Novel Insights into Lectin Binding Patterns in the Nasopharyngeal Tonsil of Buffaloes (*Bubalus bubalis*)

Pawan Kumar*

Department of Veterinary Anatomy, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, India

Abstract: *Background*: The present study investigates the specificity of lectin binding in the nasopharyngeal tonsil of six healthy adult buffaloes (*Bubalus bubalis*), a species not extensively studied regarding its immune system. Lectins, proteins that bind specifically to carbohydrates, are used to identify and characterize different cell types that may have roles in immune responses. This study explores how lectins bind to various cells within the nasopharyngeal tonsil, shedding light on cellular differentiation, interactions, and the potential functional roles of these cells in mucosal immunity.

Methods: A total of 21 biotinylated lectins, grouped into five categories based on their carbohydrate specificity (N-acetylglucosamine, N-acetylgalactosamine, galactose, glucose/ mannose, and fucose), were used to probe the nasopharyngeal tonsil tissue. Lectin histochemistry was applied to identify the binding patterns of these lectins to different cell types within the tissue, including epithelial cells, lymphoid cells, and specialized structures such as M-cells and P-cells. The study also involved the detection of vimentin filaments to explore potential immune responses within the tissue.

Results: Lectin histochemistry revealed a dynamic epithelial composition of the nasopharyngeal tonsil, consisting of pseudostratified columnar ciliated epithelium and lymphoepithelium, with distinct adaptations in the follicle-associated epithelium (FAE). The FAE exhibited M-cells, which are believed to play a role in antigen processing. Additionally, a new class of cells, termed P-cells, was identified based on their lectin-binding patterns, which share similarities with M-cells but are distinct in their function. Lectins targeting N-acetylglucosamine exhibited varying affinities for M- and P-cells, while lectins recognizing N-acetylgalactosamine selectively bound to cilia and goblet cells. Lectins targeting galactose produced complex staining patterns in mucous glands and lymphoid tissues. Specific binding was also observed in lymphoid cells with lectins recognizing glucose/mannose and fucose groups. Vimentin filaments in lymphocytes and specialized epithelial cells suggest an involvement in immune response mechanisms.

Conclusion: This study provides new insights into structural organisation landscape of the buffalo nasopharyngeal tonsil, highlighting the role of lectin-binding patterns in identifying specialized cells and tissues. The M-cells and discovery of P-cells and the detailed lectin-binding profiles may contribute to understanding the cellular dynamics of mucosal immunity. Additionally, the structural details uncovered in this study may serve as a valuable reference for comparative research on mucosal immunity across different species, advancing our understanding of antigen recognition and immune responses at mucosal surfaces.

Keywords: Buffalo, Galactose, Glucose/mannose, Lectin, Nasopharyngeal tonsil, N- acetylgalactosamine, N-acetylglucosamine, Vimentin.

INTRODUCTION

Lectins are glycoproteins or proteins that possess the ability to bind specifically to terminal carbohydrates on sugar chains, making them different from typical antigens that might trigger immune reactions. These lectins have emerged as valuable histochemical tools for cell differentiation studies [1]. The nasopharyngeal tonsil is lined by a pseudostratified columnar ciliated epithelium with goblet cells and features a follicle-associated epithelium (FAE). Within the FAE, specialized microfold (M) cells have been identified in the nasopharyngeal tonsils of horses, pigs, and buffaloes [2-4]. M-cells play a crucial role in generating mucosal immune responses by transcytosis antigens from the free epithelial surface of the tonsil. M-cells can be distinguished from adjacent cells in the FAE by their dome or flat apical surface, reduced size of microvilli, and close association with intraepithelial lymphocytes [2]. The apical membrane of M-cells exhibits higher levels of cholesterol [5] and contains varying levels of alkaline phosphatase and esterase enzymes [6, 7]. In the horse nasopharyngeal tonsil, terminal (1-3)-linked galactose epitopes demonstrated by lectin GS1B4 and vimentin filaments have been reported as additional characteristics of M-cells [2]. Detailed studies on the histoarchitecture of the nasopharyngeal tonsil have been reported in horses [2] and buffaloes [4]. These studies provide valuable insights into the structural organization and functional aspects of the tonsils in different animal species.

Through lectin histochemistry, this study aims to identify specific markers associated with distinct cell types, especially those situated on the exposed apical surface of M-cells and P-cells. The identification may

*Address correspondence to this author at the Department of Veterinary Anatomy, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, India; Tel: +919466402637; E-mail: pawanrajoria2000@rediffmail.com, pawanrajoria2000@gmail.com

ISSN: 1927-5196 / E-ISSN: 1927-520X/25

shed light on early pathogenesis by probing the interactions between microorganisms and the unique glycoconjugate receptors in the future. The expression of glycoconjugates specific to the FAE and M-cell surfaces may serve as an experimental model for developing mucosal vaccines targeting M-cells to prevent infections [8]. The apical membrane conjugates of the FAE, particularly M-cells, may serve as initial binding sites for lectins and microbial surface proteins [9]. Furthermore, the expression of glycoconjugates differs significantly in the FAE and lymphoid tissue between mice and humans [10]. Due to the limited literature on the presence of a specific glycocalyx on epithelial cells, this study aims to investigate lectin histochemistry in the nasopharyngeal tonsil of adult buffaloes. The goal is to identify distinct glycohistochemical epitopes on the respiratory epithelium and follicle-associated epithelium (FAE) with M-cells and P-cells, as well as components of the propria-submucosa, which could offer valuable insights for future research on immune responses.

MATERIALS AND METHODS

Animals

The present study was conducted on six clinically healthy adult buffaloes (*Bubalus bubalis*), aged 5-6 years, of a local mixed breed, irrespective of sex. The heads were obtained from Ghazipur Municipal Slaughterhouse, New Delhi, India, immediately after euthanizing the animals using the bolt gun method. A sagittal section of the head was made with an electric saw to collect tissues from the nasopharyngeal tonsil, which were then preserved in liquid nitrogen. Permission from the Institutional Animal Ethics Committee was not required since the animal heads were obtained from a slaughterhouse.

Frozen Tissue Preparation

Small pieces of the frozen nasopharyngeal tonsils were embedded in Poly Freeze tissue freezing medium (Sigma) to form blocks. Frozen sections of 6-8 µm thickness were obtained using a cryostat and collected on glass slides treated with a 2% solution of 3-(amino-propyl)triethoxysilane. The slides with sections were stored in a freezer at -20°C.

Lectins

A panel of 21 lectins, classified into five broad groups based on their terminal sugars, was used (Table **1**).

Lectin Cytochemistry

The slides were thawed and air-dried at room temperature for 30 minutes. The sections were fixed in acetone for 20 minutes at room temperature, air-dried, and then rehydrated in phosphate-buffered saline (PBS) at pH 7.4 for 10 minutes. The frozen sections were rimmed with a PAP pen (Sigma) to safeguard the chemicals. The sections were treated with 0.3% H_2O_2 in PBS for 30 minutes to block endogenous peroxidase activity. After washing with PBS, the sections were incubated in 1% Blocker™ bovine serum albumin (Thermo) in PBS for 45 minutes to block the binding of non-specific antigens. Separate sections were incubated with biotinylated lectins (Vector Lectin Kit I BK-1000; Kit II BK-2100; Kit III BK-3000, Biotinylated; Table **1**) at a concentration of 10 µg/ml in 0.2% gelatin-PBS for 1 hour in a humidification chamber at room temperature. Following three washes with PBS (5 minutes each), the sections were incubated with Streptavidin Alexa Fluor™ 488 conjugate (Invitrogen) (2 µg/ml in PBS) for 30 minutes and then washed twice with distilled water (5 minutes each). The sections were mounted with coverslips using Mowiol containing 2.5% 1,4-diazabicyclo [2.2.2]octane (DABCO). Control tonsil tissue sections were treated as described above, except for the incubation step with biotinylated lectin, to eliminate the possibility of non-specific binding. The sections were examined using a fluorescent microscope (Olympus CX-41 Magnus), and appropriate photographs were recorded with a CMOS digital camera (Lumenera Infinity 1).

Vimentin Histochemistry

Frozen sections were cut as described earlier for the lectin histochemistry and were permeabilized with 0.2% Triton X-100 (Sigma) for 3 minutes and then treated with mouse anti-vimentin (mAb V9) (Sigma) diluted 1:40 in PBS for 1 hour. This was followed by incubation with fluorescein-conjugated anti-mouse immunoglobulin G (IgG) at a dilution of 1:100 for 1 hour. After washing with PBS, the sections were mounted in Mowiol and examined using a fluorescent microscope.

