**Immunopathogenesis of infectious bronchitis virus Q1 in specific pathogen free chicks**

B. Manswr1,2, C. Ball1, A. Forrester1, J. Chantrey1,3, K. Ganapathy1

1Institute of Infection and Global Health, University of Liverpool, Leahurst, UK

2Faculty of Veterinary Medicine, Diyala University, Iraq

3 Institute of Veterinary Science, University of Liverpool, Leahurst, UK

Corresponding author Tel.: +44 151 7946019; fax: +44 151 7946005.

E-mail address: [gana@liverpool.ac.uk](mailto:gana@liverpool.ac.uk)

# **ABSTRACT**

The immunopathogenesis of avian coronavirus, infectious bronchitis virus (IBV) Q1, was investigated in specific pathogen free chicks. Following infection, chicks exhibited respiratory clinical signs and reduced body weight. Oropharyngeal (OP) and cloacal (CL) swabs were collected at intervals and found to be RT-PCR positive, with a greater number of partial-S1 amino acid changes noted in CL swabs compared to OP swabs. In tracheal, kidney and proventriculus tissues, IBV viral load peaked 9 days post infection (dpi) in the trachea and kidneys, and 14 dpi in the proventriculus. At 28 dpi, ELISA data showed that 63% of infected chicks seroconverted. There was significantly higher mRNA up-regulation of IFNα, TLR3, MDA5, LITAF, IL-1β and IL-6 in the trachea compared to the kidneys. Findings presented here demonstrate that this Q1 isolate induces greater lesions and host innate immune responses in chickens’ tracheas compared to the kidneys

**KEYWORDS:** Infectious bronchitis virus, avian coronavirus, specific pathogen free, pathogenesis, immune responses, gene transcription

# **1. INTRODUCTION**

The emergence of newinfectious bronchitis virus (IBV) genotypes (avian coronavirus) may have been influenced by continuous genetic mutations and recombination events [1]. Several of the emerging variant viruses have economic importance due to their ability to cause disease and production losses. While the respiratory tract is the main site of infection, , depending on the IBV strain , non-respiratory organs such as the female reproductive tract, kidneys and proventriculus can also be infected [2]. Though a number of infectious bronchitis (IB) vaccines and vaccination programmes are available for poultry farmers, the protection conferred against the newly emerged variant IBVs is lacking, mainly due to substantial antigenic differences between the emerging and existing vaccine viruses.

In the period between 1996 and 1998, a novel IBV variant was detected in Chinese layer flocks, which was later characterized as a Q1 genotype [3]. Infection was associated with respiratory distress, proventriculitis, decreased egg production and diarrhoea. Since the original report, several Q1-like IBV strains have been detected in countries such as Taiwan (2005) [4], Italy (2011) [5], Chile (2008-2009) [6], Argentina [7], and Colombia [8]. The Q1-like strain identified by Toffan et al. (2013) had 100% similarity with the original Q1 described by Yu et al. (2001), and 99.2% similarity with another Chinese Q1 variant CK/CH/LDL97I/97 [9]. Further emergence of Q1 has been recorded in three countries in the Middle East (Iraq, Jordan, and Saudi Arabia) [10]. A study by Ganapathy et al (2015) found that Q1 represented around 11% of the genotypes circulating in this area [11].

Alongside the agent (IBV) and environment, underlying immune responses also determine the degree of pathology and rate of recovery from illness. Following IBV infection, a number of innate immune response pathways are activated in the chicken host. Cytoplasmic surfaces of host immune cells express pattern recognition receptors (PRR). One family of receptors are toll-like receptors (TLRs), which are responsible for recognising conserved structures, including the spike glycoprotein [12]. Type I IFNs demonstrate prominent individual anti-proliferative, anti-viral and pro-apoptotic actions despite sharing similar receptors [13, 14]. Melanoma differentiation associated protein 5 (MDA5) also induces interferon-beta (IFN-β) transcription [15]. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and lipopolysaccharide-induced tumor necrosis factor (TNF)-α (LITAF), are important aspects of innate immunity, which bind to specific cell surface receptors to initiate cascades of intracellular signalling [16]. The IL-1β molecule, produced by epithelial cells and macrophages, induces T cell and macrophage activation, thus recruiting cells to the site of infection through chemotaxis [17]. IL-6 is produced by endothelial cells, T cells and macrophages, and promotes acute phase responses and differentiation of both B and T cells [18]. TNF-α is produced by T cells, macrophages and natural killer cells, and helps localise inflammation and endothelial cell activation at the site of infection [19].

