryos and Newborn Chicks

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Abstract

Chicken embryo mortality has been attributed to bacterial infections resulting in a decline in hatchability, and significant losses in the broiler industry. The aim of this study was to investigate the vertical transmission of specific bacterial pathogens from breeder hens to subsequent broiler flocks. In our sampling of 360 commercial broiler eggs, we isolated *Enterococcus gallinarum* and *Enterococcus faecalis*, from yolks of 17-day incubated eggs. *Enterococcus avium* was recovered by culture enrichment from 1 of 31 unincubated yolks. From 297 eggs yolks from broiler breeders incubated for 17 days we isolated *E. faecalis, Globicatella sanuinis,* and *E. gallinarum,* in some cases in high numbers. We then performed microbiological sampling of day-of-hatch chicks for broilers from a commercial hatchery. Culture enrichment and sampling of intestine and residual yolk sac suggested only yolk sac was likely to yield bacterial growth, and could be sampled directly by swabs onto growth medium. The most common isolate from sampling 30 newly hatched broiler chicks was *E. faecalis* from 16 different chicks. However, *E. coli* was recovered as a mixed infection with *E. faecalis* in 3 of those 16. Residual yolk sacs were then swab-sampled from day-of-hatch broiler breeder chicks. *E. faecalis* was recovered from 7 out of 40 chicks. Thus, yolk infections by Enterococcus species should be of concern in the broiler industry, and could be contributing to reduced hatchability, early chick death, and transmission of bacteria from hens to subsequent flocks.

Keywords: broiler; yolk infection; microbiology; hatchability; vertical transmission

Introduction

The ever-increasing demand for chicken meat and eggs has necessitated modern poultry farming to meet the growing needs of consumers. While chicken breeding has undoubtedly enhanced chicken production, it also presents challenges in terms of animal health and welfare [1]. One of these challenges lies in the incidence of embryonic mortality during the incubation period, which can result in significant economic losses [2]. This early-stage mortality can be attributed to a range of infectious and non-infectious agents [2–7] , reducing hatchability. In 2023, the USDA National Agricultural Statistics Service reported a recent 3% decline in the hatchability of broiler chicks in the United States [8], highlighting hatchability as an urgent concern.

Some studies have reported contamination of eggs by *Salmonella* Enteriditis during egg formation in the reproductive tract [9–11]. Studies have suggested that Salmonella present in the oviduct could contaminate the albumen, inner and outer shell membranes or the eggshell [9,12,13] implicating the albumen as the most probable site of contamination. Others, found the yolk as the most frequently contaminated, suggesting that the ovary is the first site of bacterial colonization [4,9,11,14–17]. A wide range of aerobic bacterial genera have been isolated from non-viable chicken embryos including Escherichia, Micrococcus, Salmonella and Staphylococcus [3,18].

Avian pathogenic *E. coli* present a range of infections, including yolk sac infections, omphalitis, respiratory infections, swollen head syndrome, pericarditis, airsacculitis, perihepatitis, arthritis, osteomyelitis, septicemia, and cellulitis [19]. In addition, Enterococcus species, which are known commensals in the gastrointestinal tracts of chickens, have also been associated with a range of pathological issues in broiler chickens [20–26]. Notably, *Enterococcus cecorum* has been linked to emerging and economically significant infectious diseases in the poultry industry, including sepsis in young chicks, as well as osteomyelitis and spondylitis in older chickens [22,25,27–31].

Therefore, the primary objective of this study was to isolate and identify the specific bacteria associated with yolk infections in embryos and newly hatched chicks, in commercial broilers and broiler breeders.

Materials and Methods

Media

Media and supplements included CHROMagar Orientation (DRG International, Springfield Township, NJ), tryptic soy broth and bacteriological agar (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ); chicken serum (Life Technologies, Gaithersburg, MD). Media abbreviations: CO-CHROMagar Orientation; TSA – tryptic soy agar; TSB - tryptic soy broth; TSBcs - TSB + 2% chicken serum .

Bacterial Cultures

All cultures from this work were grown from single colonies. Cultures were archived in 40% glycerol at -80°C. Working stocks were passaged monthly on TSA slants which were stored at 4°C.

