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Research article

Clarification of a unique mucosal vaccination route for improved systemic and mucosal immune response in broiler

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ABSTRACT

There are often outbreaks of infectious diseases on farms, which not only disrupt production but also cause significant economic losses. Vaccines are given to prevent the spread of these infectious diseases, but they produce only systemic antibodies or antibodies in the mucosa of a particular system. So, a new mucosal vaccination route is needed where the vaccine will produce antibodies in the systemic circulation as well as in the mucosa of many systems. In our study, the cloaca was targeted because it is associated with the mucosa of many systems. Whole-mount and routine histological staining show both lymphatic nodules and diffuse lymphatic tissues in the lamina propria of cloaca. These lymphatic tissues are made up of Bu-1+ B-cells, $CD3^{+}$ T-cells, and KUL01+ macrophages and monocytes. So, this is a new mucosa-associated lymphoid tissue, named cloaca-associated lymphoid tissue (CALT). The CALT contains antigen-presenting cells (dendritic cells, macrophages, B cells, MHC II molecules, and T cells) and is equipped with blood vessels and high endothelial venules, which indicate its functional status. More antibodies were produced in the treatment group compared to the vehicle control group after administration of the infectious bursal disease (IBD) and the Newcastle disease (ND) vaccine through cloaca. In addition, the cloaca-associated route produces a higher number of antibodies than the other traditional routes, which reveals the uniqueness of this route. Cloacal-vaccinated chickens showed less damage to the follicle and epithelium of the bursa of Fabricius compared to other groups, indicating its lower cytotoxic effect. Therefore, the cloaca-associated mucosal vaccination route produces more antibodies than other mucosal vaccination routes, which will protect the chickens on the farm to a greater extent.

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1. Introduction

Poultry farming is integral to global food security, providing a vital source of protein for human consumption. Through efficient protein conversion, it offers a sustainable solution to meet the growing demand for animal protein. But the global demand for poultry meat is projected to rise by double in 2050 due to a rapidly expanding population and increasing prosperity among the countries [1]. To meet the high demand for protein, the poultry industry has been forced to intensify production in many countries. However, infectious diseases continue to pose significant challenges to poultry health and productivity [2]. In recent times, poultry farms have experienced various types of diseases in developing countries as a result of globalization and the potential persistence and spread of disease agents via domestic and wild reservoirs [3]. These infectious diseases can have significant economic impacts, leading to decreased productivity, increased mortality, and the need for costly treatments or interventions.

Over the last few decades, we have seen a broad spread of Newcastle disease (ND) and infectious bursal disease (IBD), giving instances of how these diseases have a detrimental effect on society at large and the chicken production industry in particular [4,5]. To prevent these infectious diseases, vaccination is the most recognized and successful method [6]. Nowadays, farmers use different types of commercial vaccines to prevent poultry diseases. However, outbreaks of diseases occurred in vaccinated birds, indicating the ineffectiveness of vaccination [7]. Therefore, the quest for more effective and convenient vaccination methods remains a crucial aspect of ensuring flock health and productivity.

The route of vaccine administration plays a pivotal role in determining the efficacy, safety, and overall success of immunization programs. Traditional vaccination methods primarily rely on parenteral injection, where the vaccine is administered intramuscularly or subcutaneously. While these methods have been successful in controlling many diseases, they may not optimize immune responses at mucosal sites, which are often the primary sites of pathogen entry [8].

Mucosal surfaces, such as the respiratory, gastrointestinal, and urogenital tracts, are armed with specialized structures called mucosa-associated lymphoid tissue (MALT) [9] and mechanisms that can mount a robust immune response against invading pathogens [10]. MALTs are highly organized lymphoid tissues found at various sites of the mucosa and provide antibody-producing plasma cells, which confer protection against diseases [11]. MALT comprises the immune system related to mucous, which can operate independently of the systemic immune system [12]. The main function of the MALT is to produce and secrete specific immunoglobulin A (IgA) antibodies for antigens along the surface of the mucosa. This function of MALT is currently being used to develop mucosal vaccines.

The determination of mucosal vaccination routes might be most effective for inducing immune responses [13]. Since numerous pathogens use a mucosal port of entry in the host body, an immune response must be induced at the point of entrance to neutralize them [14,15]. Recent advances in vaccine research have shown that mucosal administration of vaccines can elicit strong immune responses at the site of pathogen entry, leading to enhanced protection. It has been demonstrated that vaccines administered through mucosae are effective in inducing serum antibodies as well as secretory IgA antibodies and cytotoxic T-cell responses at mucosal sites [16]. In addition, a successful mucosal vaccine elicits both local and systemic immune responses [17].

However, mucosal vaccination through a single route doesn't cover all mucosal surfaces in different systems. For example, oral immunization can induce strong immune responses in the gut but is relatively less efficient for the respiratory and genital systems [18,19]. Intranasal immunization induces immune responses in the respiratory and genital tracts but is less effective for gut immunity [20]. As the nasal and ocular administration faced several safety concerns, a new area of research based on the cloacal delivery of vaccines emerged because it has a connection with the digestive and urogenital systems. Therefore, an attempt to develop a new mucosal vaccination route that will facilitate the development of antibodies on all mucosal surfaces and serum would be beneficial.

As a whole, this study explores a new mucosal vaccination route in broiler chickens, focusing on the cloaca-associated lymphoid tissue (CALT), inducing systemic immune responses, and comparing this unique route with other mucosal routes.

Table 1
Vaccination schedule.

Day of Vaccination	Vaccines	Treatment groups				
		Eye + Eye	Eye + DW	Clo + Clo	Clo + DW	Control
4th day	ND + IB Komipharma, South Korea	1 drop in the eye	1 drop in the eye	1 drop in cloaca	1 drop in cloaca	unvaccinated
8th day	IBD LRI, Banglaesh	1 drop in the eye	1 drop in the eye	1 drop in cloaca	1 drop in cloaca	unvaccinated
16th day	IBD LRI, Banglaesh	1 drop in the eye	Drinking water	1 drop in cloaca	Drinking water	unvaccinated
23rd day	ND Komipharma, South Korea	1 drop in the eye	Drinking water	1 drop in cloaca	Drinking water	unvaccinated

Clo = cloaca, DW = drinking water, ND= Newcastle disease, IB = infectious bronchitis, IBD = infectious bursal disease, LRI = livestock research institute.

