

Comparative Pathology of Experimental *Avibacterium Paragallinarum* Infection in Chicken and Japanese Quail

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Abstract

Introduction

Avibacterium paragallinarum (*Av.paragallinarum*) is the causative agent of infectious coryza (IC) in chicken, an economically devastating disease of poultry industry. Despite the availability of effective vaccines against IC, the infection is rampant in unorganized poultry sector. Moreover, reports of wider host susceptibility in poultry farms and under field conditions are also emerging. *Av.paragallinarum* affects the upper respiratory tract of birds and has a predilection for the nasal turbinates. However, no study has systematically evaluated the early pathological changes and mucosal immune responses during *Av. paragallinarum* infection in the nasal turbinates of chicken. Furthermore, the use of Japanese quail as an alternate experimental animal model than chicken in IC remain unexplored. Here, we examined clinical signs, gross and histopathological changes at the nasal turbinates following experimental *Av. paragallinarum* infection in chicken and Japanese quail.

Methods

We developed a comprehensive scoring system for recording gross and histopathologic lesions during infection.

Results

Our data show that chicken have a higher susceptibility to *Av.paragallinarum* infection than the Japanese quail. Japanese quail had significantly lower gross as well as histopathology score in nasal turbinates as compared to infected chicken. Importantly, chicken at an early age i.e. 2.5 weeks were able to induce mucosal immune responses against *Av. paragallinarum* infection.

Conclusion

Our findings suggest that there are demonstrable differences in the disease pathology and host immune response to *Av. paragallinarum* infection in chicken and Japanese quail and warrant further investigation for the use of Japanese quail as an animal model for IC.

1. Introduction

Infectious coryza(IC),also known as Snot is a bacterial infection of poultry caused by *Avibacterium paragallinarum* (*Av. paragallinarum*). IC is a disease of economic importance in several parts of the world and predominantly affects the upper respiratory tract of chicken (Wahyuni et al. 2018; Sun et al., 2018; Balouria et al., 2019).The disease is mostly seen in intensive poultry farming operations where infectious droplets are transmitted by aerosols or through contaminated water or contact with asymptomatic carrier birds, which increases the risk of disease outbreak. Infection is known to inflict myriad of losses like drop in egg production, increased culling rates in growing chicken, poor body weight gain, especially in broiler chicken and continual problem of respiratory tract infection in birds of all ages (Droual et al., 1990;

Blackall, 1999). Unorganized farming system and multispecies rearing practices are also attributed for the high risk of transmission of the disease among flocks and farms, when located within very close geographic proximity (Crispo et al., 2018). Such progressive infection is not surprising, as most of the recovered birds become carrier and persistently shed the bacteria in the environment through infected nasal secretions.

Reports of disease outbreaks of IC in poultry other than chicken have been well described (Priya et al., 2012; Wahyuni et al., 2018; Mohammad and Sridevi, 2019). Therefore, appropriate preventive measures such as vaccination is required to prevent such disease outbreaks. Despite the availability of vaccines, IC persists in several commercial farms. One of the major reasons for the failure of existing vaccines is the absence of local pathogenic serotypes of *Av. paragallinarum* in vaccines, which lacks cross protection between vaccine and field strains (Bragg et al., 1997; Jacobs et al., 2003 Chukiatsiri et al., 2009). The impact of *Av. paragallinarum* infection in chicken has been described (Anjaneya et al., 2013; Ali et al., 2013; Trujillo-Ruiz et al, 2016). However, a detailed investigation of early pathological changes at the nasal turbinates and host-induced mucosal immune responses in chicken is lacking. Earlier studies suggest that *Av. paragallinarum* has the potential to infect Japanese quail under field conditions (Thenmozhi and Malmarugan, 2013; Wahyuni et al., 2018) but the gross and histopathological features of infection remain unclear. Japanese quail has a shorter generation interval and demands less housing space with meagre feed requirements than the chicken. Therefore, Japanese quail could serve as an alternative animal model to chicken for larger IC vaccination studies where a higher statistical power is desirable.

Here, we experimentally infected chicken and Japanese quail with *Av. paragallinarum* to investigate early pathological features of infection in the two species and to validate the potential of Japanese quail as an alternate disease model of IC. We evaluated the clinical signs post-infection and early pathological changes in nasal turbinates to establish a causal relationship between pathogen and the bird species with respect to the time of infection. Our results show that experimental *Av. paragallinarum* infection elicits characteristic disease pathology and induces mucosal immunity at an early age i.e. 2.5 weeks of age in chicken.

2. Materials And Methods

The work presented here belongs to a larger experimental study and some results obtained from the study has been published previously (Balouria et al., 2019). The information detailed here is based upon the same experimental set up, birds (chicken and Japanese quail), challenge route besides sampling programme, which was already published. However, the results presented here are novel and have not been published elsewhere. For easy understanding, some of the experimental details from the published paper are presented here to provide a complete overview of the current work.

2.1. Birds, their management and ethical approval

Healthy day-old chicken (White leghorn layer, BV-300 strain) and Japanese quail (*Coturnix coturnix japonica*) were procured from local unit of Venkateshwara Hatcheries Pvt. Ltd., Ludhiana, India and Central Poultry Development Organization, Northern region, Chandigarh, India, respectively. Birds were obtained on the same day to maintain age uniformity and to minimize experiment-induced biasness. Prior to their arrival, the bird cages, feeders, waterers and rooms were fumigated with mixture of potassium permagnate and formalin solution. The birds were examined for vaccination against Marek's diseases at the delivery portal. On reaching experimental site, birds were kept separately in two different rooms and provided antibiotic-free feed and water *ad libitum*. A strict hygienic management was performed during the period of experiment to avoid secondary bacterial infections. No birds from either species showed any respiratory signs before infection challenge. Disease-free status of the birds was confirmed after negative growth of *Av. paragallinarum* from infra-orbital sinus swabs of two birds from each species prior to the start of the experiment. All experimental procedures were approved by local Institutional Animal Ethics Committee (IAEC), GADVASU, Ludhiana, India and performed according to their regulations and guidelines.

