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Rīga Stradiņš University

Renāta Vīksne (Klagiša)

Role of Biofilms in Pathogenesis and Clinical Course of Recurrent Tonsillitis and Peritonsillar Abscess

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences
Sector – Clinical Medicine
Sub-Sector – Otorhinolaryngology

Riga, 2023

Renāta Vīksne (Klagiša)

ORCID 0000-0001-9855-5546

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The Doctoral Thesis was developed at Rīga Stradiņš University, Department of Otorhinolaryngology, Department of Biology and Microbiology, Latvia

Supervisors of the Doctoral Thesis:

Dr. med., Professor **Juta Kroiča**,
Rīga Stradiņš University, Latvia

Dr. med., Associate Professor **Ligija Ķīse**,
Rīga Stradiņš University, Latvia

Official Reviewers:

Dr. med., Professor **Ilze Akota**,
Rīga Stradiņš University, Latvia

Dr. biol., Professor **Una Riekstiņa**,
University of Latvia

Dr. med., Professor **Eugenijus Lesinskas**,
Vilnius University Hospital Santaros Clinic, Lithuania

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Secretary of the Promotion Council:

Dr. med., Professor **Ingrīda Čēma**

Table of Contents

Abbreviations

Abbreviation	Title in English	Title in Latin
A	Armpit	
Ae		*Acinetobacter ewoffi*
Aju		*Acinetobacter junii*
Ajo		*Acinetobacter johnsoni*
A. johnsoni		*Acinetobacter johnsoni*
AK	Amikacin	
AMP	Ampicillin	
AMS	Ampicillin/sulbactam	
Ap		*Acinetobacter pitti*
A. pitti		*Acinetobacter pitti*
AUG	Amoxicillin/clavulanic acid	
ASO	Antistreptolysin O	
BP	Benzylpenicillin	
C	Crypt	
C	Chloramphenicol	
C. albicans		*Candida albicans*
CAZ	Ceftazidime	
CD	Clindamycin	
CIP	Ciprofloxacin	
CRP	C-reactive protein	
CRO	Ceftriaxone	
CTX	Cefotaxime	
E	Erythromycin	
ESBL	Extended spectrum beta-lactamase	
EUCAST	European Committee on Antimicrobial Susceptibility Testing	
ETP	Ertapenem	
F	Female	
FOX	Cefoxitin	
GM	Gentamicin	
H. influenzae		*Haemophilus influenzae*
HIV	Human immunodeficiency virus	
I	Intermediate resistant	
IMP	Imipenem	
IQR	Interquartile range	

IU	International unit	
Kp		*Klebsiella pneumoniae*
K. pneumoniae		*Klebsiella pneumoniae*
LB	Luria – Bertani broth	
LEU	White blood cells	
LEV	Levofloxacin	
M	Male	
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry	
MEM	Meropenem	
MRSA	Methicillin-resistant *Staphylococcus aureus*	
MSSA	Methicillin-sensitive *Staphylococcus aureus*	
N	Nasal vestibule	Vestibulum nasi
NC	Negative control	
NOR	Norfloxacin	
N. subflava		*Neisseria subflava*
OD	Optical density	
ODc	Optical density`s cut-off value	
PSCUH	Pauls Stradiņš Clinical University Hospital	
Pa		*Pseudomonas aeruginosa*
P. aeruginosa		*Pseudomonas aeruginosa*
P. intermedia		*Prevotella intermedia*
PTA	Peritonsillar abscess	
R	Resistant	
RSU	Rīga Stradiņš University	
RT	Recurrent tonsillitis	
S	Sensitive	
Sa		*Staphylococcus aureus*
S. aureus		*Staphylococcus aureus*
S. agalactiae		*Streptococcus agalactiae*
S. anginosus		*Streptococcus anginosus*

S. dysgalactiae		*Streptococcus dysgalactiae*
S. epidermidis		*Staphylococcus epidermidis*
S. pneumoniae		*Streptococcus pneumoniae*
S. pyogenes		*Streptococcus pyogenes*
S. viridans		*Streptococcus viridans*
SD	Standard deviation	
Spp.	Species	
Sl		*Serratia liquefaciens*
S. liquefaciens		*Serratia liquefaciens*
SXT	Trimethoprim/sulfamethoxazole	
TE	Tonsillectomy	
TZP	Piperacillin/tazobactam	
TSB	Tryptic Soy Broth	
T	Throat	

