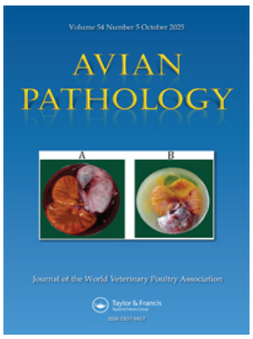

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ORIGINAL ARTICLE

Recombinant auto-bioluminescent *Escherichia coli* to monitor the progression of *Escherichia coli* infection in the embryonated chicken eggs

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) infections in poultry adversely affect health and production, with public health implications. This study assessed the potential of bioluminescence imaging for real-time, noninvasive tracking of microbial progression in 12-day-old chicken embryos inoculated with an APEC strain or its derivatives integrated either with *lux*ABCDE or *ilux2* operon. Eggs were imaged daily for bioluminescence detection, with dead embryos sampled immediately and survivors killed at 5-days post-inoculation (dpi). The eggs were opened, and egg contents were imaged for bioluminescence. Yolks were sampled for *E. coli* isolation and quantification. The results showed lethality rates of 100%, 93.3%, and 80% in embryos inoculated with native strain, *lux*ABCDE, or *ilux2,* respectively. Bioluminescence analysis showed increased bioluminescence signal strength over time preceding embryo death. Surviving embryos exhibited a sequential reduction in signal strength. A strong positive correlation was found between bioluminescence signal intensity *in ovo* and *ex ovo*, with *ilux2*-APEC-infected eggs showing a higher luminoscore than *lux*ABCDE-APEC. The *E. coli* load in yolks of APEC-inoculated eggs showed a positive trend over time. Overall, bioluminescence imaging of *ilux2* operon-labelled bacteria enabled more efficient real-time detection and monitoring of *E. coli in ovo*. Multiple imaging sessions on the same embryo throughout the experiment allowed precise monitoring of infection progression without sequential culling. This offers a controlled platform for evaluating antimicrobial treatment efficacy in an *in ovo* model that closely resembles an *in vivo* chicken model. It can also be used to study infection patterns of other pathogens, especially those that pose risks to public health.

RESEARCH HIGHLIGHTS

- Bioluminescence imaging enabled real-time, noninvasive tracking of a bioluminescent APEC infection in embryonated chicken eggs over time.
- Bioluminescence signals showed contrasting patterns for dead and surviving embryos.
- The *ilux2*-APEC showed a higher luminoscore than *lux*ABCDE-APEC in inoculated embryonated chicken eggs.
- The *in ovo* bioluminescent signal from intact eggs effectively reflects the *ex ovo* signal following the take out of yolk and embryo.

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APEC; bioluminescence imaging; noninvasive tracking; microbial progression; *in ovo*; *ex ovo*

Introduction

Escherichia coli (*E. coli*) is a Gram-negative bacterium that typically exists as a normal commensal in the gut of humans and animals, including poultry (Abdelhamid, Nekouei *et al.*, 2024). However, certain strains can potentially cause a wide range of extraintestinal infections in poultry. These infections, collectively referred to as avian colibacillosis, include colisepticaemia, coligranuloma, peritonitis, swollen head syndrome, osteomyelitis/synovitis, omphalitis, orchitis, and salpingoperitonitis (Nolan *et al.*, 2020). Avian colibacillosis is caused by avian pathogenic *Escherichia coli* (APEC) and is recognized as one of the most prevalent bacterial infections affecting poultry, causing significant economic losses in the poultry industry. The potential impact of APEC also extends to food safety and public health (Mellata, 2013; Saidenberg *et al.*, 2022; Abdelhamid, Hess *et al.*, 2024). While various serogroups have been identified among APEC strains, serogroups O1, O2, and O78 are still regarded as the most prevalent (Nolan *et al.*, 2020).

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Extensive research has been focused on identifying genetic traits in APEC that enhance its pathogenicity and defining the pathotype of *E. coli* isolates based on the presence of unique genomic markers (Gomis *et al.*, 2001; Ewers *et al.*, 2005; Nakazato *et al.*, 2009; Wang *et al.*, 2014; Abdelhamid, Hess *et al.*, 2024). However, characterizing APEC and differentiating it from commensal avian *E. coli* based on genomic traits is still challenging (Palmieri *et al.*, 2023). Moreover, some virulence genes are only expressed when *E. coli* interacts with the host (Dziva & Stevens, 2008). Besides the pathogenicity test in live chickens, which is cumbersome, the chicken embryo lethality assay remains one of the key methods for determining the virulence of *E. coli* isolates (Kunert Filho *et al.*, 2015).

The chicken embryo lethality assay has been employed to study the virulence of various bacterial pathogens, including *E. coli*, *Enterococcus* species, *Clostridium perfringens, Campylobacter, and Salmonella* species (Field *et al.*, 1986; Alnassan *et al.*, 2013; Blanco *et al.*, 2017; Zhang *et al.*, 2020; Rezaee *et al.*, 2021; Dolka *et al.*, 2022). Despite the advantages of this assay – (such as versatility, viability, speed, sensitivity, low cost, and relative simplicity) (Fonseca *et al.*, 2021), it only reveals the outcome of the treatment's effect, i.e. whether embryos are alive or dead, without allowing monitoring of the infection progression over time.