2.2. Bacterial culture and inoculum preparation

Av. paragallinarum isolate (PDRC/2016/HPg/B/789) serotyped as serovar B (using Kume scheme) was used for challenge infection and obtained from Poultry Diagnostic and Research Centre (PDRC) of Venkateshwara Hatcheries Ltd, Pune, India. For routine culturing, the isolate was grown in Hemophilus test medium (HTM) broth and HTM agar plate (Hi Media Laboratories Pvt. Ltd, Mumbai, India) supplemented with 1% (v/v) filter-sterilized, heat-inactivated horse serum, and 0.0025% (w/v) reduced nicotinamide adenine dinucleotide. Ten percent sheep blood agar with *Staphylococcus aureus* as feeder culture was additionally used (Quinn et al., 2011) to confirm the purity of organism. The challenge bacterial suspension was prepared by inoculating dew-drop like colonies onto HTM broth at 37°C for 24 h. The bacterial culture was centrifuged at 25°C (4000 x g) for 10 min. The obtained bacterial pellets were washed three times in sterile PBS (pH 7.4) and subsequently ten-fold serially diluted to determine bacterial cell concentrations. The colony forming units (CFU) in bacterial suspension used for experimental infection was 1.5×10^8 CFU/ml.

2.3. Experimental design

At the age of 2 weeks, chickens and Japanese quails were segregated into two groups i.e. "Mock-inoculated group" containing 5 birds and "Infected group" with 23 birds. Birds were then acclimatized for 4 more days. Four naive birds i.e. 2 from each species were humanely euthanized prior to the day of challenge inoculation. Around 1 and 0.5 ml of the bacterial inoculum was injected per bird through various routes (Table 1). The clinical signs were recorded at least three times a day and graded as 1 for mild, 2 for moderate, and 3 for severe intensity for each parameter (Table 2). The decalcification, gross sectioning of nasal turbinates, and the side of nostrils considered for examination was as described earlier (Balouria et al., 2019). For histopathological description, we preferred cross-sections over longitudinal sections and therefore 3 birds were used at each time point.

2.4. Gross and histopathological examination

The pathological changes in nasal turbinates were carefully examined across all the groups including naive control at each of the mentioned time points (Table 1) and the intensity of gross lesions were subsequently recorded through semi-quantitative scoring (Table 3). The nasal turbinates and other tissues after decalcification with Osteosoft® were fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned at 4-5µm thickness. The sections were stained with haematoxylin and eosin stain and evaluated for histopathological changes. The histopathological changes noted in nasal turbinates were graded (scored on a 0 to 3 scale) (Table 4). The mean severity index for each section of nasal mucosal surface based on histopathological scoring criteria from both chicken and Japanese quail was also recorded. The bright field microscopy was performed for recording histopathologic changes with Nikon CFi (Nikon Corporation, Japan) and photographs were captured by DS Fi-2 camera (Nikon Corporation).

2.5. Statistical Analysis

The data was analyzed using Prism Software version 8.4.2 (Graph Pad Software, Inc, San Diego, USA). Prior to analysis, the data was subjected to normality test (Gaussian distribution) and found to be normally distributed. Two different statistical tests were applied. Firstly, a simple regression analysis was applied to assess the intensity (trend) of infection and the difference of intensity between species. Secondly, the effect of independent variables (time point after inoculation and species difference) on the outcome of dependent variables (degree of gross pathology in all regions of nasal turbinates, degree of histopathologic changes within middle turbinates and infra-orbital sinus, and cellular infiltrations) was measured by multivariate linear mixed model i.e. twoway ANOVA with Sidak and Tukey as post hoc test. For the data on clinical signs, chi-square test (χ^2) was applied. Statistical results were considered significant when p values were below 0.05.

3. Results

Clinical signs development

In chickens, the clinical signs developed as early as 12 h post-infection (hpi) and were characterized by mild unilateral nasal discharges in almost half of the birds. However, only few birds exhibited moderate degree of bilateral nasal discharge. Ocular discharge was milder in form and noticed sporadically in few birds, while mild swelling of either infra or supra orbital sinus (Figure 1A-B), mild redness of conjunctiva, and peri-orbital areas was seen in nearly 18% of the infected birds. Majority of birds had unilateral clinical signs in the beginning of infection and by 24hpi there was a slight increase in intensity of nasal discharge with bilateral involvement. A bilateral redness in conjunctiva was also common at 24 hpi (Figure 1C-D). By 48 hpi, the intensity and regional involvement declined to mild form with unilateral appearance. The only clinical signs that prevail til 3 daypost infection (dpi) were mild nasal discharge, where both unilateral and bilateral flow of secretions was observed after pressing of nostrils. On the contrary, Japanese quail was resistant to infection and seldom showed any major clinical signs. However,

conjunctival redness was noticed only in few birds 24 and 48 hpi. A statistically significant association of clinical signs with respect to species vulnerability was noticed in chicken (Supplementary Table 1).

Gross pathology

The chicken revealed redness of turbinates and nasal cavity along with mucus accumulation 12 hpi with varying severity index (Table 3, score level 1-3) that continued up to 7 dpi. The degree of redness in nasal turbinates varied from intense (chocolate) red discoloration of mucosal folds (in turbinates) with haemorrhagic foci or haemorrhagic blotch to dark red or highly pinkish discolored turbinates. At 24 hpi, the chickens showed redness of folds (in turbinates) without haemorrhages (score level 2). The severity of lesion gradually waned down to pink coloration of mucosal folds (turbinates) (i.e. mild intensity; score level 1) at 3dpi. A copious sero-mucus accumulation within nasal passages was noted by 48 hpi (score level 3) that persisted for a day but subsequently observed as scanty secretion adhering to mucosal folds (turbinates) (score level 1), which continued upto 7 dpi. However, mucus secretions were not opaque at any of the considered time points. In chicken, mild to rare moderate degree of swelling of infraorbital sinus as well as supraorbital cavities (score level 1 and 2) was noticed at 48 hpi. On sectioning, such sinus cavities were found to be filled with sticky to clear fluidly mucus exudates. Conjunctival haemorrhages appeared infrequently in chicken and were consistent on 48 hpi with diffuse to patchy redness of surface comprising haemorrhagic foci (score level 3). In contrast, the Japanese quail exhibited mild degree of redness in mucosal folds (turbinate) and nasal cavity (score level 1) that persisted upto 48hpi. No major changes were noticed thereafter in Japanese quail.