Embryo Microbial Sampling

Fertilized broiler eggs were procured from a commercial hatchery in Arkansas in July 2022 while fertilized broiler-breeder eggs were procured from a primary breeder in September 2022 (company names withheld subject to Non-Disclosure Agreements). The eggs were washed in dilute dish soap 2% bleach mixture and then thoroughly rinsed with tap water. Eggs were stored for up to one week at 18 ^{o}C at 60% humidity.

For egg yolk enrichment cultures, 15 ml of TSBcs was added to a sterile 100 ml wide-mouth, media bottle. Unincubated eggs were cleaned, exterior rinsed with 95% ethanol, and air dried. Using ethanol rinsed, gloved hands, eggs were gently cracked and as much as possible of the egg albumen was discarded. The yolk was then transferred to the media bottle, vigorously mixed to break the yolk, and incubated overnight at 37°C with continuous shaking. On the next day, a sterile swab was used to streak the yolk mixture onto a CO plate which was incubated overnight at 37 ^{o}C.

For embryo sampling, eggs were set in groups of 90 eggs on successive days. Incubation was in an auto-rotated incubator (NartureForm Hatchery Technologies LLC, Jacksonville, FL), maintained at 37.2°C and 60% relative humidity. On the 18th day of incubation, the eggs were candled, surface sterilized with 95% ethanol, and allowed to air-dry in a laminar flow hood. Using ethanol rinsed, gloved hands, each egg was then broken into a sterile (rinsed with 70% ethanol and oven-dried) plastic weigh boat. Embryos were scored as dead/sick-looking embryos, live embryos, or non-fertile/early dead. Using sterile forceps and scissors, the abdominal cavity of each embryo was opened. For liver sampling, a portion of the liver was excised and rubbed over the surface of a CO plate. For yolk sac sampling the membrane was cut and the contents sampled by insertion of a sterile cotton swab which was then streaked over the surface of a CO plate. Plates were incubated overnight at 37°C under 5% CO_2.

Microbial Sampling of Day-of-Hatch Chicks

Day-of-hatch broiler chicks were acquired from a local commercial hatchery in September 2024. Scissors were used to remove the head, wings and legs, then the dermis was peeled off and the carcass sprayed with 95% ethanol. Carcasses were either sampled immediately or placed individually in plastic zip lock bags and frozen at -80 °C; until thawed for microbiological evaluation. For sampling the carcass was sprayed with 95% ethanol, then placed in a sterile weigh boat in a laminar flow hood to dry. Using sterilized forceps and scissors the abdomen was opened. Sampling was either through collection of tissue samples with sterile scissors and forceps or with sterile cotton swabs. Tissue samples were transferred into 5 ml TSB, incubated with shaking overnight, and then streaked with a loop onto CO plates for individual colonies. Swabs of internal surfaces or residual yolk sac contents, were spread directly onto CO plates that were incubated at 37 °C overnight for colony identification.

Evaluation of Microbial Sampling

In all cases, CO plates were evaluated after incubation overnight. Representative colonies were purified for single colony isolation by restreaking on CO plates for verification of uniform colony color and morphology.

Paramagnetic Bead DNA Extraction for PCR

DNA from cultures were extracted by lysis with NaOH and DNA capture with magnetic beads based on published procedures [32], using either: 1) a sterile toothpick to sample individual colonies into 90 µL of sterile deionized water in a 1.5 mL microfuge tube, or 2) 20 µL of an overnight culture in TSB was pelleted in a 1.5 mL microfuge tube and resuspended in 90 µL of sterile deionized water. Cells were lysed by addition of 10 µL of 1 M NaOH and the mixture incubated at room temperature for 10 minutes. Silica coated paramagnetic beads (5 µL; PureSil-Silica beads, BioChain Institute Inc., Newark, CA) were added, along with 100 µL of bead binding buffer. The resulting mixture was vortexed, then incubated for 10 minutes at room temperature. The paramagnetic beads were subsequently captured using a magnetic rack. The supernatant was carefully pipetted off and discarded. Following this, the captured beads were released from the rack and washed with 150 µL of 70% ethanol followed by magnetic capture and solution discarded– this rinse step was performed twice. Microfuge tubes were opened, and the beads were allowed to air-dry for 6 minutes. Subsequently, 50 µL of Te (10 mM TrisCl, 0.1 mM EDTA, pH 7.5) was added to the beads and incubated at room temperature for 5 minutes. The tubes were returned to the magnetic rack to capture the beads, and the eluate was collected into a new 1.5 mL microfuge tube for subsequent qPCR analysis.

