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Study of CD11c and CD169^{+} genes expression using immunohistochemistry technique in broilers infected with CRD

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Abstract

Avian Chronic Respiratory Disease (CRD) syndrome is a respiratory infection in chickens caused by many etiological agents, including *Mycoplasma spp. Escherichia coli* and some viruses, leading to pneumonia, air sacculitis, and septicemia, which are associated with high morbidity and mortality. This study aimed to evaluate immunological aspects, including marker expression for dendritic cells (CD11c) and respiratory macrophages (CD169+), in CRD broiler farms using immunohistochemistry, and to assess some pathological changes in these broilers. Fifty tracheal and lung histopathological section samples were from CRD-infected broilers used to detect pathological changes and to evaluate marker expression of dendritic cells (CD11c) and respiratory macrophages (CD169+). The results revealed severe tracheitis with vascular congestion, extensive epithelial cell necrosis, and inflammatory cell infiltration. The lung showed severe serofibrinous pneumonia with increased fibrous tissue, inflammatory cell infiltration, hyperemia, and blood vessel congestion, as demonstrated by hematoxylin and eosin staining. An immunohistochemistry study revealed intense expression of CD169+ macrophage cells and moderate expression of CD11c DCs in the lung sections of infected birds with CRD and moderate expression of CD169 macrophages and CD11c DCs in trachea sections of infected birds with CRD in comparison to lung sections of normal birds that revealed very weak to negative expression for each of them, respectively. It concluded that immunohistochemistry can be used to detect marker expression in dendritic cells (CD11c) and respiratory macrophages (CD169+), which determine the immunological state in birds infected with CRD.

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Introduction

The poultry industry is considered one of the most essential sources of white meat for humans, in addition to its fundamental role as a guarantor of nutritional security worldwide, as it provides an abundant and economical source of animal protein (1-3). Avian Chronic Respiratory Disease (CRD) considered one of the critical diseases that intimidate poultry industry in the world (4,5) that due to many reasons, first: their high morbidity and mortality rate, second: it need significant effort and expensive antibiotic treatments to improve performance of birds finally: many of infected pathogens causing CRD in birds are risky to public health and have a zoonotic importance (6). In addition to decreased egg production, reduced egg hatchability, and decreased weight gain and feed consumption (7,8). Many causative agents contribute to the development of CRD in poultry, including many viruses, such as avian pneumovirus, infectious bronchitis virus IBV, Newcastle disease virus ND, and even the virus of avian influenza, in addition to many bacterial causes such as *Mycoplasma gallisepticum* (9), *Escherichia coli* (10), *Chlamydophila psittaci*, *Staphylococcus spp*, *Pasteurella multocida*, *Bordetella avium*, *Riemerella anatipestifer* (5). CRD is nowadays

considered one of the most challenging diseases for broilers in Iraq (11). Immunohistochemistry is one of the essential techniques used to detect antigens, specific amino acids, or proteins, as well as causative agents, in infected cells or tissues (12,13). It can detect specific antigens by using particular antibody markers labeled with specific stains (12,14). Immunohistochemistry is nowadays one of the essential techniques that combines immunological principles with histological tools to specifically detect target antigens or proteins in tissue sections (15). It is a powerful technique capable of detecting foreign substances within the architecture of any tissue (16), and, when used to detect specific antigens or surface proteins in some cells, it requires the use of specific monoclonal antibodies as markers for these structures (16). Immunohistochemistry and histopathology are widely used in many fields as important tools for assessing changes in normal tissue, even during surgery. Zedan *et al.* (17) used Immunohistochemistry and histopathology successfully to assess a new surgical method to improve hernia tissue healing after a specific operation in sheep. Grabowska *et al.* (18) pointed that CD169 macrophage cells was on of the critical cells that contribute in the capture of each bacteria, virus, and dead cells both from blood and lymphatic fluids that act as filter prevent spreading and at the same time enables continuous production of antigens and transferring them to each of DCs and B lymphocytes to activate the acquired immune response. CD11c was an important marker for dendritic cells (19). The function of CD11c is somewhat unclear, but it definitely contributes to many immunological functions, such as phagocytosis, cytokine production, cell migration, and the adhesion of some cells to endothelium. Expression of surface CD11c, along with other surface proteins such as MHC II, CD80, and DEC205, which are associated with the absence of phagosomal acidification, allows the identification of a novel population of lung-specific DCs in interstitial tissue spaces of the parabronchial walls (19).