With respect to development of overall pathological changes noticed across the turbinates, significantly higher overall gross pathological lesion score was observed in chicken (slope, $p = 0.05$) (goodness of fit, $r^2 = 0.21$) as compared to Japanese quail (goodness of fit, $r^2 = 0.011$) (Figure 2A). Furthermore, a significantly higher overall pathologic lesion score was noticed at 12 ($p < 0.05$), 24 ($p < 0.05$) and 48 hpi ($p < 0.01$) in chicken as compared to Japanese quail (Figure 2B). The observed redness in the nasal turbinates and higher mucus secretion in chicken are considered to be the major factors for the visible gross pathological changes, where bacteria led to significant mucus secretion at 12 ($p < 0.05$) and 48 hpi ($p < 0.001$). The redness in nasal turbinates was however found to be host driven and was dominant at 24 hpi ($p < 0.05$) (Supplementary Table 2).

Histopathological changes

Following infection challenge in chicken, mild haemorrhages were noticed 12 hpi within posterior turbinates and inferior nasal meatus. These haemorrhages became moderate in intensity by 24 hpi in posterior turbinates, inferior nasal meatus, and infra-orbital sinus and continued until 7 dpi. The loss of cilia from respiratory epithelium covering middle turbinates, inferior nasal meatus, and infra-orbital corresponded to an initial invasion and tissue damage by the bacteria. These changes were predominant in infra-orbital sinus and less observed in posterior turbinates. Like deciliation, a similar trend was noticed for epithelial damage as well. However at 7 dpi, a gradual improvement i.e. regeneration/stratification of

epithelium was noticed. Mucus (alveolar) gland activity in the form of glandular hyperplasia and hypertrophic changes was first noticed at 24 hpi with maximum effect noted in the mucosal folds of middle turbinates and fairly at inferior nasal meatus, which subsequently waned after 48 hpi (Figure 3A1-A2). Besides haemorrhages, heterophilic infiltration in mucosal folds was prominently seen in middle turbinates at 12 hpi. Later, a mild heterophilic infiltration was observed at posterior turbinates (*within bowman's gland*), inferior nasal meatus, and infra-orbital sinus. Among all anatomical sites, heterophilic infiltrations were predominant at middle turbinates especially at *propria mucosa* and around submucosal blood vessels. Lymphoid cell infiltration was noted almost in an equitable proportion to heterophils in first 24 hpi at all sections of nasal cavity, with an exception to inferior nasal meatus, where naturally accumulated lymphoid cells as nasal associated lymphoid tissues (NALT) exists. A moderate infiltration was noted in posterior turbinates and infra-orbital sinus from 3dpi until 7 dpi (Figure 3A3-A4). Formation of small lymphoid follicles as secondary NALT was commonly seen in *propria mucosa*, submucosal connective tissues of middle turbinates, nasal septum, and infra orbital sinus at 48 hpi and 3 dpi (Figure 3A4 inset).

Japanese quail also exhibited haemorrhages as foremost histopathological change from 12 hpi onwards and predominantly within the middle turbinates. No other regions of nasal cavity showed any distinct areas of haemorrhages (Figure 3B1). The loss of cilia from epithelial surface was milder than chicken, though a moderate loss (as sloughing of superficial epithelium) was reported at 48 hpi from middle turbinates. Further, the loss of epithelium was observed in middle turbinates and fairly in infra-orbital sinus as mild degenerative changes limited to upper 1/3rd of epithelial thickness at 12 hpi and upto 3 dpi (Figure 3B2). Intriguingly, on 3 dpi, the Japanese quail were found to exhibit concurrent changes i.e. fibroplasia at submucosal connective tissue with mild epithelial cell regeneration at some places (Figure 3B3). By 7dpi, regenerative changes was observed (Figure 3B4). Only mild mucus gland involvement was noticed in middle turbinates, inferior nasal meatus and infra-orbital sinus post 24 hpi. Unlike chicken, a mild heterophilic infiltration was predominately noticed in inferior nasal meatus and infra-orbital sinus at 12 hpi. No infiltration of heterophils was noticed in posterior turbinates.

Though lymphoid cell infiltration was mild in nature, however, in comparison to heterophils, they were more prominent and prevalent in all the compartments of nasal cavity starting at 12 hpi until 7dpi. An insignificant formation of secondary NALT was observed in infra-orbital sinus on 3 dpi. On comparison between birds, a strong trend in lymphoid cell activity particularly in NALT (in inferior nasal meatus) and formation of secondary NALT as protective response was noted in chicken. Similarly, lymphoid cell infiltration in other areas like *propria mucosa*, sub mucosal connective tissues of nasal septum, and posterior turbinates was observed. However in Japanese quail, such pathologic responses were meager during early stage of infection and remained unaffected at later stages. Heterophilic infiltration and alveolar mucus gland activity was more pronounced in chicken at an early stage of infection, which proceeded quickly to the basal level after 3dpi. In addition, the loss of epithelium was predominant in chicken than Japanese quail.

Impact of infection and host response on the outcome of histopathological changes

Infection with *Av. paragallinarum* induced distinct histopathological changes in chicken and Japanese quail especially in middle turbinates (Intercept = 9.18) (Figure 4A) and infra-orbital sinus (Intercept = 7.99) (Figure 4B). As compared to the middle turbinates, an increasing trend in tissue damage in infra-orbital sinus was noted in chicken as infection progressed (Figure 4C and D). Both time as well as host factor determined the development of histopathological changes in middle turbinates and infra-orbital sinus (Supplementary Table 3), where chicken had significant higher histopathological scores in middle turbinates by 24 hpi ($p < 0.01$) (Figure 4C) and in infra-orbital sinus at 3dpi ($p < 0.05$) (Figure 4D). Infection-affiliated changes e.g. heterophilic and lymphocytic infiltration were governed by the time of infection and species involved (Supplementary Table 4). Chicken had a significantly higher heterophilic infiltration than Japanese quail especially at 24hpi ($p < 0.001$). Pathological changes like loss of epithelium, lymphoid cell infiltration, NALT hyperplasia and haemorrhages were found to be mainly host driven and were significantly higher at 72 hpi in birds. However, in chicken haemorrhages were visible at 24 hpi that peaked on 72 hpi (*data not shown*).

