

# Determining Presence of Yolk Sac and Intestinal Bacterial Colonization in Pre-hatch Chicken Embryos

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### Summary and Implications

Chicks hatch with a sterile gastrointestinal tract and receive the first colonization of intestinal bacteria from the shell and hatching environment. Bacteria may translocate from the shell to remaining yolk and/or the developing embryo pre-hatch due to penetration of the protective bloom on the unwashed shell's surface. The objective of this study was to determine the presence or absence of bacterial DNA via PCR in the gastrointestinal tract of pre-hatch and hatched chicken embryos. Pre-hatch chicken embryo digestive tracts and yolk sacs sampled in a biosafety cabinet on days 13, 20, and 21 of incubation showed no bacterial presence using sensitive 16s PCR. Further research needs to be done in this area, potentially utilizing sterile sampling and cultures to confirm the apparent sterility of the inner egg environment as well as impact of time post-hatch and diversity of bacterial colonization on future production.

### Introduction

It is widely accepted that gastrointestinal microbial colonization in chickens does not develop pre-hatch, but there is room for doubt as the shell is made of porous calcium carbonate that has been subjected to warm, dark, and humid conditions for 21 days. The egg environment may not be entirely sterile; bacteria could translocate from the shell to the internal yolk or the embryo during embryonic development, yet work identifying presence of microbial colonization pre-hatch, specifically the remaining yolk sac and the embryo intestine, has not been verified using current molecular biology techniques. This experiment focused on collecting fertile eggs from a laying hen colony housed at the Iowa State Poultry Farm for three days, incubating using commercial incubators, and then sampling yolk and tissue during the incubation process as well as chicks shortly post-hatch. The objective of this study was to sample unwashed, conventionally-incubated chicken embryos in a sterile environment to identify any natural bacterial colonization pre-hatch.

### Materials and Methods

Semen was collected from seven different variety breed roosters and pooled to inseminate three Bovans White laying hens at the Iowa State Poultry Farm. This experiment was approved by the Iowa State University Institutional Animal Care and Use Committee.

Subsequent fertile eggs were collected for 14 days, then eggs were set for incubation. On days 13, 16, and 20 of incubation, three eggs for each time period were brought to the lab and sampled in the biosafety cabinet. All tools used were placed under UV light for 15 minutes and then soaked in bleach for 15 minutes prior to sampling. Eggs were dipped in bleach before being opened, and then the entirety of the embryonic gastrointestinal tract as well as a portion of the yolk sac were collected using the sterile tools, stored in a microcentrifuge tube, stored in -80 degrees Celsius. On day 21, three recently hatched chicks and one chick mid-hatch were euthanized using cervical dislocation and then brought to the biosafety cabinet and sampled in the same fashion. Samples collected from each embryo or chick included two yolk sac samples and two total tract intestinal samples. Following sampling and freezing at -80C, DNA was extracted using the MOBIO DNeasy PowerLyzer PowerSoil Kit (Qiagen, The Netherlands) and measured using the Nanodrop 2000 (Thermo Fisher Scientific, MA). Following Nanodrop validation of nucleic acid presence, DNA was amplified using the Invitrogen Platinum Taq DNA Polymerase Kit (Thermo Fisher Scientific, MA). Qualitative PCR for bacterial product 16s was run for 38 cycles with an annealing temperature of 55 degrees Celsius, and then the PCR product was visualized on 1.5% agarose gels. Data was collected using Microsoft Excel to organize information and create graphs.

### Results and Discussion

Across all time points, (days 13, 16, 20, & 21), Nanodrop results showed significantly higher amounts of nucleic acid (in nanograms per microliter) in the embryonic intestinal samples compared with yolk sac samples (**Tables 1 and 2**). Day 13 samples showed no positive results for bacterial presence in the egg via 16s PCR (**Figure 1**). Day 16 PCR results showed possible contamination in a negative control (water extracted with samples using the extraction kit) and thus could not be used for further evaluation. Day 20 PCR results (**Figure 2**) showed one faint positive band on one of the 2 yolk samples collected from that embryo at that time point, but no other positives were detected. Day 21 PCR results (**Figure 3**) showed no positive bands besides the positive controls, indicating that there was no detectable

bacteria present in the intestines or yolk sac of recently hatched chicks.