We aimed to use immunohistochemistry in this study to examine immunological aspects, including gene expression of dendritic cell (CD11c) and respiratory macrophage (CD169+) in selected respiratory tissues, and their roles in the immune response in CRD-infected broilers.

Materials and methods

Ethical approval

The tissue samples of current study were collected according to Committee approval by the Institutional Animal Care, University of Mosul, College of Veterinary Medicine under authority no. UN.VET.2022.037.

Samples collection

Fifty lung and tracheal samples from each of CRD-diseased broilers and healthy control samples were collected from different broiler farms, all birds were euthanised ethically. All samples were placed in 10% formalin solution in special plastic containers for histopathological analysis and immunohistochemical staining after tissue section preparation (20).

Sample preparation and histopathology

All samples under study were prepared and subjected to traditional histopathology sectioning by the following general steps: first, dehydration; then clearing and impregnation; and finally, embedding in paraffin. After tissue sections were prepared, they were stained with hematoxylin and eosin (21). All slides were examined by a microscope eyepiece grid using 20-400 magnification power, and results were recorded by a blind pathologist (22). Histopathological changes were graded on a scale of 3 to calculate the lesion score for each sample (23,24), and the expression of CD11c and CD169 on the dendritic cell and macrophage cell surfaces was detected.

Immunohistochemistry analysis

A specific technique, avidin-biotin immunoperoxidase, was used to obtain immunohistochemistry sections from previously prepared tissues. These sections were first rehydrated after deparaffinization, then deactivated, and finally prepared as immunohistochemistry sections using an IHC protocol. A 3% hydrogen peroxide-methanol solution was used for 10 minutes to block endogenous peroxidase. Washed by PBS. The section was blocked secondly for 30 minutes using 0.5% goat serum.

Immunohistochemical staining was performed according to (25) by adding specific polyclonal antibodies against CD11c and CD169 at a dilution of 1:300 for each, according to the manufacturer's instructions (ELABSCIENCE®, USA). These polyclonal antibodies were detected using a Poly-HRP (Elabscience, USA) detection system (26). Slides were incubated at 4 °C overnight, then stained with the DAB system, with hematoxylin as a counterstain. Finally, they were dehydrated and prepared as ready slides for examination under a microscope (27,28).

Statistical analysis

The data were analyzed using median and IQR (Inter-Quartile-Range) and the Mann-Whitney Test between the two groups, with a significance level of $p \leq 0.05$, using computer statistical software (SigmaPlot Version 12.5).

Results

Histopathology of the lung

The lung section photomicrograph stained with H&E (from non-infected control group broilers without any symptoms of CRD) showed standard architecture of the parabronchi, parenchyma with atria, air capillaries, and blood vessels. While a section photomicrograph (from a

group of infected broilers with CRD) showed severe pneumonia characterised by interstitial infiltration of inflammatory cells, exudation with pus in the lung interstitium and bronchi surrounded by inflammatory cells, congestion of blood vessels, and increased fibrous tissue (Figure 1).

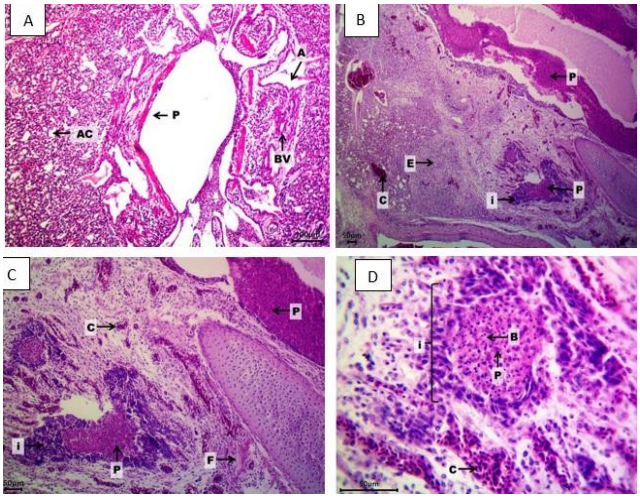

Figure 1: Photomicrographs of broiler lungs. A: Control group showing normal architecture of the para bronchi (P), parenchyma with atria (A), air capillaries (AC), and blood vessels (BV). B: CRD Infected broiler group showing severe pneumonia characterised by interstitial infiltration of inflammatory cells, exudation (E), pus in the lung interstitium and bronchi (P) surrounded by inflammatory cells (i), congestion of blood vessels (C). C: CRD group showing focal inflammatory reaction of the pus in the lung interstitium and bronchi (P) surrounded by inflammatory cells (i), increased fibrous tissue (F), and congestion of blood vessels (C). D: CRD group showing focal inflammatory reaction (i) with the pus (P), bacteria (B), and congestion of blood vessels (C). H&E stain, [A: 100X; B: 40X; C: 100X; 400X].