Several reporter systems, such as green fluorescent protein (GFP), bioluminescent enzyme firefly luciferase, and bacterial luciferase, are available for bacterial tracking both *in vivo* and *ex vivo*. The *lux* operon is unique in that it allows bacteria to act as a light source, synthesizing all components required to emit light from endogenous sources (Engebrecht *et al.*, 1983). This system is sensitive, making it ideal for various applications including microbial detection, gene expression assessment, antimicrobial drug studies, environmental monitoring, and intracellular metabolic function assessment (e.g. ATP) (Syed & Anderson, 2021). The rapid growth in bioluminescence application has led to the development of improved systems, such as *ilux* (Gregor *et al.*, 2018) and *ilux2* (Gregor, 2022), with the latter being the brightest version to date, based on an *in vitro* approach. Previously, only a single study has reported the use of bioluminescent non-pathogenic *E. coli* DH5α in the context of chicken embryo inoculation to evaluate bacterial colonization in various visceral tissues post-necropsy (Castañeda *et al.*, 2019).

This study aimed to explore the potential of a bioluminescence imaging system to continuously monitor the infected embryonated chicken eggs without opening the eggshell by tracking a *lux*-tagged APEC in real-time. Additionally, the brightness of the bioluminescence produced by *E. coli* tagged with two different bioluminescent operons was evaluated in embryonated chicken eggs.

Materials and methods

Bacterial isolates and preparation for egg inoculation

One parent strain and two genetically modified strains derived from the same strain were used for embryonated chicken egg inoculation. The parent strain *E. coli* PA14/17480/5-ovary, serotype O1:K1 (APEC) was isolated from the ovary of a laying chicken with colibacillosis and met all criteria as an APEC strain based on *in vitro* and *in vivo* traits (Abdelhamid *et al.*, 2020; Rezaee *et al.*, 2021). The *lux*ABCDE-*E. coli* PA14/17480/5¬ovary (*lux*ABCDE-APEC) was created by insertion of the *lux*ABCDE operon into the 16S locus of the parent APEC strain's chromosome following a previously published protocol (Riedel *et al.*, 2007; Abdelhamid *et al.*, 2020). For the *ilux*2-*E. coli* PA14/17480/5-ovary (*ilux*2-APEC), the pGRG25 vector was used for transposition of the *ilux*2 operon into the attTn7 site of the parent APEC strain's chromosome as described earlier (Gregor, 2022). PCR and next-generation sequencing were performed to verify that the *lux* operons are correctly integrated at the specified site in both *lux*-tagged APEC strains without disrupting essential genes associated with virulence. To prepare the inocula, all three *E. coli* cultures were washed and resuspended in phosphate-buffered saline (PBS). Bacterial concentrations were quantified before and after inoculation as colony-forming units (CFU) by direct plating of 100 µl of serial dilutions in duplicates on LB agar, as previously described in detail (Paudel *et al.*, 2023). Broth cultures of *lux*-tagged *E. coli* isolates were visualized for bioluminescence emission using an *in vivo* imaging system (IVIS) instrument that allows the capture of signals using the advanced camera (Lumina LT, PerkinElmer, Rodgau, Germany).

Infection of embryonated chicken eggs

Applied methods were based upon standard diagnostic procedures for isolating and multiplying avian pathogens which are not covered by the national law for animal experiments in Austria (Tierversuchsgesetz 2012 – TVG 2012). As such, the approval of the Animal Ethics Commission was not required under Directive, 2010/63/EU regulations (Directive, 2010).

Experimental design

Sixty White Leghorn specified pathogen-free (SPF) fertile eggs (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany) were divided into four groups of 15 eggs per group. All eggs were incubated under standard conditions (37°C, 65% relative humidity). On day 12 of incubation, all eggs were candled to confirm embryo viability. Afterwards, embryonated

eggs in groups 1, 2, and 3 were inoculated with 0.1 ml of 2.2–2.7 × 10^2 CFU/ml of PA14/17480/5ovary, *lux*-ABCDE-APEC, or *ilux2*-APEC, respectively, via the allantoic route as previously described (Guy, 2015). Using the same route, embryos in group 4 were inoculated with 0.1 ml of PBS. Following inoculation, all eggs were incubated and candled daily to assess embryo mortality.

Bioluminescence imaging and quantification

All embryonated eggs were imaged daily under the IVIS instrument with a binning of 16 (large) and a f/stop of 1 (IVIS Lumina LT, PerkinElmer, Rodgau, Germany). A binning factor of 16 means that 16 pixels are merged into a single pixel, enhancing signal strength, while the f/stop indicates aperture size; low f/stop permits more light to pass to the sensor, specifically f/stop 1 signifies a wide aperture that maximizes light capture. Each egg was set on a stand and imaged twice, once vertically with the air cell on the top and once with horizontal positioning for a side image capture (called *in ovo* imaging hereafter). In the case of embryo death, the eggs were aseptically opened and all the internal contents, including embryos, were placed on a sterile dish, and investigated again by IVIS (called *ex ovo* imaging hereafter). Live embryos were returned to the incubator and this process was repeated daily for 5 days post-inoculation (dpi). On day 5, embryos that remained alive were imaged and then chilled at 4°C for 4 h. Afterwards, eggs were opened, and internal contents, including embryo, were imaged.