2. Materials and method

2.1. Birds and management

For this study, 150-day-old Lohman Meat (Indian River) unsexed broiler chicks were purchased from a local hatchery (Kazi Farms Ltd.) on a pre-order basis. On arrival at the farm, broiler chicks were randomized into five different groups: Control (C), Eye + Eye, Eye + Drinking Water (Eye + DW), Cloaca + Cloaca (Clo + Clo), and Cloaca + Drinking Water (Clo + DW), and each group contained 15 birds with two replications. The birds were reared on a litter-based poultry farm with proper ventilation and a standard lighting program. Feed and water were supplied *ad libitum*.

2.2. Vaccination

We followed the vaccination schedule as per shown in Table 1.

2.3. Sample collection

At age 30 days, the birds were anesthetized with chloroform-soaked cotton, and blood was drawn from the wing vein for serological examination in all groups. We randomly selected four birds from each group for sampling. Following a cervical dislocation, the bursa of Fabricius (BF) and cloaca were collected. The collected bursa of Fabricius was trimmed into small slices and fixed with 10 % Neutral Buffer Formalin (NBF) for histopathological examination.

2.4. Whole-mount staining

Cloaca was collected during sampling, and a ventral section was given to the cloaca. Then the cloaca was flattened with the pins, with the mucosa on the upper side. We poured a hematoxylin solution on the cloacal mucosa and soaked it for 8–10 min to visualize lymphoid tissues. Then the cloaca was examined at a 4–5 min interval.

2.5. Histopathological examination

Paraffin blocks of the bursa of Fabricius samples were sectioned at a thickness of 3 μm using a microtome and stained with hematoxylin and eosin (H&E) to examine the general histological structure.

2.6. Histoplanimetry

We used a Euromex BS.1153-EPLi (S/N-EC 2221114) microscope with image focus alfa software to measure the follicular area of Fabricius' bursa at 400X magnification. Digital microscopic images were captured over randomly selected follicles from separate groups (H and E-stained sections). ImageJ was used to count the number of cells per 1000 μm^2 area (https://imagej.net/ij/).

2.7. Immunofluorescence

NBF-fixed paraffin blocks were cut at 2 μm thickness and stained with periodic acid Schiff-hematoxylin (PAS-H) to examine the cellular characteristics of the cloacal mucosa. Immunodetection of cellular markers was performed as previously reported by Masum et al. (2021) [9] for B cells (Bu 1), T cells (CD3), macrophages/monocytes (KUL01), dendritic cells (CD 1), and MHCII.

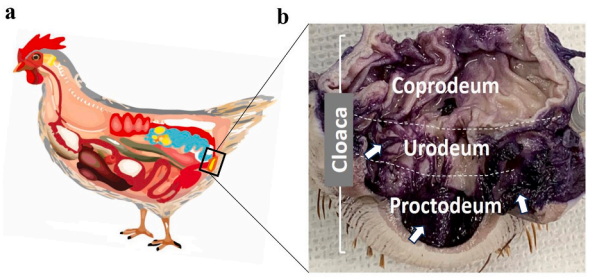

Fig. 1. Gross detection of lymphatic tissue in cloaca.
(a) The digestive, urinary, and genital systems are connected to the cloaca (box area). (b) The wall of the cloaca shows a deep blue color in whole-mount staining with hematoxylin, suggesting the presence of lymphatic aggregations (white arrows). Proctodeum shows a deeper blue color than that of c oprodeum and urodeum. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.8. Serological examination

The collected blood samples were centrifuged to separate serum for testing the ND and IBD antibody titers. A micro-titer hemagglutination inhibition (HI) test was performed to get the antibody titer against ND. A commercial ELSA kit (Bio-Check, UK) was used to determine the antibody titer against IBD according to the manufacturer's instructions.

2.9. Statistical analysis

The values are expressed as the mean ± standard error. The results were statistically analyzed using a Tukey pairwise comparison test ($P < 0.05$).

3. Result

3.1. Gross detection of lymphatic tissue in the cloaca

We examined the cloacal mucosa for the detection of the lymphoid tissue cluster (LTC) (Fig. 1a and b). Whole-mount hematoxylin staining was performed to visualize the lymphoid tissue in the cloacal mucosa. The lymphoid cell nuclei took on a hematoxylin color, and hematoxylin-positive dark blue patches appeared, indicating the LTCs on the cloacal mucosa (Fig. 1b). The examined area showed abundant dark blue spots throughout the cloacal mucosal surface. We observed larger dark-blue spots in the proctodeum than in the coprodeum and urodeum, which indicated more LTC in the proctodeum (Fig. 1b).

3.2. Microscopic clarification of the lymphoid tissue in the cloaca

Hematoxylin and eosin (H & E) staining was performed to evaluate the histological features of the cloaca. We observed the presence of lymphoid tissue (LT) in the lamina propria (LP) just underneath the epithelium of the proctodeum (Fig. 2a). LTs consist of diffusely

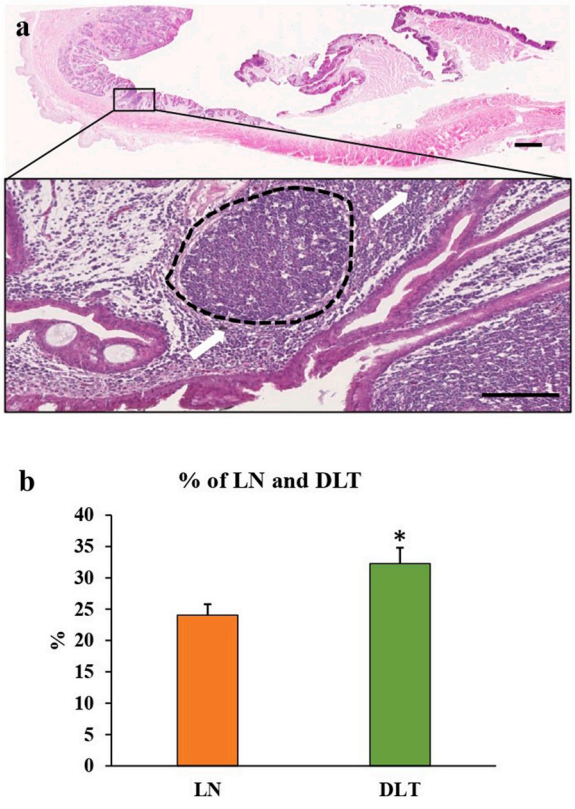

Fig. 2. Histological clarification of lymphoid tissue in cloaca.
(a) The histological section of Cloaca confirms the presence of LN (dashed circle) and DLT (arrows) in the lamina propria. H & E stain. (b) Percentage of LN and DLT in the examined lamina propria of Cloaca.
Values are expressed as mean ± s.e. Significant difference from the other group is indicated by * (*$P < 0.05$, Tukey Pairwise Comparison test). N = 4. LN = lymphatic nodule, DLT = diffuse lymphatic tissue, and H & E = hematoxylin and eosin. Bars = 100 μm.

disseminated and more compactly aggregated lymphoid cells. Lymphatic nodules (LN), ovoid-shaped and composed of aggregated lymphoid cells, are found in the LP, as is diffuse lymphoid tissue (DLT), which is scattered along the LP (Fig. 2a). The percentage of DLT was significantly higher than the LN in the lamina propria of cloaca (Fig. 2b).