To date, little to no information is available on immunopathogenesis of Q1, particularly on outlining the host innate immune responses. We addressed this through an infection model using SPF chicks, with collection of samples at intervals. This highlighted the immune responses, the distribution of viruses, and the inherent molecular changes in the Q1 virus.

# **2. MATERIALS AND METHODS**

**2.1 Ethical statement**

All experimental procedures were performed according to the UK legislation on governing experimental animals under the project licence P8E4FC2C9. Experimental procedures were approved by the University of Liverpool ethical review process.

## **2.2 Chick welfare and management**

Commercially available fertile SPF eggs were incubated and hatched at the University of Liverpool, United Kingdom. Chicks were reared until 28 days of age in a high-biosecurity poultry unit (University of Liverpool), meeting requirements of UK legislation. Birds were reared on deep litter (wood shavings), with water and feed provided *ad libitum*.

## **2.3 Q1-like infectious bronchitis virus**

An IBV Q1 virus strain (reference number 37089/2013) was used to infect the chickens in this study. Strain details, including the pathogenicity in broiler chicks, has been previously reported [12]. The virus was propagated in embryonated white leghorn SPF eggs before use. Following propagation, virus-rich allantoic fluid was collected (5th passage) and titrated in trachea organ cultures (TOCs). The titre inoculum used was 106.5 ciliostatic doses (CD)50/ml. The inoculum was confirmed to be free from other avian viruses such as Newcastle disease virus, avian metapneumovirus, avian influenza and infectious bursal disease virus by RT-PCR, and confirmed to be free from bacteria, fungi and Mycoplasmasby culture [20, 21].

## **2.4 Experimental design**

After hatching, 80 SPF chicks were randomly allocated into two groups, with 40 chicks per group. At day-old, chicks received either 100 µl virus free allantoic fluid (Group 1) or 100 µl 104.5 CD50/bird of IBV Q1 (Group 2) via the oculo (50µl) and nasal (50µl) route. The inoculum was administered as drops, using a sterile pipette and filter tips. Treatment groups were kept in separate, negative pressure rooms with controllable temperature and humidity. Clinical signs were observed daily [2] by the lead researchers and ten chicks from each group were weighed weekly. Oropharyngeal (OP) and cloacal (CL) swabs were collected from ten birds at 0, 3, 7, 9, 14, 21, 28 days post infection (dpi) for virus detection by RT-PCR. At 1, 21 and 28 dpi, blood was collected from five birds and sera were subjected to ELISA. At 1, 3, 7, 9, 14, 21, 28 dpi, five chicks were humanely euthanised by injection of sodium pentobarbitone by the wing vein, and the trachea and kidneys scored for gross lesions [22]. Trachea and kidney samples were collected in RNA*later* (Qiagen, Crawley, UK) for quantification of IBV RNA and measurement of host gene expression. Trachea, kidney and proventriculus samples were collected in formalin and optimum cutting temperature (OCT) media for histological examination [23].

## **2.5 Clinical and pathological signs**

Clinical signs were recorded daily for each group [2]. Mild signs were considered as coughing, head shaking and nasal scratching, and severe signs considered as depression, along with gasping or ruffled feathers. As per the project licence, chicks exhibiting severe signs were humanely removed from the study.At necropsy, gross lesions observed on the trachea, kidney and proventriculus were recorded as previously described [2].