RESULTS

The nasopharyngeal tonsil exhibited a lining of pseudostratified columnar ciliated epithelium consisting of basal, ciliated, and goblet cells. This epithelium was referred to as lymphoepithelium when it was associated with the underlying lymphoid tissue in the propria-submucosa. The follicle-associated epithelium (FAE)

Table 1: Lectins used for the Present Study with Specific Sugar Moieties

Lectin	Common Name	Acronym	Major sugar moieties/ specificity
N-acetylglucosamine group			
Triticum vulgaris	Wheat germ	WGA	N-acetyl-D-glucosamine and Sialic acid (sia) (β-GlcNAc)
Succinylated *Triticum vulgaris*	Wheat germ, succinylated	s-WGA	N-acetylglucosamine (β-GlcNAc)
Lycopersicon esculentum	Tomato lectin	LEL	[GlcNAc]1-3, N-Acetylglucosamine
Datura stramonium	Datura	DSL	[GlcNAc]1-3, N-Acetylglucosamine
Solanum tuberosum	Potato lectin	STL	N-acetylglucosamine (β-GlcNAc)
Griffonia (Bandeiraea) *simplicifolia* lectin II	African shrub	GSL II	α- or β-linked N-Acetylglucosamine
N-acetylgalactosamine group			
Glycine max	Soybean	SBA	α-linked N-acetylgalactosamine
Dolichos biflorus	Horse gram	DBA	α-linked N-acetylgalactosamine (α-GalNAc)
Ricinus communis	Castor bean	RCA	N-acetylgalactosamine
Vicia villosa agglutinin	Hairy vetch	VVL	α- or β-linked terminal N-acetylgalactosamine
Griffonia simplicifolia lectin I	African legume	GSL I	α-linked N-acetylgalactosamine
Galactose group			
Griffonia simplicifolia isolectin B4	Africa shrub legume	GS1B4	Terminal (1-3)-linked galactose epitopes
Arachis hypogaea	Peanut agglutinin	PNA	Galactose-β(1-3) N-acetyl galactosamine Gal-β(1-3)-GalNAc
Artocarpus integrifolia	Jackfruit	Jacalin	Galactose, β(1,3) N-aetylgalactosamine
Erythrina crisagalli	Coral tree	ECL	Galactose, N-acetylgalactosamine, Lactose β -Gal>β-GalNAc
Phaseolus vulgaris Erythroagglutinin	Red kidney beans	PHA-E	Galactose, Complex structures
Phaseolus vulgaris Leucoagglutinin	Red kidney beans	PHA-L	Galactose, Complex structures
Glucose/Mannose group			
Canavalia ensiformis Concanavalin A	Jack bean	Con A	Terminal α-D-mannosyl and α-D-glucosyl groups *α-Man>α-Glc*
Lens culinaris agglutinin	Common lentil	LCA	α-linked Mannose, Glucose specific for *α-Man>α-Glc*
Pisum sativum agglutinin	Pea	PSA	α-linked mannose, glucose *specific for α-Man>α-Glc*
Fucose			
Ulex europaeus agglutinin	Furze gorse	UEA	α-linked fucose

Gal: Galactose; GalNAc: N-acetyl-D-galactosamine; GlcNAc: N-acetyl-D-glucosamine; Sia: Sialic acid (N-acetylneuraminic acid).

showed specialized modifications characterized by the absence of columnar ciliated and goblet cells, infiltration of lymphoid cells, and the presence of microvilli and M-cells. Furthermore, there was another modification of the epithelium that may or may not have an association with lymphoid tissue, and it contained specialized cells called potential cells (P-cells), which shared characteristics with M-cells based on lectin histochemistry. These newly discovered P-cells appeared to be potential cells that may play a more efficient role than M-cells. The propria-submucosa primarily consisted of lymphoid tissue, including isolated lymphoid cells, diffuse lymphoid aggregations, and follicles. It also contained mucous glandular acini and blood vessels. The frozen sections of the tonsil were individually incubated with 21 biotinylated lectins. Consequently, photographs of the negative control sections were not included in the analysis. The comprehensive selectivity of various lectins in different constituents of the nasopharyngeal tonsil has been shown in Table **2**. The negative control sections consistently showed a dark black appearance without

Table 2: Specificity of Different Lectins in Structural Components of the Nasopharyngeal Tonsil of Buffalo

N-acetyl glucosamine group											
Lectin	**Epithelium**							**CT**	**LT**	**GL**	**EN**
	B	**S**	**C**	**G**	**FAE**	**M**	**P**				
WGA	-, ±	-	+++	-	+	++	++	-, +	± Few ++*	++, ±	+
s-WGA	±	-	++	+++	-	+		±	-	+++, ±	++
LEL	++	-	++	-	++	++	+++	++	+ Few +*	++, ±	+++
DSL	+	-	++	-	-	++	+++	+	++	+, ±	+++
STL	++	-	++	-	++	++	+++	++	++ Few +*	++, ±	+++
GSL II	±	-	-	-	-	-	-	+++	-	++, ±	-
N-acetyl galactosamine group											
SBA	-	-	-	+++	-	-	-	++	±	+++, ±	++
DBA	-	-	+	++	-	++	++	±	-	+++, ±	++
RCA	±	-	±	++	++	-	-	+++	++	++, ±	+++
VVL	-	-	+	++	-	-	-	++	-	++, ±	+++
GSL I	++	++	+++	+++	++	+++	++	+	++ Few ++*	+++	+++
Galactose group											
GS1B4	++	-	++	-	-	+	+++	+	++* Few ++*	+++, ±	++
PNA	-	-	±	-	-	-	±	+++	±*	++, ±	+
Jacalin	++	±	+	++	+	+	++	+++	+++*	+++, ±	++
ECL	-	++	++	-	-	-	-	++	+++* +**	+++, ±	++
PHA-E	+++	-	+	-	++	++	+++	+++	++* +**	±	+
PHA-L	+	-	±	-	++	+	+++	+++	±	±	+
Glucose/Mannose group											
Con A	-	++	++	-	+++	+	+	++	++* ±**	-, ±	++
PSA	-	-	-	-	-	-	-	+++	+* +**	+, ±	+
LCA	-	-	-	-	-	-	-	+++	++* +**	+++, ±	+
Fucose											
UEA	-	-	++	++	++	-	-	±	±	++, ±	-

B: basal cells; S: supporting cell; C: ciliated surface, G: goblet cells; FAE: follicle-associated epithelium. M: M-cell; P: P cell; CT: connective tissue; LT: lymphoid tissue; GL: glands; EN: endothelium.
±: weak to absent; +: weak; ++: moderate; +++: strong; *: inter and parafollicular; **: germinal centre.

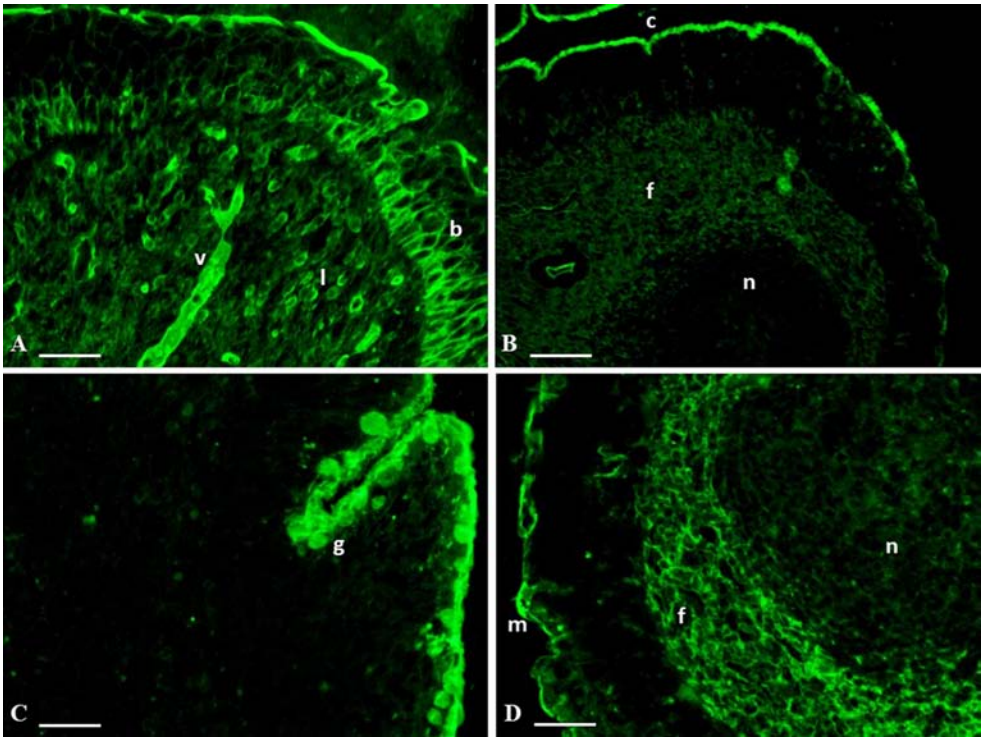

Figure 1: Photomicrograph showing binding affinities and patterns of lectins from the N-acetylglucosamine group within the nasopharyngeal tonsil of the buffalo. **A.** STL (bar 200 µm); **B.** WGA (bar 200 µm); **C.** s-WGA (bar 200 µm); **D.** WGA (bar 200 µm). Note: **b.** basal cells; **c.** ciliated surface; **f.** parafollicular area; **g.** goblet cells; **l.** lymphocytes; **m.** M-cells; **n.** germinal center; **v.** blood vessel.

any fluorescence, confirming the absence of non-specific binding of streptavidin (Figure **Control**).