4. Discussion

Av. paragallinarum infection in poultry especially in chicken has been studied extensively. However, a complete understanding of pathological changes immediately after infection is still lacking. Moreover, the disease pathology in other species of birds than chicken is not known. Here, we elucidate, for the first time, the kinetics of pathological changes due to *Av. paragallinarum* infection in the nasal turbinates of chicken and Japanese quail. We developed a comprehensive scoring system for recording clinical signs, gross and histopathological lesions during *Av. paragallinarum* infection in chicken and Japanese quail. Following experimental infection, the prominent clinical signs in chicken included unilateral and bilateral nasal discharge while Japanese quail exhibited only conjunctival redness for a short duration. The development of clinical signs in chicken was noticed a little earlier than the previously reported studies. This could be possibly due to the use of younger birds (<2.5 weeks of age) and intra-sinus route of inoculation in our study. In chicken, ocular discharges and mild swelling of infraorbital sinus were also observed but were less severe than other reports (Anjaneya et al., 2013; Xu et al., 2019). This could be due to our use of a mild to moderate pathotype of *Av. paragallinarum* that did not severely affect architecture of infected organs. Therefore, the bacterial strain, pathogenicity, and host susceptibility could govern outcome of clinical signs and antagonistic effect to infection (Jacobs et al., 2003; Zhao et al., 2010). A multiple infusion of infection through direct intra-sinus inoculation of birds over a period of three days for the establishment of infection and development of clinical signs has been attempted previously (Anjaneya et al. 2013). However, we successfully reproduced reasonable level of infection in both chicken and Japanese quail without multiple inoculation of the birds.

We speculate that the lack of clinical signs in Japanese quail is predominantly associated with host factors including an increased level of lipid peroxidation by the epithelial surface and leucocytes in systemic circulation (Diptesh et al., 2020). This mechanism could promote protection of Japanese quail against infection, besides the existential anatomical complexities at nasal turbinates of Japanese quail (Cevik-Demirkan et al., 2007). Using an *in silico* analysis, an anti-oxidant mechanism as an early response

strategy against *Av. paragallinarum* has been reported in chicken (Boucher et al., 2014). In our earlier work, we found that Japanese quail is tolerant to this oxidative stress mechanism and uses an undefined mechanism for disease resistance (Diptesh et al., 2020).

The trend in the development of gross pathological lesions in nasal turbinates was almost similar to other studies (Bragg 2002; Anjaneya et al., 2013; Ali et al., 2013) with a slight variation in severity and intensity. The severity of lesions in our study mainly ranged from mild to moderate degree with rare occurrence of severe intense lesions. In addition to the pathogenic strain type, we also speculate that some host-oriented inherent immunological factors like early serum/tissue bactericidal activity could govern the outcome of infection (Byarugaba et al., 2007). Statistical analysis during early host pathogen interaction reveals significant impact of *Av. paragallinarum* in chicken as compared to Japanese quail. In chickens, the gross pathological changes begin at 12 hpi and peaked at 48hpi, which suggest that chicken is a more susceptible host than Japanese quail. Among various host-associated pathological variables, mucus or nasal secretions were found to be markedly influenced by the infection and a significantly higher mucus outflow was observed during first 48 h. Importantly, we have previously observed lodgement of bacteria across alveolar mucoid gland, their luminal surface, and at the base of epithelial lining of glands by immunohistochemistry (IHC) (Balouria et. al. 2019; Ahmed et al, 2020). Such changes were commonly observed in middle turbinates and inferior nasal meatus. Other prominent change was redness in turbinates, an invariable host response at an early stage i.e. within 12-24 hpi following intra-sinus inoculation arising from the damage inflicted upon penetration of needle. Intriguingly, redness of turbinates continued further resulting into more inflammatory disturbances at later time points i.e. 24 and 72 hpi. A similar effect was observed in Japanese quail but only for ~48 h. Microscopically, the noted redness in turbinates was not merely associated with bacterial-inflicted damage but also as a result of bacterial adhesion to ciliated epithelial cells resulting in epithelial cell damage and inflammation. A similar loss of epithelium was previously observed in chicken following infection of chicken with field isolates of *Av. paragallinarum* and the authors suggested the epithelium loss as a possible cause of narrowing of nasal passages and airways (Anjaneya et al., 2013). However, such epithelial damage was not noticed in Japanese quail in our study, despite bacterial adhesions with ciliated surface, which implies poor bacterial attachment on the respiratory mucosal surface. Future studies are required to know if any host-derived factor is responsible for the disease resistance in Japanese quail or if the moderately pathogenic bacterial strain used in our study failed to induce adequate cellular pathological changes *Av. paragallinarum* is capable of producing outer membrane vesicles (OMVs) with secretory proteins that can contribute to its pathogenicity through formation of biofilms (Ramon Rocha et al., 2006). The role of OMVs in the pathogenicity of *Av. paragallinarum* in Japanese quail needs verification. On the contrary, in chicken, *Av. paragallinarum* can induce biofilm formation through OMVs at nasal turbinates, which augments its pathogenicity. This was well supported from our earlier histological observation (IHC) of co-aggregation of bacteria at respiratory epithelial surface, tissue edema, and partial lysis/necrosis of epithelial cells at nasal turbinates in chicken (Balouria et al., 2019; Singh, 2020).

Heterophils are the major polymorphonuclear leukocytes in birds that act against most bacterial infections and respond early to pathological insults (Krams et al., 2012). In the present study, heterophils were found as early as 12 hpi in Japanese quail than chicken, which shows differential ability of the Japanese quail in mounting a quick immunological response to combat infection. In a parallel experiment, we observed an increased tissue-affiliated heterophil to lymphocyte ratio in nasal turbinates of infected Japanese quail that was associated with a rise in haemagglutinin antibody response (Diptesh et al. 2020). This is in contrast to a report where a weaker antibody response in the host (i.e. Great tits - *Parus major*) was reported with high heterophil to lymphocyte ratio due to infection or infection-associated stress (Krams et al., 2012).

Unlike Japanese quail, the chickens in the present study had little higher proportion of lymphocytes as compared to heterophils. Despite this, chicken could not mount a reasonable antibody response. However, secondary lymphoid follicle formation in the form of secondary NALT was observed only in chicken. The NALT formation probably resulted from contigual presence of bacterial antigen(s) causing modification and re-activation of lymphoid cells for more pronounced immune protection. To the best of our knowledge, there are no reports on the formation of secondary lymphoid cell follicle as secondary NALT at sub mucosal region of nasal turbinates in IC. Therefore, while evaluating the cellular changes, we carefully excluded primary NALT from this characteristic observation. Due to the absence of local lymph nodes, avian species mostly rely on localized sub-epithelial and sub mucosal lymphoid aggregates, like primary NALT (as in case of upper respiratory tract), for antigen uptake, processing and induction of an immune response. NALT are the primary storehouse of T cells, confer cell-mediated immunity (Kuper et al., 1992; Oshima and Hiramatsu, 2000), and undergo conditional hyperplasia. However, our recent data show that NALT can extend humoral immunity to chicken through inducing B cell and IgA responses (*Manuscript under review*). Therefore, histologically observed NALT hyperplasia on 72 hpi and thereafter in both the species, is because of long, persistent presence of bacteria or bacterial antigens, which invariably triggered re-organization, clustering and packaging of both B and T cells for better immune protection. In addition, the presence of bacteria at later time points corroborated with our successful re-isolation of bacterial colonies as late as 7 dpi. We hypothesize that *Av. paragallinarum* can influence mucus secretion from the host and reduce the viscosity of mucus gel to more liquid form. A watery mucus could enable bacteria to migrate within luminal surface to reach deeper peri-glandular tissues. A similar situation has also been described for *Helicobacter pylori*, which hydrolyses acidic mucin into neutral mucin in gastric glands that facilitates its easy migration within mucus (Celli et al. 2009). Thus, the changes in composition of mucin might have governed direct interaction of *Av. paragallinarum* with tissues leading to alveolar mucus gland hyperplasia and hypertrophic changes.

