Respiratory Tract Associated Lymphoid Tissues in Native Chicken (*Gallus gallusdomesticus*) of Bangladesh

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List of Acronyms

BALT: Bronchus associated lymphoid tissue.

et al: And his associate.

GALT: Gut associated lymphoid tissue.

IEL: Intraepithelial lymphocytes.

ILF: Isolated lymphoid follicles.

MALT: Mucosa associated lymphoid tissue.

RALT: Respiratory tract associated lymphoid tissue.

Abstract

The respiratory system of birds exhibits unique adaptations, including the presence of air sacs. Birds, particularly those in scavenging management systems, face constant challenges from inhaled particles, microorganisms, and aerosols. To combat these challenges, the avian respiratory mucosa is equipped with immune-competent cells and various lymphoid tissues. This study focuses on the role of Respiratory tract associated lymphoid tissue (RALT) in the immune response to respiratory pathogens and the postnatal development of RALT in native scavenger chickens (*Gallus gallus domesticus*) as they age. Histological examinations of the trachea and lung in male and female native chickens at different age groups (Day 1, 30, 90, and 180) were conducted. Significant variations in IELs were observed at Day 180 in trachea of both male and female chickens ($P \leq 0.05$). Also, there was significant variations in IELs at Day 180 in lungs of male native chickens. This increase in IELs may be attributed to their scavenging nature and continuous exposure to pathogens.

Additionally, aggregated lymphoid tissues were present in the lamina propria of the respiratory tract, but significant variations were observed only in the trachea of female native chickens at Day 180.

This study contributes to a better understanding of avian respiratory immunology. It highlights the unique immune responses and lymphoid structures in the respiratory system of native scavenger chickens and underscores the importance of considering these factors in chicken health management.

Keywords: Chicken, Respiratory Tract, Lymphoid tissue, RALT, Histology.

Chapter 1: Introduction

The respiratory system of birds in general is unique, having several changes in anatomy from the respiratory system of mammals. Air sacs and a different bronchial tree structure are only a few instances of its differences. Particles and particle-associated micro-organisms are inhaled as unavoidable constituents of the tidal air. Intensive management systems for poultry are frequently associated with high loads of dust and pathogens in the environment and therefore pose a particular stress to the respiratory system (Glisson, 1998; Villegas, 1998). Also, antigens can be intentionally delivered to the respiratory tract in the form of aerosols. The respiratory immune system has developed strategies to remove inhaled particles and to adequately respond to those micro-organisms that succeed in crossing the epithelial barrier for maintaining its integrity and functions. (Sonja Kothlow et al. 2008).

To effectively combat these challenges, the avian respiratory mucosa is equipped with some immune-competent cells. Such as Respiratory-tract associated lymphoid tissue (RALT), bronchus-associated lymphoid tissue (BALT).

Central organs of the lymphatic system in birds are the bursa of Fabricius (responsible for maturation and differentiation of B lymphocytes) and thymus (responsible for T lymphocytes maturation). Beside central organs there are numerous peripheral organs (also referred to as secondary) of the lymphatic system such as head – associated lymphoid tissue (HALT), gut – associated lymphoid tissue (GALT), bronchus – associated lymphoid tissue (BALT), spleen, cecum tonsils and others (Rumińska et al. 2008). These structures are commonly (directly or indirectly) involved in the immune response occurring in the respiratory mucosal system of birds.

Mucosa-associated lymphoid tissue (MALT) including those in the respiratory tract, are uniquely designed to initiate immune responses to mucosal pathogens. (McGhee, et. al. 1992).

Past research has reported the presence of mucosa-associated lymphoid tissue (MALTs), isolated lymphoid follicles (ILF), and lamina propria-associated lymphocytes in various mucosal layers of the respiratory system yet absent in the muscularis mucosa. Moreover, the prevalence of lymphoid tissues within the respiratory mucosa is likely to change significantly with the age of the avian species.