CD169

The results showed that lung sections of non-infected control group broiler showed very weak expression of CD169 (1+) in the immunohistochemical sections of these lungs while in group of infected broiler lungs with CRD showed intense expression of CD169 (3+) in immunohistochemical sections of their lungs stained with hematoxylin stain and examined under each of 100X and 400 X by light microscope (Figure 2 and Table 1).

CD11c

The results showed that lung sections from the non-infected control group broiler showed negative CD11c expression (0) in immunohistochemical sections. In contrast, the group of infected broiler lungs with CRD showed moderate CD11c expression (2+) in immunohistochemical sections, stained with hematoxylin and examined at 100X and 400X under a light microscope. (Figure 3 and Table 2).

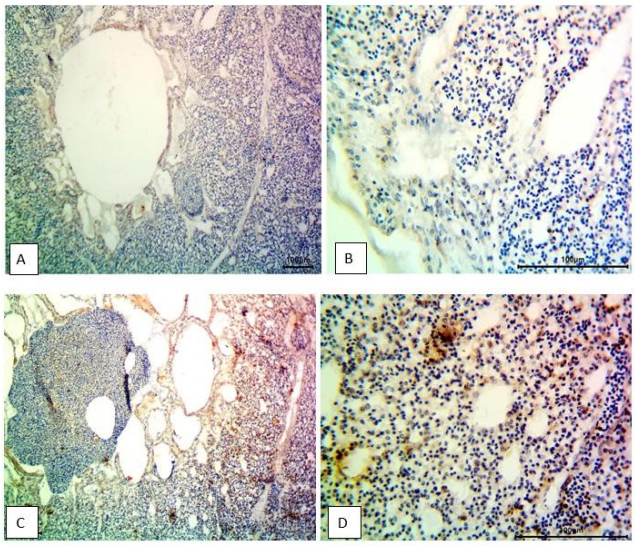

Figure 2: A: Immunohistochemical expression of CD169 in the control group of non-infected broiler lungs showing very weak expression (1+); hematoxylin; 100X, B: 400X, C: Immunohistochemical expression of CD169 in the lungs of group-infected broiler with CRD showing intense expression (3+); hematoxylin; 100X, D: 400X.

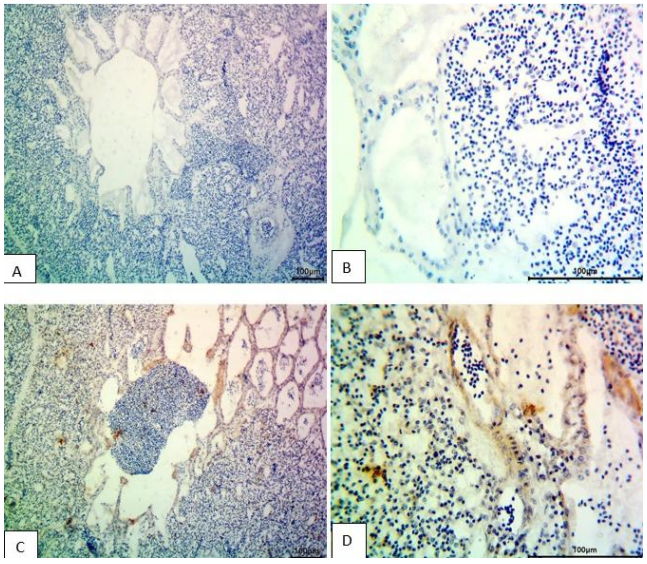

Figure 3: A: Immunohistochemical expression of CD11c in the control group of non-infected broiler lungs showing negative expression (0-); hematoxylin; 100X, B: 400X, C: Immunohistochemical expression of CD11c in lungs of group infected broiler with CRD showing moderate expression (2+); hematoxylin; 100X, D: 400X.

Table 1: The intensity scores of the immunohistochemical expression of CD169 between groups

Groups	Intensity Scores
Healthy non-infected control group	1 (1)
CRD-infected broiler group	3 (1) *
P-Value	<0.001

Data expressed as Median & IQR (Inter-Quartile-Range) (N= 50). * Significant difference between groups at P≤0.05. Mann-Whitney Test.