Introduction

Recurrent tonsillitis (RT) is a recurrent inflammation of the palatine tonsils predominantly, or even exclusively, caused by bacteria (Zautner et al., 2010; Cavalcanti et al., 2019; Buname et al., 2021). Episodes of tonsillitis are characterised by fever, sore throat and odynophagia, reddening of yawn, swelling of the palatine tonsils with or without plaque, as well as cervical lymphadenopathy (Buname et al., 2021). Discomfort in the throat, increased accumulation of debris in the crypts of the palatine tonsils, halitosis and cervical lymphadenopathy may persist between episodes of tonsillitis (bin Abu Bakar et al., 2018). The diagnosis of RT is made clinically based on medical history (Windfuhr et al., 2016a; Sykes et al., 2020). The diagnosis of RT can be made if there are more than 2 episodes of tonsillitis within a 12-month period (Burton et al., 2014). Antibiotics are used for treatment of episodes of RT (Windfuhr et al., 2016a; Katkowska et al., 2017). Tonsillectomy is indicated for patients with RT in the case of 7 episodes of tonsillitis per year, or 5 episodes of tonsillitis per year for 2 consecutive years, or 3 episodes of tonsillitis per year for 3 consecutive years (Paradise et al., 1984; Sykes et al., 2020).

Inflammation from the palatine tonsil tissue can spread to the adjacent peritonsillar tissue and form an abscess in the peritonsillar space (Windfuhr et al., 2016a; Peter Sell et al., 2023). A peritonsillar abscess (PTA) is the most common purulent complication of tonsillitis (Klug, 2017). The diagnosis of PTA is made clinically. A patient with PTA is characterised by fever, unilateral sore throat, unclear speech, sometimes trismus, involvement of the descending lymph nodes of the neck in the inflammatory process (Klug, 2017). On examination one-sided reddening of the pharyngeal mucosa and palpably stiff and painful swelling in the peritonsillar and adjacent soft palate area are observed, but uvula is deviated to the contra-lateral side (Slouka et al., 2020). PTA is in most cases a unilateral process. Treatment of PTA requires antibiotic therapy and surgical

treatment – incision and drainage of the abscess or acute tonsillectomy (Windfuhr et al., 2016b; Klug, 2017). The reasons for the ineffectiveness of conservative therapy are still not clear.

Episodes of RT decrease the quality of life and lead to financial losses due to temporary disability and medical expenses (Windfuhr et al., 2016b; Tzelnick et al., 2020). The risk of repeated courses of antibacterial therapy represents promotion of bacterial resistance. When using surgical treatment and anaesthesia, there are risks such as bleeding, blood aspiration, injury to adjacent structures, prolonged wound healing, etc. (Windfuhr et al., 2016b).

There are several reasons for the failure of antibiotic therapy: difficulties in identifying the bacteria that cause tonsillitis, low concentration of antibiotics in tonsillar tissues, intracellular retention of pathogen in antigen-presenting cells like macrophages, specific bacterial resistance mechanisms, for example, formation of biofilms (Pichichero & Casey, 2007; Zautner et al., 2010; bin Abu Bakar et al., 2018). Several studies have been conducted characterising the spectrum of pathogens involved in the aetiology of RT and PTA, the aetiology is often polymicrobial (Zautner et al., 2010; Klug, 2017). A microbiological analysis of palatine tonsil samples and identification of the aetiological agent are quite challenging due to the patient's diversity of microorganisms present in the normal oral microbiota (Windfuhr et al., 2016a; Dickinson et al., 2020). Palatine tonsils have the highest microbial diversity with significant individual variation (Aas et al., 2005; Jorgensen et al., 2015; Ivaska et al., 2020). The surface of the tonsils is covered with normal oral microbiota, which is not usually involved in the aetiology of tonsillitis, although autoinfection with oral microbiota is possible (Windfuhr et al., 2016a; Haq et al., 2017). In areas with high bacterial colonisation, the clinical and etiological significance of isolated bacteria should be assessed (Vaikjärv et al., 2016). The aetiology of tonsillitis is caused by bacteria located in the parenchyma or crypts of the palatine tonsils, and not by

bacteria on the surface (Khadilkar & Ankle, 2016). Tonsillar crypts are narrow, branching passages or folds in the tonsillar tissues. For microbiological analysis, samples from palatine tonsil crypts are considered to be more accurate and relevant compared to tonsillar surface swabbing (Haq et al., 2017; Dickinson et al., 2020),

The tonsillar tissue of RT patients has a high prevalence of *Staphylococcus aureus* (*S. aureus*), so *S. aureus* is considered to be the main aetiological factor of RT (Brook & Foote, 2006; Zautner et al., 2010; Katkowska et al., 2017; Kostić et al., 2022). Nevertheless, the role of this pathogen in the pathogenesis of RT exacerbations, abscess formation and antibacterial resistance is unclear. Asymptomatic carriage of *S. aureus* complicates microbiological analysis (Chmielowiec-Korzeniowska et al., 2020). *S. aureus* is capable of acquiring broad-spectrum antibacterial resistance, however, in RT studies, *S. aureus* does not show high antibacterial resistance, so other protective mechanisms such as biofilm formation are being studied (bin Abu Bakar et al., 2018; Cavalcanti et al., 2019; Katkowska et al., 2020; Kostić et al., 2022). Studies have confirmed the ability of *Klebsiella pneumoniae (K. pneumoniae)* to form persistent biofilms. Due to *K. pneumoniae* biofilms, they are able to colonise the respiratory tract, nasopharynx, and tonsils (Alasil et al., 2013; Wang et al., 2020).