N-Acetylglucosamine Group

The lectins in this group, including WGA, s-WGA, GSL II, LEL, DSL, and STL, exhibited varying affinities for the N-acetylglucosamine group in cytoplasm and membranes of different cells in the respiratory epithelium. All lectins in the group showed weak binding in basal cells, except for lectin LEL, STL, which displayed moderate binding for 1-3, N-acetylglucosamine (Figure **1A**). Ciliated cells in the epithelium did not show affinity for N-acetylglucosamine; however, free ciliated surface exhibited a moderate to strong binding (Figure **1B**). Goblet cells were specifically labeled by the lectin s-WGA (Figure **1C**). The various cells in the FAE demonstrated moderate positive affinity for N-acetylglucosamine with all the lectins except for s-WGA and GSL II. M-cells in the FAE (Figure **1D**) and P-cells exhibited moderate to strong binding with all the lectins, with P-cells displaying a stronger reaction (Figure **2A, B**). In the connective tissue of the propria-submucosa, these lectins displayed a weak to moderate positive binding reaction, except for lectin s-WGA, which showed no binding. The lectins of the group were able to demonstrate the presence of lymphoid cells, particularly lymphocytes, in the subepithelial and interfollicular areas (Figure **1A, D; 2A, B**), except for lectins s-WGA and GSL II. However, the majority of lymphoid cells in the germinal center could not be detected by any of the lectins of the N-acetylglucosamine group. Mucous acini showed a strong reaction with an increased intensity towards the luminal surface by the lectin s-WGA (Figure **2C**). The secretions of the mucous acini displayed weak to moderate concentrations of the N-acetylglucosamine group by the remaining lectins (Figure **2D**). The striated muscular tissue presented a strong binding only for the lectin DSL. The endothelium and tunica intima of the blood vessels were strongly highlighted by lectins LEL, DSL, and STL, while lectins s-WGA, WGA, and GSL II showed moderate to weak binding.

N-Acetylgalactosamine Group

The lectins in the N-acetylgalactosamine group (SBA, DBA, RCA, VVL) did not bind to the basal and ciliated cells of the ciliated respiratory epithelium, except for GSL I, which exhibited moderate affinity (Figure **3A**). Lectins DBA, VVL, and GSL I specifically highlighted the cilia present on the free surface of the epithelium, with GSL I showing the strongest binding

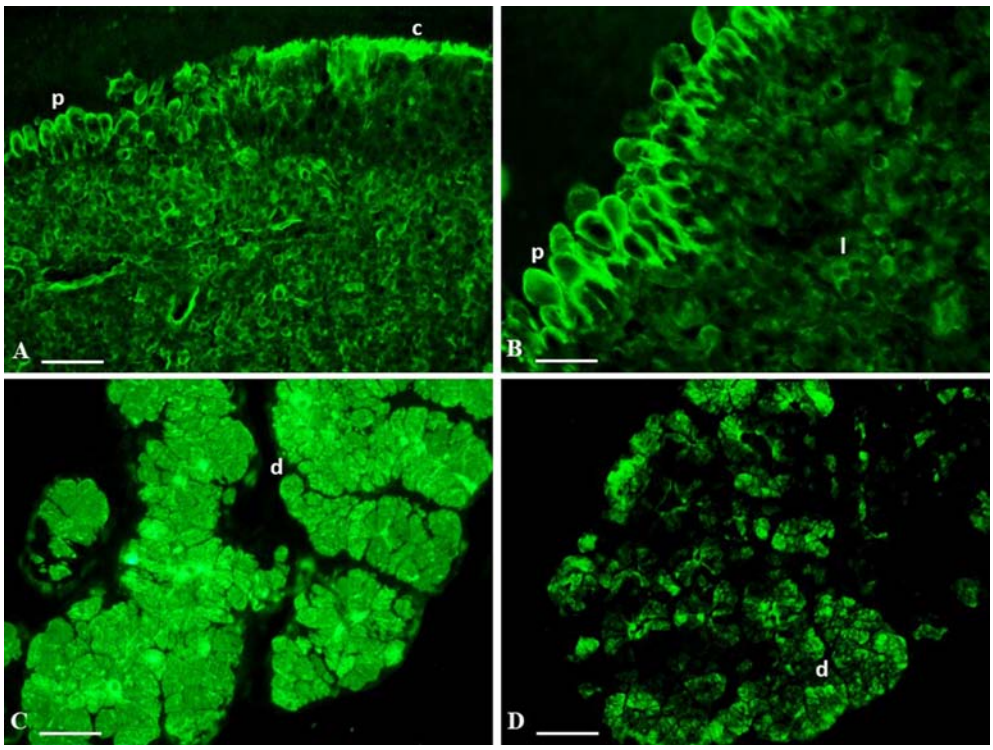

Figure 2: Photomicrograph showing binding affinities and patterns of lectins from the N-acetylglucosamine group in the nasopharyngeal tonsil of the buffalo. **A.** LEL (bar 200 µm); **B.** DSL (bar 100 µm); **C.** s-WGA (bar 200 µm); **D.** STL (bar 200 µm). Note: **c.** ciliated surface; **d.** glandular tissue; **l.** lymphocytes; **p.** P-cells.

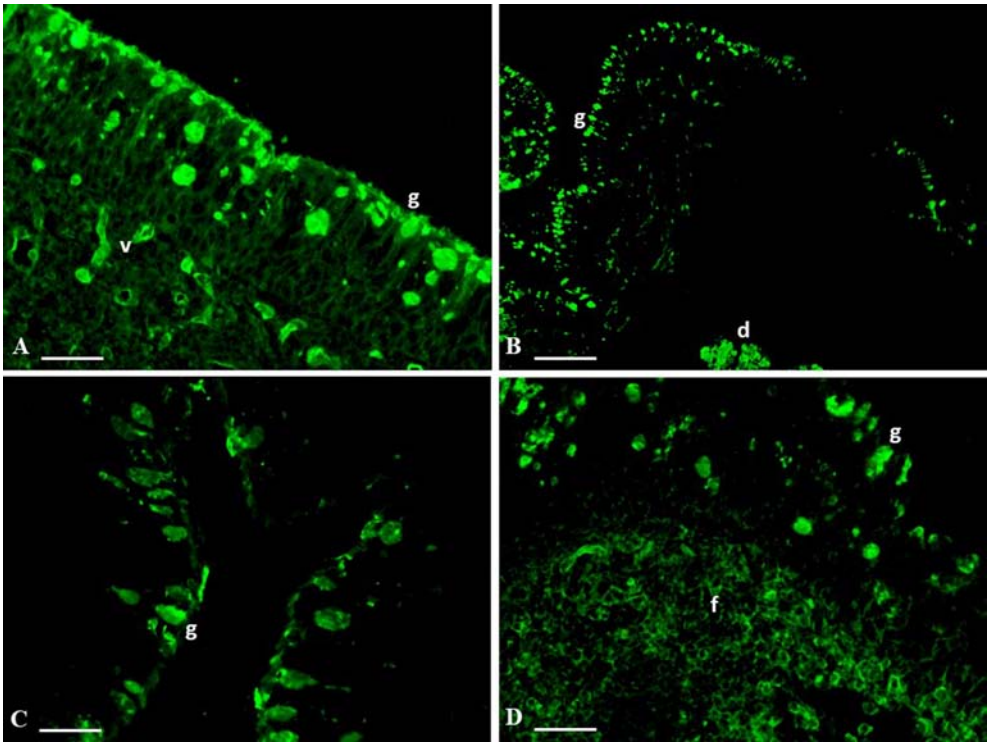

Figure 3: Photomicrograph showing binding affinities and patterns of lectins from the N-acetylgalactosamine group in the nasopharyngeal tonsil of the buffalo. **A.** GSL-I (bar 200 µm); **B.** SBA (bar 400 µm); **C.** DBA (bar 200 µm); **D.** RCA (bar 200 µm). Note: **d.** glandular tissue; **f.** parafollicular area; **g.** goblet cells; **v.** blood vessel.