In the context of poultry in our country, the native scavenger chicken breed, *Gallus gallus domesticus,* stands as a pivotal subject of study. This indigenous breed, recognized as the primary local chicken population in Bangladesh (Faruque et al., 2010). exhibits unique scavenging behaviors. Due to their scavenging nature and distinct ecological niches, these chickens are anticipated to host a richer population of immune-competent cells within their RALT, compared to high-yielding chicken breeds bred for commercial purposes. (Islam et al., 2008; Khan et al., 2007). However, as much of the research in avian immunology has concentrated on high-yielding breeds, data regarding the growth and distribution of RALT in native scavenger chickens remains scarce. While birds roam continuously, pathogens, dust, aerosols, air-transmitted microorganisms enter the respiratory tract continuously. The local immunity of the respiratory mucosal system is ensured by non-specific defensive reactions (e.g., by mechanical removal of impurities by movements of the respiratory epithelial cilia system) on one hand, and by precise mechanisms employed by immunocompetent cells (B and T lymphocytes) on the other. (M. Śmiałek et al. 2011).

Previously, many research papers were published about native chickens but not much attention was given to respiratory tract associated lymphoid tissue. Therefore, this study is designed to evaluate the role of chicken RALT in immune responses to respiratory pathogens and postnatal growth and development of RALT in various segments of the avian respiratory tract as native scavenger chickens age.

Chapter 2: Materials and Methods

Study area and study population: The research was carried out in Chattogram's Hathazari upazilla. The deshi chicken (*Gallus gallus domesticus*) was used as the study population.

Collection of Native chicken: A total of 24 chicken (both male and female) were collected from Hathazari. The chickens were reared in a scavenging system. The chickens were divided by age group Day (D) 1, Day 30, Day 90, and Day 180. (3 chickens from each group). The chickens were routinely sacrificed at D1, D30, D90, and D180 by excess chloroform inhalation. Then the respiratory tract was dissected out carefully from both male and female native chickens. In the respiratory tract the following two portions were examined histologically: trachea and lung.

Preparation of permanent slide: Tissues were fixed in 10% neutral buffered formalin for routine histology. Then the tissues were dehydrated in ascending graded alcohol. After completing dehydration, the tissues were cleaned by xylene until the alcohol from tissue was replaced. Then the tissues were embedded in paraffin and cut into 4-6µm sections using sliding microtome machine with the help of disposable carbon blade. Then the tissues were stained with hematoxylin and eosin for viewing under the light microscope as described previously by Cardiff et al (2014).

Histomorphometry: To obtain histological data, a microscopic study of lymphoid tissues of the respiratory tract at different aged chickens was conducted. Photomicrographs were taken using a photomicroscope (AmScope Trinocular Compound Microscope with 1.3 MP Camera, Model T490 B-MT) and AmScope image measuring software (x86, 3.7.3036 version).

Data Analysis: All the data were recorded in an Excel sheet (Microsoft Excel 2019). Then the data were stored in Statistical software, STATA-14.2 (STATA Corp., Texas, USA) for statistical analysis. An unpaired sample t-test was performed to compare the means of the different variables between two groups. (Value of day 1 chicks were considered as

baseline) A p-value equal to or less than 0.05 *(P≤0.05)* was considered significant for this test. Results were expressed as arithmetic mean ± standard deviation (Mean ± SD).

Chapter 3: Results

The present study histologically showed that the trachea is lined by ciliated, pseudostratified columnar epithelium containing numerous, simple alveolar mucous glands. In the posterior portion of trachea, the mucous glands are replaced by goblet cells. A lamina propia and submucosa is present. The submucosa is rich in elastic fibers. The trachea is supported by a complete cartilaginous ring. Lymphoid follicles and scattered lymphocytes are found in the lamina propria. The current study found that the frequency of IELs in the trachea of male native chicken was 2,3,5, and 7 for Day (D)1, D30, D90, and D180 respectively (Table 1). The frequency of aggregated lymphoid tissue in the trachea of male native chicken was 1 (D1), 2 (D30), 2 (D90), and 4 (D180) (Table 1). The frequency of IELs and aggregated lymphoid tissue in the trachea of female native chicken was recorded as 2 & nil in D1, 4 & 2 in D30, 5 & 3 in D90, 6 & 4 in D180(Table 2). The mean value was 1.40±0.548 in the trachea of male chickens in 180 days (Table 3) which is the highest mean± SD value in trachea of both male and female chickens. The mean± SD value was increased with age in both males and females. The mean of IELs in the trachea of both male and female native chickens has a significant variation at Day 180 ($P \leq 0.05$) (Table 3&4). In the case of aggregated lymphoid tissue in the trachea only female native chickens have a significant variation at Day 180($P \leq 0.05$) (Table 6).

