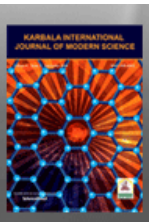

Karbala International Journal of Modern Science

Volume 11 | Issue 3 Article 9

Role of Staphylococcus aureus and Streptococcus pyogenes Biofilms on the Alternation of Cellular Immunity in Pediat-ric Tonsillitis Patients

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Abstract

This study examines the effect of *Staphylococcus aureus* and *Streptococcus pyogenes* biofilms on cellular hematological parameters and distribution and phenotyping of cellular immunity in mucosal tissue of tonsils. Thirty healthy controls and fifty pediatric tonsillitis patients participated in the research. Thirty isolated *S. aureus* and *S. pyogenes* were tested for biofilm-forming capability (BFC). Hematological parameters were assessed before tonsillectomy, and 9 tonsil samples were evaluated using hematoxylin and eosin stain to investigate the histopathological alterations. Immunohistochemistry (IHC) staining was carried out for detecting dendritic cells (CD1a), neutrophils (CD15), macrophages (CD68), helper T cells (CD4), and cytotoxic T cells (CD8). Hematological parameters showed a significant increase in total WBC count in pediatric tonsillitis patients infected with *S. aureus*, mixed bacteria, and *S. pyogenes* groups. Both *S. aureus* and *S. pyogenes* had the ability to form biofilms with different capacities. 72% of the *S. aureus* isolates showed a moderate BFC compared to 23% strong and 5% with weak ability. While 50% of *S. pyogenes* showed strong BFC compared to 37% moderate and 13% with weak ability. The biofilms' effect on immune cells CD1a and CD15 showed weak positive staining, while CD68, CD4, and CD8 showed positive to strong positive staining. This study confirmed the capability of *S. aureus* and *S. pyogenes* to form biofilms with different effects on the immune cells in palatine tonsils of children, and the decreased number of dendritic cells (CD1a) and neutrophils (CD15) influence phagocytosis and antigen processing and presenting, which confirmed the association between BFC and the persistent nature of infection. Understanding a pathogen's BFC may guide clinicians to implement more aggressive or combination therapies to improve patient management and outcomes in the treatment strategies.

Keywords

S. aureus; S. pyogenes; BFC; Hematological parameters; Immunohistochemistry; Palatine tonsils

RESEARCH PAPER

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Abstract

This study examines the effect of *Staphylococcus aureus* and *Streptococcus pyogenes* biofilms on cellular hematological parameters and distribution and phenotyping of cellular immunity in mucosal tissue of tonsils. Thirty healthy controls and fifty pediatric tonsillitis patients participated in the research. Thirty isolated *S. aureus* and *S. pyogenes* were tested for biofilm-forming capability (BFC). Hematological parameters were assessed before tonsillectomy, and 9 tonsil samples were evaluated using hematoxylin and eosin stain to investigate the histopathological alterations. Immunohistochemistry (IHC) staining was carried out for detecting dendritic cells (CD1a), neutrophils (CD15), macrophages (CD68), helper T cells (CD4), and cytotoxic T cells (CD8). Hematological parameters showed a significant increase in total WBC count in pediatric tonsillitis patients infected with *S. aureus*, mixed bacteria, and *S. pyogenes* groups. Both *S. aureus* and *S. pyogenes* had the ability to form biofilms with different capacities. 72 % of the *S. aureus* isolates showed a moderate BFC compared to 23 % strong and 5 % with weak ability. While 50 % of *S. pyogenes* showed strong BFC compared to 37 % moderate and 13 % with weak ability. The biofilms' effect on immune cells CD1a and CD15 showed weak positive staining, while CD68, CD4, and CD8 showed positive to strong positive staining. This study confirmed the capability of *S. aureus* and *S. pyogenes* to form biofilms with different effects on the immune cells in palatine tonsils of children, and the decreased number of dendritic cells (CD1a) and neutrophils (CD15) influence phagocytosis and antigen processing and presenting, which confirmed the association between BFC and the persistent nature of infection. Understanding a pathogen's BFC may guide clinicians to implement more aggressive or combination therapies to improve patient management and outcomes in the treatment strategies.