It is believed that biofilm-forming bacteria are involved in the pathogenesis of RT (Kostić et al., 2022). Studies using a scanning electron microscope revealed a statistically significant presence of biofilms in the group of RT patients compared with the control group, 80 % (16/20) and 45 % (9/20), respectively (Woo et al., 2012). Bacteria located in a multi-layered mature biofilm are protected from the host's immune system – macrophages and antibodies, and are also resistant to antibiotics (Archer et al., 2011; Lister & Horswill, 2014; Hamilos, 2019). The properties of biofilms explain the ability

of pathogens to survive in tonsillar tissue despite repeated courses of antibacterial therapy (Archer et al., 2011; Lister & Horswill, 2014; Moormeier & Bayles, 2017). RT exacerbations are explained by the presence of free planktonic bacteria, but chronic symptoms between episodes of tonsillitis are explained by bacteria present in crypts and biofilm colonies that can resist the host's immune defence system for a long time (bin Abu Bakar et al., 2018).

Aim

Identification of aetiological agents of RT and PTA in the crypts and parenchyma of palatine tonsils, determination of their antibacterial resistance and ability to form biofilms, as well as assessment of their significance in the pathogenesis and clinical course of RT and PTA.

Tasks

1. To isolate and identify microorganisms present in the crypts of the palatine tonsils of patients with RT and PTA, to evaluate the biofilm-forming ability of *S. aureus* and *K. pneumoniae*, antibacterial resistance and their role in the development of RT and PTA.
2. To compare the presence of bacteria in the surface and crypt samples of palatine tonsils in RT patients, to evaluate their role in the aetiology of RT.
3. To identify and characterise the presence of the most important pharyngeal and respiratory pathogens in the crypts of palatine tonsils of healthy individuals.
4. To evaluate and compare the frequency and clinical significance of *S. aureus* colonisation in RT patients in tonsillectomy material and carriage of *S. aureus* one year after surgery.

Hypotheses

The aetiological agents of RT and PTA are localised in the crypts or parenchyma of the palatine tonsils and not on the surface of the tonsils, and in the clinical course of RT and PTA, the type and variety of bacteria, their ability to form a biofilm and antibacterial resistance play an important role.

Novelty

An extensive identification of the etiological agents of RT and PTA was carried out using matrix-assisted laser desorption/ionisation – time-of-flight mass spectrometer (MALDI-TOF MS) system and VITEK-2 Compact device. For the optimal and accurate sampling of the crypts of the palatine tonsils, a punch biopsy needle was designed, patented, and used for the first time (Klagisa et al., 2021a). A study of the palatine tonsil microbiota of healthy individuals was conducted. The presence of biofilm-forming strains in RT and PTA patients was assessed and their relationship with antibacterial resistance was evaluated. For the first time, RT patients were observed one year after tonsillectomy, as well as the efficiency of tonsillectomy was evaluated.

Author's Contributions

The author of the Thesis has examined, selected, consulted, treated and operated on all patients and healthy individuals who participated in the study, took biopsy specimens of the palatine tonsils and material for bacteriological examination, personally conducted a bacteriological examination of all samples (isolation and identification of microorganisms, determination of antimicrobial sensitivity and biofilm formation), analysed the obtained data, as well as prepared materials for obtaining a patent for a biopsy needle (Patent No.: LVP2020000055).

Ethical Aspects

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Rīga Stradiņš University on 30 November 2017, Permit No. 49/30.11.2017 and on 10 September 2020, Permit No. 6-1/09/22. All patients and healthy volunteers involved in the study signed an informed consent form to participate in the study.

1 Materials

In a prospective study that included RT and PTA patients and healthy individuals (see Figure 1.1), a medical examination was carried out, data were collected on their health, episodes and frequency of tonsillitis, as well as antibacterial therapy used. A microbiological testing of the palatine tonsils was carried out – a variety of microorganisms was identified, their antibacterial sensitivity and ability to form biofilms was evaluated, a comparison was made of the method of obtaining samples of the palatine tonsils with the puncture method from the crypts of the palatine tonsils and a swab from the surface of the palatine tonsils. The incidence of *S. aureus* was assessed in patients one year after tonsillectomy. The palatine tonsil samples were obtained at the Otorhinolaryngology Clinic of the Pauls Stradiņš Clinical University Hospital (PSCUH). Microbiological studies were carried out in the laboratory of the Department of Biology and Microbiology, Rīga Stradiņš University and in the bacteriological laboratory of PSCUH.