3.3. Cellular characterization of LT in cloaca

We characterized the lymphoid cells in the LT of the cloacal mucosa by immunofluorescence technique using different cell markers. Various types of immune cells were found in the cloacal mucosa, including Bu-1+ B cells, $CD3^+$ T cells, and KUL01+ macrophages and monocytes. At first, we observed Bu-1+ B-cells, which were densely dispersed throughout the LN as well as in the DLT (Fig. 3a–c). No aggregation was noticed, and Bu + B-cells were scattered all over the LN and DLT. Additionally, B-cells were localized in the epithelium, LP, and surrounding areas of LN (Fig. 3a–c). Similar localization was noticed in the $CD3^+$ T cells that were diffusely distributed in the LN and DLT (Fig. 3d–f). KUL01+ macrophages were localized throughout the LN and surrounding area of the LN (Fig. 3g–i). KUL01+ macrophages were also observed in the DLT, LP, and submucosa (Fig. 3g–i). These structures reflect the similar structural organization of MALT. We have named these LTs CALT (cloaca-associated lymphatic tissue) after their location in cloaca.

3.4. Antigen presentation

To make the functional activity more apparent, we evaluated the antigen presentation in CALT (Fig. 4). We characterized the colonization of different types of immune cells in CALT through immunofluorescence, in which dendritic cells, macrophages, and B cells are key antigen-presenting cells (APCs) responsible for capturing antigens in peripheral tissues. We observed that the same LN contained $CD3^+$ lymphocytes, KUL01+ macrophages, CD1.1+ dendritic cells (Fig. 4a–c), and MCH-II-positive molecules (Fig. 4d–f).

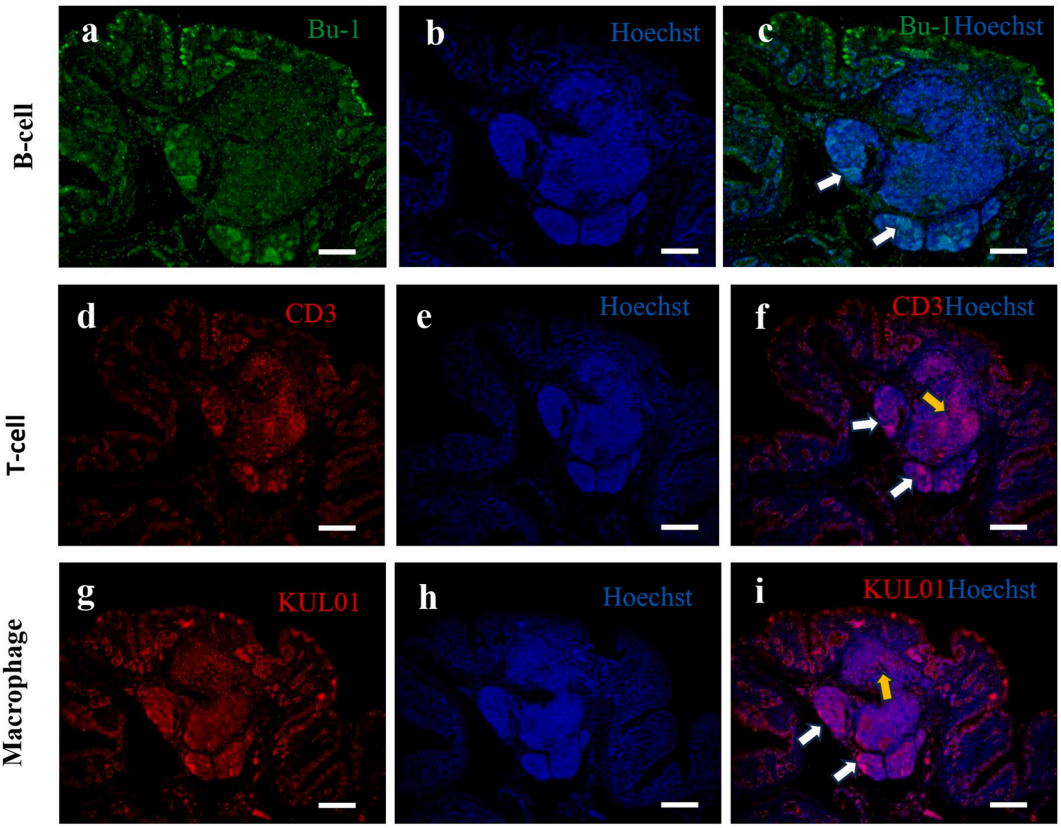

Fig. 3. Cellular Characterization of LT in cloaca
(a-c) Bu-1+ B-cells are present throughout the LN and DLT of cloaca (white arrows). IF. (d–f) $CD3^+$ T-cells are present in the LNs (white arrows) but dispersed in the DLT (yellow arrow). IF. (g–i) KUL01+ Macrophages are scattered throughout the LN (white arrow) and DLT (yellow arrow), IF. LT = lymphatic tissue, LN = lymphatic nodule, DLT = diffuse lymphatic tissue and IF = immunofluorescence. Bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