(Figure **3A**). The secretions of goblet cells were strongly positive for N-acetylgalactosamine with all the lectins in the group (Figure **3B-D; 4A-B**). The regions of FAE, including M-cells, did not show positive binding

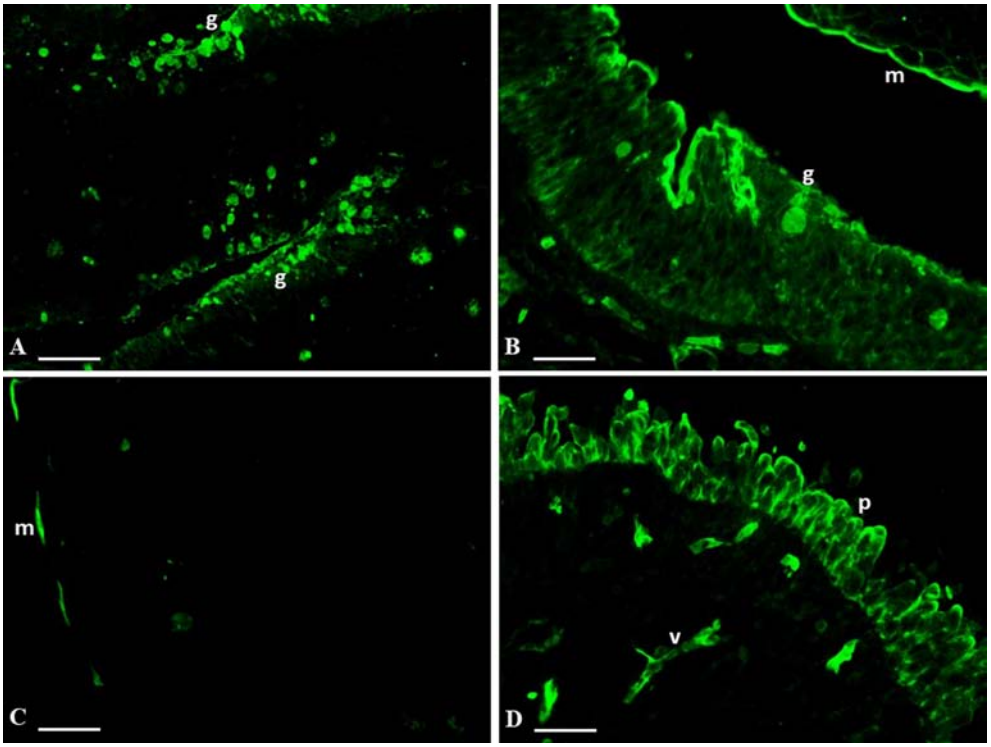

Figure 4: Photomicrograph showing binding affinities and patterns of lectins from the N-acetylgalactosamine group in the nasopharyngeal tonsil of the buffalo. **A.** VVL (bar 400 µm); **B.** GSL-I (bar 200 µm); **C.** DBA (bar 200 µm); **D.** GSL-I (bar 200 µm). Note: **g.** goblet cells; **m.** M-cells; **p.** P-cells; **v.** blood vessel.

for lectins SBA, DBA, VVL, and RCA. However, RCA displayed binding similar to a thin rim-like structure near the free surface of the epithelium, which may represent mucous coat spreading from adjacent goblet cells. GSL I, followed by DBA, were the only lectins in this group that bound to M-cells and P-cells (Figure **4B-D**). The connective tissue showed stronger staining with lectins RCA and VVL, followed by SBA, DBA, and GSL I. The binding of lectins RCA and GSL I to a few lymphocytes indicated the presence of a specific lymphocyte subpopulation of the lymphoid tissue. However, not all lymphocytes were stained, and the lymphocytes in the germinal center did not show positive binding. The mucous glandular acini displayed strong binding for N-acetylgalactosamine with all the lectins, although a few acini showed weak to moderate affinity (Figure **5A-C**). All the lectins strongly stained the endothelium and tunica intima of blood vessels (Figure **5D**). Small capillaries were entirely stained, and high endothelial venules along with lymphocytes were also highlighted. Only lectin DBA highlighted binding to the striated muscles in the deeper propria-submucosa.

Galactose Group

GS1B4, PNA, Jacalin, ECL, PHA-E, and PHA-L, which all have galactose groups, showed varying reactions to different cells and exhibited minor variations in the epithelium. The basal cells exhibited intense staining with the lectin PHA-E, followed by GS1B4 and Jacalin (Figure **6A-C**). The remaining lectins in the group did not show reactivity towards these cells. The ciliated surface of the pseudostratified columnar ciliated epithelium showed weak to moderate concentrations of galactose. The secretions of the goblet cells were negative for this group with all the lectins except Jacalin, which showed moderate affinity (Figure **6C**). The follicle-associated epithelium (FAE) consistently showed a positive reaction for the lectins PHA-E and PHA-L. The M-cells within the FAE and P-cells exhibited a more positive reaction compared to the adjacent cells (Figure **6D**; **7A-C**). The connective tissue showed moderate to intense positive reaction galactose with PNA, Jacalin, PHA-E, and PHA-L and a weaker binding reaction with other lectins. The lymphoid cells present in the epithelium and the subepithelial connective tissue, including interfollicular and parafollicular areas, were distinctly labeled by these lectins. Similarly, the interfollicular and parafollicular areas also showed reactivity to these lectins. However, the lymphoid cells of the germinal center showed negative staining (Figure **7D**). The acini of the mucous glandular tissue were strongly positive for galactose, as demonstrated by the lectins GS1B4, PNA, Jacalin, and ECL (Figure **8A-B**), with a weaker

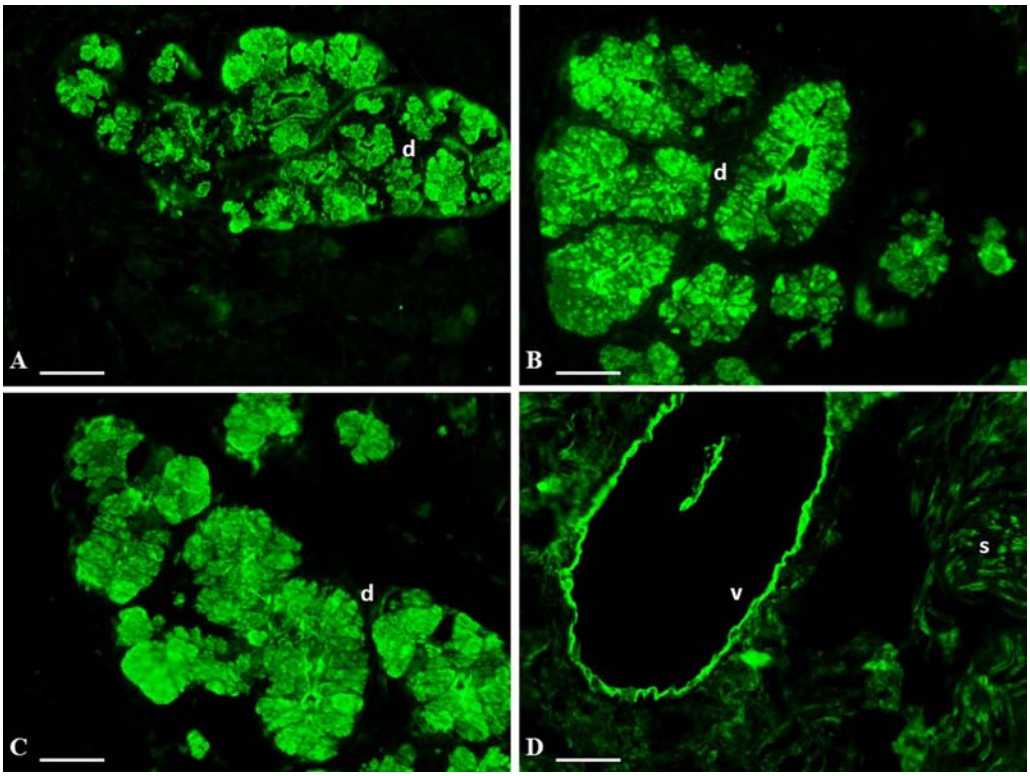

Figure 5: Photomicrograph showing binding affinities and patterns of lectins from the N-acetylgalactosamine group in the nasopharyngeal tonsil of the buffalo. **A.** SBA (bar 200 µm); **B.** DBA (bar 200 µm); **C.** GSL-I (bar 200 µm); **D.** RCA (bar 200 µm). Note: **d.** glandular tissue; **s.** striated muscles; **v.** blood vessel.