RT and PTA patients were included in the study in the period from 2018 to 2020, when they received treatment at the Otorhinolaryngology Clinic of PSCUH. Inclusion criteria were as follows: diagnosis of RT or PTA, received surgical treatment (bilateral tonsillectomy), no antibacterial therapy received during the last 4 weeks (Klagisa, Racenis, Broks, Balode, et al., 2022). RT was defined according to the *Paradise* criteria as 7 tonsillitis episodes per year, 5 tonsillitis episodes per year for 2 consecutive years, or 3 tonsillitis episodes per year for 3 consecutive years despite receiving appropriate antibacterial therapy (Paradise et al., 1984; Windfuhr et al., 2016a, 2016b). PTA was diagnosed clinically on the basis of patient complaints and examination data: unilateral palpably stiff and painful swelling of the peritonsillar region and soft palate with hyperaemia tonsillar pillars, sometimes trismus, neck lymphadenopathy, sore throat, unclear speech, fever (Klagisa, Racenis, Broks,

Balode, et al., 2022). The diagnosis of RT and PTA abscess was made at PSCUH after consultation with a certified otorhinolaryngologist.

All RT patients underwent elective bilateral tonsillectomy, with the last episode of tonsillitis exacerbation being not earlier than 4 weeks prior to the surgery. All PTA patients underwent immediate bilateral tonsillectomy during exacerbation of tonsillitis.

The study did not include patients with haematological diseases (thrombocytopenia or coagulopathy), primary or acquired immunodeficiency, dental infection, if antibiotic therapy was received within the last 4 weeks or it was started prior to obtaining a sample of the palatine tonsil for microbiological examination, if other PTA treatment methods were used, such as incision and drainage of the PTA, or there were contraindications for tonsillectomy (contraindications for surgery or general anaesthesia), or the patient refused to participate in the study, was unable to provide written informed consent (Klagisa, Racenis, Broks, Balode, et al., 2022). Patients with oncological disease, active autoimmune disease, taking immunosuppressive drugs (prednisolone > 10 mg/day or equivalent medications) for more than 4 weeks were not enrolled in the study (Klagisa, Racenis, Broks, Balode, et al., 2022). Information about patients' comorbidities was obtained by referral from the general practitioner, as the otorhinolaryngologist is a secondary health care specialist, and after a consultation with a certified otorhinolaryngologist and anaesthesiologist at the PSCUH.

The healthy individuals enrolled in the study were students of the Faculty of Medicine of Rīga Stradiņš University. Students were enrolled in the study in 2019 from 1 October to 31 December, when they attended the study course in Otorhinolaryngology. The students enrolled in the study had no comorbidities, tonsillopathy, signs of upper respiratory tract infection during the study period, had not received antibiotic therapy for at least 4 weeks prior to enrolment in the

study, based on the information provided by the students on their health status (Viksne et al., 2023). The study did not include students who had received antibacterial therapy in the last 4 weeks prior to participating in the study, with carious teeth, periodontal diseases, dental prostheses, based on the information provided by the students about their health status, or the ones who did not want to participate in the study (Viksne et al., 2023).