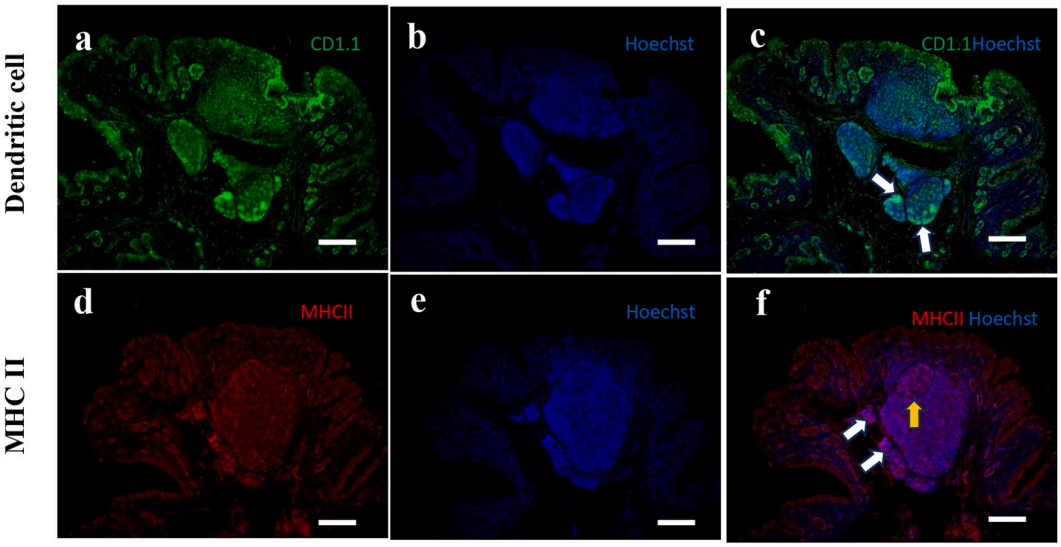

Fig. 4. Antigen presentation in CALT
(a-c) CD1.1+ Dendritic cells are localized in the LN of cloaca (white arrows). IF. (d–f) MHCII + cells are also localized in the LN (white arrows) and DLT (yellow arrows) of cloaca. IF. CALT = cloaca-associated lymphoid tissue, LN = lymphatic nodule, DLT = diffuse lymphatic tissue, and IF = immunofluorescence. Bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Identification of vascular networks in CALT

We examined the vascular network in cloacal lymphoid tissue and found blood vessels (Fig. 5 a') and high endothelial venules (HEV) (Fig. 5 b') in the submucosa. This submucosa containing HEV indicated a close immunological association of the cloacal lymphoid tissue with other lymphoid organs.

3.6. Clarification of cloaca as a novel mucosal vaccination route

We determined the immune response of cloacal vaccination through serum antibody levels against the ND and IBD vaccines (Fig. 6). The antibody titer against the ND vaccine in the cloacal vaccination group was significantly higher than that in the vehicle control group (Fig. 6a). A similar result was found in antibody titers against the IBD vaccine (Fig. 6b). The results of this study indicate that cloacal vaccination can induce robust immune responses via the production of serum antibodies.

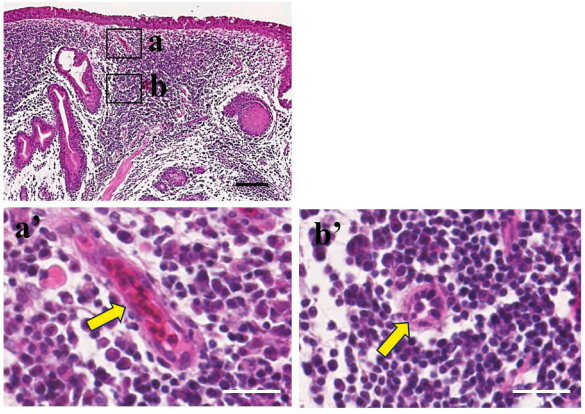

Fig. 5. Vascular system in CALT
(a) CALT has a vascular network. (a') A blood vessel is present in the CALT. (b') A high endothelial venule is present in the CALT. H & E stain. CALT = cloaca-associated lymphoid tissue; H & E = hematoxylin and eosin. Bars = 100 μm.

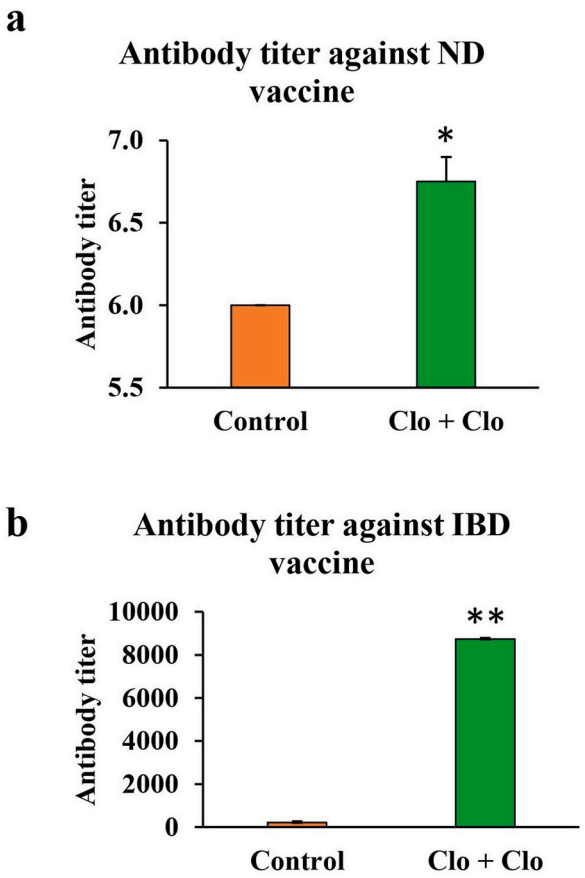

Fig. 6. Clarification of immune response after vaccination through Cloaca
(a) Antibody titer against the ND vaccine in vehicle control and cloacal vaccinated (received vaccine antigen) birds. HI. (b) Antibody titer against the IBD vaccine in vehicle control and cloacal vaccinated (received vaccine antigen) birds. ELISA. A significant increase in antibody titers was noticed following the cloacal vaccination.
Values are expressed as mean ± s.e. Significant difference from the other group is indicated by * (*P < 0.05 and **P < 0.01, Tukey Pairwise Comparison test). N = 4. ND = Newcastle disease; IBD = infectious bursal disease; Clo = cloaca; HI = hemagglutination inhibition; ELISA = enzyme-linked immunosorbent assay.

3.7. Comparison of the cloacal route with other mucosal vaccination routes

The antibody titers against the ND and IBD vaccines in different groups are presented in Fig. 7. We found a higher ND antibody titer in the Clo + Clo vaccination route than the traditional routes (Eye + Eye, Eye + DW), and the value was significantly higher than both the control and Clo + DW groups (Fig. 7a). We found the highest IBD antibody titer in the Clo + DW vaccination group, and it was significantly higher than the rest of the groups (Fig. 7b). The antibody titers of Clo + Clo and Eye + DW were significantly higher than those of the control and Eye + Eye vaccination groups (Fig. 7b).