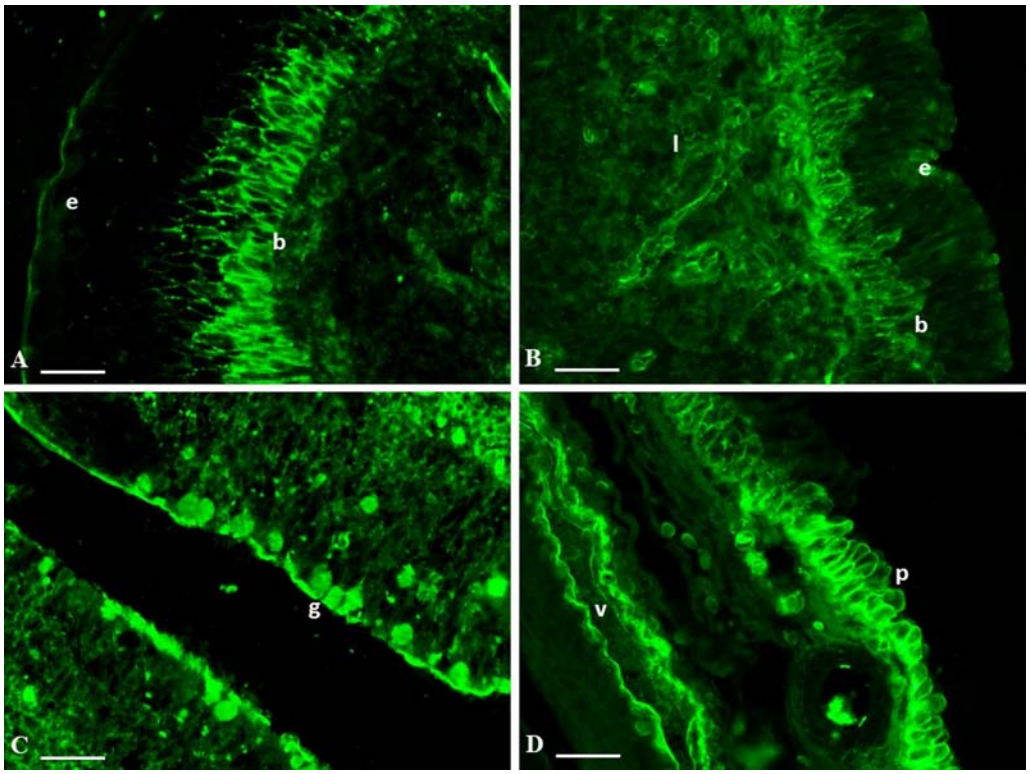

Figure 6: Photomicrograph showing binding affinities and patterns of lectins from the galactose group in the nasopharyngeal tonsil of the buffalo. **A.** PHA-E (bar 200 µm); **B.** GS1B4 (bar 200 µm); **C.** Jacalin (bar 200 µm); **D.** GS1B4 (bar 200 µm). Note: **b.** basal cells; **e.** respiratory epithelium; **g.** goblet cells; **l.** lymphocytes; **p.** P-cells; **v.** blood vessel.

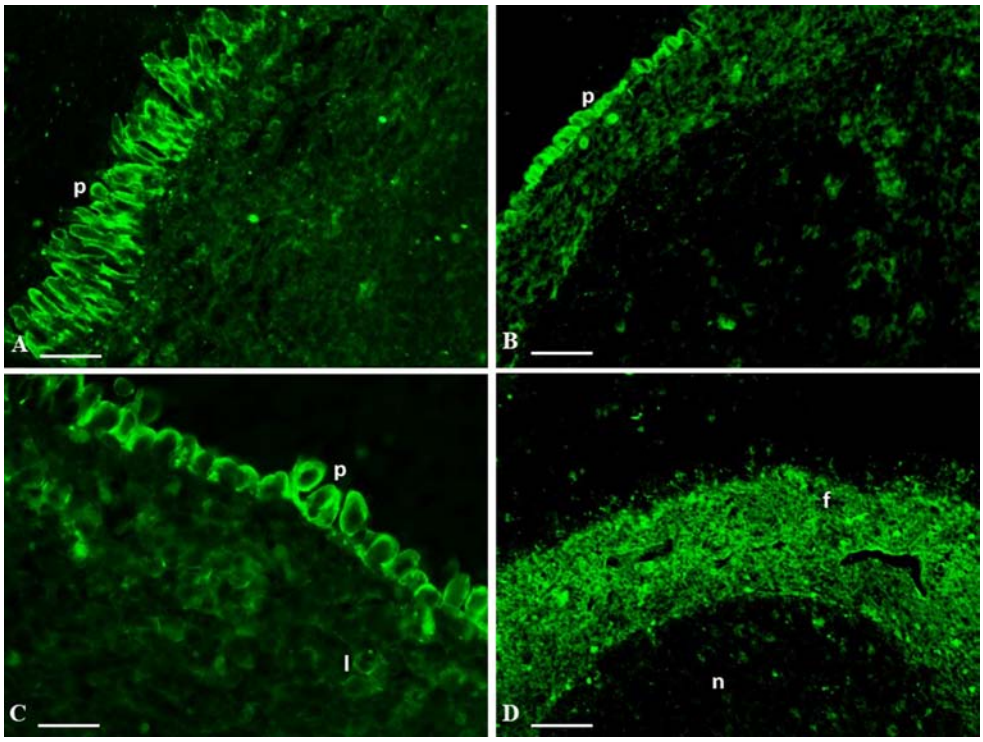

Figure 7: Photomicrograph showing binding affinities and patterns of lectins from the galactose group in the nasopharyngeal tonsil of the buffalo. **A.** PHA-E (bar 200 µm); **B.** Jacalin (bar 200 µm); **C.** PHA-E (bar 400 µm); **D.** Jacalin (bar 400 µm). Note: **f.** parafollicular area; **l.** Lymphocytes; **n.** germinal center; **p.** P-cells.

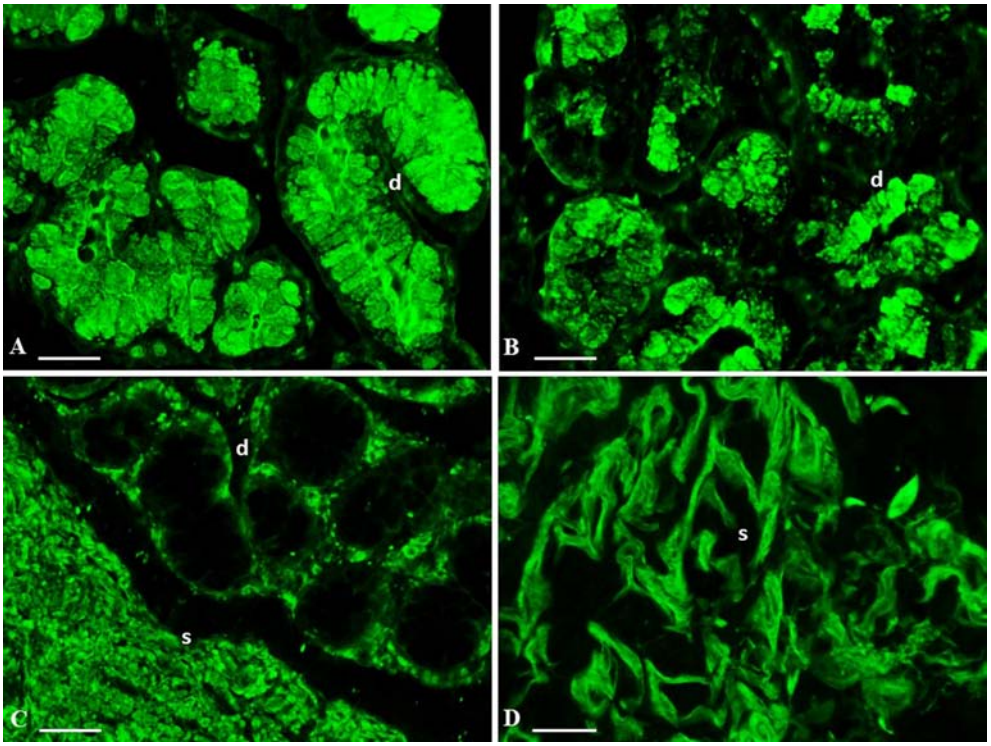

Figure 8: Photomicrograph showing binding affinities and patterns of lectins from the galactose group in the nasopharyngeal tonsil of the buffalo. **A.** Jacalin (bar 200 µm); **B.** ECL (bar 200 µm); **C.** PHA-E (bar 200 µm); **D.** PHA-L (bar 200 µm). Note: **d.** glandular tissue; **s.** striated muscles; **v.** blood vessel.

intensity of binding with PHA-E and PHA-L lectins (Figure **8C**). The peripheral portions of the acini in the region of myoepithelial cells were also highlighted by the lectins. The tunica intima, comprising the