Using Living Image software (version 4.5.5; PerkinElmer), the bioluminescent signal was analysed to quantify the amount of bioluminescence released by *lux*-tagged *E. coli* isolates either *in ovo* (with intact eggshell) or *ex ovo* (egg content after the removal of eggshell). Following the acquisition of each image, light intensities were retrieved as a pseudo-colour spectrum, where blue colour represented the lowest signal and red colour represented the strongest intensity. Regions of interest (ROI) in the optical image were manually marked by free drawing, and corresponding measurements were then displayed (Supplementary Figure 1). The bioluminescent signal was reported as total flux (radiance: photons(p)/second (sec)/cm^2/steradian(sr)), where steradian represents the photons emitted from a unit solid angle of a sphere. The photon flux per ROI is named luminoscore (Cosette *et al.*, 2016).

Assessment of weight and pathological lesions of embryos

Macroscopic lesions in embryos were recorded after bioluminescence imaging. To obtain the yolk-free body mass, the yolk sac and membranes were removed, and the embryo was cleaned and weighed.

Re-isolation of E. coli

For bacteriology sampling, embryos were first sprayed with 70% alcohol and dissected with clean sterile instruments. The heart, lung, liver, and brain from embryos were directly streaked on LB agar plates. For *E. coli* quantification in the yolk, 1 ml of the yolk was collected, mixed with PBS, and serially diluted 10-fold. Diluted suspensions were then plated in duplicate on LB agar plates. Samples from the negative control group, either for direct plating or quantification, were also plated on LB agar plates. All plates were then incubated aerobically at 37°C overnight. Agar plates from groups 2 and 3 were visualized under the IVIS instrument, and bioluminescent *E. coli* colonies were counted and expressed as $\log_{10}$-CFU/ml.

Statistical analysis

All analyses were performed in R (version 4.1.2) (R Core Team 2021). Survival of the embryos until 5 dpi was evaluated via a logistic regression model [function *glm*, option "family = binomial (link = 'logit')"]. The survival status (alive, dead) was fitted as a binary response and the group was fitted as a fixed categorical effect. To avoid complete separation (all embryos in group 1 died and all embryos in group 4 survived), a pseudo-observation with the response "alive" and a pseudo-observation with the response "dead" was included for each group. The overall significance of the group effect was evaluated via the function *anova* in R. Multiple pairwise comparisons between groups were accounted for via the false discovery rate (FDR) method (package *emmeans*, version 1.8.5, function *emmeans*, options "pairwise ~ group, adjust = 'fdr', type = 'response'") (Lenth 2023).

Daily trends of $\log_{10}$-transformed luminoscore for embryos with intact eggshell, with air cells either horizontal or vertical, were evaluated via separate linear mixed models in R (package lme4, version 1.1.32, function *lmer*, options "REML = FALSE") (Bates, 2014). The group and the interaction between the final viability status of the egg and the day post-inoculation were fitted as fixed effects. The egg per group was fitted as a random intercept to account for the covariance structure in the data as each egg was measured multiple times. The day post-inoculation was fitted as a correlated random slope within the random intercept, and assumptions about the residuals were visually evaluated. Multicollinearity was evaluated via variance-inflation factors (package *car*, version 3.1-1, function *vif*) (Fox and Weisberg 2019) on a model without the interaction. Pairwise contrasts

between groups or viability statuses on the $\log_{10}$ scale from each model (package *emmeans*, function *emmeans*, options "pairwise ~ group, type = 'response', lmer.df = 'satterthwaite'") were (separately for each predictor) collectively accounted for via the FDR method (function *p.adjust*). Trends of the luminoscore across the days post-inoculation for each viability status were evaluated, compared between the two statuses and collectively accounted between and within status via the FDR method (package *emmeans*, functions *emtrends*, options "pairwise ~ status, var = 'dpi', adjust = 'fdr' and *test*, options 'emtrends, adjust = 'fdr', lmer.df = 'satterthwaite'").

Effects on the final $\log_{10}$-transformed luminoscore for embryos with intact eggshell, whether the air cell was horizontal or vertical or after the eggshell removal, were evaluated via separate ANCOVA models in R (function *lm*). The group was fitted as a fixed categorical effect and the day post inoculation as a covariate. Assumptions about the residuals were visually evaluated. Multicolinearity was evaluated via variance-inflation factors (package *car*, version 3.1-1, function *vif*) (Fox and Weisberg 2019). Pairwise contrasts between groups on the $\log_{10}$ scale from each model (package *emmeans*, function *emmeans*, options "pairwise ~ group, type = 'response'") were collectively accounted for via the FDR method (function *p.adjust*).