## **2.6 Histopathology**

Trachea and kidney tissues fixed in 10% buffered formalin were processed for histological examination. Fixed tissues were embedded in paraffin blocks and then sectioned at 5 μm thickness. Slides were stained with Haematoxylin and Eosin (H&E) [24]. For the trachea, five tracheal rings were scored per sample, with the average score taken from five samples per time-point. For kidney samples, the average score was taken from the five samples per time-point. Proventriculus samples were frozen in OCT media [23], cut into 5 μm sections using a pre-cooled cryostat, and mounted on glass slides. Slides were then subjected to H&E staining as previously described. Histological changes were scored as follows: 0 = no change, 1 = mild, 2 = moderate, 3 = severe [25].

## **2.7 Molecular detection of IBV from swabs**

For detection of IBV, using the phenol-chloroform method, RNA was extracted from the Q1 inoculum, and OP and CL swabs, [26, 27]. Following extraction, RT-PCR was conducted to detect a partial IBV S1 gene (393bp) as described previously.

Positive amplicons were purified by adding 0.99 μl shrimp alkaline phosphatase and 0.15 μl exonuclease (Affymetrix Ltd., High Wycombe, UK). Samples were incubated at 37°C for 30 min, then 80°C for 10 min to remove extraneous material. Purified samples were sent for commercial Sanger sequencing of the part-S1 gene of IBV, using the forward primer SX3+ (Source BioScience Ltd, Nottingham, UK).

**2.8 Swabs (OP and CL) amino acid comparison of partial S1 gene sequences**

Sequences were first cleaned using ChromasPRO v1.7.3 (<http://technelysium.com.au/>) and then aligned using ClustalW [28] in MEGA X [29]. Basic local alignment search tool (BLAST) comparisons were carried out against the sequence obtained from the Q1 inoculum, with amino acid changes arising from non-synonymous nucleotide variations recorded and visualised using Circos [30]

**2.9 IBV quantitative RT-PCR**

Viral RNA was quantified from the trachea, kidney and proventriculus. Total RNA was extracted from all tissues (30 mg per tissue) using the RNeasy Plus Mini kit (Qiagen), according to manufacturer’s instructions [31], and quantification of viral RNA was conducted [32]. All reactions were performed using the One-Step RT-PCR Qiagen kit and 40 ng of total RNA per reaction. Amplification plots were analysed and the cycle threshold (Ct) value determined. The Ct values were converted to log relative equivalent units (log REU) of viral RNA using a standard curve generated from 10-fold dilutions of extracted RNA from M41 infected allantoic fluid (106 EID50) as previously described [33].

## **2.10 Virus isolation from proventriculus**

Virus isolation was attempted from proventriculus samples taken at 3, 7 and 9 dpi [3, 5]. Tissues were ground aseptically, and stored at -70 °C until processed for RNA extraction. Ground tissues were centrifuged and 0.2 ml from the supernatant was inoculated into embryonated SPF eggs and incubated at 37 oC. Allantoic fluid was collected after 48 hours and used to inoculate the second passage. After another 48 hours of incubation, allantoic fluid was recovered and re-inoculated into SPF eggs for 5-days of incubation. After the third passage, allantoic fluid was collected and subjected to RNA extraction and RT-PCR. Positive amplicons were submitted for partial sequencing of the S1 gene as previously described (section 2.7 and 2.8).

# **2.11 Host gene expression**

Host gene expression analysis was carried out on trachea and kidney samples collected at 1, 3, 7, 9, and 14 dpi. Seven genes were analysed using qRT-PCR; type I interferon (IFN-α and IFN-β), toll like receptor 3 (TLR3), melanoma differentiation associated protein 5 (MDA5), pro-inflammatory cytokines (IL-6 and IL-1β) and lipopolysaccharide-induced tumour necrosis factor (TNF)-α factor (LITAF). All primers and conditions were as previously described [24]. Data were normalised using a relative standard curve method to 18S ribosomal RNA expression [34] and presented as the fold-change difference in gene expression of Q1-infected against the sham-inoculated samples.