3.8. Histopathological findings

The bursal follicle exhibited a distinct morphology, with a lighter-stained center medulla surrounded by a darker-stained cortex (Fig. 8a–e). The typical BF folicole was present in the control and Eye + Eye groups. The BF folicole of Eye + DW was smaller than the control group, while Clo + Clo and Clo + DW had the larger and even follicole (Fig. 8a–e). Importantly, the cloaca vaccination groups (Clo + Clo, Clo + DW) had a higher follicular area than the traditional vaccination groups (Eye + Eye, Eye + DW) (Fig. 8f). We also counted the number of immune cells in BF and found a higher number of cells in cloacal vaccination groups than the control and traditional vaccination groups (Fig. 8g).

3.9. Cytotoxic effects in the bursa of Fabricius following vaccination

We performed a histopathological examination of the BF to reveal the cytotoxic effects of the IBD and ND vaccines delivered through different mucosal routes. The bursa of Fabricius in the control group showed a very mild degree of histopathological changes

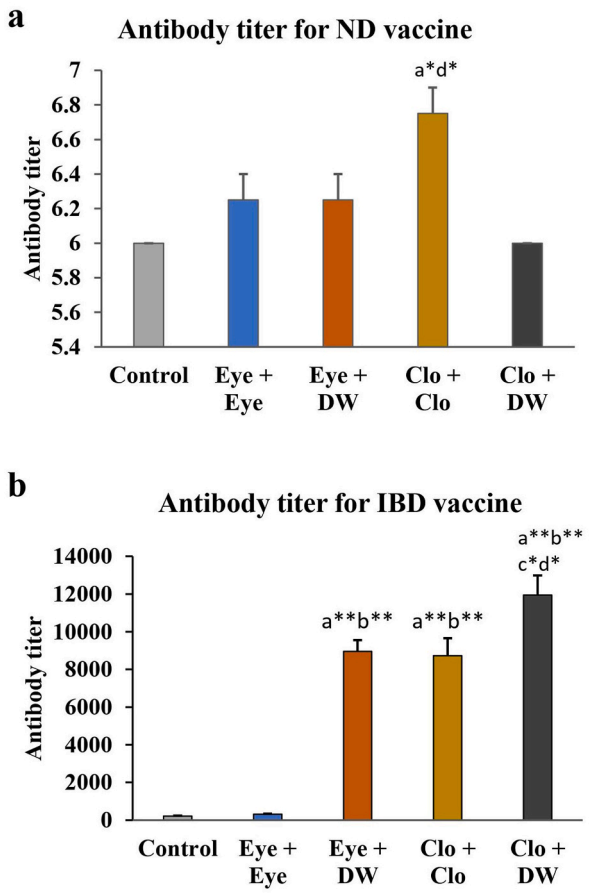

Fig. 7. Comparison of immune responses between traditional and cloacal mucosal vaccination routes
(a)Antibody titers for ND in different groups. A significant increase in antibody titers was observed in Clo + Clo compared to the other groups. HI. (b) Antibody titers for IBD in different groups after vaccination. ELISA. The Clo + DW group showed the highest level of antibody titer compared to the other groups.
Values are expressed as mean ± s.e. Significant difference from the other group is indicated by * (*P < 0.05 and **P < 0.01, Tukey Pairwise Comparison test). N = 4. a, b, c, d, and e denote Control, Eye + Eye, Eye + DW, Clo + Clo, and Clo + DW, respectively. ND = Newcastle disease; IBD = infectious bursal disease; Clo = cloaca; DW = drinking water; HI = hemagglutination inhibition; ELISA = enzyme-linked immunosorbent assay.

with a well-developed lymphoid follicle and mild to moderate lymphoid cell depletion (Fig. 9a and b**).** A very small number of intraepithelial vacuoles were found in the control group (Fig. 9c). The Eye + Eye vaccination group showed moderate lymphoid depletion in the cortex and medulla (Fig. 9d and e**)**. Lymphoid follicles exhibited mild atrophy, and severe intraepithelial vacuole and cyst formation were observed (Fig. 9f**)**. The Eye + DW group exhibited large vacuoles in the bursal follicle and moderate intraepithelial vacuole formation (Fig. 9g–i). The Clo + Clo vaccination group showed similar histopathological features as the control (Fig. 9j–l). The most severe histopathological lesions were observed in the Clo + DW group (Fig. 9m). These birds exhibited marked lymphoid cell depletion and large vacuole formation in the cortex and medulla of the bursal follicle (Fig. 9n). Large intraepithelial cyst/vacuole formations were also noticed in this group (Fig. 9o). The results of this study indicate that different vaccination routes produce different degrees of histopathological changes in the bursa of Fabricius in broilers. Severe cytotoxic effects were seen in the Eye + DW and Clo + DW groups based on lymphoid cell depletion and vacuole formation in the bursal follicle and epithelium (Fig. 9p and q).

4. Discussion

Infectious diseases pose a threat to public health, the poultry industry, and the global economy, which is responsible for the fall in meat production [21]. Generally, pathogens that cause these infectious diseases enter the body through the mucosal route. Mucosal vaccines are capable of preventing these diseases by triggering an immune response at the mucosal surface. Traditional mucosal vaccinations in chickens rely on the oculo-nasal or oral route, which confers antibody production in the respective system only. As cloaca is connected to the digestive and urogenital systems, we clarified LT in the cloacal wall of broilers as a novel MALT and demonstrated cloaca as a unique mucosal vaccination route that would protect a broad mucosal area.

Firstly, the presence of LT in the cloaca of broilers was examined by using a whole-mount technique with hematoxylin. In the