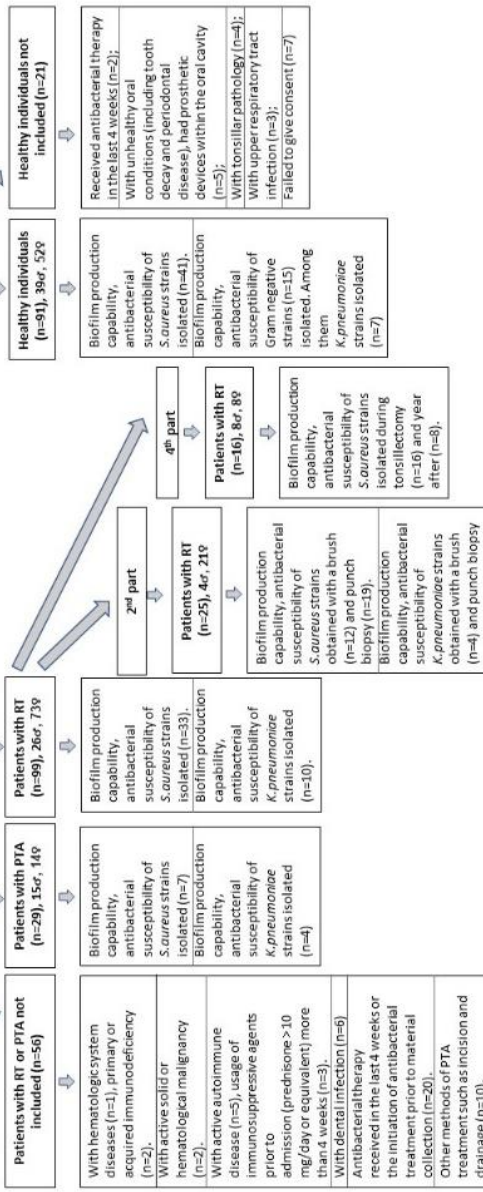

Figure 1.1 **Inclusion of patients in the study. Schematic diagram**

Samples for microbiological analysis were obtained as follows: with a sterile brush from the crypts of the palatine tonsils (Kito brush, reference number 0640, Kaltek srl, Padova, Italy) (2nd and 3rd part); with a sterile punch biopsy needle a fragment of palatine tonsil tissue with a crypt and its contents during tonsillectomy (1st, 2nd, 4th part); with a sterile cotton swab from the nasal vestibule (lat. *vestibulum nasi*), axillae and niches after tonsillectomy (4th part). A punch biopsy needle with a prolonged and curved handle and a circular blade was designed and used to obtain an optimal sample size of a tissue fragment of the palatine tonsil for the study purposes (see Figure 1.2), patent No. LVP2020000055, (Klagisa et al., 2021a). The obtained samples were placed in Amies transport media and transported to the laboratory at room temperature within 24 h.

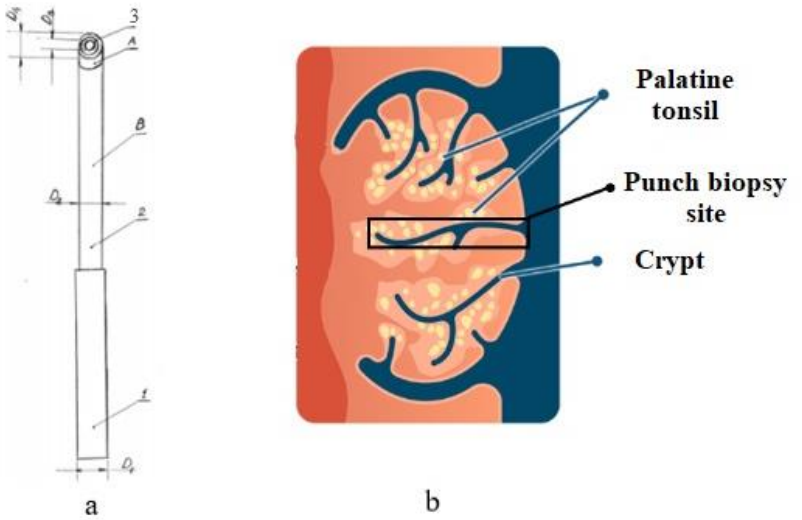

Figure 1.2 **Schematic diagram of the punch biopsy needle (a) and biopsy site of palatine tonsil (b)**

The punch biopsy needle consists of a blade (3) and a holder (2) with two parts (A) and (B) forming an angle of 125° between them.

2 Methods

2.1 Microorganism identification

The obtained samples were cultivated under aerobic conditions at 36 ± 1 °C for 24–48 h on Columbia blood agar, Mannitol salt agar, MacConcey agar, and Sabouraud dextrose agar plates (Liofilchem, Italy). A Brucella blood agar plate was used for cultivation of anaerobic microorganisms in an anaerobic bag system incubated at 36 ± 1 °C for up to 5 days. A Columbia blood agar medium with an optochin disc incubated at 36 ± 1 °C for 24–48 h with a CO_2 indicator was used for the cultivation of *Streptococcus pneumoniae*. For *Haemophylus* spp. cultivation Chocolate agar plate incubated in a CO_2 incubator at 36 ± 1 °C for 24–48 h with an oleandomycin disc was used. Isolated pure cultures were subjected to Gram staining and microscopy. Identification of microorganisms was performed using a Microflex LT (Bruker Daltonics flexAnalysis version 3.4, Bruker Daltonics GmbH & Co. KG, Bremen, Germany) MALDI–TOF MS system or using VITEK-2 Compact device (BioMérieux, Marcy – l'Étoile, France) (Klagisa, Racenis, Broks, Balode, et al., 2022; Viksne et al., 2023).

The normal oral microbiota was defined according to the guidelines of the European Society of Clinical Microbiology and Infectious Diseases (Cornaglia & Courcol, 2012).

2.2 Detection of biofilms using the crystal violet method

Isolated Gram-positive strains were suspended in trypticase soy broth (TSB) with 1 % glucose, and Gram-negative strains were suspended in Luria-Bertani (LB) broth incubation at 37 °C for 16–18 h. The inoculated TSB and LB broths were diluted with sterile TSB or LB broths, respectively, in a ratio of 1:100. Then, 150 μL of the diluted broth was transferred in a sterile manner with

a multichannel pipette into a sterile 96-well plate (Thermo Scientific™ Nunc MicroWell 96-Well Microplates, flat bottom, Thermo Fisher Scientific, Roskilde, Denmark). Each plate contained 11 strains and 1 negative control (sterile broth), 8 wells for each strain, each experiment was performed in 3 plates (triplicated). The inoculated plates were cultivated in aerobic conditions at 37 °C for 48 h. After incubation, all wells of the plates were drained without the use of a pipette by gently pouring the liquid into a clinical waste bag. Each well of the plate was rinsed 3 times with sterile 250 µL 0.9 % saline. After washing, staining with crystal violet solution was performed by adding 150 µL of 0.1 % crystal violet solution to each well. In 15 minutes, the dye was gently poured off and each well was washed 3 times with 250 µL distilled water. Finally, 150 µL of 96 % ethanol was added to each well. Then, the optical densities (ODs) of the wells were measured with a microplate spectrophotometer (Tecan Infinite F50, Mannendorf, Switzerland, with Magellan™ reader control and data analysis software V 6.6) at a wavelength of 570 nm (Klagisa, Racenis, Broks, Balode, et al., 2022; Klagisa, Racenis, Broks, Kise, et al., 2022; Reisner et al., 2006; Viksne et al., 2023).