Overall, our data show that chicken is the most favourable host for *Av. paragallinarum* infection and has a high tendency to acquire the infection from the environment. Japanese quail does not seem to act as a host species favouring the amplification of this pathogen. Due to the use of mild to moderate pathotype of *Av. paragallinarum*, we could not induce infection similar to that under field conditions in both chicken and Japanese quail. Based on the pathological changes observed in the current study, Japanese quail could not be used as a novel animal model for IC. However, we found that younger chicken i.e. chicken

≥2.5 weeks of age are capable of eliciting local immune response to the infection. Further research efforts are required to verify the potential of Japanese quail as a novel animal model for IC and to understand the ability of Japanese quail to resist ongoing infection and its susceptibility to the infection following breach of immunity or immunosuppression.

Declarations

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Declarations statement

Ethics Approval

All experimental procedures were approved by local Institutional Animal Ethics Committee (IAEC), GADVASU, Ludhiana, India with approval no. GADVASU/2014/IAEC/23/006 and performed in compliance to their regulations and guidelines.

Consent to participate

Not applicable.

Consent for publication

All the authors agreed to the content of the article and declare no competing interest.

Authors Contribution

Authors contributions	AB	SD	HSB	MMC	SKD	AT
Reserach concept and design	—	Y	—	—	—	Y
Isolates provided	—	—	—	Y	—	—
Conduct of experiment collection of tissues / recording of lesions /assembly of data	Y	Y	Y	—	—	—
Data analysis and interpretation	—	Y	—	—	Y	—
Histopathology and slide interpretation	—	Y	Y	—	—	—
Writing of article	—	Y	—	Y	—	Y
Final approval of article	Y	Y	Y	Y	Y	Y

Y: Yes; —: No

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MMC: Milind M Chawak; SKD: Shakti Kanta Dash; AT: Aneesh Thakur.

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Competing interest: No potential conflict of interest was reported by the author(s).

Data availability: Data leading to the findings of this study will be available from corresponding author upon reasonable request.

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Tables

Table 1: Experimental set up

Group	No. of Birds	Inoculum and Volume	Distribution of inoculum	Sampling time point(s)
			(@ 1.5 x10 ⁸ CFU/ml/bird)	Post-challenge (Hours/days)
Naïve Chicken	2	None		0
Naïve Japanese quail	2	None		0
Sterile broth-inoculated Chicken	5	Sterile <i>Haemophilus</i> broth (1 ml / bird)		12 hrs, 1, 2, 3, 7
Sterile broth-inoculated Japanese quail	5	Sterile <i>Haemophilus</i> broth (0.5 ml / bird)		
Infected Chicken	23 ^a	<i>Haemophilus</i> broth + <i>Avibacterium paragallinarum</i> (1 ml / bird)	0.5ml right intra-sinus	12 hrs, 1, 2, 3, 7
			0.2 ml left intranasal*	
			0.2 ml left ocular*	
			0.1 ml right ocular	
Infected Japanese quail	23 ^b	<i>Haemophilus</i> broth + <i>Avibacterium paragallinarum</i> (0.5 ml / bird)	0.25 ml right intra-sinus	12 hrs, 1, 2, 3, 7
			0.1 ml left intranasal*	
			0.1 ml left ocular*	
			0.05 ml right ocular	

Day 0 was the day before challenge.

^{a&b} Total Number of birds in duplicate cages.

* Distribution sites (left side) considered for histo-anatomical localization of bacterial antigen.

Table 2: Scoring system for the evaluation of clinical signs in chicken and Japanese quail experimentally infected with *Avibacterium paragallinarum* (Balouria, 2017).

Clinical signs and its description	Intensity	Score
A) Nasal discharge		
Pressing of nostrils results in flow of scanty nasal secretions.	+ (Mild)	1
Partial or intermittent flow of nasal secretions or sticking/drying of previous fresh exudates near nostrils.	++ (Moderate)	2
Excessive, free flow of secretions from nasal passages without pressing of nostrils.	+++ (Severe)	3
B) Ocular discharge		
Flooding of orbital area with clear ocular secretions.	+ (Mild)	1
Partial flow of ocular secretions as evident by sticking/drying of immediate ocular secretions to nearby skin surface.	++ (Moderate)	2
Continual or intermittent free flow of ocular secretions from ocular orbit.	+++ (Severe)	3
C) Facial swelling		
Bilateral or unilateral swelling of triangular fossa (infra-orbital sinus).	+ (Mild)	1
Bilateral or unilateral swelling of sinus with elevation of facial /orbital surface.	++ (Moderate)	2
Both side swelling with prominent evidence of raised bumps over facial surface/orbital surface.	+++ (Severe)	3
D) Conjunctival redness		
Mild red coloration of conjunctival surface.	+ (Mild)	1
Rose red coloration of conjunctival surface.	++ (Moderate)	2
Prominent dark red coloration of conjunctival surface.	+++ (Severe)	3

Table 3. Scoring system for the evaluation of gross lesions in chicken and Japanese quail experimentally infected with *Avibacterium paragallinarum*.