Differences in the $\log_{10}$-transformed bacterial load in yolk were evaluated via an ANCOVA model in R (function *lm*) that included the interaction between the group and the day post-inoculation. The control group (group 4) embryos that were assessed only at 5 dpi were not included to avoid multicollinearity (evaluated via variance-inflation factors with the function *vif* using a model without the interaction). Assumptions about the residuals were visually evaluated. Trends of the bacterial load across the days post inoculation for each group were evaluated, compared between the three groups and accounted for between and within groups via the FDR method (package *emmeans*, functions *emtrends*, options "pairwise ~ group, var = 'dpi', adjust = 'fdr' and *test*, options 'emtrends(…), adjust = 'fdr'").

Correlation tests were performed between bacterial load in the yolk and the body-weight of embryos (without the negative control group 4), and between the three types of luminoscores (final luminoscores *in ovo* horizontal/vertical, *ex ovo*) via Spearman's rank correlation rho (function *cor.test*, options "method = 'spearman', exact = FALSE"). Multiple pairwise correlation tests were collectively accounted for via the FDR method (function *p.adjust*).

Significance for all analyses was declared at a 5% FDR cut-off. For visualization of the results from all statistical analyses, we used ggplot2 (R package *ggplot2*, Version 3.4.1, function ggplot2) (Wickham 2016).

Results

Embryo mortality

The embryo mortality rates in APEC-inoculated groups (1, 2, 3) and the PBS-inoculated group (4) are shown in Table 1. The highest embryonic mortality occurred at 2 dpi, when 9/15, 8/15, and 8/15 embryos from groups 1, 2, and 3, respectively, died. Differences in survival probability among groups 1, 2, and 3 until 5 dpi were not statistically significant but it was significantly lower in all infected groups than in the negative control (Figure 1).

In ovo *bioluminescence imaging*

Applying IVIS, bioluminescence was only observed in embryonated chicken eggs from groups 2 and 3 that were inoculated with *luxABCDE*-APEC and *ilux2*-APEC, respectively. Embryos in groups 1 and 4 that were inoculated with the parent APEC strain or PBS, respectively, showed no bioluminescence. The spectral data of the bioluminescence signal followed the same trend when capturing images of the same egg with different air cell orientations, either horizontal or vertical. Different patterns of bioluminescence were observed among embryos following APEC infection (Figure 2). Eggs with dead embryos (1–4 dpi) exhibited the highest luminoscore on the day of death (Figure 2(A–C)), while eggs with live embryos recorded the lowest luminoscore at 5 dpi (Figure 2(D)). This resulted in a significantly higher luminoscore of the dead embryos in both air cell positions (horizontal air cell FDR = 1.3e-05, predicted dead/alive luminoscore ratio = 16.1; vertical air cell FDR = 2.13e-05, predicted dead/alive luminoscore ratio = 8.48). Furthermore, bioluminescence signals exhibited contrasting trends over time for dead and surviving embryos in both air cell positions. Specifically, the bioluminescence increased for dying embryos, whereas it decreased for those that survived (Figure 3(A,B)).

In ovo *and* ex ovo *bioluminescence quantification*

Final luminoscores were significantly higher in group 3 (inoculated with *ilux2*-APEC) than group 2 (inoculated with *lux*ABCDE-APEC) either *in ovo* (while eggshell is intact, horizontal air cell FDR = 0.0042, predicted group 3/group 2 luminoscore ratio = 5.43; vertical air cell FDR = 0.047, predicted group 3/group 2 luminoscore ratio = 2.93) or *ex ovo* (egg content after eggshell removal, FDR = 0.011, predicted group 3/group 2 luminoscore ratio = 3.83).

Strong positive correlations were retrieved between *in ovo* bioluminescence intensity of captured images for eggs with horizontal and vertical air cell

Table 1. Embryo mortality by day, and total mortality following infection with APEC isolates.

Group	*E. coli* isolate used for inoculation	Inoculum (CFU/ml)	No. of dead embryos, days post-inoculation					Totals	% deaths
			1	2	3	4	5		
1 ($n = 15$)	*E. coli* PA14/17480/5-ovary (APEC)	2.6×10^2	5	9	1	0	0	15/15	100
2 ($n = 15$)	*lux*ABCDE-APEC	2.7×10^2	4	8	2	0	0	14/15	93.3
3 ($n = 15$)	*ilux2*-APEC	2.2×10^2	0	8	3	1	0	12/15	80
4 ($n = 15$)	PBS controls	–	0	0	0	0	0	0/15	0

orientations (rho = 0.925, FDR = 1.84e-12), as well as between the *in ovo* and the *ex ovo* bioluminescence intensities (rho = 0.833 and 0.844, FDR = 2.59e-08 and 1.65e-08 for horizontal and vertical air cell orientations, respectively).

Pathological lesions

The most noticeable gross lesions in dead embryos were bad smelling fluid, yolk turbidity, and generalized body congestion, mostly localized in the head and around the eye. Hyperaemic yolk sacs with congested blood vessels that occasionally had black foci were also noticed in dead embryos. Surviving infected embryos and negative control embryos showed no pathological changes (Figure 4).