2.3 Biofilm calculation

Mean OD values for each strain were calculated and expressed as numbers. The optical density cut-off value (ODc) was defined as 3 standard deviations (SDs) above the mean OD of the negative control, and it was calculated for each plate separately. Strains were divided as follows: $OD \leq ODc$ = non-biofilm forming strain, $ODc < OD \leq 2 \times ODc$ = weak biofilm producer, $2 \times ODc < OD \leq 4 \times ODc$ = moderate biofilm producer, and $4 \times ODc < OD$ = strong biofilm producer (Klagisa, Racenis, Broks, Balode, et al., 2022; Klagisa, Racenis, Broks, Kise, et al., 2022; Stepanović et al., 2007; Viksne et al., 2023).

2.4 Antibacterial Susceptibility Testing

Susceptibility testing was performed by the *Kirby-Bauer* disk diffusion method. Overnight cultures were suspended in physiological saline to 0.5 McFarland units (McFarland Densitometer DEN-1, Biosan, Latvia). The suspensions were uniformly inoculated on the surface of Mueller-Hinton agar (Oxid, UK) using a sterile cotton swab. Standardised discs containing antimicrobial agents at a given concentration were placed on inoculated agar plates. The following disks were used for *S. aureus* strains: cefoxitin 30 µg, ceftriaxone 30 µg, benzylpenicillin 1 international unit (IU), ampicillin 2 µg, ampicillin/sulbactam 10 µg/10 µg, amoxicillin/clavulanic acid 20 µg/10 µg, norfloxacin 10 µg, amikacin 30 µg, erythromycin 15 µg, clindamycin 2 µg and chloramphenicol 30 µg (Liofilchem, Italy). The following discs were used for *K. pneumoniae* and *Serratia liquefaciens (S. liquefaciens)* strains: amoxicillin/clavulanic acid 20 µg/10 µg, piperacillin/tazobactam 30 µg/6 µg, cefotaxime 5 µg, ceftazidime 10 µg, ertapenem 10 µg, imipenem 10 µg, meropenem 10 µg, ciprofloxacin 5 µg, gentamicin 10 µg and trimethoprim/sulfamethoxazole 1.25 µg/23.75 µg (Liofilchem, Italy). The following discs were used for *Acinetobacter spp.* strains: imipenem 10 µg, amikacin 30 µg, gentamicin 10 µg, trimethoprim/sulfamethoxazole 1.25 µg / 23.75 µg, ciprofloxacin 5 µg and levofloxacin 5 µg (Liofilchem, Italy). The plates were incubated for 16–18 h at 36 ± 1 °C (Klagisa, Racenis, Broks, Balode, et al., 2022; Viksne et al., 2023). After incubation, the diameter of the bacterial colony retention zone around each of the antibiotic disks was measured according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard “Clinical breakpoints and dosing of antibiotics” (Version 10.0, January 2020) (Anonymous, 2020).

3 Statistical Analysis

The *Kolmogorov–Smirnov* test was used to test the normal data distribution. Central tendencies of normally distributed data are characterised using mean values with SD, while data not conforming to normal distribution are characterised using interquartile range (IQR) median values.

Differences between groups were determined using:

1. *Kruskal-Wallis* test for independent samples (age differences between patients in RT and PTA groups, differences between biofilm formation abilities of 4 strains of *S. aureus*);
2. *Pearson* χ^2 test (correlations between the presence of Gram-positive microbes and the ability to form biofilms);
3. *Fisher's* exact test (correlations between the identification of *S. aureus, K. pneumoniae* in brush and punch biopsy needle samples, correlations between the number of episodes of tonsillitis and comorbidities; the number of episodes of tonsillitis and the presence of *S. aureus* in palatine tonsil biopsy samples or the presence of *K. pneumoniae* in palatine tonsils biopsy samples; the number of episodes of tonsillitis and the level of *S. aureus* biofilm formation, or the level of *K. pneumoniae* biofilm formation; and the number of episodes of tonsillitis and the antibacterial resistance of *S. aureus* or the antibacterial resistance of *K. pneumoniae*);
4. *Mann-Whitney* U test (differences between the biofilm-forming abilities of 2 strains of *S. aureus*).
5. *McNemar* test (comparison of methods, relationships between identification of *K. pneumoniae* and *S. aureus* in brush and puncture biopsy samples).