Bacterial Isolate Species Identification

The Illumina 16S metagenome primers which amplify the V3-V4 region of the 16S rDNA gene, F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGA; R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAG; were synthesized by Integrated DNA Technologies (Coralville, IA USA). PCR (20 µL) included 2 µL of 10x Taq buffer (500 mM TrisCl pH 8.3, 10 mM MgCl2, 3mg/ml bovine serum albumin), 0.2 µL of 20 mM dNTPs, 0.2 µL of 50 µM primers, 1 µL of 20X EvaGreen® Dye (Biotium Inc. Fremont, CA), and 0.1 µL of 40U/µL Taq Polymerase. Triplicate reactions were performed in 96-well plates and analyzed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc. CA). Cycling parameters were an initial denaturation step at 90°C for 45 seconds, followed by 5 cycles of denaturation at 90°C for 15 seconds, annealing at 71.5°C for 15 seconds, and extension at 72°C for 60 seconds. Afterward, 35 cycles were performed with identical parameters, with plate reads following each cycle. Subsequently, a High-Resolution Melt (HRM) analysis was carried out, which entailed incubation at 72°C for 120 seconds, 90°C for 60 seconds, and 65°C for 120 seconds. The melt gradient was from 70°C to 90°C, in increments of 0.1°C for 5 seconds with a plate read.

The amplified PCR products were purified with RapidTip® (Chiral Technologies, West Chester, PA, USA) and shipped to Eurofins Genomics LLC (Louisville, KY) for capillary sequencing. Sequence data were inspected and edited using SeqBuilder (LaserGene v 17.3, DNAStar, Madison, WI) and subsequently used for BLASTn searches at NCBI of the bacterial 16S rDNA database.

Results

Egg Microbiology

This project was initially conceived and implemented to screen for vertical transmission of *Enterococcus cecorum* which has recently been reported as causing early onset sepsis, in broiler-breeders and broilers [30,33]. We obtained fertile eggs from two specific flocks that had experienced *E. cecorum* outbreaks in 2022. The first evaluation was for fertile broiler-breeder eggs (from a broiler-breeder parent flock). The second evaluation was for fertile broiler eggs.

We first investigated microbial contamination of unincubated eggs through direct enrichment culture of whole yolks. As a positive control we spiked an additional yolk with ca. 10^3_CFU of *E. cecorum* 1415 [30,34] which, after the overnight enrichment, and streaking onto a CO plate produced a uniform population representative of *E. cecorum* (i.e., very small white colonies with green halo). For the 31 yolks examined by culture enrichment, 30 were negative for growth while one yolk produced a uniform culture identified as *Enterococcus avium*.

We incubated 297 broiler-breeder eggs for 18 days. Candling revealed 21 as nonfertile or very early dead, so these early deads were sampled for yolk sac only. One yolk produced a lawn of very small green colonies evaluated as *Globicatella sanguinis*. One yolk produced a single colony of *Enterococcus faecalis*. One yolk produced a green lawn of *Enterococcus gallinarum*. For the developed eggs, 37 embryos were evaluated as dead or stressed and were sampled for liver and yolk sac microbiology. There were no colonies from liver tissue samples. One yolk produced a lawn of *E. gallinarum* and one a lawn of *E. faecalis*. For 239 normal embryos, all were sampled for yolk sac microbiology with 175 sampled for liver tissue microbiology. One liver sample produced a single colony determined to be *Streptococcus salivarius*. Two yolk sac samples produced single colonies determined to be *Lactobacillus cremoris,* and a Bacillus species (licheniformis or safensis). One yolk sample produced 10 colonies determined to be *E. gallinarum*.