Yolk-free body-weight

On the last day of the experiment (5 dpi), the body-weight of embryos that survived the infection with APEC was lower than those inoculated with PBS (Supplementary Figure 2).

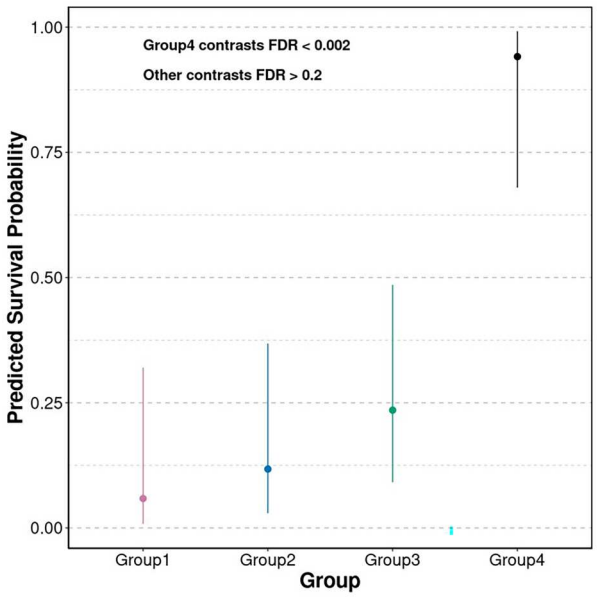

Figure 1. Estimated probabilities and their 95% confidence intervals from the logistic regression model were used to test for differences in the survival of chicken embryos until 5 days post-inoculation. We accounted for multiple testing via the FDR method and declared significance at the FDR cut-off of 5%. Groups: group 1-inoculated with parent APEC; group 2-inoculated with *lux*ABCDE-APEC; group 3-inoculated with *ilux*2-APEC; and group 4-inoculated with PBS.

Bacterial re-isolation

Pure cultures of *E. coli* were re-isolated from the yolk and systemic organs of all *E. coli*-inoculated embryos, but not from the PBS-inoculated group. *E. coli* load in the yolk showed positive trends across time for *E. coli*-inoculated groups that were significant only for groups 1 and 2 (Figure 5, FDR = 0.0031, 0.0027 and 0.0812 for groups 1, 2, and 3, respectively). These trends were not significantly different between the groups (FDR > 0.1). *E. coli* was re-isolated from the lung, heart, liver, and brain of all *E. coli*-inoculated embryos, either dead or alive. All the cultured plates from groups 2 and 3 were visualized under the IVIS for the detection of bioluminescent colonies. No correlation was found between the *E. coli* load in the yolk and the body-weight (rho = 0.1817, FDR = 0.407)

Discussion

Despite extensive research on *E. coli* in poultry, defining the APEC pathotype remains challenging (Al-Kandari & Woodward, 2019). The embryo lethality assay is commonly used to assess the pathogenicity of *E. coli* isolates (Rezaee *et al.*, 2021; Joseph *et al.*, 2024). Recently, embryonated chicken eggs have gained popularity as an alternative model for biomedical research as they are ethically more acceptable than experiments on hatched birds or mice (Mesas *et al.*, 2024; Nelogi *et al.*, 2024). In addition to pathogens, *in ovo* inoculation has been used in several studies to investigate the effects of probiotics, peptides, and immune stimulants on microbial challenges and antibiotic resistance (Cox *et al.*, 1998; De Oliveira *et al.*, 2014; Cuperus *et al.*, 2016; Tsiouris *et al.*, 2021; Sarfraz *et al.*, 2022). However, most of these studies rely on daily mortality rates and lack information on the dynamic changes in microbial growth and infection progression. Some studies randomly select and kill embryos to assess the impact of infection or treatments, which also makes it impossible to track infection progression in the same embryo over time. Therefore, in the current study, we aimed to use bioluminescence imaging to monitor the progression of APEC isolates in embryonated chicken eggs in real-time without the need to kill the embryo and to open the egg during the incubation time.

The bacterial luciferase *lux* system allows for the expression of the biosynthetic enzymes that generate