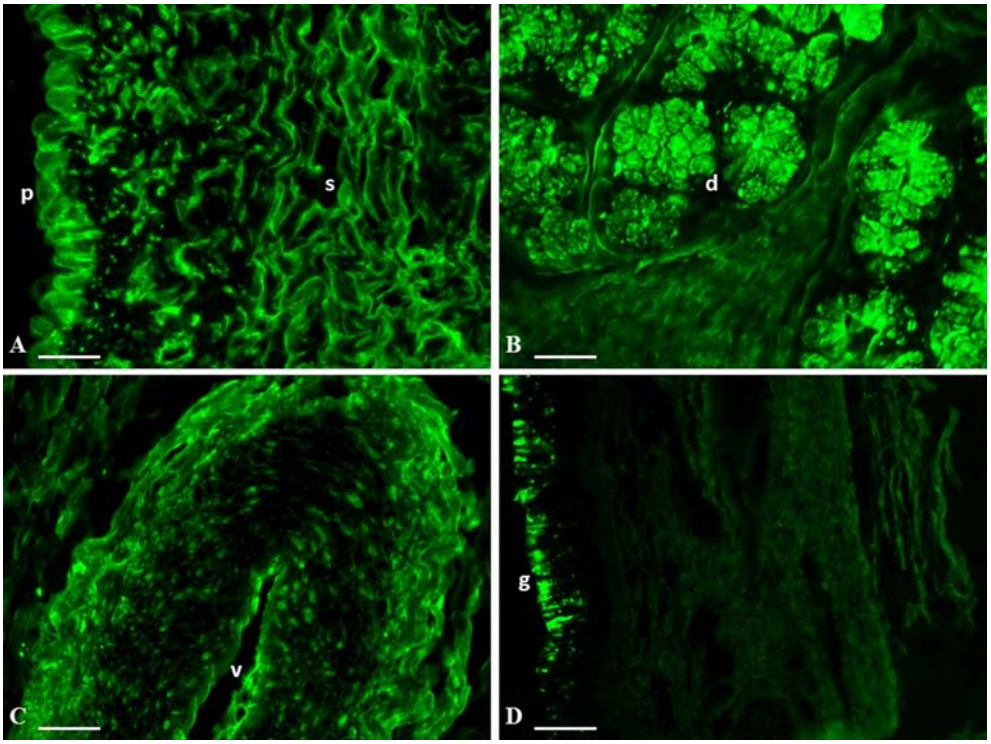

Figure 9: Photomicrograph showing **b**inding affinities and patterns of lectins from the glucose/mannose and Fucose groups in the nasopharyngeal tonsil of the buffalo. **A.** Con A (bar 200 µm); **B.** LCA (bar 200 µm); **C.** Con A (bar 200 µm); **D.** UEA (bar 200 µm). Note: **d.** glandular tissue; **g.** goblet cells; **p.** P-cells; **s.** striated muscles; **v.** blood vessel.

endothelium and the internal elastic lamina, showed moderate positivity for the galactose group with the lectins GS1B4, Jacalin, and ECL, whereas a weak binding was noticed with PNA, PHA-E, and PHA-L lectins. However, a strong reaction was observed towards the tunica externa of the blood vessels. The striated muscles demonstrated positive staining only with GS1B4 and PHA-L lectins (Figure **8D**).

Glucose/Mannose Group

The lectins belonging to the glucose/mannose group (Con A, LCA, and PSA) did not bind to any cell types of the pseudostratified columnar ciliated epithelium except for the lectin Con A, which showed moderate affinity for mannosyl and glucosyl groups in the supporting cells and towards the free ciliated surface. The M-cells of FAE and P-cells also showed moderate binding with Con A (Figure **9A**). The connective tissue in the subepithelial region and around the glandular tissue displayed moderate binding with all the lectins. Additionally, the LCA lectin demonstrated staining in the muscular tissue present in the deeper portion of the propria submucosa. The isolated lymphocytes infiltrating the epithelium, diffuse lymphoid tissue, and lymphoid follicles in the superficial portion exhibited moderate binding. However, the intensity of the binding reaction was stronger in the interfollicular areas. These lectins are also bound to the lymphoid cells of the germinal center. The mucous acini of the glandular tissue showed a moderate-to-strong affinity for mannose and glucose by the LCA lectin (Figure **9B**) and a weaker binding with the PSA lectin. Some of the acini exhibited a more intense positive binding reaction towards their luminal surfaces. The tunica intima and externa of the blood vessels were strongly labeled by these lectins, especially Con A (Figure **9C**). Additionally, some of the smooth muscles in the tunica media were also highlighted.

Fucose Group

The lectin UEA, representing the fucose group, is primarily bound to the goblet cells and free ciliated surface of the respiratory epithelium (Figure **9D**). The regions of the FAE, M-cells, and specialized P-cells did not show binding with the lectin. The connective tissue of the propria submucosa exhibited weak reactivity, while the striated muscles showed moderate binding for UEA. The lymphoid tissue and their cells did not demonstrate binding with this lectin. The mucous glandular acini displayed moderate to strong binding, with comparatively more intensity towards the luminal surface. UEA did not bind to any portion of the blood vessels.

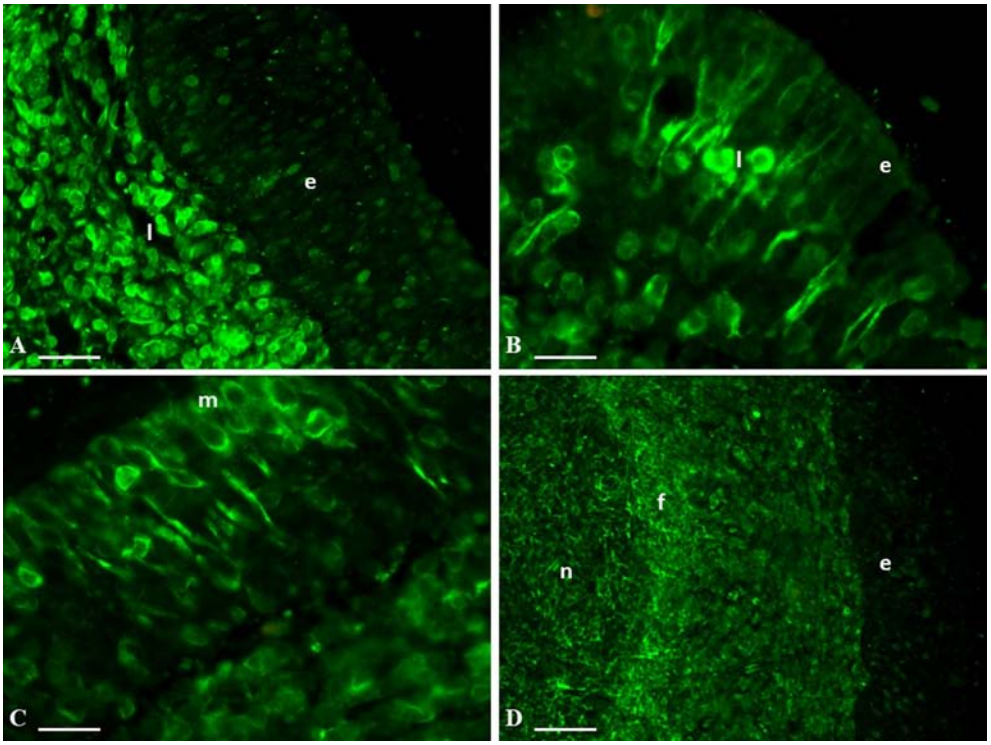

Figure 10: Photomicrograph showing binding affinities and patterns of vimentin in the nasopharyngeal tonsil of the buffalo. **A.** (bar 200 µm); **B.** (bar 100 µm); **C.** (bar 100 µm); **D.** (bar 200 µm). Note: **e.** respiratory epithelium; **f.** parafollicular area; **l.** lymphocytes; **m.** M-cells; **n.** germinal center.

Vimentin

The basal, ciliated, and goblet cells of the respiratory epithelium were devoid of vimentin filaments. However, the infiltrated lymphocytes showed a strong presence (Figure **10A-B**). Similarly, the regions of the follicle-associated epithelium (FAE) were also devoid of these filaments, except for a few M- and P-cells, which were rich in these filaments (Figure **10C**). The lymphoid cells present in the subepithelial connective tissue, interfollicular areas, and parafollicular areas showed strong positivity for vimentin filaments (Figure **10D**). However, only a few cells in the germinal center displayed the presence of these filaments. In contrast, all the lymphoid cells in some of the germinal centers were strongly positive, and the concentration of the filaments increased towards the corona of the germinal center.