Table 2: The intensity scores of the immunohistochemical expression of CD11c between groups

Groups	Intensity Scores
Healthy non-infected control group	0 (1)
CRD-infected broiler group	2 (0) *
P-Value	<0.001

Data expressed as Median & IQR (Inter-Quartile-Range) (N=50). * Significant difference between groups at P≤0.05. Mann-Whitney Test.

Trachea photomicrograph

The photomicrograph of the trachea in the control group (non-infected broilers) showed the standard architecture of the epithelial layer, cartilage, muscular layer, and adventitia. A photomicrograph of the trachea in infected broilers with Chronic Respiratory Disease CRD group showed necrosis of the epithelial cells lining the mucosa, infiltration of inflammatory cells, severe congestion of blood vessels, and degeneration of the chondrocytes in the cartilages (Figure 4).

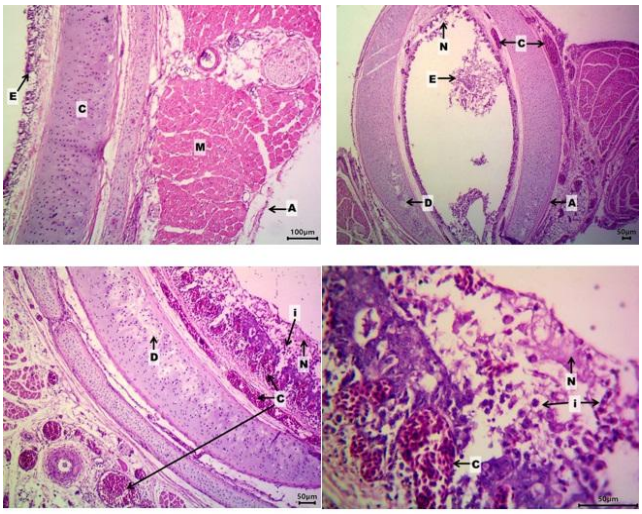

Figure 4: A: Photomicrograph of trachea in control group non-infected broilers showing standard architecture of the epithelial layer (E), cartilage (C), muscular layer (M), and adventitia (A), H&E stain, 100X. B: photomicrograph of trachea in group of broilers infected with Chronic Respiratory Disease CRD showing tracheitis characterized by inflammatory exudate in the lumen with infiltration of inflammatory cells (E), necrosis and sloughing of the epithelial cells lining mucosa (N), severe congestion of blood vessels and hemorrhage (C), degeneration of the chondrocytes in the cartilages (D) and atrophy of muscularis (A) H&E stain, 40X. C: photomicrograph of CRD showing necrosis of the epithelial cells lining mucosa (N), infiltration of inflammatory cells (i), severe congestion of blood vessels (C), and degeneration of the chondrocytes in the cartilages (D). H&E stain, 100X, D: photomicrograph of CRD showing necrosis of the epithelial cells lining mucosa (N), infiltration of polymorph nuclear inflammatory cells (i), and severe congestion of blood vessels (C). H&E stain, 400X.

CD169

The results showed that the trachea of non-infected control group broilers showed very weak CD169 expression (1+) in immunohistochemical sections. In contrast, the group infected broiler with CRD showed moderate CD169 expression (2+) in immunohistochemical sections of the trachea stained with hematoxylin (Figure 5).

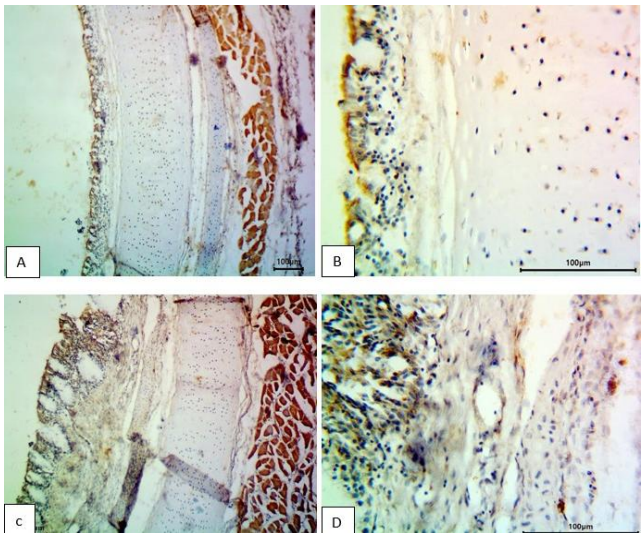

Figure 5: A: Immunohistochemical expression of CD169 in the trachea of a control group non-infected broiler, showing very weak expression (1+); hematoxylin; 100X, B: 400X, C: Immunohistochemical expression of CD169 in the trachea of a group-infected broiler with CRD showing moderate expression (2+); hematoxylin; 100X, D: 400X.