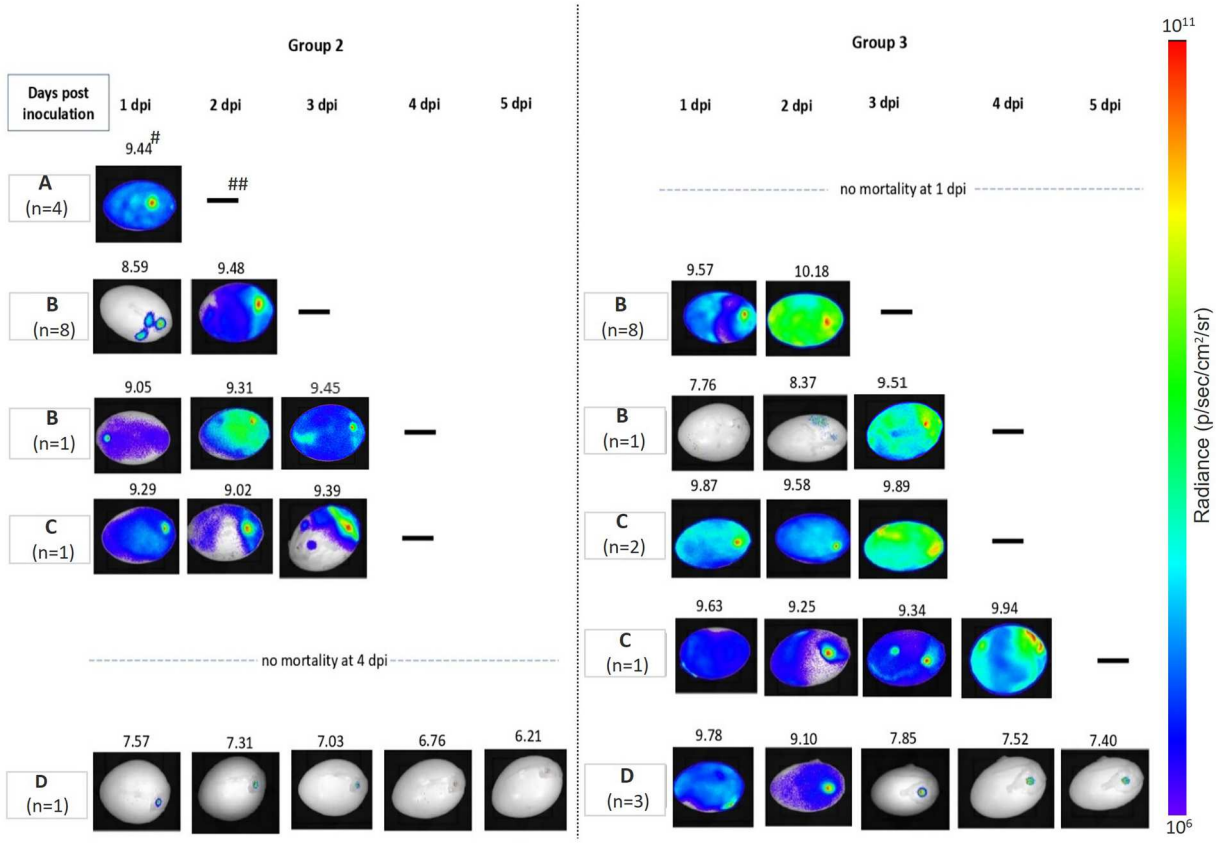

Figure 2. Representative images (horizontal orientation) of embryonated chicken eggs inoculated either with *lux*ABCDE-APEC (group 2), or with *ilux*2-APEC (group 3) showing varying patterns and levels of the bioluminescent signal at different days post-inoculation. Egg pictures in the same row represent the same egg over different dpi. (A, B, C) represent *in ovo* bioluminescent patterns linked to embryo death over days, and D represents the pattern linked to embryo survival up to 5 dpi. (A) Egg with a dead embryo showing strong bioluminescence and high luminoscore; (B) Embryonated chicken egg showing bioluminescence at 1 dpi, which increased over time; (C) Embryonated chicken egg exhibiting bioluminescence at 1 dpi that decreased in intensity at 2 dpi and then increases later; (D) Embryonated chicken egg showing a continuous decrease in bioluminescent intensity from 1 dpi until the end of the experiment. The different rows represented by patterns B and C show embryonated chicken egg death at different dpi. *n*, represents the number of eggs per pattern. #The luminoscore is represented as $\log_{10}$ per image.##No image because the embryo died the previous day.