DISCUSSION

The present study on the nasopharyngeal tonsil of buffalo is the first of its kind to involve a panel of 21 lectins, broadly subdivided into N-acetyl glucosamine, N-acetyl galactosamine, galactose, glucose/mannose, and fucose groups. The study aimed to reveal the binding patterns of different glycoconjugates targeting various sugar moieties in the nasopharyngeal tonsil of buffaloes. The lectins within each group exhibited nearly identical binding patterns to specific cells or tissues, with only minor variations. The specificity of each lectin to different structural components not only helped eliminate the possibility of non-specific binding but also supported the observations on the control frozen sections.

The goblet cells of the pseudostratified columnar ciliated epithelium did not react with lectins from the N-acetyl glucosamine group, except for one lectin (s-WGA), indicating the absence of β-GlcNAc and sialic acid N-acetyl glucosamine in the secretions of the epithelium. However, a moderate to strong binding for acetyl glucosamine in the mucous glandular acini suggested that their composition differed from that of the goblet cells in the epithelium. The lesser staining with the lectin DSL, which had a similar terminal sugar, could not be confirmed. However, sialylation resulted in a strong positive reaction, as demonstrated by the lectin s-WGA. The moderate to strong presence of sialic acid towards the free ciliated surface indicated that it may be due to the spreading of secretions from the glandular ducts opening towards the free surface. A higher content of sialic acid plays a significant role in protecting and defending the respiratory epithelium against viruses and toxins [11]. Additionally, the

expression of these lectins may assist in trapping foreign particles/organisms and presenting them to adjacent M-cells and P-cells. Similar staining patterns of these lectins toward the free surface of the epithelium in sheep have been attributed to the expression of sugar residues present in the mucous toward the free surface and partial expression in the cell membrane of cilia or microvilli of the epithelial cells [12, 13].

The basal cells were delineated by the majority of the lectins. A moderate reaction in the FAE, M-cells, and newly discovered P-cells indicated that glucosamine and sialic acid were required for the growth of these cells, and it also reflected differences in their compositions compared to the surface epithelium. The similar lectin staining pattern in M-cells and newly discovered P-cells suggests that the P-cells might be playing a physiological role similar to M-cells. This hypothesis is supported by the fact that the associated lymphoid tissue also showed similar affinity for these lectins, although further functional studies are needed for verification. A negative correlation was also observed between the goblet cells and the lymphoid tissue for the lectin s-WGA. The lesser binding with the lectin DSL, which had a similar terminal sugar, could not be confirmed. The expression in the basal cells confirmed that acetyl glucosamine was required for the maintenance of the epithelium. These lectins, except GSL II, may be utilized as endothelial markers for vascular studies.

The lectins with N-acetyl galactosamine carbohydrate moieties strongly bound to the secretions of goblet cells and mucous acini in the propria-submucosa, indicating similarity in their compositions. The mucous plays a significant physiological role in waterproofing, lubrication, and protection of the mucosal surface against ingested infectious agents and their toxins [14]. The structure of the mucous mimics receptor sites for microorganisms on the epithelial surface, facilitating the trapping and removal of foreign agents [15]. A similar binding pattern was observed in the respiratory mucosa of sheep [12]. The lectins DBA and VVL exhibited a stronger positive binding in the goblet cells of the villi compared to those of the FAE in humans [10].

The remaining cell types of the respiratory epithelium, FAE, and lymphoid tissue did not exhibit these terminal sugars. However, lectins DBA and GSL I demonstrated moderate binding affinity for the FAE, M-cells, and P-cells. The lymphoid tissue did not show binding with these lectins, except for GSL I and RCA. The tunica intima of blood vessels exhibited the presence of acetylgalactosamine. These lectins may be good candidates for exploring the role of endothelial glycocalyx in blood circulation, similar to lectins from the glucosamine group. The secretions of mucous acini showed a strong positive reaction for N-acetyl galacto-samine with all the lectins, with a slightly lower intensity for RCA and VVL lectins. A similar binding pattern was observed in the respiratory mucosa of sheep [12]. The terminal sugars of lectins DBA and VVL exhibited a stronger positive binding in the goblet cells of the villi compared to those of the FAE in humans [10].

The binding affinities of lectins in the galactose group resembled those in the glucosamine group, although with lesser intensity. The basal cells were specifically marked by GS1B4 and PHA-E lectins and, to a lesser extent, by lectin Jacalin. The latter was only lectin, showing a positive binding reaction with goblet cells. The positive reaction of all lectins to the ciliated surface was attributed to mucous secretions originating from glandular acini. Lectins PHA-E and PHA-L also showed moderate binding to the FAE, M-cells, and P-cells. The expression of terminal α (1-3)-linked galactose epitopes helped identify mature M-cells in the pharyngeal tonsil of the hamster [16] and the horse [12]. This unique binding pattern of lectins has been utilized to deliver test antigens to the intestinal mucosal immune system [8, 9] and the nasopharyngeal lymphoid tissue of the hamster [16]. A similar pattern was observed in mice using the GS1B4 lectin [10].

The connective tissue of the propria-submucosa showed reactivity to all lectins having galactose sugar, although with varying intensities. The lymphoid tissue, particularly the lymphocytes in the subepithelial area and interfollicular areas, exhibited a stronger binding, whereas those in the germinal centers were weakly positive for these sugar moieties. This difference in staining intensity suggests a higher binding affinity of these lectins for T-lymphocytes than B-lymphocytes. The mucous acini showed strong positive binding for galactose with lectins that did not exhibit presence in goblet cells, and vice-versa, except Jacalin in this group. The epithelium was delineated, although with less intensity compared to the glucosamine group. These lectins, except for Jacalin, showed similar binding patterns in the respiratory epithelium and associated submucosa of sheep [12]. Macrophages in germinal centers in humans reacted with mannose and GalNAc-specific lectins, which also labeled macrophages in the paracortical region in mice [10].

The lectins PSA and LCA, which recognize glucose and mannose surface epitopes, did not mark the respiratory epithelium or FAE. In contrast, Con A exhibited moderate binding to certain cell types of the pseudostratified columnar ciliated epithelium, excluding goblet cells, and strong binding to the FAE, with lesser intensity towards M-cells. Varying reactivity of all lectins was observed in the connective tissue, glandular acini, and endothelium, as reported in sheep, except for LCA [12]. The staining pattern of the lymphoid tissue indicated a predominance of T-lymphocytes over B-lymphocytes.

The lectin UEA, which recognizes fucose sugar, is bound with the secretions of goblet cells, the free ciliated surface, a few cells of the FAE, and the mucous acini of the glandular tissue. The binding of UEA-I in the secretory granules of goblet cells and the periciliary layer suggests the incorporation of α-L-fucose residues into glycoproteins during the later stages of development in rats [17]. However, UEA did not bind to any other cell types in the epithelia, including endothelial cells of blood vessels, as previously reported in rat and rabbit Peyer's patches. However, UEA-1 and WBA are specifically bound to M-cells in Peyer's patches of mice, indicating variations in the cytoplasmic or membranous chemical composition of M-cells across different species [9, 10]. In humans, the lectins Con A and PNA showed a similar pattern of binding to goblet cells and mucous glands, in contrast to UEA-I, Jacalin, and s-WGA lectins [18]. The epithelium may possess its own antimicrobial defense mechanisms through the secretion of lysozyme, lactoferrin, α -defensins, and the production of other chemicals and proinflammatory cytokines upon interaction with foreign particles. The expression pattern of defensins on a particular surface may influence the type and density of microflora hosted [19]. The differential expression pattern of receptors for N-acetyl glucosamine, galactose, glucose, and mannose moieties in the secretions of goblet cells and mucous glands may reflect a two-tier protection mechanism against invading microorganisms.

Vimentin, a protein filament, is a normal constituent of mesenchymal cells. It has been recognized as a marker for M-cells in rabbits, pigs, cattle, and horses due to its unique presence towards the apical surface of these cells [2, 20-22]. However, a detailed study on the ileum of rabbits revealed that the vimentin-containing epithelial cells of the villi were actually cup cells and not M-cells [23]. In the present study, vimentin filaments were observed in a few M or P-cells, suggesting that vimentin staining alone was not a confirmatory technique for identifying M or P-cells in the nasopharyngeal tonsil of buffaloes. In various studies conducted on different animal species, vimentin has been investigated as a potential marker for identifying specific cell types within the tonsils, particularly M-cells. Additional markers, such as lectins and specific cell surface proteins, have been utilized in conjunction with vimentin staining to enhance the accuracy of M-cell identification [20, 22]. Combining multiple markers may provide a more comprehensive and reliable approach for identifying and characterizing M-cells in different species.