## **2.12 Serology**

Serum samples were analysed using a commercial IBV ELISA kit (IDEXX, UK) according to manufacturer’s instructions. Serum with a sample to positive (S/P) ratio greater than 0.2 was considered positive.

# **2.13 Statistical analysis**

Statistical analysis was carried out in GraphPad Prism version 6. For parametric and non-parametric data, the *t*-test and Mann-Whitney test were respectively used to determine significant differences between the control and Q1 infected groups at the same time points. Differences between groups were considered significant at P<0.05.

# **3. RESULTS**

## **3.1 Body weight**

There was no significant difference in body weight between the control and Q1-infected group in the first week. From 14 dpi, we saw a significant decrease (P<0.05) in the average weekly body weight of chicks in the infected group at 14, 21 and 28 dpi compared to the control group (Figure 1).

## **3.2 Clinical signs and gross pathological lesions**

Mild clinical signs appeared within infected birds at one dpi and continued until 17 dpi. These signs included nasal scratching, head shaking and snicks. A single bird from the infected group died at 5 dpi and on necropsy, presented with swollen kidneys and ureters filled with urate deposits. There were no clinical signs or mortality in the control group during the study period.

Tracheal lesions were noted in all infected chicks until 14 dpi, peaking at 7 dpi. Lesions comprised of clear to caseous exudate, with or without hyperaemia. Birds showed signs of oculo-nasal discharge and periocular swelling at 14 dpi, with cloudy and turbid air sacs found at 9 dpi. In the kidney, gross lesion scores peaked at 9 dpi, consisting of paleness, swelling and urate deposition. In the proventriculus, there was dilation and thickening in the mucosa of one bird at 14 dpi. There were no gross lesions observed in the control group.

## **3.3 Histopathological changes**

In the infected group, tracheal lesions mainly consisted of de-ciliation and epithelial degeneration, decreased mucous cells and heterophil infiltration. Lesions started early at 1 dpi and remained until 21 dpi. The greatest tracheal histopathology scores were seen at 9 and 14 dpi (Figure 2 A1). In the kidneys, histological changes started at 1 dpi and peaked at 3-9 dpi (Figure 2 A2). Kidney lesions consisted of poorly defined sub-acute interstitial lymphoid infiltration and more discrete chronic lymphoid nodules. In the proventriculus, there were no significant microscopic lesions other than the presence of interstitial lymphoid nodules (Figure 2 B2). There were no histological changes in the control group.

**3.4 Detection of Q1 from p****ooled oropharyngeal (OP) and cloacal (CL) swabs**

All OP and CL swabs were IBV-positive by RT-PCR, with partial S1 gene sequences obtained at all sampling points. For the OP swabs collected at 3, 7, 14 and 28 dpi, amino acid (AA) sequences showed 100% identity to the original inoculum. For the 9 dpi OP swabs, there were 27 AA substitutions (Figure 3). At 21 dpi, there were 15 AA substitutions and two AA deletions: tyrosine (Y) and asparagine (N) at positions 306 and 307 within the S1 gene. In the CL swabs, there were 11, 30, 19 and 41 AA alterations at 3, 7, 9 and 21 dpi respectively. At 9 dpi, within the S1-gene, there were three AA deletions; tyrosine (Y), asparagine (N) and phenylalanine (F) at position 306, 307 and 308 respectively.

The most persistent amino acid changes occurred at positions 347 (n=5; arginine into lysine), 348 (n=4; leucine to glutamine), 350 (n=4; leucine to phenylalanine) and 368 (n=4; proline to arginine) (Figure 3). While these mutations were only present at 9 dpi in the OP swabs, the same variations were present at 3, 7, 9 and 21 dpi in the CL swabs. By 28 dpi, all substitutes and deletions were absent, and the detected strain had 100% identity to the initial inoculum.