CD11c

The results showed that the trachea of the control group non-infected broilers showed very weak expression of CD11c (1+) in the immunohistochemical section of these tracheae while in group infected broilers with CRD showed moderate expression of CD11c (2+) in immunohistochemical sections of their trachea stained with

hematoxylin stain and examined under each of 100X and 400 X by light microscope (Figure 6).

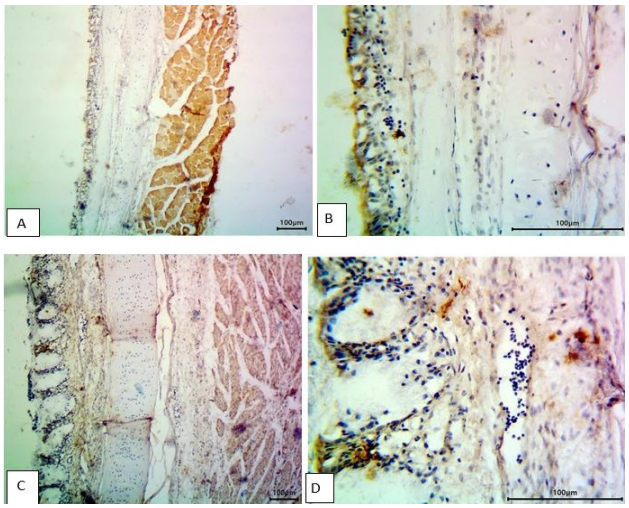

Figure 6: A: Immunohistochemical expression of CD11c in trachea of control group non-infected broiler showing weak expression (1+); hematoxylin; 100X, B: 400X, C: Immunohistochemical expression of CD11c in trachea of group infected broiler with CRD showing moderate expression (2+); hematoxylin; 100X, D: 400X.

Discussion

The poultry industry today is one of the most critical, fast-paced industries in the world, providing people with their animal protein requirements. One of the most common diseases that can cause considerable losses in this sector is CRD (29). Causative organisms' colonies along the respiratory tract reach the trachea and alveoli of the lung (30). Our results revealed the presence of severe histopathological changes both in the lung and bronchi of infected broilers with CRD compared with non-infected broilers that showed no histopathological lesions. Many studies support our results (31-36), indicating that these histopathological changes contribute to the clinical signs of CRD in affected birds. The use of immunohistochemistry to study gene expression in immunological cells that contribute to the immune response against the causative agents of CRD in broilers yielded successful results. Immunohistochemistry is nowadays one of the more relevant and sensitive methods (34) and a powerful tool for detecting foreign substances within the architecture of any tissue (16). Our results using immunohistochemistry showed a significant difference in the lungs of broilers infected with CRD, with intense CD169 expression and moderate CD11c expression. In contrast, the trachea of the same broilers showed moderate expression of each CD169 and CD11c in comparison to the lung and trachea of the control group non-infected broilers that showed negative expression for each of them.

CD169 is an essential marker for macrophages that sometimes act as a second-phase orchestrator of innate protection, transporting pathogens to the T-cell zone in the spleen (37). Macrophages bearing the CD169 marker play essential roles in processes such as antigen presentation, inflammation, viral infections, and phagocytosis (38). CD169 bearing macrophages has many crucial functions on immune response, the first one their ability to play a pivotal role in enhancing viral replication and producing large number of viral proteins that are important in synthesis of adequate antigens to induce immune response against them (39,40), indeed it has dual action in promoting each of innate and acquired immune response (40-44), in addition to their ability in activation of CD8+ T-cells which is responsible for cell-mediated immunity CMI (44) and their ability to balance B-cell function that is responsible for humoral immunity (45).