CONCLUSIONS

The histological structure of the nasopharyngeal tonsil in buffalo showed the presence of respiratory epithelium and juxtaposed lymphoepithelium in close proximity to underlying lymphoid tissue, highlighting the multifaceted nature of this mucosal immune site. With its unique M-cells, the FAE suggests their vital role in potential antigen-processing mechanisms within this tissue. The discovery of P-cells may contribute to the immune function of the nasopharyngeal tonsil independently of lymphoid tissue influence. The binding affinities of lectins targeting the N-acetyl glucosamine, N-acetyl galactosamine, galactose, glucose/mannose, and fucose groups have revealed specific binding patterns within the tonsil. (indicating their involvement in antigen detection and processing) (which may offer valuable insights into the complex interactions between immune cells, mucosal surfaces, and pathogens.) (The presence of vimentin filaments, primarily within lymphocytes and select epithelial cells, suggests their potential involvement in immune responses.) (Further studies are needed to explore and validate alternative markers that can enhance the identification and characterization of M and P-cells in different species, including buffaloes.) Furthermore, it provides a data base that will serve as a useful tool for investigating changes in glycosylation associated with diseases, including infections that impact the tonsils. Further studies are required to explore and validate alternative markers that could improve the identification and characterization of M-cells and P-cells across various species, including buffaloes.

ACKNOWLEDGEMENTS

The author sincerely acknowledges the award of the research grant received via No. HSCST/R7D/2019/ 2573-76 Dt. 11.09.2019 by the Haryana State Council for Science and Technology, Panchkula, India.

CONFLICT OF INTEREST

The author does not have any conflict of interest.

FUNDING STATEMENT

The author received no funding from a private agency to carry out this research.

ABBREVIATIONS

Con A = Canavalia ensiformis Concanavalin A

DBA = Dolichos biflorus

DSL = *Datura stramonium*

ECL = *Erythrina crisagalli*

FAE = Follicle-associated epithelium

Gal = Galactose

GalNAc = N-acetyl-D-galactosamine

GlcNAc = N-acetyl-D-glucosamine

GS1B4 = Griffonia simplicifolia isolectin B4

GSL I = Griffonia simplicifolia lectin

GSL II = Griffonia (Bandeiraea) simplicifolia lectin II

IgG = Immunoglobulin G

Jacalin = Artocarpus integrifolia

LCA = Lens culinaris agglutinin

LEL = *Lycopersicon esculentum*

M-cells = Microfold/membranous cells

PBS = Phosphate-buffered saline

P-cells = Potential cells

PHA-E = Phaseolus vulgaris Erythroagglutinin

PHA-L = Phaseolus vulgaris Leucoagglutinin

PNA = Arachis hypogaea

PSA = Pisum sativum agglutinin

RCA = Ricinus communis

SBA = Glycine max

Sia = Sialic acid (N-acetylneuraminic acid)

STL = *Solanum tuberosum*

s-WGA = Succinylated Triticum vulgaris

UEA = Ulex europaeus agglutinin

VVL = *Vicia villosa* agglutinin

WGA = Triticum vulgaris

REFERENCES

[1] Ibrahim D, Nakamuta N, Taniguchi K, Taniguchi K. Lectin histochemical studies on the vomeronasal organ of the sheep. J Vet Med Sci 2013; 75(8): 1131-7. https://doi.org/10.1292/jvms.12-0532

[2] Kumar P, Timoney JF, Sheoran AS. M cells and associated lymphoid tissue of the equine nasopharyngeal tonsil. Equine Vet J 2001; 33(2): 224-30. https://doi.org/10.2746/042516401776249697

[3] Ranjit R, Kumar P, Singh G. Histology, histochemistry, and scanning electron microscopy of nasopharyngeal tonsil of the young pigs. Indian J Vet Anat 2015; 27: 29-32.

[4] Girgiri IA, Kumar P. Histology, histochemistry, and ultrastructure of the nasopharyngeal tonsil of the buffalo (*Bubalus bubalis*). Anat Histol Embryol 2019; 48(4): 375-83. https://doi.org/10.1111/ahe.12452

[5] Madara JL, Bye WA, Trier JS. Structural features of and cholesterol distribution in M-cell membranes in guinea pig, rat, and mouse Peyer's patches. Gastroenterology 1984; 87: 1091-103. https://doi.org/10.1016/S0016-5085(84)80069-4

[6] Smith MW, James PS, Tivey DR. M cell numbers increase after transfer of SPF mice to a normal animal house environment. Am J Pathol 1987; 128(3): 385-9.

[7] Owen RL, Bhalla DK. Cytochemical analysis of alkaline phosphatase and esterase activities and of lectin-binding and anionic sites in rat and mouse Peyer's patch M cells. Am J Anat 1983; 168: 199-212. https://doi.org/10.1002/aja.1001680207

[8] Giannasca PJ, Giannasca KT, Falk P, Gordon JI, Neutra MR. Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. Am J Physiol Gastrointest Liver Physiol 1994; 267: G1108-21. https://doi.org/10.1152/ajpgi.1994.267.6.G1108

[9] Clark MA, Jepson MA, Simmons NL, Booth TA, Hirst BH. Differential expression of lectin-binding sites defines mouse intestinal M-cells. J Histochem Cytochem 1993; 41: 1679-87. https://doi.org/10.1177/41.11.7691933

[10] Sharma R, van Damme EJ, Peumans WJ, Sarsfield P, Schumacher U. Lectin binding reveals divergent carbohydrate expression in human and mouse Peyer's patches. Histochem Cell Biol 1996; 105: 459-65. https://doi.org/10.1007/BF01457659

[11] Murray MC, Bhavanandan VP, Davidson EA, Reinhold V. Modification of sialyl residues of glycoconjugates by reductive amination. Characterization of the modified sialic acids. Carbohydr Res 1989; 186: 255-65. https://doi.org/10.1016/0008-6215(89)84039-X

[12] Ibrahim D, Nakamuta N, Taniguchi K, Yamamoto Y, Taniguchi K. Histological and lectin histochemical studies on the olfactory and respiratory mucosae of the sheep. J Vet Med Sci 2014; 76: 339-46. https://doi.org/10.1292/jvms.13-0436

[13] Scocco P, Lepri E, Mercati F, Vitellozzi G, Mechelli L, Ceccarelli P. Glycohistochemical characterization of histologically normal nasal mucosa and enzootic nasal tumor of sheep. Am J Vet Res 2012; 73: 1128-36. https://doi.org/10.2460/ajvr.73.8.1128

[14] Forstner JF. Intestinal mucins in health and disease. Digestion 1978; 17: 234-63. https://doi.org/10.1159/000198115

[15] Abraham SN, Beachey EH. Host defenses against adhesion of bacteria to mucosal surfaces. Host Def Mech 1985; 4: 63-88.

[16] Giannasca PJ, Boden JA, Monath TP. Targeted delivery of antigen to hamster nasal lymphoid tissue with M-cell-directed lectins. Infect Immun 1997; 65: 4288-96. https://doi.org/10.1128/iai.65.10.4288-4298.1997

[17] Castells MT, Ballesta J, Madrid JF, Aviles M, Martinez-Menarguez JA. Characterization of glycoconjugates in developing rat respiratory system by means of conventional and lectin histochemistry. Histochem 1991; 95: 419-26. https://doi.org/10.1007/BF00266971

[18] Paulsen FP, Tschernig T, Debertin AS, Kleemann WJ, Pabst R, Tillmann BN. Similarities and differences in lectin cytochemistry of laryngeal and tracheal epithelium and subepithelial seromucous glands in cases of sudden infant death and controls. Thorax 2001; 56: 223-7. https://doi.org/10.1136/thorax.56.3.223

[19] Schroder JM. Epithelial peptide antibiotics. Biochem Pharmacol 1999; 57: 121-34. https://doi.org/10.1016/S0006-2952(98)00226-3

[20] Gebert A, Hach G, Bartels H. Co-localization of vimentin and cytokeratins in M-cells of rabbit gut-associated lymphoid tissue (GALT). Cell Tissue Res 1992; 269: 331-40. https://doi.org/10.1007/BF00319625

[21] Gebert A, Rothkötter HJ, Pabst R. M cells in Peyer's patches of the intestine. Int Rev Cytol 1996; 167: 91-159. https://doi.org/10.1016/S0074-7696(08)61346-7

[22] Lelouard H, Reggio H, Mangeat P, Neutra M, Montcourrier P. Mucin-related epitopes distinguish M cells and enterocytes in rabbit appendix and Peyer's patches. Infect Immun 1999; 67(1): 357-67. https://doi.org/10.1128/IAI.67.1.357-367.1999

[23] Ramirez C, Gebert A. Vimentin-positive cells in the epithelium of rabbit ileal villi represent cup cells but not M-cells. J Histochem Cytochem 2003; 51: 1533-44. https://doi.org/10.1177/002215540305101113

Received on 18-02-2025 Accepted on 20-03-2025 Published on 30-03-2025

https://doi.org/10.6000/1927-520X.2025.14.06