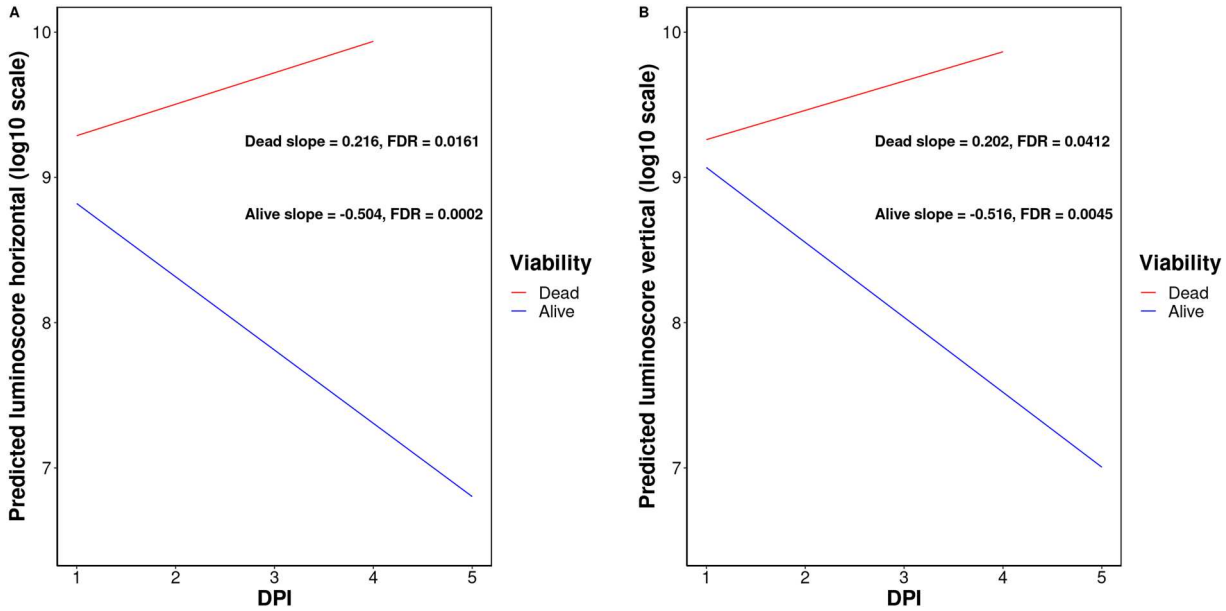

Figure 3. Daily (dpi) trends of bioluminescence ($\log_{10}$ scale) for dying and surviving embryos at horizontal (A) and vertical (B) air cell positions, evaluated via a linear mixed model. We accounted for multiple testing via the FDR method and declared significance at an FDR cut-off of 5%. The figure was created via the functions *ref_grid* and *emmip* (package *emmeans*).

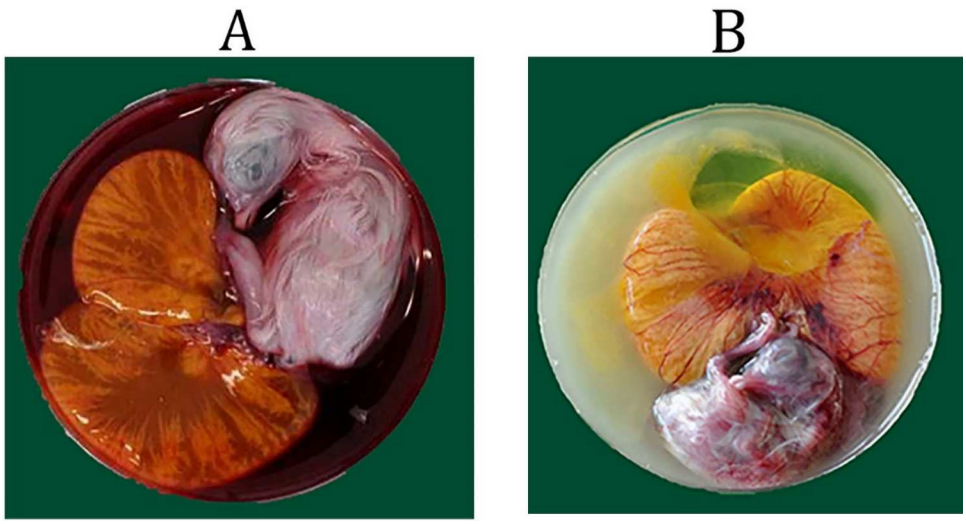

Figure 4. (A) Normal embryo without pathological changes (PBS-inoculated, 5 dpi); (B) Congested dead embryo with hyperaemic blood vessels and necrotic foci in the yolk sac (APEC-inoculated, 3 dpi).

bioluminescence without external substrate, making it an excellent tool for real-time bacterial monitoring (Gregor *et al.*, 2018, 2022; Hao *et al.*, 2024). Using bioluminescence imaging to track the same host throughout the experiment reduces the number of hosts required and prevents variability (Wang *et al.*, 2016). The present study demonstrated that *lux*-tagged *E. coli* isolates could be monitored over time without killing individual embryos and opening eggs until the death of the embryo or termination of the study.

The *lux*-tagged APEC strains, used for infection in the current study, were previously used in *in vivo* studies conducted by our group to track the bacteria after inoculation, and differentiation from commensal isolates (Abdelhamid *et al.*, 2020; Paudel *et al.*, 2021; Paudel *et al.*, 2023; Abdelhamid, Hess *et al.*, 2024). However, tracking of these *lux*-tagged isolates in living chicken models is limited to *ex vivo* tissues at a single time-point or bacterial re-isolation on agar plates. No studies have successfully tracked infections in the bird's body throughout its lifetime after systemic infection, likely due to the hindrance of bioluminescent signal emission by feathers (Wellawa *et al.*, 2022; Abdelhamid, Hess *et al.*, 2024). Hence, the purpose of the current study was to overcome this limitation using embryonated chicken eggs.