Results were considered statistically significant if the significance level (p) value was < 0.05. Statistical analysis was performed using IBM SPSS Statistics version 26 (Chicago, IL, USA) and Microsoft Excel 10 (Microsoft, Redmond, Washington, USA).

4 Results

4.1 Analysis of microorganism colonisation, biofilm production, and antibacterial susceptibility in RT and PTA patients

The study is described in the manuscript of Renāta Klagiša, Kārlis Rācenis, Renārs Broks, Arta Olga Balode, Ligija Ķīse and Juta Kroiča *Analysis of Microorganism Colonisation, Biofilm Production, and Antibacterial Susceptibility in Recurrent Tonsillitis and Peritonsillar Abscess Patients. International Journal of Molecular Sciences.* 2022, 23, 10273. doi.org/10.3390/ijms231810273

4.1.1 Patient Data

128 patients were enrolled in this study, of which 29 patients were diagnosed with PTA, and 99 patients with RT. Study patients underwent tonsillectomy at PSCUH between 2018 and 2020. The RT patients group included 26 (26 %) men and 73 (74 %) women in the age range from 20 to 72 years old with a mean age of 32.94 years (± 11.19). The PTA patients group included 15 (52 %) men and 14 (48 %) women in the age range from 18 to 58 years old with a mean age of 32.4 years (± 12.2). Age and gender ratios of patients are provided in the Table 4.1. (Klagisa, Racenis, Broks, Balode, et al., 2022)

Table 4.1

Characteristics of the study population

Parameter		RT	PTA	p value
Gender	Males, n (%)	26 (26 %)	15 (52 %)	p = 0.061
	Females, n (%)	73 (74 %)	14 (48 %)	p = 0.061
Age	Age (range, mean ± SD), years	20–72, 32.94 ± 11.19	18–58, 32.4 ± 12.2	p = 0.279
	Age (median, IQR), years	31, 10	31, 16	–
Laboratory indicators	CRP, median, mg/L	1.17	85.5	p = 0.001
	WBC, median, ×109/L	6.52	12.97	p = 0.001

SD – standard deviation; IQR – interquartile range; CRP – C-reactive protein; WBC – white blood cell; RT – recurrent tonsillitis; PTA – peritonsillar abscess.

According to our data, women predominated in the RT group, while the gender ratio was more balanced in the PTA group (see Table 4.1). There was no age difference between patients in the RT and PTA groups (*Kruskal-Wallis* test, p = 0.617). The patients with PTA had elevated white blood cell counts (WBCs) and C-reactive protein (CRP) levels in blood samples, and patients with RT had normal WBCs and CRP levels. These differences were statistically significant (Klagisa, Racenis, Broks, Balode, et al., 2022).

4.1.2 Diversity of isolated microorganisms

Of the 128 patient samples tested, a positive cultivation result (at least 1 pathogen or potential pathogen) was detected in 60 patients (60.6 %) in the RT group and 24 patients (82.8 %) in the PTA group (Pearson χ^2 test, p = 0.027). The highest diversity of microorganisms was found in RT group patients. The cultivation result was negative, i.e. only normal oral microbiota was found in 39 patients (39.4 %) in the RT group and 5 patients (17.2 %) in the PTA group. Regardless of the patient group, the most frequently isolated pathogenic bacterium was *S. aureus*, isolated as the sole microorganism or together with other potentially pathogenic microorganisms (Klagisa, Racenis, Broks, Balode,

et al., 2022). In the RT group, *S. aureus* was isolated in 33 of 99 (33.3 %) cases, and in the PTA group, in 7 of 29 (24.14 %) cases. Gram-positive bacteria predominated, but at least one Gram-negative bacterium was detected in 22 of 99 (22.2 %) patients in the RT group and 8 of 29 (27.6 %) patients in the PTA group. The most common Gram-negative bacterium was *K. pneumoniae*; it was isolated in 10 of 99 (10.1 %) RT cases and 4 of 29 (13.4 %) PTA cases. *Candida* species were isolated and predominated in PTA patients, where they were found in 14 of 29 (48.3 %) cases and mostly as monocultures (*Fisher's* test, $p < 0.001$) (Klagisa, Racenis, Broks, Balode, et al., 2022).

4.1.3 Biofilms

At least 1 biofilm-producing strain was found in 37 of 99 (37.4 %) RT and 8 of 29 (27.6 %) PTA cases (Pearson χ^2 test, $p = 0.332$). Moderate or strong biofilm producers were detected in 16 of 37 cases of RT and in 2 of 8 cases of PTA. In the RT group, among the 33 *S. aureus* strains isolated, 5 were strong, 8 were moderate, and 15 were weak biofilm producers, but 5 were not producing biofilms at all. In the PTA group, among the 7 *S. aureus* strains isolated, 2 were weak biofilm producers, and 5 were not producing biofilms. In the RT group, among the 10 *K. pneumoniae* strains isolated, 6 were weak biofilm producers, and 4 were not producing biofilms. In the PTA group, among the *K. pneumoniae* 4 isolated strains, 1 was a weak biofilm producer, and 3 were not producing biofilms (Klagisa, Racenis, Broks, Balode, et al., 2022). The biofilm mean ODs of all isolated strains of *S. aureus* and *K. pneumoniae* are overlooked in Figure 4.1 and 4.2.