For the second evaluation of broiler eggs we incubated for 18 days and only yolk sac microbiology was investigated. We scored 22 eggs as nonfertile or early dead, with one producing a mixed lawn of green colonies of *E. faecalis* and pink colonies of *Escherichia coli*. The 36 yolks from stressed or possibly dead embryos produced no colonies. For 302 live embryos, one gave a green lawn of *E. faecalis*, one gave a single colony of *Streptococcus gordonii*, and one gave four colonies of *Staphylococcus pasteuri*.

Day of Hatch Microbiology

In September 2024, broiler chicks were obtained from a commercial hatchery on day-of-hatch, with no provision of water or food. First, we sampled eight carcasses for intestine and residual yolk sac through overnight enrichment in TSB. Single colony streaking of the overnight broth culture determined there was limited microbiological diversity. One chick produced a uniform culture of *Staphylococcus warneri* (white colonies), two produced uniform cultures of *E. faecalis* (green colonies), two produced mixed cultures of *S. warneri* and *E. faecalis*, one produced a mixed culture of *E. faecalis* and *E. coli* (purple colonies), and one a mixed culture of *E. coli* (both white colonies and purple colonies) and *E. faecalis* (green colonies). The second round of sampling was for four carcasses using direct swab streaking on CO plates for peritoneal fluid, and residual yolk swab, each onto two plates with one incubated at ambient CO_2 and one at 5% CO_2. Additionally, small intestine tissue and residual yolk sac were sampled into TSB for broth overnight enrichment. Peritoneal swabs for 2 of 4 embryos produced single green colonies which were not evaluated further. Three of four small intestine TSB enrichments were turbid, all four residual yolk sac TSB enrichments were turbid, and residual yolk sac direct swabs onto CO plates were the same whether incubated at ambient or 5% CO_2. One chick gave no colonies for direct residual yolk sac sampling, one gave a single green colony, one gave 7 green colonies determined to be *E. faecalis* and one gave a mixed lawn of white colonies of *E. coli* and green colonies of *E. faecalis*. We then evaluated 18 remaining carcasses only by residual yolk sac swabs spread on CO plates that were incubated at ambient CO_2. Eight of the 18 gave only green colonies diagnosed as *E. faecalis*: three produced single colonies, one produced 2 colonies, one produced 3 colonies, one produced 5 colonies, one produced 16 colonies, and one produced a lawn.

In January of 2025, surplus male chicks from a broiler breeder female line were similarly obtained on day-of-hatch, with no provision of food or water. Microbiology sampling was assessed as before but only by direct swab sampling of the residual yolk sac streaked on CO plates. Seven of 40 dissected chicks produced colonies from the swab smears. Six chicks produced only green colonies with counts of 1, 3, 6, 8, 15, and 120, where representative colonies were diagnosed as *E. faecalis* based on 16S sequencing. The seventh chick yielded 45 green colonies and 7 purple colonies diagnosed as *E. faecalis* and *E. coli*, respectively.

Discussion

Concerns in evaluating eggs, embryos and chicks include random contaminants from the instruments or investigators, especially when using broth enrichment methods. Our initial methods (direct yolk cultures) were designed to screen for *E. cecorum.* Out of 31 yolks we failed to find any *E. cecorum* but did recover *E. avium* from one yolk. We then sampled an additional 297 eggs from the same set, after 18 days of incubation by direct sampling onto CO plates. From 21 infertile or early dead yolks we recovered three species: *G. sanguinis*, *E. faecalis,* and *E. gallinarum.* Although the *E. faecalis* was only a single colony and thus could be a contaminant, the other two were lawns indicating a high level of growth for these two species, likely contributing to the failure of the embryo to develop. From 37 developed embryos evaluated as stressed or recent dead, we recovered a lawn of *E. gallinarum* from one embryo and a lawn of *E. faecalis* from another yolk sample, but no colonies from liver tissue samples. From 239 live embryos we sampled 175 for liver and got only a single colony of *S. salivarius* from one liver which could be a random contaminant. From the 239 live embryos only one yolk sample produced colonies, 10 colonies of *E. gallinarum.* Thus, we conclude that there is sufficient evidence for yolk infections by *E. gallinarum* and *E. faecalis*. Clearly one yolk was infected with *G. sanguinis,* since it produced a lawn from direct swab sampling without enrichment. Finding this species is surprising since this species has been primarily associated with rare human infections [35,36] and has not been previously associated with chickens. Based on 16S rDNA this species is closely related to Streptococcus and Enterococcus [37,38] but distinguishable by specific phenotypes and at the genome sequence level.