## **3.5 Viral load in trachea, kidney and proventriculus**

Viral RNA was detected in all tissues on all sampling days, however, each tissue exhibited a different expression pattern (Figure 4). Viral load peaked in the trachea at 7 dpi (0.533 log10 REU), kidneys at 9 dpi (0.121 log10 REU) and proventriculus at 14 dpi (0.202 log10 REU). In the trachea, there was a significantly higher (P<0.05) viral load at 1-14 dpi when compared to 21-28 dpi. For the kidney tissue, viral load was significantly higher (P<0.05) at 1-21 dpi compared to 28 dpi.

**3.6 Virus isolation**

Virus re-isolation was attempted from the proventriculus at 3, 7, 9 dpi in SPF eggs. The virus was detected from all samples at all time points, and on sequencing, isolates had > 99% amino acid similarity to the initial inoculum.

## **3.7 Humoral anti-IBV antibody levels**

Serum samples at 1 dpi showed no detectable antibodies against IBV in either the control or infected groups. At 21 dpi, 43% of infected birds were ELISA positive against IBV, which increased to 63% by 28 dpi (data not shown). Controls remained ELISA negative throughout the study period.

## **3.8 Host gene expression analysis in the trachea**

***3.8.1 IFN-α and IFN-β mRNA expression***

There was significant up–regulation (P<0.05) of IFN-α mRNA expression at 7 dpi (Figure 5 A), whereas IFN-β mRNA was significantly up-regulated at 1 and 3 dpi (Figure 5 B).

# ***3.8.2 Pro-inflammatory cytokines transcription profile***

# Expression of IL-1β and IL-6 was only significantly up-regulated (P<0.05) at 7 dpi in the infected group compared with the control group (Figure 5 C & 5 D). In comparison, there was significant up-regulation (P<0.05) in LITAF expression during all sampling days (Figure 5 E).

***3.8.3 Expression of mRNA of TLR3 and MDA5***

There was significant up-regulation in TLR3 expression (P<0.05) for all sampling days compared to the control group (Figure 6 A), whereas MDA5 was only significantly up-regulated at 3 dpi (Figure 6 B).

## **3.9 Host gene expression analysis in the kidney**

***3.9.1 IFN-α and IFN-β mRNA expression***

There was significant (P<0.05) up-regulation identified in both IFN-α and IFN-β expression levels at all dpi in the infected group compared with the control group (Figure 5 A & 5 B).

***3.9.2******Pro-inflammatory cytokines transcription profile***

There was significant up-regulation (P<0.05) at 3-9 dpi for IL-1β, and 7-9 dpi for IL-6 in the infected group compared with control group (Figure 5 C & 5 D). Similar to the trachea, there was significant up-regulation (P<0.05) in LITAF for all sampling days (Figure 5 E).

***3.9.3 Expression of mRNA of TLR3 and MDA5***

There was significant up-regulation in both TLR3 and MDA5 expression (P<0.05) for all sampling days compared to the control group (Figure 6 A & 6 B).

## **4. DISCUSSION**

The pathogenesis and innate immune responses of several IBV strains have been previously published [35-37]. To further improve knowledge on the immunopathogenesis of Q1, attempts were made to examine host-Q1 virus interactions, including the induction of innate and humoral immune responses.

Chicks infected with Q1 exhibited a significant decline in average body weight from 14 dpi. Reduction in body weight following IBV infection has been previously reported for a number of strains, including the Australian T-strain [38], QX [39] and M41 [40]. While previous work has demonstrated an association between tracheal lesions and body weight reduction [41], it appeared to be strain-dependent, with variations in the association witnessed between five isolates. Furthermore, the body weight differences were found only for the first 11 days of infection. Although no specific reasons could be outlined for the prolonged reduction in body weight (up to 28 dpi) of Q1 infected chicks in this study, it may have been caused by damage to the kidney tubular epithelial cells [42], leading to irregularities in electrolyte and fluid transport. An increase in urinary water excretion can also occur, causing an increase of urine osmolality and high fractional excretion of sodium, calcium and potassium [38].