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1. Introduction

Tonsils are lymphoid tissues that make up *Waldeyer's tonsillar ring* and the mucosa-associated lymphoid tissue of the children's throat. They are in charge of immunological monitoring of the upper respiratory tract and guard the mucosa of the alimentary canal against different infections [1]. Tonsillitis is the inflammatory and immunological response to bacterial or viral infections, bacterial infection in children accounts for approximately 30 percent of cases [2]. The production of biofilms by these bacteria, through clustering of bacteria in self-proteins, DNA, or polysaccharides, plays an important function in growing the bacterial virulence and adhesion in the initial stage of infection [3]. Bacterial infections and antibiotic tolerance are thought to be significantly influenced by the bacterial formation of biofilms, which prolongs the duration of inflammation and chronic infections [4]. *Staphylococcus aureus* has the ability to form multilayered, mature biofilms, and metabolic adaptability with pharmic resistance likely aids its survival within tonsillar tissues and significantly contributes to the persistence of recurrent infections [5,6]. *Streptococcus pyogenes* was also discovered to be an otopathogen

Received 27 April 2025; revised 19 June 2025; accepted 22 June 2025.
Available online 15 July 2025

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https://doi.org/10.33640/2405-609X.3418

that produces biofilms in the nasopharynx. The capacity of *S. pyogenes* to develop mature biofilms and microcolonies both in vitro and in vivo was just recently recognized. Because of their physical characteristics, physiology, and composition, biofilms pose a serious threat [7,8]. The oral cavity serves as the initial location of *S. aureus* and *S. pyogenes* infection and can develop there and remain mostly intracellularly as biofilm in the palatine tonsils [9]. Cellular immunity, such as macrophages, lymphocytes, and plasma cells, may all play a pathogenic role, perhaps as a result of infection removal and an immunological response. Furthermore, antigen-presenting cells (APCs) (macrophages and dendritic cells) are thought to be significant immune response regulators that activate CD4 and CD8 T lymphocytes in response to infectious pathogens [10]. The goal of this research was to examine the ability of *S. aureus* and *S. pyogenes* to form biofilms within the pediatric tonsillar tissue and their effect on cellular hematological parameters and distribution, phenotyping of CD1a dendritic cells, CD15 neutrophils, CD68 macrophages, CD4 T lymphocytes, and CD8 T lymphocytes.

2. Material and methods

2.1. Blood sample collection

Three ml blood samples were collected from 50 pediatric patients (26 males and 24 females), with an average age of 7.40 ± 2.72 years, and 30 healthy children as controls (17 males and 13 females), with a mean age of 6.50 ± 2.48 years, and then put in an EDTA tube for hematological investigation (lymphocyte, monocyte, granulocyte, hemoglobin, and platelet counts). The patients were classified into three groups: *S. aureus* (22 samples), *S. pyogenes* (8 samples), and mixed bacteria (20 samples).

2.2. Tosillopharyngeal swab

Mannitol salt agar and blood agar were used to cultivate the swab samples, then incubated under aerobic state for 24 h at 36 ± 2 °C. Gram staining was used to identify the isolated microorganisms. Bacteria were tested for DNase test agar, coagulase test, antibiotics like bacitracin, and catalase test, and the results were confirmed using a VITEK® 2 Compact system (bioMérieux, Inc., Durham, NC, USA).

2.3. Assay for quantitative biofilm formation

The microtiter plate (MTP) method [11] was employed to assess the capacity of the discovered 30 bacterial isolates of *S. aureus* and *S. pyogenes* to produce biofilm, despite slight adjustments [12]. In essence, tested bacteria were inoculated in 5 ml of nutrient broth (NB) (Neogen, USA) and cultivated overnight at 37 °C. Afterward, a bacterial inoculum of 1:100 was prepared in NB supplemented with 2 % glucose. A multichannel pipette was used to transfer 200 μL of the diluted sample into a sterile 96-well MTP. Wells with only NB were consumed as control. Then, MTP was maintained at 37 °C for 24 hrs in a stationary state. After this, the wells were rinsed three times with sterile phosphate buffer saline (PBS), the planktonic broth culture was removed, and the plates were desiccated in an oven at 55 °C for 20 mins. After adding 200 μl of 1 % crystal violet staining to each well, the plate was kept at room temperature for 10 mins. The wells were then washed with PBS, dehydrated, and eluted with 95 % ethanol solvent to spectrophotometrically quantify the generated biofilms at a wavelength of 490 nm using ELISA (Epson, Biotek, UK). For data calculation, the classification based on obtained OD values was applied according to Table 1.