Positive Nanodrop results allow us to conclude that across all time points, DNA extraction was successful and nucleic acid was present, but did not determine if DNA extracted was eukaryotic or prokaryotic. The significantly higher (200-300 nanograms/microliter) range in the intestines versus the yolk sacs are what we would expect to see considering that the intestines contain chicken cells (DNA) while the yolk sac is nutrition for the embryo (acellular). 16s PCR amplifies bacterial DNA only. Thus, the lack of positive bands on the day 13 PCR gel confirms that the DNA extracted and measured was eukaryotic and not bacterial. This leads us to conclude that there was no detectable bacterial colonization within the eggs sampled on that timepoint.

Day 20 (16s) PCR results displayed one faint positive band originating from a yolk sac. There were no positives recovered from any other samples from this time point or samples from other time points, and the band was faint in comparison with the positive controls, so it is likely that the positive originated from contamination during sampling and was an outlier. Day 21 results revealed no bacterial presence in the intestines or yolk sacs of any of the three chicks shortly following hatch nor the chick who was mid hatch during sampling (still within the egg, but had broken the shell).

Due to the preliminary nature of this project, a definitive pattern of 16s in samples to indicate bacterial presence is necessary to warrant future investigation. The lack of consistent bacteria in day 13 and 20 samples indicates that there may not be bacterial colonization before hatch, but further research using more replicates and including DNA sequencing or nested PCR must be carried out to conclude this with absolute certainty. Lack of bacterial DNA in day 21 chicks also indicates that bacterial colonization does not take place simply during the hatching process without chick access to feed or conspecifics.

Following this experiment, we cannot conclude that there is intestinal bacterial colonization in the chicken embryos pre-hatch in our testing conditions. We also cannot conclude that the process of hatching itself caused colonization within the short time span prior to sampling (hours), which implies that environmental interaction with feed or other chicks may be key factors contributing to colonization. Further research utilizing DNA sequencing or nested PCR is warranted to verify the absence of bacteria in eggs mid-incubation. Hatching with a sterile intestinal tract opens the door for producers to colonize with specifically selected bacteria that may improve growth and production.

### **Acknowledgements**

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Figure 1: Nanodrop 2000 results depicting the higher DNA concentrations observed in intestinal samples compared to yolk sac samples of day 13 embryos. 401, 402, and 403 are identification numbers of hens used for egg collection.

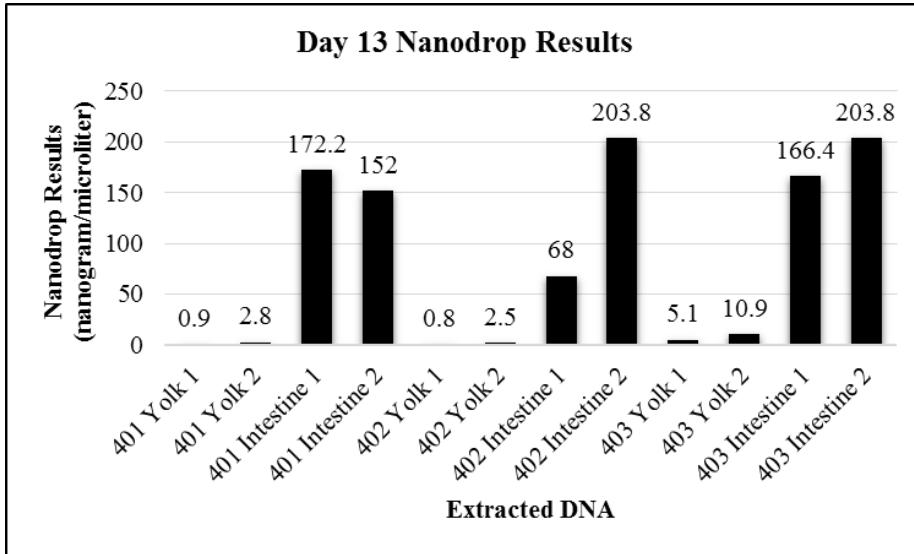


Figure 2: Nanodrop 2000 day 20 results displaying increased concentration of DNA present in intestinal versus yolk sac samples. 401, 402, and 403 are identification numbers of hens used for egg collection.