In this study, using SPF chicks, the duration and severity of lesions were found to be consistently greater in the trachea compared to the kidney. This finding is consistent with those reported for other classical and variant IBV strains, including Q1 infection of commercial broilers [5, 35, 39, 43-46]. A high severity of tracheal histological changes was seen at 9 and 14 dpi, mainly manifested by loss of cilia, mononuclear cell infiltration and epithelial hyperplasia. Similar findings were reported previously in Q1-like infected SPF chicks [3], however, in the earlier study, inoculation was given to 2, 7 or 16 week-old birds. Kidney samples showed interstitial lymphoid infiltration and mild lymphoid follicles similar to those previously reported in broiler chickens [5]. There were minimal histopathological changes in proventriculus samples, which mainly comprised of lymphoid follicles. The findings in the proventriculus is contradictory to those reported by Toffan et al. (2013), where they found diffuse epithelial necrosis, associated with infiltration of heterophils, lymphocytes and plasma cells in the lamina propria. The kidney lesions reported herewith in SPF birds were much milder compared to those found in commercial broilers. This could have been due to exacerbating factors in the field, such as co-infection with other pathogens, ammonia, temperature changes, dust, type of feed and other management practices in commercial broiler farms. The lesions reported for the Q1 isolate in this study are similar to those caused by other IBV strains [3, 24, 35, 47]

To date, there are limited studies focusing on the partial-S1 gene changes in virulent IBV strains detected in OP or CL swab samples. This is as most studies report on vaccine strains [26, 48]. Despite a greater viral load and histopathological lesions in the trachea, a greater number of amino acid changes were found in the CL swab compared to the OP swab sequences. This may be influenced by the microenvironment in the intestine compared to the upper respiratory tract [49], rather than replication-induced asymmetry [50]. Since the S1 protein is associated with attachment to host cells and is the primary target of virus neutralisation [51, 52], mutations in the S1-gene may be advantageous to the survival of Q1 in the gastrointestinal tract. This is more important for the six amino acid variations detected at multiple timepoints. Of the six, the only mutation to not persist until 21 dpv in the cloaca was also the only variation with a change in the hydrophobic properties (position 336; leucine to glutamine) [53]. By using Sanger sequencing, the current study reported genetic variations from the consensus sequence obtained from the majority virus population at each sampling point. However, future work examining the present sub-populations may provide further insight into genetic changes that occur following IBV infection.

The IBV Q1 strain was successfully re-isolated from the proventriculus, with the recovered isolate being more than 99% similar to the original Q1 inoculum, thereby highlighting the possibility of using this tissue for IBV detection or isolation [39, 54]. It was not clear if this detection indicates primary replication in the proventriculus or merely those found in the blood vessels. Despite viral RNA being detected in both swab and tissue samples, only 63% of birds seroconverted by 28 dpi, which was the end-point for the experiment. Therefore, the possibility of further increase in the number of birds becoming IBV-positive could not be ruled out.

In recent years, much attention has been given to understanding the early immune responses towards IBV isolates [24, 36], as this could improve our knowledge of disease mechanisms, which could ultimately be utilized for better control strategies. To monitor early immune responses following Q1 infection, TLR3, MDA5, IFN-α**,** IFN-β, IL-1β and IL-6 mRNA expressions were examined. For TLR3, significantly higher expressions was found at all sampling days compared to the control, with the expression peaking in the infected group at 7 dpi. This finding echoes data seen in similar studies reporting up-regulation of TLR3 following IBV infections in chickens, particularly during the early stage of infection (1-8 dpi) [24, 36, 55, 56]. However, the magnitude and onset in the current study differs from others, possibly due to variations in the IBV strain, inoculum dosage, route of administration and age of birds at the time of infection [24, 36, 55, 56]. There was a lower magnitude of TLR3 expression in the kidney compared to the trachea, which may be associated with the lower viral load. As infected cells distinguish virus components by PRRs, up-regulation may lead to early immune responses. Elevation of TLR3 is associated with an increase of inflammatory cytokines in response to viral pathogenesis and is also associated with the subsequent deterioration of infected tissue through the downstream cascade antiviral state [57, 58].