2.4. Histopathological and immunohistochemistry (IHC) analysis

Tonsillar tissue from 9 pediatric patients obtained at surgery was fixed in 10 % formalin solution and sent as fresh specimens to the histopathological laboratory for processing and analysis. The palatine section was prepared, embedded in paraffin, and stained with hematoxylin and eosin (Thermo Scientific, USA) and viewed under the microscope (Olympus BX40, Japan). The most typical areas in the palatine tonsil specimens were stained with IHC staining for antibodies against CD1a for recognition of dendritic cells (DCs) (clone 010 Fisher MS-1858-R7), CD15 for recognition of neutrophil cells (clone MC-480 Fisher MA1-002), CD68 for recognition of macrophage cells (clone KP1 F1-sher MA5-13324), CD4 for the recognition of helper T cells (clone 4B12, Fisher MA5-12259), and CD8 for recognition of cytotoxic T cells (clone SP16, Fisher MAS-14548). Prepared monoclonal antibodies for usage (from Thermo Fisher Scientific, Tudor Road, Manor Park, Runcorn, Cheshire WA7 1 TA, UK) were used. The

Table 1. Categorization of bacterial biofilm degree by MTP procedure [13].

Mean OD values	Adhesion	Biofilm formation
<0.120	Non	Non \ weak
0.120–0.240	Moderately	Moderate
>0.240	Strong	High

resulting sections were examined using a high-resolution color microscope camera at magnified powers of 100x and 400x. Each slide was histopathologically evaluated and approved by two specialist pathologists. Based on the cell membrane's staining intensity, immunological markers and immune cell response characteristics were analyzed. Staining intensity was evaluated for no expression (negative), mild (weak positive), moderate (positive), or intense (strong positive).

2.5. Statistical analysis

GraphPad Prism Software 8.0.1 was used to perform descriptive analysis and multiple comparisons, and the Shapiro–Wilk, Kolmogorov, and D'Agostino-Pearson omnibus tests were used to assess the data's normality. For quantitative variables with abnormal distributions and to compare groups, the Kruskal–Wallis test was employed. A p-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Bacterial biofilm forming capacity (BFC)

Tonsillitis in children is significantly influenced by bacterial biofilm production, especially by *S. aureus*, *S. pyogenes*, or mixed infection of both [14]. Interestingly, we found that a significant number (72 %) of *S. aureus* strains exhibit a moderate ability compared to 23 % strong and 5 % weak ability to form biofilms (Table 2). Many researchers proved that the capability of *S. aureus* to produce biofilms aids in recurrent and chronic infections, as well as treatment failure, and may help in its persistence in tonsillar tissue even after the inflammation has subsided [4,15]. A greater number (50%) of *S. pyogenes* bacteria have a stronger ability to produce biofilms compared to moderate and weak strains (37 % and 13 %, respectively) (Table 2). Maddocks and his colleagues found that more than 90 % of invasive and noninvasive strains *of S. pyogenes* have the ability to generate biofilms during infection [16]. Our result was consistent with the findings reported by Matysik and Kline, who verified *that S. pyogenes* under static conditions form a biofilm composed of chaining cocci [8].