Types of gross lesions and their description	Intensity	Score
<i>A) Redness of turbinates</i>		
Red or pinkish colouration of mucosal surfaces of nasal turbinates	+ (Mild)	1
Marked red discoloration of mucosal surfaces of nasal turbinates (without hemorrhagic impression)	++ (Moderate)	2
Dark intense red colouration with hemorrhagic impression	+++ (Severe)	3
<i>B) Mucus secretion/accumulation</i>		
Scanty and sticky mucus secretions adhering to mucosal surface on one or both sides of nasal turbinates	+ (Mild)	1
Moist mucus accumulation on both sides of nasal turbinates	++ (Moderate)	2
Copious accumulations of watery/frothy secretions on both sides of nasal turbinates	+++ (Severe)	3
<i>C) Swelling of infra-orbital/ supra-orbital sinus</i>		
Bilateral or unilateral swelling as bumpy raised surface along with mild redness of skin surface with or without mild sticky mucus exudates inside.	+ (Mild)	1
Bilateral or unilateral swelling as bumpy raised surface along with apparent/noticeable redness of skin /surface with fluidly clear mucus exudates inside.	++ (Moderate)	2
Swelling on both sides with prominent evidence of red and raised bumps over facial surface/orbital surface with partially clear or opaque mucus exudates inside.	+++ (Severe)	3
<i>D) Conjunctival haemorrhages</i>		

Localized areas of redness with or without scattered petechial haemorrhages in conjunctival surface.	+ (Mild)	1
Patches of redness with or without haemorrhagic impression in conjunctival surface.	++ (Moderate)	2
Diffuse areas of intense red coloration of conjunctival surface with haemorrhagic changes.	+++ (Severe)	3

Table 4: Scoring system for the evaluation of histopathological changes in nasal turbinates of chicken and Japanese quail experimentally infected with *Avibacterium paragallinarum*

Types of histopathological lesions and their description	Intensity	Score
A) Loss of mucosal epithelial cells		
Focal or small sized multifocal damages as epithelial thinning or desquamation (sloughing) of cells, cellular degeneration or necrosis without denudation to sub epithelial connective tissues.	+ (Mild)	1
Widespread damage in the form of loss of epithelial cells due to desquamation (sloughing) or cellular degeneration or necrosis leading to partial denudation to sub epithelial connective tissues with cellular infiltration.	++ (Moderate)	2
Complete loss of epithelial cells with exposed sub epithelial connective tissues, accompanied by massive haemorrhages and cellular infiltrations.	+++ (Severe)	3
B) Loss of cilia/deciliation		
Focal to multifocal attenuation of cilia or absence from epithelium or fur-like arrangement of cilia at some places.	+ (Mild)	1
Widespread attenuation or absence or sloughing of cilia.	++ (Moderate)	2
No traces of cilia noted due to attenuation of epithelium or due to loss of associated epithelium.	+++ (Severe)	3
C) Alveolar mucoid gland activity		
Hypertrophy of alveolar mucous gland with accumulated secretions or mild goblet cell hyperplasia.	+ (Mild)	1
Hypertrophy of alveolar mucous gland, goblet cell hyperplasia and beginning of stratification of mucus glands.	++ (Moderate)	2
Massive hyperplasia of glands noted with resultant stratification along with goblet cell hyperplasia.	+++ (Severe)	3
D) Heterophilic cell infiltration		
Heterophils infiltration in few places with confined distribution to epithelial surface or sub epithelial tissues.	+ (Mild)	1
Small sized multifocal or widespread heterophils infiltration at intra-epithelial region and in sub epithelial region.	++ (Moderate)	2
Wide and dense infiltration of heterophils across epithelium, sub epithelial connective tissues or upto <i>muscularis mucosa</i> .	+++ (Severe)	3
E) Lymphoid cell infiltration		
Lymphoid cell infiltration in few places with confined distribution around mucoid gland, interglandular region and sub epithelial connective tissues.	+ (Mild)	1
Multifocal to widespread lymphoid cell infiltration at intraepithelial region, interglandular region and upto sub epithelial connective tissue.	++ (Moderate)	2

Types of histopathological lesions and their description	Intensity	Score
Marked lymphoid cell aggregation [~ secondary nasal-associated lymphoid tissue (NALT) formation], dense infiltration across the epithelial surface, sub epithelial connective tissues extending to <i>muscularis mucosa</i> (not involving NALT of inferior nasal meatus).	+++ (Severe)	3
F) <i>Vascular congestion/ Hyperaemic changes</i>		
Only vascular congestion noted without dilatation of blood vessels.	+ (Mild)	1
Vascular congestion noted as dilatation of blood vessels, along with increase in their numbers.	++ (Moderate)	2
Marked dilation of blood vessels; often accompanied with or without focal or occasional areas of haemorrhages.	+++ (Severe)	3
G) <i>Haemorrhages</i>		
Occasional focal foci of haemorrhages especially around the blood vessels.	+ (Mild)	1
Focal to large widespread areas of haemorrhages.	++ (Moderate)	2
Diffuse areas of haemorrhages.	+++ (Severe)	3
H) <i>Nasal associated lymphoid tissue hyperplasia</i>		
Expansion of primary lymphoid zone with vascular congestion and haemorrhages.	+ (Mild)	1
Expansion of lymphoid zone (with or without germinal centre formation); often associated with encroachment to surrounding tissues.	++ (Moderate)	2
Profuse expansion of lymphoid zone with complete replacement of neighbouring tissue details with or without germinal centre formation.	+++ (Severe)	3

Supplemental Data

Supplemental Tables 1-4 are not available with this version

Figures

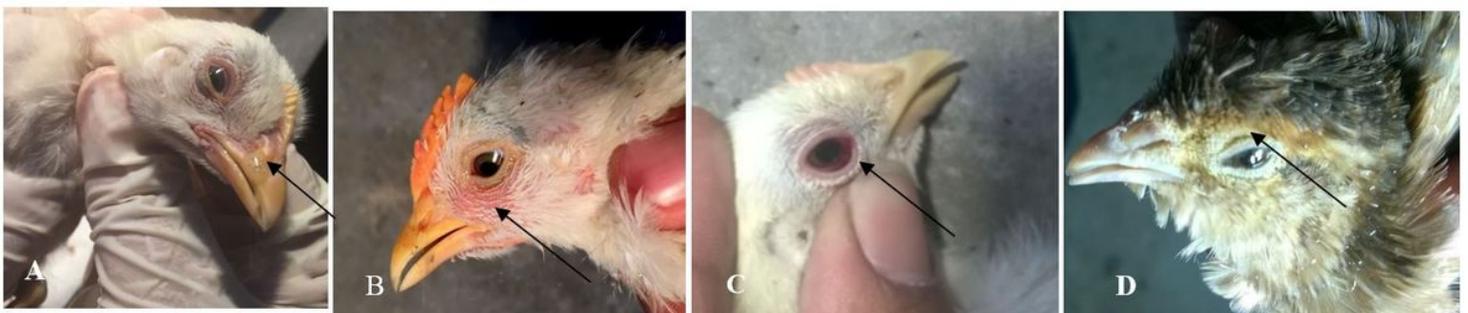


Figure 1

Development of clinical symptoms and pathologic severity noted in nasal turbinates after infection with *Avibacterium paragallinarum* serovar B in chickens and Japanese quail. A. Mucous secretions from nostrils (score 2) at 24 h post infection (hpi) in chicken. B. Mild, unilateral swelling of infra-orbital sinus at 48 h pi (score 1) in chicken. C. Congestion in the conjunctival surface in both eyes at 24 hpi (Score 2) in chicken. D. Mild peri-orbital swelling and ocular secretions in Japanese quail at 24 hpi (score 1).