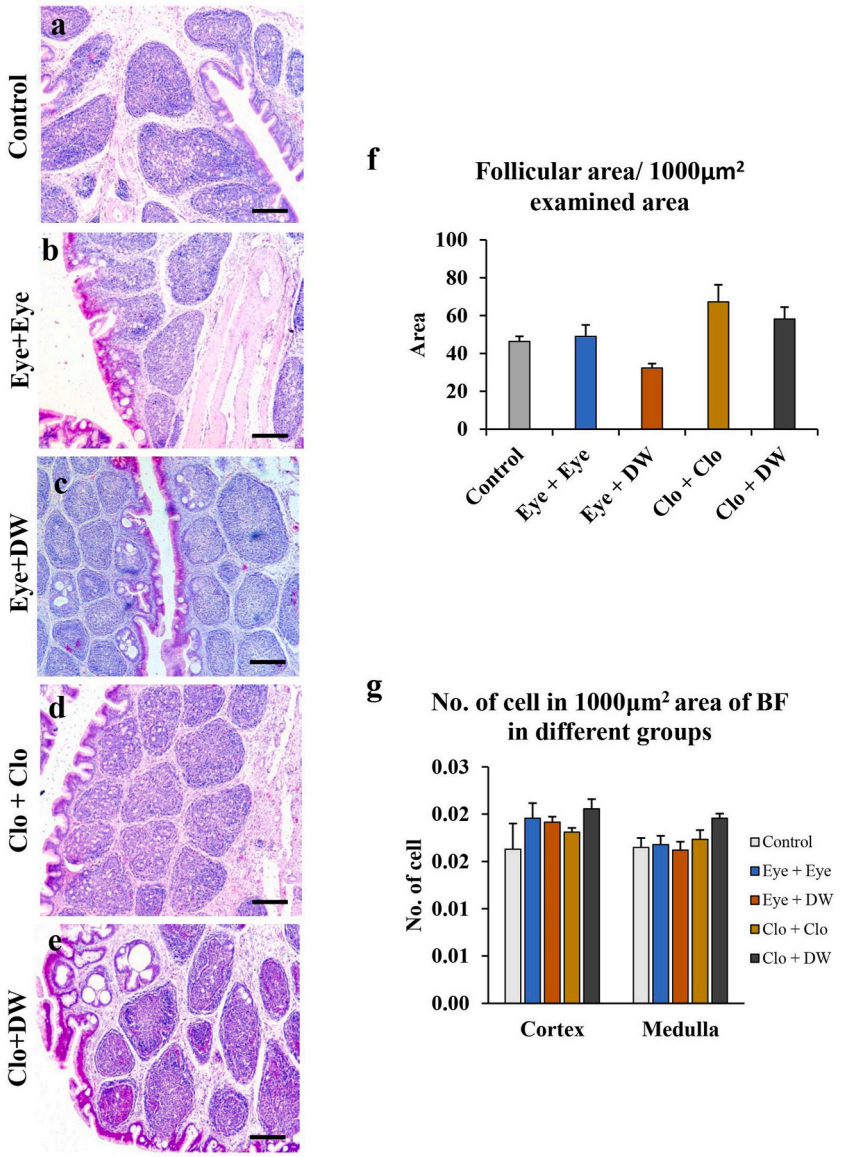

Fig. 8. Histological examination of bursa of Fabricius in different group
(a–e) Histological sections of the bursa of Fabricius in different groups. (a–b) Typical BF present in the control and Eye + Eye groups. (c) smaller BF than the control present in the Eye + DW group. (d) Comparatively larger and even BF is present in the Clo + Clo group. (e) A larger BF is present in the Clo + DW group. H & E stain. (f) Follicular area in the examined area of the bursa of Fabricius in different groups. (g) Number of cells in BF in different groups. Values are expressed as mean ± s.e. Significant difference from the other group is indicated by * (*P < 0.05 and **P < 0.01, Tukey Pairwise Comparison test). N = 4. a, b, c, d, and e denote Control, Eye + Eye, Eye + DW, Clo + Clo, and Clo + DW, respectively. Clo = cloaca; DW = drinking water; H & E = hematoxylin and eosin, BF = bursal follicle. Bars = 100 µm.

previous studies, the use of the wholemount technique with hematoxylin staining proved to be a valuable tool for visualizing the LT [22]. After whole mounting with hematoxylin, hematoxylin-positive dark blue patches were observed in the mucosa of cloaca, indicating the presence of LT (Fig. 1). Furthermore, microscopic examination was performed with H and E staining to understand the microscopic structure of the LT that was found after whole-mount staining. Microscopic examination revealed that the LP of the cloacal mucosa contained clusters of LT in a well-defined area known as LN. In addition, some lymphoid cells are dispersed throughout the connective tissue of LP as DLT (Fig. 2). These DLTs were amorphous and lacked a distinct border with the surrounding tissue. The organizational features of these LTs are similar to those of MALT, which are found in the mucosal lining of the gastrointestinal tract, respiratory tract, and urogenital tract [23–25].

To understand the cellular composition of LNs and DLTs, we performed immunofluorescence using different cell markers. Both in LNs and DLTs in the cloacal mucosa, different types of immune cells, namely, Bu-1+ B cells, $CD3^+$ T cells, KUL01+ macrophages and