3.2. Hematological parameters among tonsillitis patients and healthy controls

A significant increase in total WBC count was observed in the *S. aureus* (8.5 ± 1.8), mixed bacteria (9.2 ± 2.6), and *S. pyogenes* (7.2 ± 1.6) groups in contrast to the control group (6.5 ± 1.4) ($p < 0.0001$). Also, the lymphocyte counts were significantly higher in the *S. aureus* (4.2 ± 0.8), mixed bacteria (4.4 ± 1.5), and *S. pyogenes* (3.8 ± 0.8) groups than in the healthy group (2.5 ± 0.7) ($p < 0.0001$). Monocyte degrees were significantly raised in the *S. aureus, S. pyogenes*, and mixed bacteria groups compared to the healthy group ($p = 0.0018$). On the other hand, granulocyte counts revealed no significant difference among the groups ($p = 0.9999$). Hemoglobin levels were comparable among all the groups ($p = 0.4004$). Platelet number showed no significant differences except for *S. aureus*, which showed a significant difference compared to the control group ($p = 0.0402$) (Table 3). A recent finding of Vintilescu and his colleague concluded that most of the children with tonsil inflammation show lymphocytosis, monocytosis, and neutrophilia [17]. On the other hand, compared to other blood parameters like granulocyte and hemoglobin, each patient group had no significant difference with the control, as shown in Table 3. However, the level of platelet count is not significant among groups except the *S. aureus* group compared to the control group. Cengiz and his team indicate no significant difference in mean platelet count and hemoglobin levels with a negative relationship between recurrent tonsillitis and value of platelet or hemoglobin in children [18].

Table 2. Biofilm forming capacity of Staphylococcus aureus and Streptococcus pyogenes.

	Degree of biofilm	No. of isolates (22)	Percentage (%)	Mean ± SE (OD)
S. aureus	Weak	1	5 %	0.1097 ± 0.000
	Moderate	16	72 %	0.1732 ± 0.008
	Strong	5	23 %	0.3389 ± 0.043
	Total	22	100 %	
	Degree of biofilm	No. of isolates (8)	Percentage (%)	Mean ± SE (OD)
S. pyogenes	Weak	1	13 %	0.1173 ± 0.000
	Moderate	3	37 %	0.1310 ± 0.057
	Strong	4	50 %	0.3177 ± 0.024
	Total	8	100 %	

Table 3. Comparison of hematological parameters between tonsillitis patients infected with S. aureus, S. pyogenes, mixed bacteria, and healthy controls.

CBC Mean ± SD Median (min–max)	22 Sample *S. aureus*	8 Sample *S. pyogenes*	20 Sample Mixed	30 Sample Control	H	*P*-value	SBG
Total WBC ($\times 10^3$/mm)							$p1 < 0.0001$****
	8.5 ± 1.8	7.2 ± 1.6	9.2 ± 2.6	6.5 ± 1.4	43.29	<0.0001	$p2 = 0.3044$ ns
	8.2 (4.4–13.9)	7.1 (4.7–11.9)	9.3 (4.6–16.3)	6.3 (4.2–9.8)			$p3 < 0.0001$****
Lymphocyte ($\times 10^3$/mm)							$p1 < 0.0001$****
	4.2 ± 0.8	3.8 ± 0.8	4.4 ± 1.5	2.5 ± 0.7	76.28	<0.0001	$p2 < 0.0001$****
	4.1 (2.5–6)	3.7 (2.2–5.9)	4.2 (1.7–7.4)	2.6 (0.8–4.7)			$p3 < 0.0001$****
Monocyte ($\times 10^3$/mm)							$p1 = 0.0018$**
	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.5 ± 0.9	16.54	0.0009	$p2 = 0.1179$ ns
	0.7 (0.2–1.3)	0.6 (0.4–2)	0.7 (0.4–1.3)	0.5 (0.2–1)			$p3 = 0.0012$**
Granulocyte ($\times 10^3$/mm)							$p1 = 0.9999$ ns
	3.6 ± 1.1	2.8 ± 0.8	4.2 ± 2.2	3.4 ± 1.1	8.61	0.035	$p2 = 0.0410$*
	3.3 (1.6–6.8)	2.7 (1.8–5.1)	3.2 (1.4–11.6)	3.2 (1.5–6.4)			$p3 = 0.9999$ ns
Hemoglobin (g/dl)							$p1 = 0.4004$ ns
	13.5 ± 1.1	13.4 ± 0.9	13.4 ± 1.1	13.2 ± 1.1	2.43	0.4882	$p2 = 0.9999$ ns
	13.5 (11.1–18.1)	13.5 (11.6–16.3)	13.4 (10.9–15.4)	13.2 (11.1–15.7)			$p3 = 0.9622$ ns
Platelets ($\times 10^3$/mm)							$p1 = 0.0402$*
	333 ± 92.1	309.6 ± 59.6	304.1 ± 95.9	283 ± 55.9	6.297	0.0980	$p2 = 0.4543$ ns
	329 (206–579)	301 (196–501)	305.5 (87–471)	273 (141–377)			$p3 = 0.7339$ ns