In this study, the lungs were found to be wedge-shaped, and poorly elastic. Lungs did not contain blind ending alveoli as mammalian lungs but anastomosing air capillaries (pneumocapillares). Each primary bronchus enters the lung and gives rise to secondary bronchi which branches into numerous parabronchi (tertiary bronchi) within the lung.

Primary bronchi are lined by ciliated pseudostratified columnar epithelium with mucous glands and goblet cells. Secondary bronchi are lined by ciliated columnar epithelium. Parabronchi are lined by cuboidal epithelium. The frequency of IELs and Aggregated lymphoid tissue in the lung of male native chickens was found 3 & 1 in D1, 4 & 2 in D30, 4 & 3 in D90, 7 & 4 in D180 (Table 1). The frequency of IELs and aggregated lymphoid tissue in the lung of female native chickens was recorded 2 & 2 in D1, 3 & 3 in D30, 3 &

3 in D90, 5 & 4 in D180 (Table 2). The mean value was increased with age in both male and female native chickens. The highest mean value was found 1.40±0.548 in the lungs of male native chickens (Table 3). The mean of IELs in the lung only male native chickens has a significant variation at Day 180 ($P \leq 0.05$).

Table 1: Frequency of Intraepithelial lymphocytes and Aggregated lymphoid tissue per 5 microscopic fields (40X) in the male respiratory system of the native chickens.

Name of the organ	Age (Days)	Intraepithelial Lymphocytes (N)	Aggregated Lymphoid Tissue (N)
Trachea	1	2	1
	30	3	2
	90	5	2
	180	7	4
Lung	1	3	1
	30	4	2
	90	4	3
	180	7	4

Table 2: Frequency of Intraepithelial lymphocytes and Aggregated lymphoid tissue per 5 microscopic fields (40X) in the female respiratory system of the native chickens.

Name of the organ	Age (Days)	Intraepithelial Lymphocytes (N)	Aggregated Lymphoid Tissue (N)
Trachea	1	2	0
	30	4	2
	90	5	3
	180	6	4
Lung	1	2	2
	30	3	3
	90	3	3
	180	5	4

Table 3: Intraepithelial lymphocytes per 5 microscopic fields (40X) in the male respiratory tract of the Native Chicken.

Name of the organ	Age (Days)	Mean± SD	P-value
Trachea	1	0.40±0.548	
	30	0.60±0.548	0.6213
	90	1.00±1.000	0.3046
	180	1.40±0.548	**0.0341**
Lung	1	0.60±0.548	
	30	0.80±0.837	0.3739
	90	0.80±0.837	0.7040
	180	1.40±0.548	**0.0161**

Table 4: Intraepithelial lymphocytes per 5 microscopic fields (40X) in the female respiratory tract of the Native Chicken.

Name of the organ	Age (Days)	Mean± SD	P-value
Trachea	1	0.40±0.548	
	30	0.80±0.837	0.1778
	90	1.00±0.707	0.3046
	180	1.20±0.837	**0.0161**
Lung	1	0.40±0.548	
	30	0.60±0.548	0.6213
	90	0.60±0.548	0.6213
	180	1.00±1.000	0.2080

Table 5: Aggregated Lymphoid tissue per 5 microscopic fields (40X) in the male respiratory tract of the Native Chicken.

Name of the organ	Age (Days)	Mean±SD	P-value
Trachea	1	0.20±0.447	
	30	0.40±0.548	0.6213
	90	0.40±0.548	0.6213
	180	0.80±0.447	0.0705
Lung	1	0.20±0.447	
	30	0.40±0.548	0.3739
	90	0.60±0.548	0.1778
	180	0.80±0.447	0.0705

Table 6: Aggregated Lymphoid tissue per 5 microscopic fields (40X) in the female respiratory tract of the Native Chicken.

Name of the organ	Age (Days)	Mean±SD	P-value
Trachea	1	0±0	
	30	0.40±0.548	0.1778
	90	0.60±0.548	0.0705
	180	0.80±0.447	**0.0161**
Lung	1	0.40±0.548	
	30	0.60±0.548	0.6213
	90	0.60±0.548	0.6213
	180	0.80±0.447	0.1778