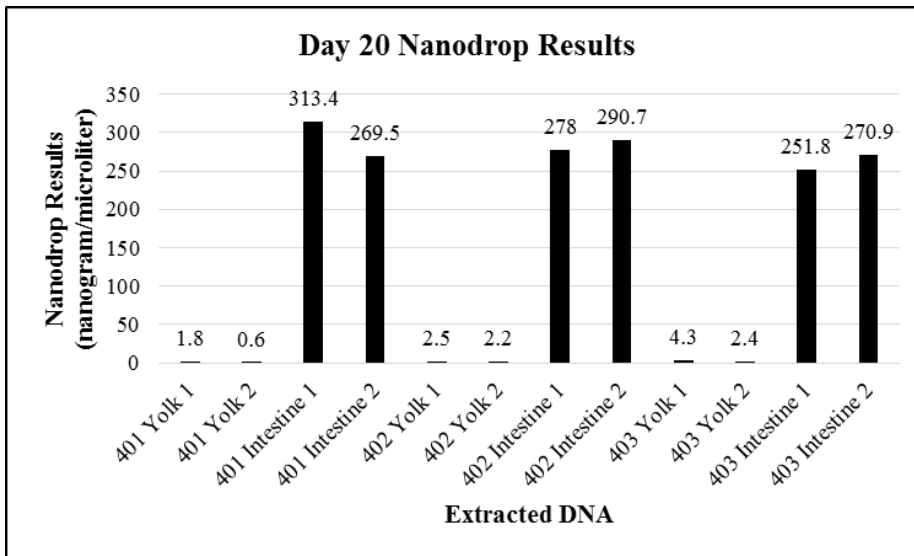
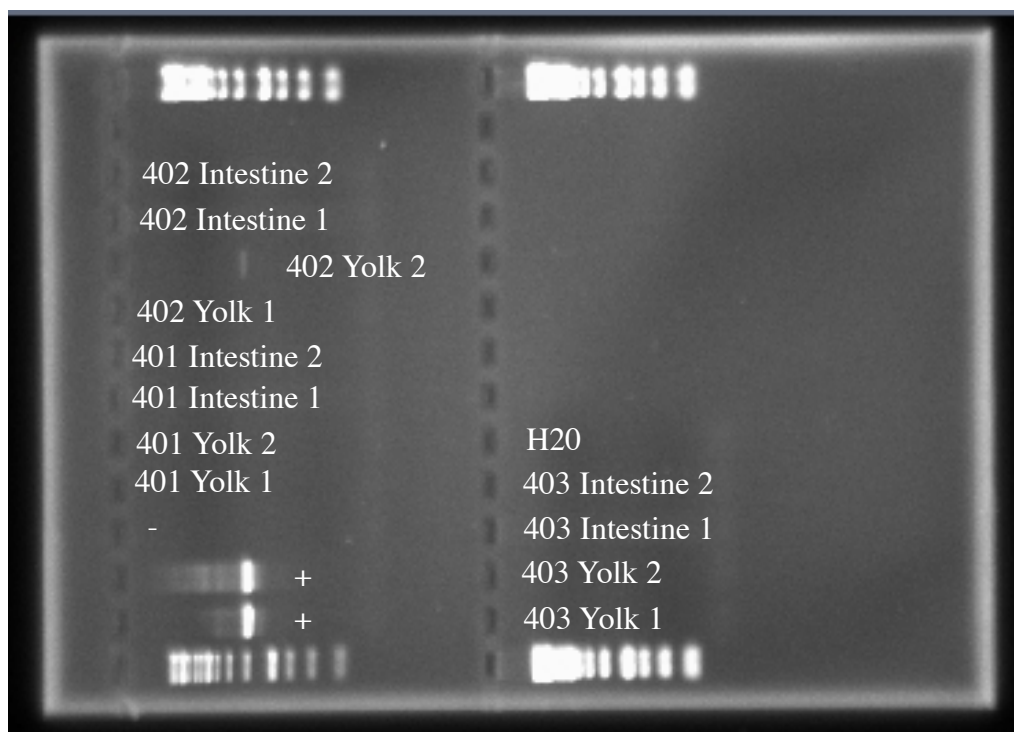


Figure 3: Day 20 16s PCR results with two positive controls and one faint band on yolk sample 2 out of hen 402.



*\*The four ladders (top left and right, bottom left and right) present are 1 kb DNA molecular weight size markers.*