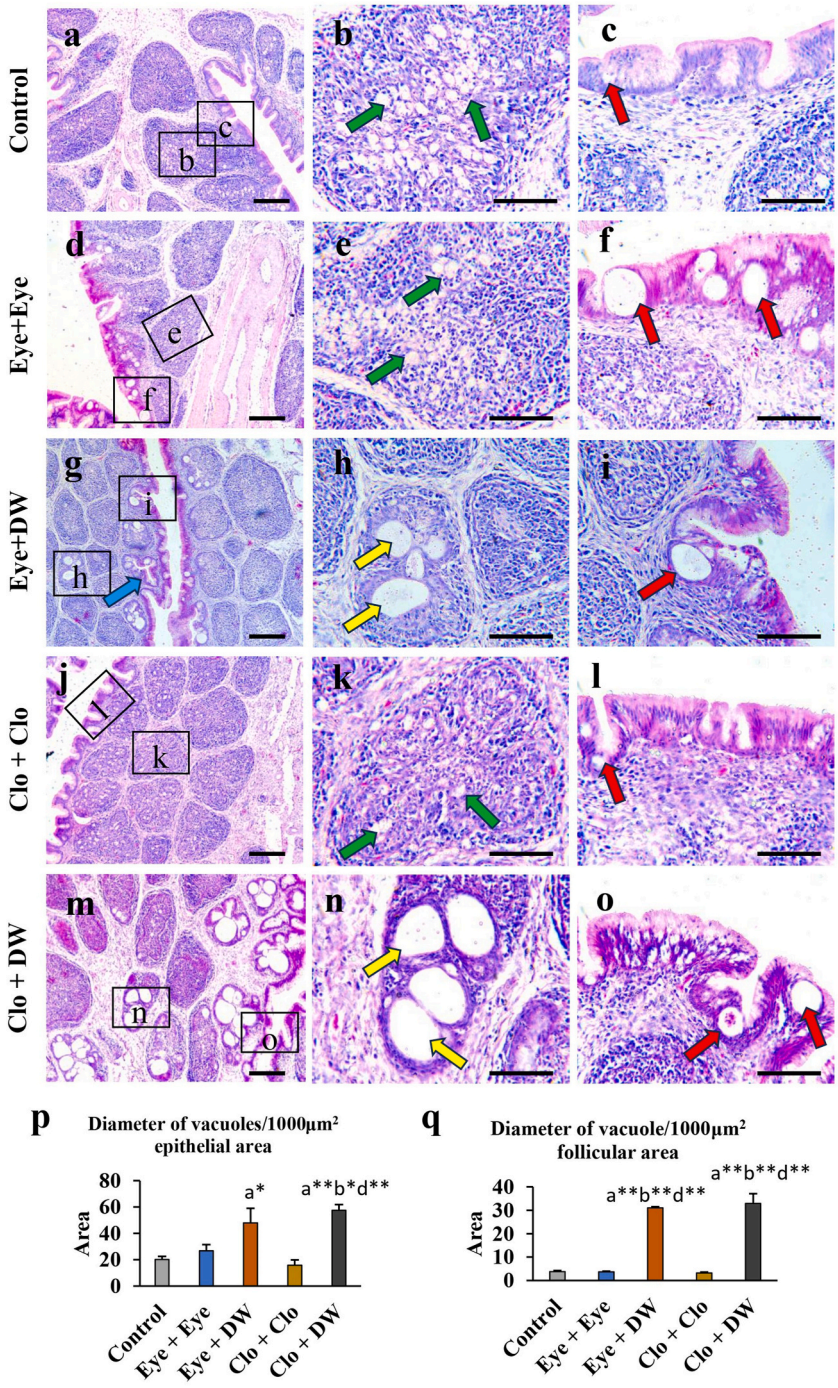

Fig. 9. Cytotoxic effects in bursa of Fabricius after administration of vaccines via different mucosal routes.
(a–c) Normal BF is present in the control, but it contains small vacuoles in both the parenchyma (green arrows) and epithelium (red arrows). (d–f) The bursal follicle contains small vacuoles (green arrows), but large vacuoles are present in the epithelium (red arrows). (g–i) Both the bursal follicle and epithelium contain large vacuoles (green and red arrows). (j–l) The bursal follicle and epithelium contain small vacuoles like control (green and red arrows). (m–o) Both the bursal follicle and epithelium contain large vacuoles (green and red arrows). H & E stain, (p) Diameter of vacuoles/1000 μm^2 epithelial area (q) Diameter of vacuoles/1000 μm^2 follicular area.
Values are expressed as mean ± s.e. Significant difference from the other group is indicated by * (*P < 0.05 and **P < 0.01, Tukey Pairwise Comparison test). N = 4. a, b, c, d, and e denote Control, Eye + Eye, Eye + DW, Clo + Clo, and Clo + DW, respectively. Clo = cloaca; DW = drinking water; H & E = hematoxylin and eosin. Bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

monocytes, CD1.1+ dendritic cells, and MHCII + APCs cells, were present (Figs. 3 and 4). This result agreed with our previous work, which illustrated that similar types of immune cells were found in MALT [24,25]. These cells are responsible for the humoral and cell-mediated immune responses. B cells produce antibodies, while T cells play various roles, including helping B cells to produce antibodies, activating other immune cells, and attacking abnormal or virus-infected cells directly [26]. As the LTs of cloacal mucosa mimic the similar structural organization of a typical MALT, we named it cloaca-associated lymphoid tissue (CALT).

As the vascular network facilitates the transport of immune cells, antigens, and antibodies to and from MALT, we examined the vascular network in the CALT. Blood vessels were observed in the connective tissue of LP (Fig. 5). Additionally, HEVs are also observed in the cloaca, which are specialized blood vessels within lymphoid tissues (Fig. 5). HEVs would facilitate the entry of lymphocytes into CALT from the bloodstream, allowing for immune responses and also being important for maintaining mucosal immunity as found in other MALTs [27,28].

We also observed dendritic cell, macrophage, and B-cell colonization in CALT (Figs. 3 and 4). So, we considered that macrophages and dendritic cells were colonized with the CALT. Macrophages and dendrite cells recognize antigens and present them to neighboring lymphocytes, which initiates an adoptive immune response by activating T cells [24,29]. The presence of lymphoid aggregates, including specialized immune cells (lymphocytes, macrophages, and dendritic cells), supports CALT as a novel MALT.

The bursa of Fabricius, a primary lymphoid organ unique to birds, is located in the cloacal proctodeum. The bursa of Fabricius facilitates the development of protective antibody responses after vaccination by ensuring B-cell maturation and activation [30]. Cloaca has a close association with the bursa of Fabricius, so vaccination through cloaca would be more beneficial to enhance the immune response against antigens. That's why we examined the immunological effect of cloacal vaccination. We evaluated antibody titers against the ND and IBD vaccines after vaccination through the cloaca and compared them with the vehicle control group (Fig. 6). A significant increase in antibody titer was found in cloacal vaccinated birds compared to the control, which ensures the robust immune response of cloacal vaccinated birds.

The measurement of antibody titers in serum is commonly used to evaluate immune responses [31]. We compared the cloacal vaccination route with other traditional mucosal vaccination routes by measuring antibody titers. We found the highest ND titers in the Clo + Clo group compared to the other groups (Fig. 7). Similar findings were demonstrated in the case of the IBD antibody titer. Broilers from group Clo + DW showed a higher antibody titer against IBD than other vaccinated groups (Fig. 7). The mean HI titers of the birds in the Clo + Clo group showed the highest antibody titers compared to the other groups (Fig. 7). Similar findings were demonstrated in the case of the IBD antibody titer. Broilers from group Clo + DW showed a higher antibody titer against IBDV than that of other vaccinated groups (Fig. 7).

Our results differed from Al-Zuhariy (2023) [32], who found a better immune response using the intraocular route in the live ND vaccine. Previous researchers reported that maternal-derived antibodies neutralize the vaccine antigen, resulting in a lower antibody titer after post-vaccination in humans, animals, and birds [33–35]. The increased immune response in cloacal-vaccinated birds may be due to the fact that the cloacal route may allow for more efficient uptake of the vaccine antigen by CALT. Therefore, increased antibody titers in cloacal-vaccinated birds compared to the others indicate that cloacal vaccination may overcome that maternal antibody interference. Further, detection of $CD4^+$, $CD8^+$ helper T cells, and cell-mediated immune response-specific cytokines in cloacal vaccinated birds will additionally prove the efficacy of cloacal vaccination.