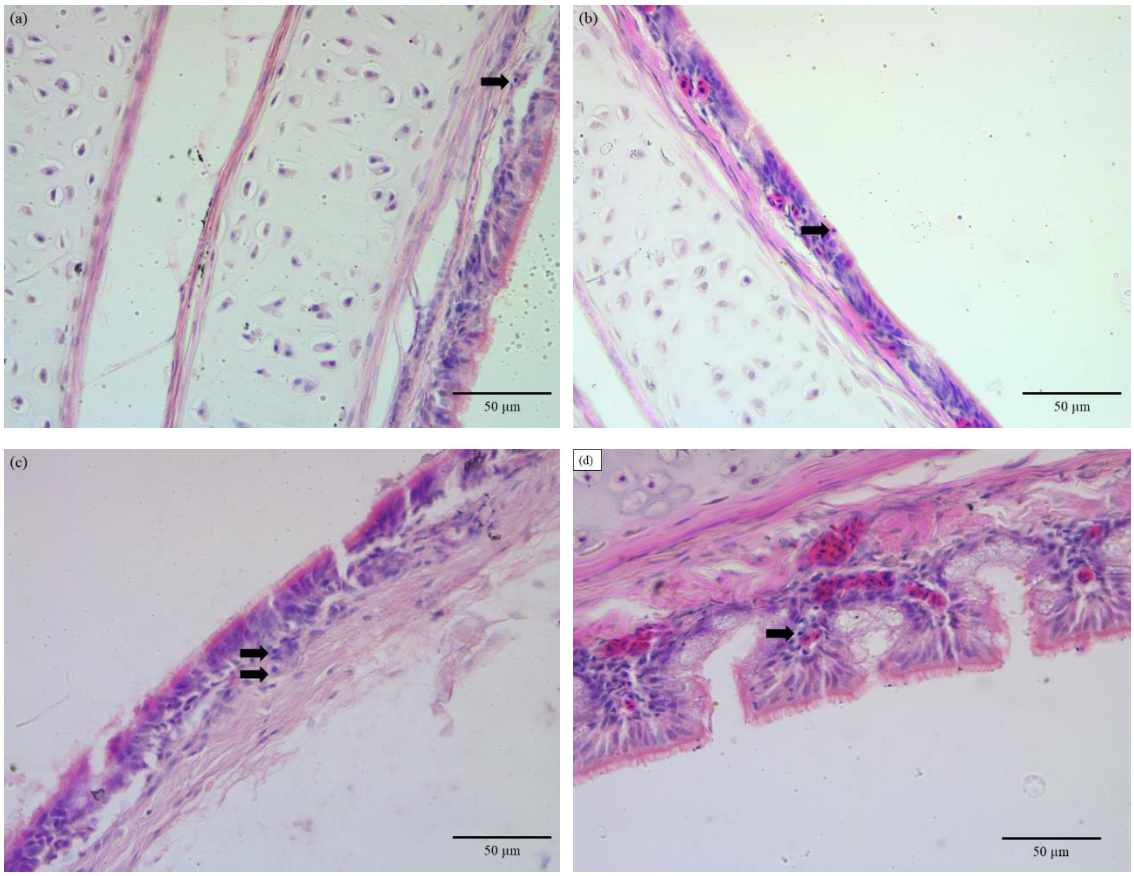

Figure 1: Hematoxylin and Eosin staining of trachea. (a)Female Chicken at Day 1 (b) Male Chicken at Day 30 (c)Male Chicken at Day 90 (d) Female chicken at Day 180; Arrow symbol indicated Intraepithelial lymphocytes in lining epithelium; Scale for Lower magnification=100µm, Higher magnification= 50µm.