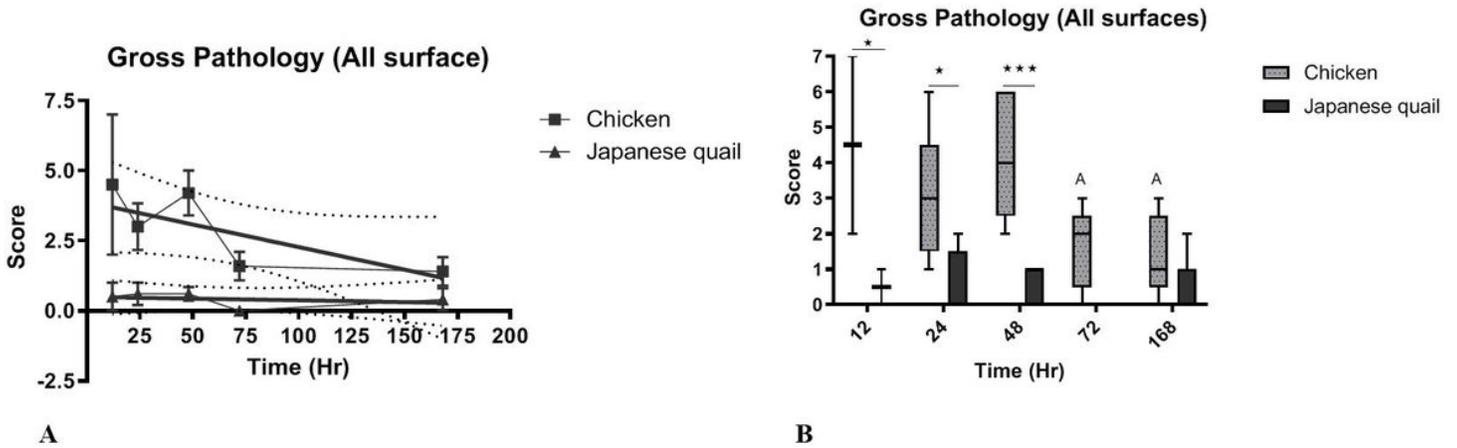


Figure 2

Effect of *Avibacterium paragallinarum* serovar B (1.5×10^8 CFU/ml per bird) on the outcome of gross pathological changes in nasal turbinates at 5 pre-considered time points after infection challenge. A. Regression analysis showed distinct and overall trend in the progression of pathologic changes between the two species following infection at various time points. A goodness of fit, $r^2 = 0.21$ was noted in chicken as compared to Japanese quail (goodness of fit, $r^2 = 0.011$). B. Multivariate linear mixed model analysis has shown distinction in the severity of lesions developed between chicken and Japanese quail. Values are mean \pm SEM ($n=3$). * indicates significant difference between chicken and Japanese quail. Alphabet indicates significant difference noted in nasal turbinates at different time point post-inoculation when compared to naive control (data not shown), which did not show any pathological changes.