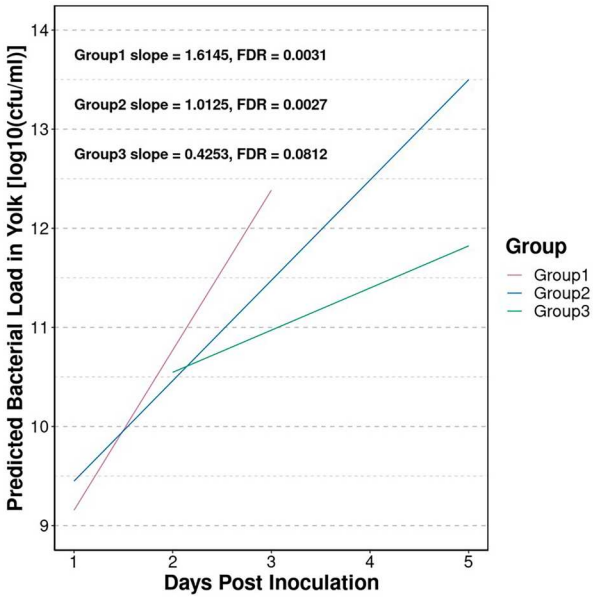

Figure 5. Daily (dpi) trends of $\log_{10}$-transformed bacterial load in yolk for each group, evaluated via an ANCOVA model. We accounted for multiple testing via the FDR method and declared significance at an FDR cut-off of 5%. The figure was created via the functions *ref_grid* and *emmip* (package *emmeans*). Groups: group 1-inoculated with parent APEC; group 2-inoculated with *lux*ABCDE-APEC; and group 3-inoculated with *ilux2*-APEC.

Unlike a previous study that focused on imaging the extra-embryonic tissues after inoculating embryos with bioluminescent non-pathogenic *E. coli* DH5α (Castañeda *et al.*, 2019), we used *lux*-tagged APEC strains separately inserted with two different *lux* systems into the same parent strain to monitor both *in ovo* (embryo with intact eggshell) and *ex ovo* (egg content including embryo with no eggshell) imaging. Our results showed a strong positive correlation between the bioluminescence signal intensity in *in ovo* and *ex ovo* imaging, indicating that *in ovo* imaging reliably represents microbial detection and progression inside the egg. The higher $\log_{10}$ luminoscore in embryos inoculated with *ilux2*-APEC compared to *lux*-ABCDE-APEC supports previous findings that chromosomal integration of improved *ilux2* operon in *E. coli* yields a bioluminescent strain with enhanced brightness (Gregor, 2022).

During real-time monitoring of *in ovo* infection, we observed differences in bioluminescent patterns in embryos inoculated with *lux*-tagged APEC. Most embryos which died during incubation displayed an increased bioluminescent signal over time, indicating active replication of APEC. However, a few embryos

that died at 3–4 dpi showed a decreased signal at 2 dpi, followed by an increase again before death. The subsequent increase in signal suggests a resurgence of bacteria overwhelming the embryo's immune defence (Hincke *et al.*, 2019; Garcia *et al.*, 2021). These dynamic changes in bioluminescent signals shed light on the interaction between embryonated chicken eggs and pathogens. Further investigation is needed to examine the immune responses, along with metabolic and proteomic profiles in embryonated chicken eggs following a similar pattern. This could provide valuable insights into the mechanisms underlying both resistance and susceptibility.

Although the mortality rate in embryos inoculated with the *lux*-tagged APEC strain (93.3% and 80% for *lux*ABCDE-APEC and *ilux2*-APEC, respectively) was lower than that of embryos inoculated with the parent APEC strain (100%), these differences were not statistically significant. Additionally, all infected groups, whether inoculated with the parent or *lux*-tagged APEC strain, exhibited the highest mortality rate at 2 dpi. This indicates that integrating the *lux* operons in the chromosomes of the parent strains does not affect their pathogenicity. The high mortality rate, as well as haemorrhage and congestion in the dead embryos from APEC-inoculated groups can be attributed to the rapid growth and multiplication of APEC in the tissues, along with the production and release of toxic substances (Nabbut & Khatib, 1978; Rezaee *et al.*, 2021).

The increasing *E. coli* load in the yolk of the *E. coli*-inoculated groups over time indicates the ability of some APEC isolates to multiply rapidly in the yolk, supported by the presence of certain bacterial virulence-related genes. These genes increase the embryonic lethality of APEC isolates and may also enhance their ability to utilize yolk nutrients (Ovi *et al.*, 2023). This finding highlights the importance of preventing the transmission of *E. coli* from hens to eggs and from eggs to hatching chicks which is crucial for poultry and human health (Christensen *et al.*, 2021).

In conclusion, this study demonstrates the effectiveness of the bioluminescence imaging system in tracking bioluminescent-labeled APEC in embryonated chicken eggs and monitoring their progression over time without invasive methods. The higher luminoscore of *ilux2*-APEC compared to *lux*ABCDE-APEC in the inoculated embryonated chicken eggs demonstrates the superior effectiveness of the *ilux2* operon in visualizing the spread of bacterial infections in living hosts with enhanced sensitivity. The applied technology can be utilized for various purposes, including evaluating the efficacy of antimicrobial treatments such as probiotics, bacteriophages, herbal products, and nanoparticles. Additionally, it can be used to detect reactive neutralizing antibodies against pathogens in an *in ovo* model, which closely resembles the *in vivo* environment of chickens or other bird models, and to assess egg disinfection and bacterial penetration through the eggshell. This system could also be further elaborated and extended to other microbial infections important for poultry and human health.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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