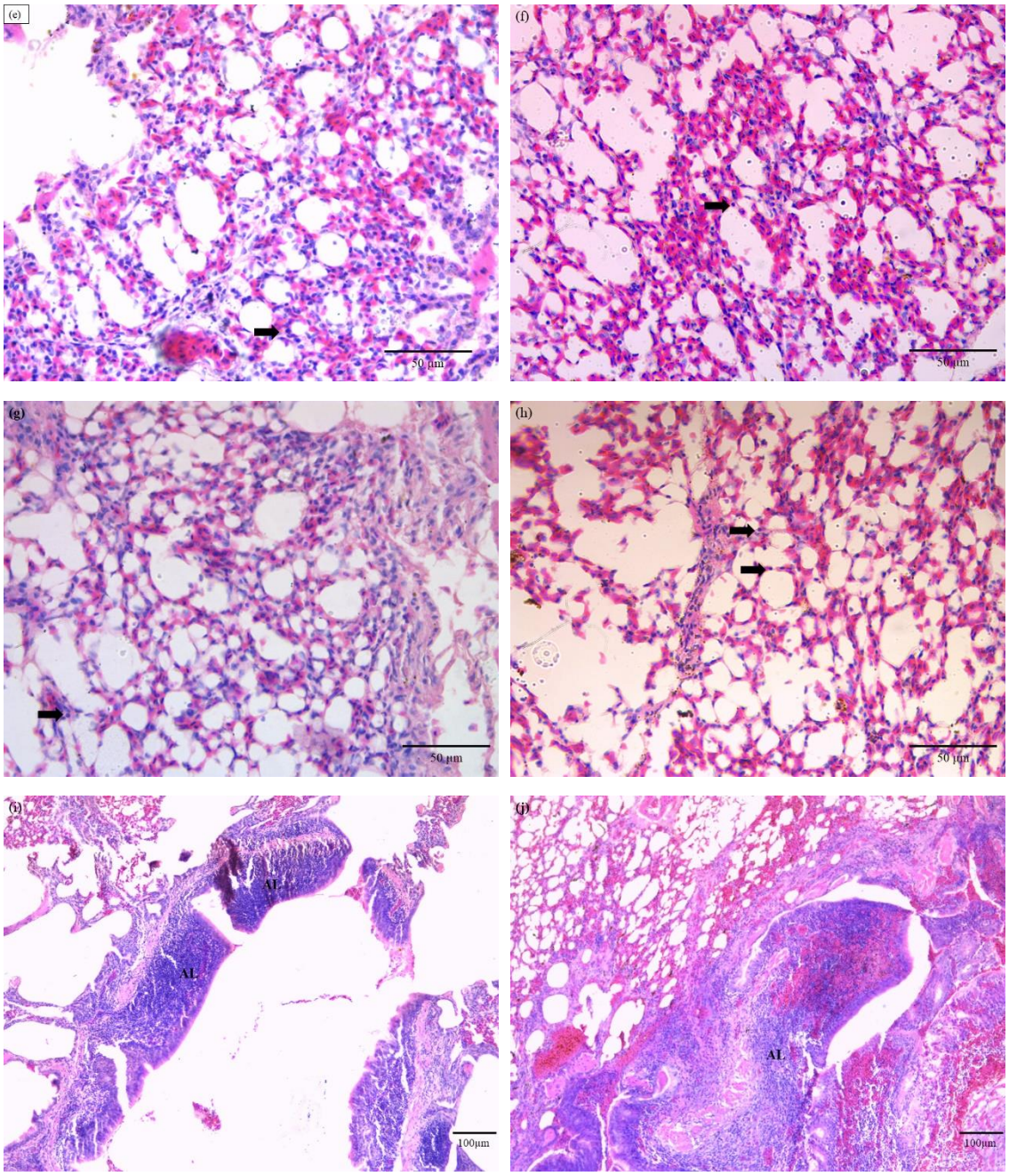

Figure 2: Hematoxylin and Eosin staining of Lung. (e)Male chicken at Day1. (f) Female chicken at Day30 (g) Male chicken at Day90 (h) Female chicken at Day180 (i)Male Chicken at Day 30 (10X) (j)Female Chicken at Day 90 (10X); Arrow symbol indicated IELs in lining epithelium; AL= Aggregated lymphocytes; Scale for Lower magnification=100µm (i-j), Higher magnification= 50µm. (e-h)

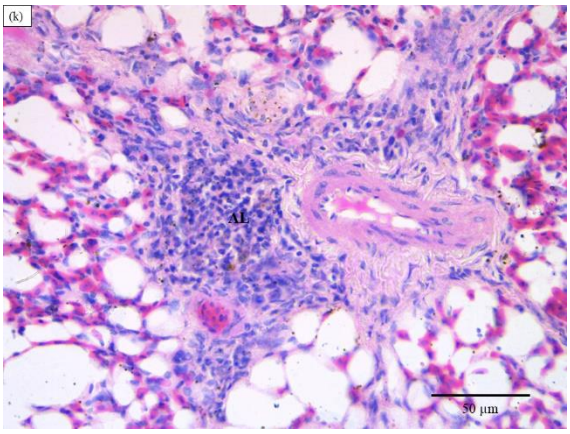

Figure 3: Hematoxylin & Eosin staining of lung at Day 180 (k) Female Chicken at Day 180. AL= Aggregated Lymphocytes; Scale for Lower magnification=100µm, Higher magnification= 50µm.