H represents the Kruskal–Wallis test, and Dunn's post hoc test was utilized for pairwise group comparisons. *p*1: *p*-value for comparing Control and *S. aureus*; *p*2: *p*-value for comparing Control and *S. pyogenes*; *p*3: *p*-value for comparing Control and mixed bacteria. SBG: Significant differences between groups. * $p < 0.05$ and ** $p < 0.01$ statistically significant, and *** $p < 0.001$ is highly significant.

3.3. Histopathological analysis

Histological examination of palatine tonsil tissue infected with *S. aureus*, *S. pyogenes*, and mixed bacteria disclosed significant pathological modifications (Fig. 1). **I**/(A) Showed bacterial colonies with the interfollicular area (red arrow), with deposition of collagen fibers (black arrow) 400x. (B) Showed hyperplasia of tonsillar epithelial cells (black arrow), diffuse infiltration of lymphocytes (red arrow), and deposition of collagen fibers in the tonsillar septa (yellow arrow) 400x. (C) Showed diffuse infiltration of lymphocytes (black arrow) and macrophages (blue arrow) and accumulation of collagen fibers in the tonsillar septa (red arrow) 100x. (D) Showed hyperplasia of tonsillar epithelial cells (black arrow), hyperplasia of M cells (blue arrow), diffuse deposition of collagen fibers (red arrow), and infiltration of lymphocytes (yellow arrow) 400x. **II**/(A) Bacterial colonies near the epithelial surface (black arrow), with infiltration of lymphocyte cells (red arrow) 400x. (B) Showed hyperplasia of tonsillar epithelial cells (black arrow), infiltration of lymphocytes (red arrow), and hypertrophy of M cells (yellow arrow) 400x. (C) Showed focal infiltration of lymphocytes (black arrow) and macrophages (blue arrow) and deposition of collagen fibers in the tonsillar septa (red arrow) 100x. (D) Showed focal infiltration of lymphocytes (black arrow) and macrophages (blue arrow) and deposition of collagen fibers in the tonsillar septa (red arrow). 100x. **III**/(A) Showed bacterial colonies at the epithelial surface (black arrow), with infiltration of inflammatory cells (red arrow). 400x. (B) Showed diffuse infiltration of lymphocytes (red arrow) and deposition of collagen fibers in the tonsillar septa (blue arrow). 400x. (C) Showed focal infiltration of lymphocytes around blood vessels (red arrow), with infiltration of other macrophages (blue arrow), between diffused depositions of collagen fibers in the tonsillar septa (yellow arrow). 400x. (D) Showed an increase in the thickness of tonsillar septa due to massive deposition of collagen fibers (black arrow) and hypertrophy of tonsillar follicles with a clear germinal center (red arrow). 100x.

The palatine tissue infected with *S. pyogenes* and mixed bacteria showed bacterial colonies at the epithelial surface, while *S. aureus* colonies were concentrated in the interfollicular area, and these findings were consistent with Radcliff and his colleague's finding that confirmed embedding and colonization of *S. aureus* within the tonsil tissue [19]. In both cases of *S. aureus* and *S. pyogenes*, hyperplasia of tonsillar epithelial cells was observed, while in the mixed bacterial cases, an increase in the thickness of tonsillar septa with massive deposition of collagen fibers and hypertrophy of tonsillar follicles was observed. A previous finding demonstrated that the presence of biofilms in infected tonsils is linked to