Microbiological surveys of yolk sacs from 360 18-day broiler embryos identified a heavy mixed infection of *E. faecalis* and *E. coli* in one nonfertile or very early dead egg. There was also a high-level infection with *E. faecalis* in the yolk of one live embryo. Therefore, our investigations of embryos from fertile eggs determined that severe yolk sac infections may affect 1 to 2 % of eggs and some of these infections affect embryos that would be included in hatching baskets or that might even produce live chicks at hatch. Thus, they could be the vector for vertical transmission of pathogenic bacteria, and thus the source for early colonization of the other hatched chicks.

Our microbiological sampling of multiple tissues from 40 each day-of-hatch broiler chicks revealed a low diversity of bacterial species. We suspect that the isolates of *S. warneri* could be contaminants in the enrichment growth. However, *E. faecalis* and *E. coli* were identified at significant levels without enrichment. We also identified a co-infection by *E. faecalis* and *E. coli* in a non-fertile or early dead broiler yolk from an 18-day embryo. In a Canadian study of early dead embryos *E. faecalis* predominated with *E. coli* being the second most prevalent but more than half the *E. coli* cases were co-infected with *E. faecalis [28]. E. faecalis* and *E. coli* have been associated with high levels of embryo mortality in chickens [39]. Further, *E. faecalis* is commonly co-isolated from chicken colibacillosis mortalities [26]. Therefore, the identification of *E. faecalis* in the majority of infections is in line with past findings, and we also see a probable synergism between *E. faecalis* and *E. coli* in embryo and day-old chick yolk infections.

Staphylococcus, Enterococcus, Enterobacter, and Escherichia have all been previously cultured from embryos and day-old chicks [3,6,40–42]. First Week Mortality (FWM) of broiler chicks on Dutch farms differed significantly between chicks originating from eggs of different breeder flocks and correlated with breeder age, egg storage length, season, and feed company for the breeders [2]. All of which could affect breeder hen colonization by opportunistic bacterial pathogens. *E. coli* has been commonly associated with yolk sac infections, and subclinical or acute septicemia, contributing to increased FWM in broilers [43]. Others have reported Enterococcal infections in non-viable chicken embryos [28]. Different lines of research have suggested Salmonella colonization of eggs could be through the egg shell, post ovulation, or in the ovary in the reproductive tract (reviewed in [11]). Our data are most consistent with bacterial contamination of the yolk, most likely from the breeder hens. Depending on the level of the initial infection this could lead to early death of the embryo, FWM, or even persistent infection of some chicks after the first week. Although we initiated these experiments to pursue the vertical transmission of *E. cecorum* from breeders to broilers, the fact that we recovered three other species of Enterococci suggests that this same vertical transmission could facilitate transmission of *E. cecorum* albeit at lower levels than the numbers of eggs examined in our surveys. With the removal of prophylactic antibiotics during early chick growth as well as in breeder flocks, opportunistic bacterial infections are not surprising. The levels of contamination that we detect could result in a 2% reduction in hatchability and increased FWM. Additionally, vertical transmission from the breeder hens to the new flock could "seed" that flock for subsequent bacterial outbreaks including sepsis, colibacillosis, or osteomyelitis (lameness).

Further pursuits should be aimed at repeated evaluation of eggs with tracking from breeder hens to determine whether specific hens are responsible for the infected egg yolks, the so-called Typhoid Mary scenario. If specific hens are responsible, then the genetics of these particular hens could be examined for whether there is genetic susceptibility. If environmental factors are more critical for which eggs are infected then it might be advisable to use probiotics during lay to reduce colonization of the hen, to see if probiotics can reduce reproductive tract colonization. Finally, vaccines for the most common egg contaminant species might be an effective strategy, with eBeam vaccines as a suitable candidate [44].

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