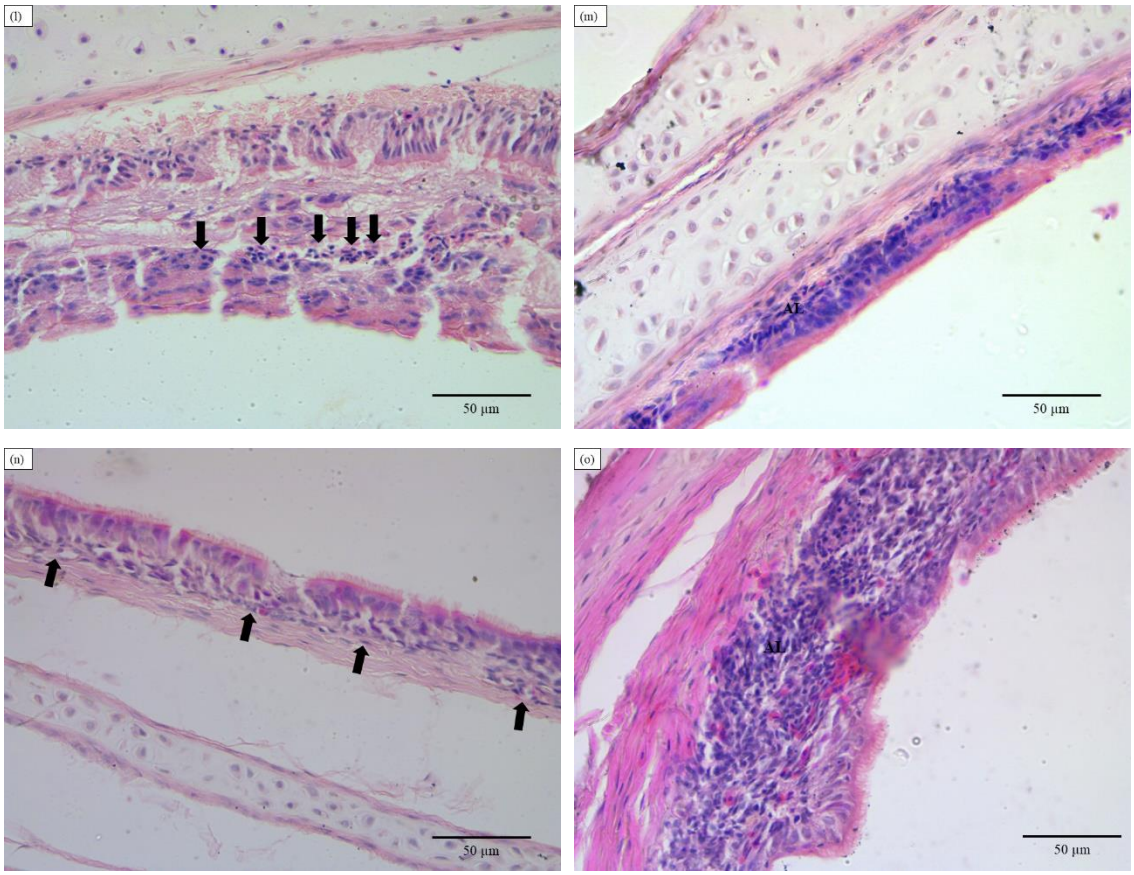

Figure 4: H&E staining of trachea (aggregated). (l)Male chicken at Day 1. (m) Female chicken at Day 30. (n) Female Chicken at Day 90. (o) Male Chicken at Day 180. Arrow symbol indicates the aggregated lymphocytes; AL= Aggregated Lymphocytes; Scale for Lower magnification=100µm, Higher magnification= 50µm.