The role of MDA5 is well established in the induction of IFN-β transcription [15]. In the current study, at 1 dpi, there was a significant up-regulation of MDA5 in the trachea, which was consistent with previous reports [24, 55]. Compared to the control, this study demonstrated significantly higher levels of TLR3 and MDA5 in the kidneys following Q1 infection. Working on IBV IS/885/00, Chhabra *et al.* (2018) reported up to five-fold up-regulation of MDA5. In comparison, up to a three-fold change was found in this study. It appears that the magnitude of MDA5 and TLR3 expression could reflect the degree of tropism of IBV strains for the kidneys [59]. It has been shown that IFN-β expression occurs at a later stage of IBV infection, coinciding with the peak of viral replication and the complementary accumulation of double stranded RNA [15]. In this study, IFN-β expression was up-regulated up to 3 dpi following the Q1 infection. This is comparable to findings by Chhabra et al. (2018) and Okino et al. (2017). However, the magnitude of fold change reported by both studies was greater compared to the one reported in the current experiment, likely due to differences in the virulence of the IBV strains. In addition to detecting up-regulation of PRRs and IFNs, the findings in this study showed an up-regulation of pro-inflammatory gene expression in both investigated tissues. In the trachea, gene expressions corresponded with the gross lesion scores, the viral load and histological changes. An increase in IL-1β and IL-6 was previously described after challenge with virulent M41, which was associated with the highest microscopic lesion scores and viral load, suggesting IL-1β and IL-6 may have contributed to tracheal lesion induction [60]. All pro-inflammatory cytokines in the current study peaked at 3-7 dpi, and were associated with greater viral load and pathology in the trachea rather than in the kidneys [61].

In conclusion, the Q1 isolate used in this study is pathogenic for SPF chicks. It induces greater early immune responses in the trachea compared to the kidneys. Following Q1 infection, an increase in the mRNA expression of TLR3, IFNs, and IL-1β indicates an early robust host response to infection.

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**List of figures**

**Figure 1**. Weekly mean body weight comparison of control (C) and infected (Q1) groups on all sampling days. Data represent the mean with error bars as standard error with significant differences indicated by different letters (*P*<0.05).

**Figure 2**. (A) Histological scores in the (A1) trachea and (A2) kidney. (B) Haematoxylin and eosin stains of: (B1) Control proventriculus; (B2) Infected proventriculus at 9 dpi.

**Figure 3.** Location and type of amino acid variations detected in OP and CL swab samples. The outer track indicates the original Q1 inoculum amino acid sequence, with each subsequent track showing amino acid changes per sample. Changes which occur at least four samples are highlighted in grey, with hydrophobicity changes marked with (+). Samples with no changes are not included.

**Figure 4**. Quantification of viral RNA, expressed as a log relative equivalent units (REU) of RNA in the trachea, kidney and proventriculus of the Q1 Infected groups. Data represent the mean with error bars as standard error with significant changes indicated by different letters (*P*<0.05).

**Figure 5**. mRNA expression of (A) IFN-α, (B) IFN-β (C) IL1-β, (D) IL- 6 and (E) LITAF mRNA expression in Q1 infected trachea and kidney. Data represent the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters (*P*<0.05). Control groups are shown with a solid black bar, infected groups are shown with a chequered bar.

**Figure 6**. Transcriptional regulation of innate viral sensing molecules (A) TLR3 and (B) MDA5 in Q1 infected chicken trachea and kidney. Data represent the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters (P<0.05). Control groups are shown with a solid black bar, infected groups are shown with a chequered bar.