Another important marker is CD11c, which belongs to the beta2 integrin family (46) and is highly expressed on the surface of many immunological cells, including macrophages, DCs, NK cells, activated macrophages, lymphocytes, and activated T cells (47-51). Despite its widespread distribution on the surfaces of the cells mentioned above, it is still widely used as a specific surface protein marker for DCs. It is mentioned in conjunction with these cells (52). We must remember also that CD11c is always used for antigen-targeting studies (49). Also, as in CD169 cells, CD11c cells have a dual effect, promoting both humoral and CMI responses. In our research, we concluded that expression of CD11c was moderate in both lung and trachea, compared with non-infected broilers that expressed it in a very weak manner, and this indicates the critical role of these cells in priming of immune response by their two arms, CMI and humoral immunity, and this agrees with the results of many researchers.

A study conducted by Castro *et al.* (53) revealed that DCs expressing CD11c act as APCs that ingest microorganisms, then process them to separate their antigenic parts and display them on their surface as antigen peptides both in MHC I and II molecules, and present them to T-cells that finally induce both humoral and CMI responses. Our final results lead us to propose a criterion: the expression of CD169 and CD11c may indicate infection by several agents, including bacteria, Mycoplasma, or even viruses, which can lead to secondary bacterial infections, the main cause of CRD in broiler farms.

Conclusion

Our study concludes that CRD infection causes severe pathological changes with serious activation of innate immune arm cells (dendritic and macrophage cells) by intense and moderate expression of CD169 and CD11c, respectively, in the lung sections and mild expression of each of CD169 and CD11c in tracheal sections of the infected

broiler and the immunohistochemistry technique can be successfully used to detect marker expression of dendritic cells (CD11c) and respiratory macrophages (CD169+) determines the immunological state in birds infected with CRD.

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Conflict of interest

There was no conflict of interest.

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دراسة التعبير الجيني لكل من عنقود التمايز ١١ج وعنقود التمايز ١٦٩+ باستخدام تقنية الكيمياء النسجية المناعية عند الأفراخ المصابة بمرض الجهاز التنفسي المزمن

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الخلاصة

تعد متلازمة مرض الجهاز التنفسي المزمن في الطيور عدوى تنفسية مهمة في الأفراخ تسببها العديد من العوامل بما في ذلك بكتريا المتفطرات، والإيشريكيا القولونية وبعض الفيروسات مسببة التهاب رئوي شديد، التهاب الحويصلات الهوائية فضلا عن تسمم الدم وتكون هذه المتلازمة مترافقة في اغلب الأحيان مع ارتفاع ملحوظ في معدل الاصابة والهلاكات في القاعات المصابة. هدفت الدراسة إلى تقييم بعض الجوانب المناعية بما في ذلك التعبير الجيني للخلايا التشجرية نوع (CD11c) والخلايا البلعمية نوع (CD169 +) في حقول أفراخ اللحم المصابة بمرض التهاب الجهاز التنفسي المزمن باستخدام تقنية الكيمياء النسجية المناعية إضافة إلى بعض التغيرات المرضية في هذه الأفراخ. حضرت المقاطع النسيجية من خمسين عينة من القصبة الهوائية والرئة من فروج اللحم المصابة ب مرض الجهاز التنفسي المزمن للكشف عن جميع التغيرات المرضية وتم استخدام طريقة الكيمياء النسجية المناعية لتقييم التعبير الجيني لخلايا (CD11c) و (CD169+). أظهرت النتائج وجود التهاب القصبة الهوائية الحاد واحتقان الدم مع نخر شديد للخلايا الظهارية المبطنة للغشاء المخاطي، وتراكم الإفرازات الالتهابية وارتشاح الخلايا الالتهابية. وأظهرت نسيج الرئة وجود التهاب رئوي حاد تميزت بإفرازات مصلية تجبنيه وزيادة في الأنسجة الليفية وتسلل الخلايا الالتهابية إلى أنسجة الرئة باستخدام صبغة الهيماتوكسيلين والايوسين. بينما كشفت تقنية الكيمياء النسجية المناعية للمقاطع النسجية

لرئة الطيور المصابة بمرض الجهاز التنفسي المزمن عن تعبير شديد لجين الخلايا البلعمية (+CD169) وتعبير متوسط لجين الخلايا التشجرية نوع CD11c مع تعبير متوسط للخلايا البلعمية (+CD169) والخلايا التشجيرية (CD11c) في المقاطع النسجية لقصبات الطيور المصابة بالمقارنة مع مجموعة السيطرة الغير المصابة.وخلصت الدراسة إلى أن تقنية الكيمياء النسجية المناعية استخدمت بنجاح في الكشف عن التعبير الجيني للخلايا التشجيرية (CD11c) والبلعميات التنفسية (CD169 +) في الطيور المصابة مرض الجهاز التنفسي المزمن في الطيور.