Chapter 4: Discussion

The present study revealed histological features of the trachea of native chickens. The findings were like Renu Yadav et al. (2022) who noticed that histologically trachea had lamina epithelial mucosa and lamina propria submucosa surrounded by a cartilaginous ring. The mucosa layer had ciliated pseudostratified columnar epithelium with intraepithelial mucous glands. These results were supported by Bienenstock et al. (1973), who reported that the avian lung exhibits both highly organized lymphoid structures and diffusely distributed lymphoid cells. In the trachea intraepithelial lymphocytes showed statistically significant variations at Day 180 for both male and female native chickens. These results were like Hadiopur, 2010, who reported that an increased number of aggregated and intraepithelial lymphocytes of birds were observed while they were affected by infectious diseases. Fagerland & Arp, (1993) further reported that there were age-related differences in the number of lymphocyte infiltration in the epithelial lining and aggregated lymphoid tissues in the lamina propria and submucosa of the respiratory tract. Almost of the IELs in the upper respiratory and bronchi are T lymphocytes. IELs often have more CD81 cytotoxic/suppressive T cells than CD41 T-helper cells. The primary function is to maintain the integrity of the mucosal barrier and defend the epithelial against pathogenic agents (Goto et al., 2000). Furthermore, it was also reported that the numbers of lymphocytes and aggregated tissue depend on aging. Infection models with *Mycoplasma gallisepticum* have shown that the tracheal mucosa is highly responsive to infections and reacts with extensive lymphocyte infiltration followed by lymphoproliferation (Gaunson et al., 2000, 2006).

Intraepithelial lymphocytes were found in lungs of both male and female native chickens; There was significant variations observed in lung of male native chickens at Day 180. These were like Van Alstine et al (1988), who reported that in turkeys, lymphoid nodules comprising the bronchus associated lymphoid tissue (BALT) are present in normal, uninfected birds; however, BALT nodules are more numerous and widely distributed in *Bordetella avium* infected turkeys, suggesting a role for BALT in respiratory immunity. The frequency of IELs in lung were increased with age in both male and female native

chickens. This may be due to their scavenging nature as they are more prone to infections. As a result, lymphocytes are produced as a first line defense against microorganisms. Bronchus-associated lymphoid tissue (BALT) takes part in bronchial immune processes and its structure, topography, and ability to perform defensive function in birds is largely age-dependent**.** (M. Śmiałek et al., 2011)

In this study, intraepithelial lymphocytes were found in the lining epithelium, and aggregated lymphoid tissue was found in lamina propia. These results were like Sonja Kothlow et al. (2008) who recorded that Lymphoid follicles and scattered lymphocytes are found in the lamina propria. The IELs in the current study ranged in size from small lymphocytes with little cytoplasm to big lymphocytes with evident cytoplasm. The result was supported by Wilson et al., (1986), who reported that cytotoxic T-cell markers are predominant in intraepithelial lymphocytes (IELs), whereas T-helper characteristics are less frequently observed.

In this study, the frequency of IELs and aggregated lymphoid tissue was increased in both males and females' respiratory system with their age. This can occur due to their scavenging nature as they are exposed to various kinds of microorganisms throughout their life. Due to exposure to various kinds of pathogens, lymphocytes originated as immunity against pathogens. Jerry R. McGhee (1998) said that Intraepithelial lymphocytes (IELs) reside between epithelial cells and are the first cells to contact luminal antigen that crosses the mucosa in an M cell-independent manner.

Aggregated lymphoid tissues were observed in the lamina propia of respiratory tract. There were no germinal centers observed in aggregated lymphocytes. But no significant variations ($P \leq 0.05$) were observed except in trachea of female native chickens at Day 180.

Chapter 5: Conclusion

The study focused on the trachea and lungs of native chickens and examined the presence of intraepithelial lymphocytes (IELs) and aggregated lymphoid tissue. The study demonstrated a significant variation in the mean of IELs in respiratory system of male and female native chickens at Day 180. The distribution of lymphoid cells and tissues significantly depends on their bird's age. This is likely attributed to their scavenging nature and continuous exposure to various microorganisms. The presence of IELs in the respiratory system is crucial for mounting an effective immune response against inhaled pathogens. There was no significant variation of aggregated lymphoid tissue except trachea of female native chicken at Day 180.

The findings in this study align with previous research, highlighting the role of lymphocytes in respiratory immunity and emphasizing the importance of these lymphoid tissues in the avian respiratory system.

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Biography

I am Abdullah Al Noman passed my DAKHIL exam in 2015 from Madrasa board and HSC exam in 2017 with GPA-5 from Chittagong board. I am currently enrolled in Chattogram Veterinary and Animal Sciences University as an intern student. As a person, I am full of enthusiasm, encouragement, and determination.

I am engaged with various extracurricular activities in and outside my university.

As a vet student, I devote my life to my patients with the hopes of becoming a successful veterinarian. I am enthusiastic about working with pet animals and being a successful pet practitioner.