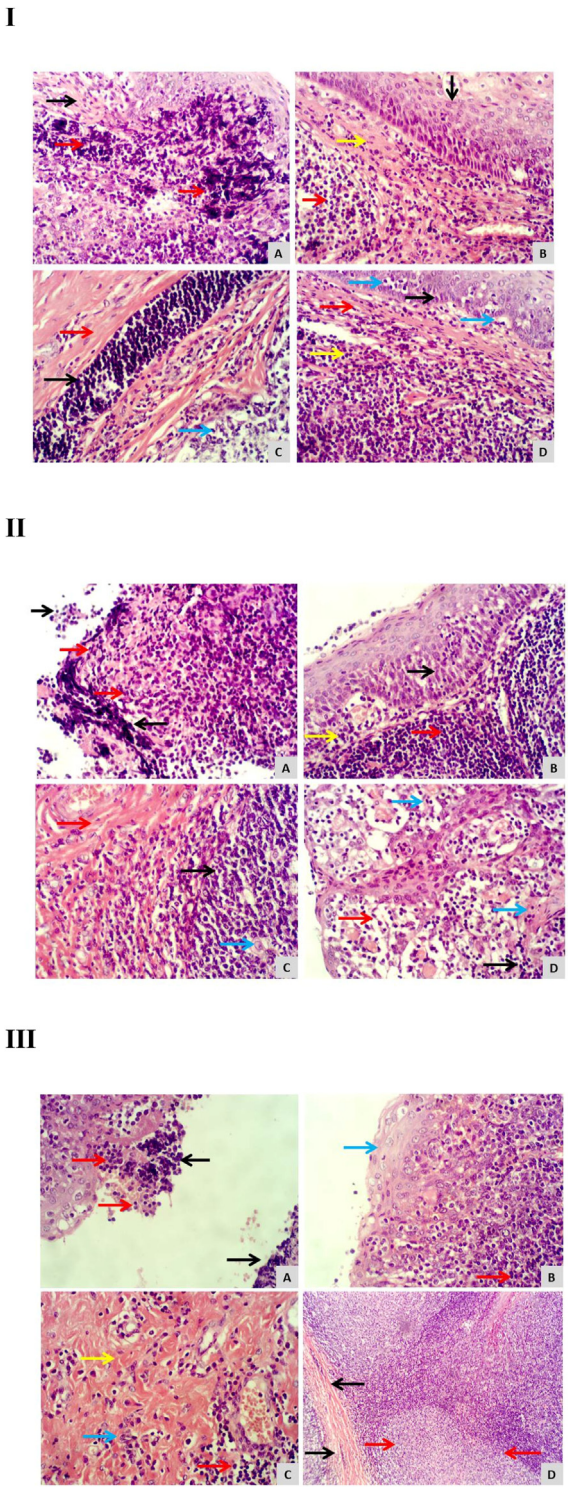

Fig. 1. Section of palatine tonsil tissue infected with I/S. aureus, II/S. pyogenes, and III/mixed bacteria (H&E).

tonsillar hypertrophy and increasing the number of tonsillar lymphatic follicles [20]. Additionally, diffuse infiltration of lymphocytes and macrophages with increased deposition of collagen fibers was identified in all cases. Muşetescu and his colleague concluded that infiltration of lymphocytes serves as a reliable histopathological marker for diagnosing chronic tonsillitis [21].

3.4. Immunohistopathological finding

We analyzed frequencies of specific immune cells by immunostaining neutrophils (CD15) and different APCs, such as macrophages (CD68) and dendritic cells (CD1a), which serve to stimulate the innate system activity and, through which, stimulate the adaptive immune system, including helper CD4 T cells and cytotoxic CD8 T cells [22]. Immunostained for CD1a dendritic cells and CD15 neutrophils, a weak positive stain was shown for all the groups (Fig. 2. A, F, K, and B, G, L). Viciani and his teams correlated the role of *S. pyogenes* in low levels of neutrophils CD15 in the tonsils of patients with obstructive sleep apnea syndrome [23]. However, Gondak and his colleagues observed severe dendritic cell depletion in the palatine tonsils and cervical lymph nodes of advanced HIV patients [24]. While the CD68 antibody for macrophages in the palatine tissue showed positive to strong positive staining in all cases (Fig. 2. C, H, M), this finding was similar to a study done by Muşetescu and his colleague, which confirmed that tonsillar tissue frequently had a greater density of CD68 cells compared to neutrophils and dendritic cells [21]. On the other hand, helper CD4 T cells and cytotoxic CD8 T cells showed positive and strong positive staining, respectively (Fig. 2. D, I, N and E, J, O). Enriched infiltration of tonsillar CD4+ and CD8+ T lymphocytes was reported by Sada-Ovalle and his team [25].