Moreover, measuring the degree of microscopic changes in the bursa of Fabricius (follicular area, follicular size, and density of cells in bursal follicles) can be an effective method of assessing immunological protection [36]. In the present study, we measured the follicular area of the bursa in a particular area after vaccination. A higher follicular area was observed in cloacal-vaccinated birds compared to the other groups, where birds were vaccinated through the ocular and oral routes (Fig. 8).

Importantly, the bursal follicular area is important for antibody production. A previous study reported that bursal follicles are composed of numerous immune cells, including macrophages, lymphocytes, and plasma cells, which are linked to immunological responses [37]. So, the higher follicular area reflects the higher immunity of vaccinated birds. Aihara et al. (2015) [38] also reported that the number of follicles is crucial for the immunoreactivity of chickens infected with the IBD virus.

Several physical responses can occur after mucosal vaccination as a part of an immune reaction. Reduced feed intake, lethargy, mild respiratory signs like sneezing and coughing, swelling, and redness at the mucosal site may occur temporarily after vaccination [39]. Interestingly, no allergic reaction was noticed in birds following the cloacal vaccination. Furthermore, histopathological examination of the bursa of Fabricius was performed to evaluate the cytotoxic effects of the vaccine at the cellular level, as vaccination also produces some cytotoxic effect or immunopathological lesion on the bursa of Fabricius [5,39,40]. We found mild degree's vacuoles in both the control and Clo + Clo groups. We found a large number of vacuoles in the Eye + DW and Clo + DW groups. This study found comparatively larger vacuoles in those groups where booster vaccination doses were administered via drinking water (Fig. 9). These vaccination-induced bursal lesions can heal, regenerate, and reduce with time [41].

Overall, cloacal vaccination has demonstrated promising results, showing higher antibody levels and fewer lesions on the bursa of Fabricius compared to traditional vaccination methods. These findings suggest that cloacal vaccination could offer a more effective and less invasive approach for stimulating immune responses in birds.

In conclusion, the cloacal mucosa contains CALT, which comprises B-cells, T-cells, and macrophages and is decorated with a fine vascular network, indicating a unique MALT. In addition, CALT harbors both antigen-presenting cells and molecules, suggesting its functional status. The proximity of CALT to the bursa of Fabricius offers a new avenue for mucosal vaccination. Importantly, vaccination through the cloacal route induces a higher range of antibodies compared to other traditional mucosal vaccination routes, suggesting it is a unique vaccination route in chickens (Fig. 10). The cloacal route is the most effective method for mass immunization of poultry, as it is easy, rapid, and convenient. This method, particularly during vent sexing, minimizes handling events, reduces stress on chicks, saves time, and reduces labor costs, enhancing herd immunity and ensuring uniform vaccination.

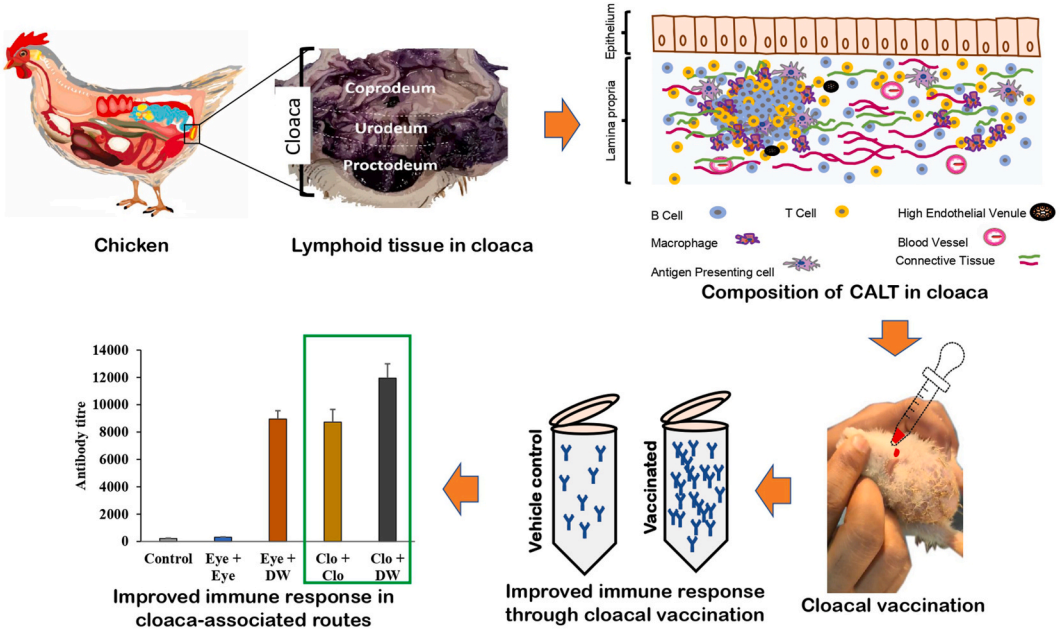

Fig. 10. Improved immune response through cloacal vaccination.
The cloacal mucosa contains CALT, which comprises B-cells, T-cells, and macrophages, and is decorated with a fine vascular network, indicating a unique MALT. In addition, CALT harbors both antigen-presenting cells and molecules, suggesting its functional status. The proximity of CALT to the bursa of Fabricius offers a new avenue for mucosal vaccination. Importantly, vaccination through the cloacal route induces a higher range of antibodies compared to other traditional mucosal vaccination routes, suggesting it is a unique vaccination route in chickens. CALT = cloaca-associated lymphoid tissue; MALT = mucosa-associated lymphoid tissue.

CRediT authorship contribution statement

Rupa Akter: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Md Abdul Masum:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Subrato Biswas:** Writing – review & editing, Writing – original draft, Software, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Md Zahir Uddin Rubel:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation. **Sujan Kumar Sarkar:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Data curation, Conceptualization. **Mohammad Saiful Islam:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Hossain M. Golbar:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Md Emtiaj Alam:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Conceptualization. **Md Abdur Rakib:** Writing – review & editing, Writing – original draft, Software, Resources, Formal analysis, Data curation, Conceptualization. **Md Zahirul Isalm Khan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethical statement

This study was authorized by the Animal Welfare and Experimentation Ethics Committee of Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh, and conducted according to compliance with institutional ethical standards. [Number of approval: SAU/AHIPHI/22/843].

Data availability

The data that support the findings of this study are within the manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Md. Abdul Masum reports financial support was provided by University Grants Commission of Bangladesh. Md. Abdul Masum reports financial support was provided by Government of the People's Republic of Bangladesh Ministry of Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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