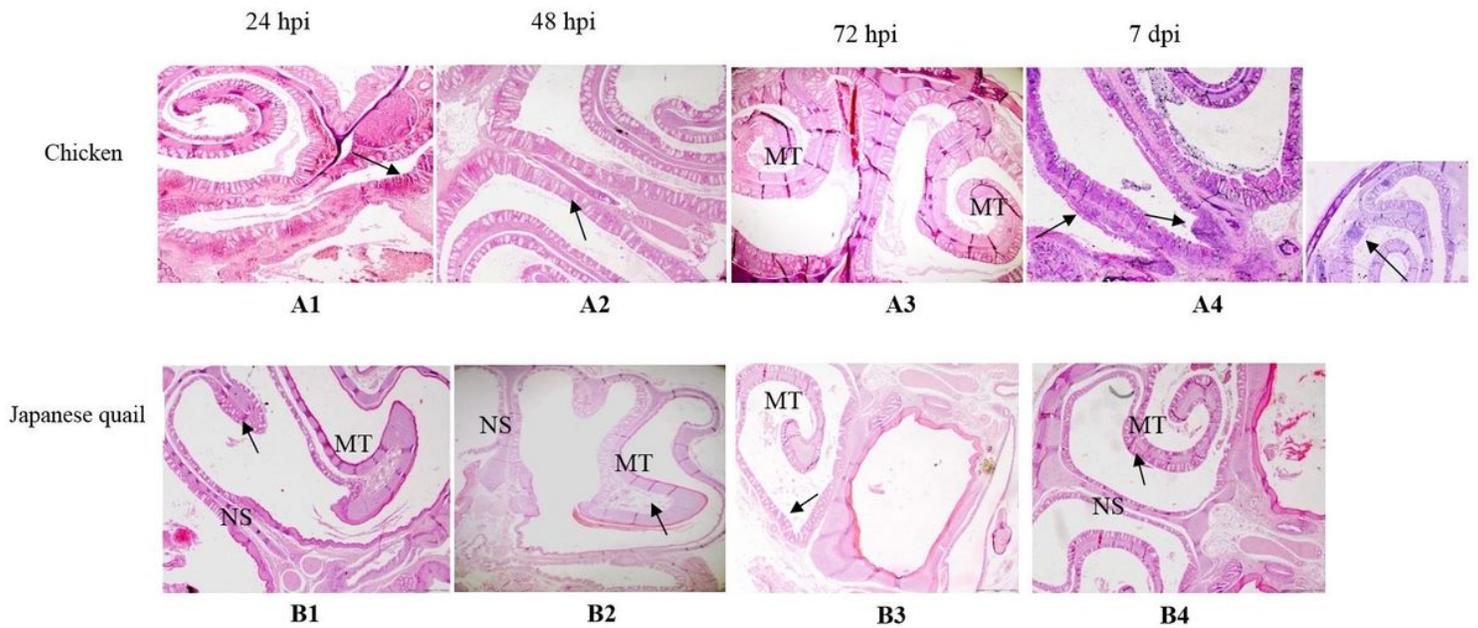


Figure 3

Histopathological changes observed in nasal turbinates of chicken and Japanese quail following experimental inoculation with *Avibacterium paragallinarum* serovar B (1.5×10^8 CFU/ml per bird). Figures in upper row indicate cross sections of nasal turbinates from chicken. A1. Haemorrhages were observed in middle turbinates (MT) and infra-orbital sinus (IOS) at 24 h post inoculation (hpi). A2. Proliferations of alveolar mucus gland and scattered multifocal haemorrhages at mucosal lining of middle turbinates noted at 48 hpi. A3. Increased mucosal thickness following inflammatory cell infiltrates and increased alveolar mucus gland in middle turbinates at 72 hpi. A4. Presence of lymphoid cell infiltrates at all major mucosal surface of inferior nasal meatus (INM) and middle turbinates (MT) on 7 day post inoculation. Figures in lower row indicate cross sections of nasal turbinates from Japanese quail. B1-B2. Congestion, edema and focal haemorrhages at sub epithelial region of anterior turbinates (AT) and middle turbinates (MT) after 24 h post inoculation (hpi) and 48 h pi. B3. Thickening of mucosa due to alveolar gland hyperplasia and fibrous tissue proliferation in sub mucosal connective tissue at 72 hpi. B4. Regenerative changes with return of normal histo-architecture of mucosal lining with hyperplastic alveolar glands on 7 day post inoculation (dpi). All images were acquired at x4 magnification.

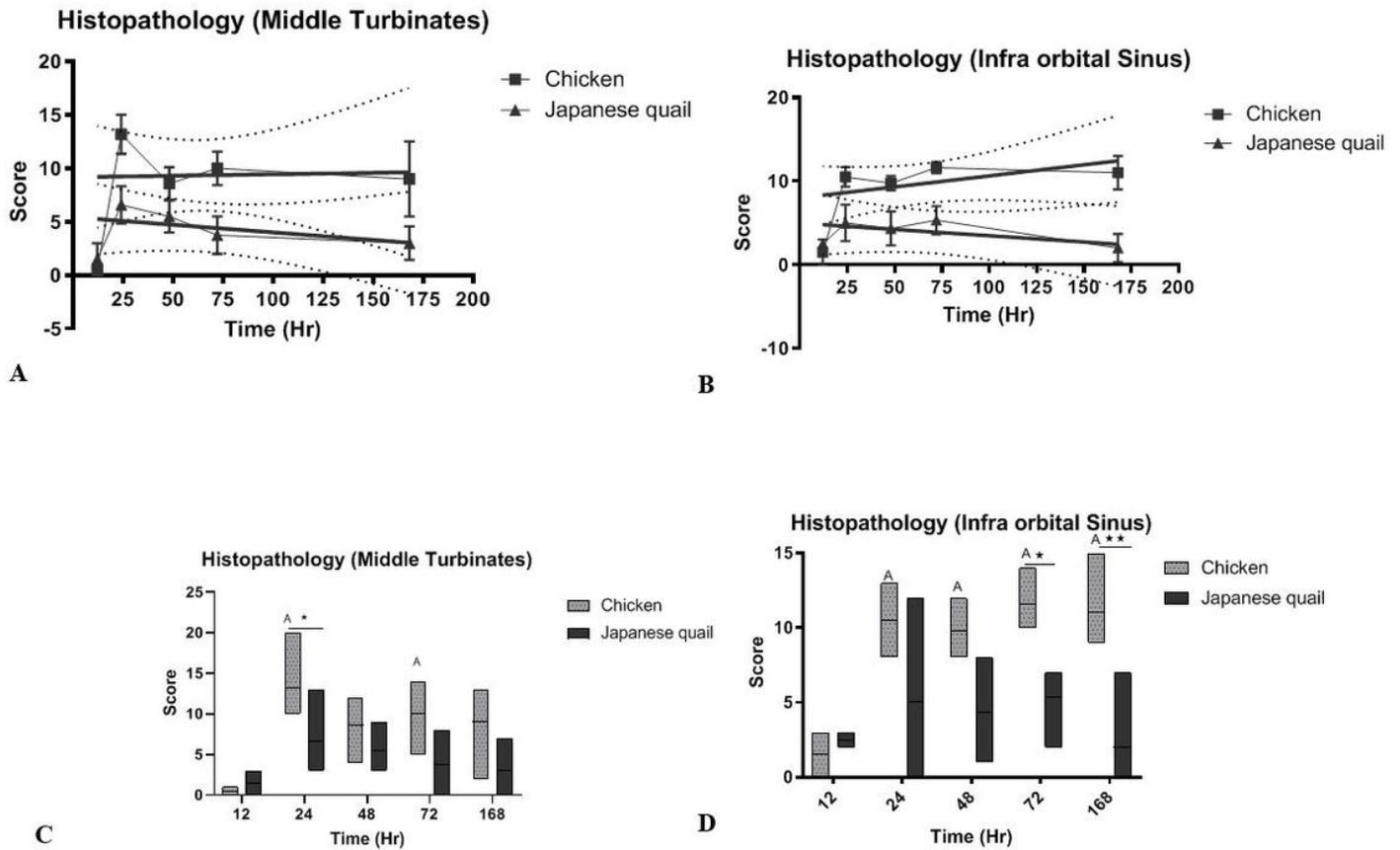


Figure 4

Effect of *Avibacterium paragallinarum* serovar B infection on the outcome of histopathological changes noted in middle turbinates and infra-orbital sinus at different time points. A-B. Regression analysis showed distinct differences in histopathological changes noted between chicken and Japanese quail following infection at various time points. An intercept = 9.18 for middle turbinates and intercept = 7.99 for infra-orbital sinus indicates these two region are most affected region of nasal turbinates following infection challenge in both chicken and Japanese quail respectively. C-D. Multivariate linear mixed model analysis has shown distinction in the severity of lesions developed between chicken and Japanese quail. Values are mean±SEM (n=3). * indicates significant difference between chicken and Japanese quail. Alphabet indicates significant difference noted in nasal turbinates at different time point post inoculation when compared to naive control (data not shown), which did not show any pathological changes.