4. Conclusion

In conclusion, our study indicated that *S. aureus* strains mostly form a moderate biofilm in contrast to *S. pyogenes*, which had strong BFC, and this ability of *S. aureus* and *S. pyogenes* to form biofilms has different effects on the immune cells in children's palatine tissue and contributes to prolonged or recurrent infections due to their increased resistance to host defenses and antimicrobial treatments. The decreased number of dendritic cells (CD1a) and neutrophils (CD15) that influence phagocytosis and antigen processing and presenting confirmed the relationship between BFC and the persistent nature of the infection. Furthermore, understanding a pathogen's BFC may guide clinicians to implement more aggressive or combination therapies to improve patient management and outcomes in the treatment strategies.

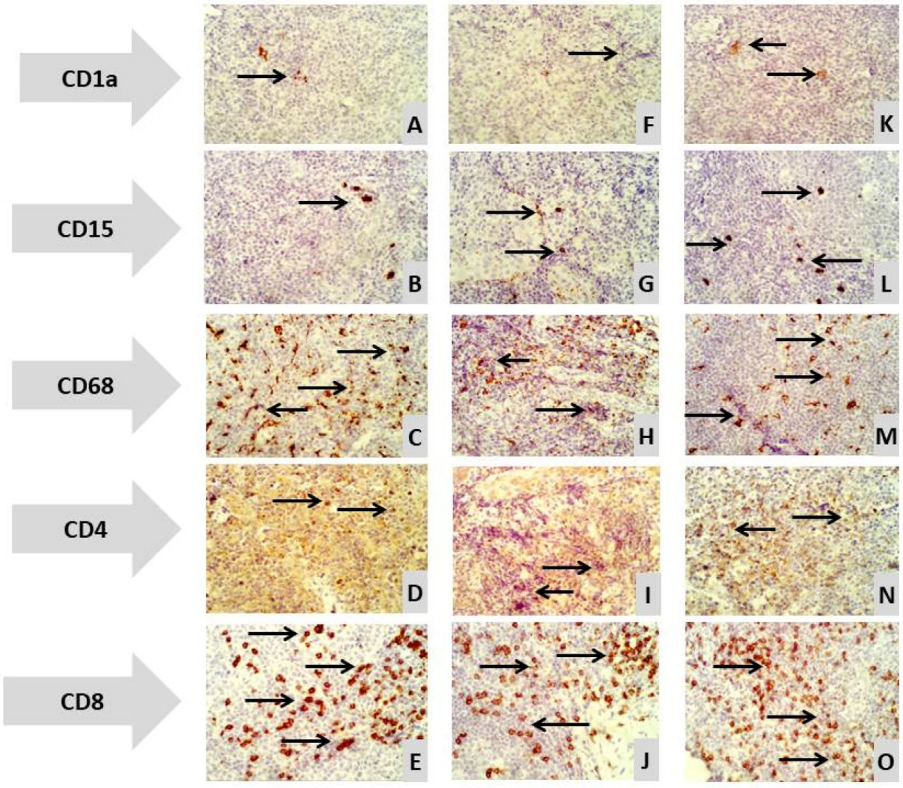

Fig. 2. Palatine tonsils infected with S. aureus (A–E), S. pyogenes (F–J), and mixed bacteria (K–O). IHC showed a weak positive staining for CD1a and CD15 antibodies (A, F, K, B, G, L). CD68 antibodies showed positive to strong positive staining (C, H, M). Both CD4 and CD8 antibodies showed positive (D, I, N) to strong positive staining in all cases (E, J, O). 400x.

Institutional review board statement

The study was carried out in a teaching hospital in Erbil from July 2024 to December 2024 in accordance with the current study's authorization and approval by the College of Science's Human Ethics Committee at Salahaddin University in Erbil (Approval No.: 45/270 Date: 30/6/2024).

Funding

The authors would like to state that no particular funding was obtained for this study.

Conflicts of interest

The authors declare that they have no conflicts of interest

Acknowledgment

The author would like to thank all of the medical and administrative personnel at the Teaching Hospital, but especially the two advisers who are histopathologists, for their help in assessing and confirming the histological and immunohistochemical results.

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