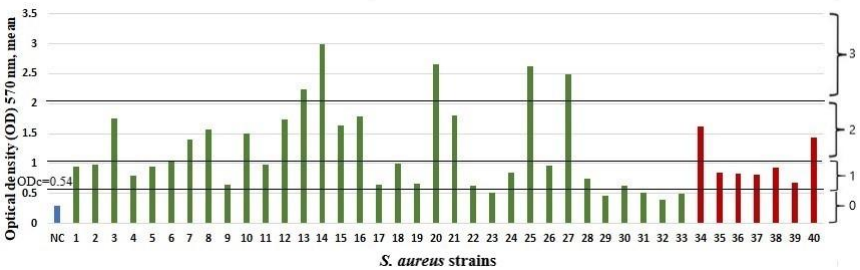

Figure 4.1 **Biofilm-production capability of *S. aureus* strains from RT and PTA patients** (Klagisa, Racenis, Broks, Balode, et al., 2022)

Green bars (1–33) represent RT patients. Red bars (34–40) represent PTA patients. Bars represent the mean OD values (measured at 570 nm wavelength). TSB with 1 % glucose was used as a negative control (NC, blue bar). The OD cut-off value (ODc) and biofilm-production-capacity levels are marked with horizontal lines. 0 – non-producers of biofilm; 1 – weak biofilm producers; 2 – moderate biofilm producers; 3 – strong biofilm producers.

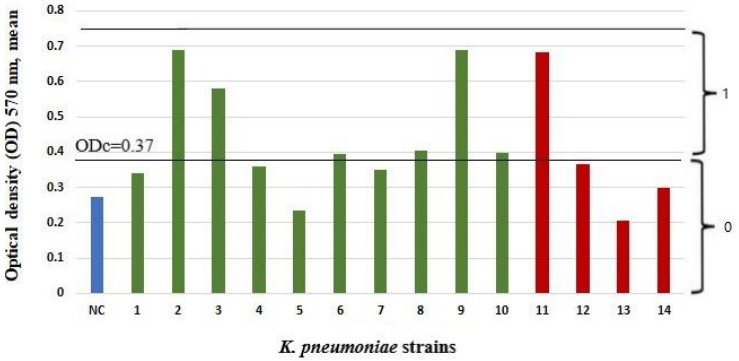

Figure 4.2 **Biofilm-production capability of *K. pneumoniae* strains from RT and PTA patients** (Klagisa, Racenis, Broks, Balode, et al., 2022)

Green bars (1–10) represent RT patients. Red bars (11–14) represent PTA patients. Bars represent the mean OD values (measured at 570 nm wavelength). LB medium as a negative control (NC, blue bar). The cut-off value (ODc) and biofilm-production-capacity levels are marked with horizontal lines. 0 – non-producers of biofilm; 1 – weak biofilm producers.

A statistically significant relationship was found between the presence of Gram-positive bacteria and the biofilm-producing phenotype in the RT group and PTA group. If a Gram-positive microbe is present, a biofilm-forming phenotype is more likely to be present as well (*Pearson* χ^2 test, $p < 0.001$). No statistically significant relationships were found between Gram-negative microbes, *Candida* spp., comorbidities, episodes of tonsillitis or history of PTA and biofilm-producing strains in the RT or PTA group. Although no statistically significant relationships were found between the presence of biofilm-forming strains or presence of *S. aureus* biofilm-forming strains and patient group, the PTA group had fewer biofilm-forming variants compared to the RT group (see Table 4.2) (Klagisa, Racenis, Broks, Balode, et al., 2022).

Table 4.2

Comparison of patients' microbiological data in the RT and PTA groups
(Klagisa, Racenis, Broks, Balode, et al., 2022)

Patients' microbiological data		**RT Group**	**PTA Group**	**p value**
Isolation rate	*S. aureus*, n (%)	33/99 (33.33 %)	7/29 (24.14 %)	p = 0.347
	K. pneumoniae, n (%)	10/99 (10.10 %)	4/29 (13.79 %)	p = 0.519
	Candida spp., n (%)	8/99 (8.08 %)	14/29 (48.28 %)	p = 0.001
Biofilms, mean OD	*S. aureus* biofilms, mean OD	1.24	1.02	p = 0.929
	K. pneumoniae biofilms, mean OD	0.44	0.39	p = 0.322
Biofilm-producing strains	Biofilm-producing strains, n	37	8	p = 0.111
	S. aureus biofilm-producing strains, n	28	7	p = 0.642
	S. aureus moderate and strong biofilm-producing strains, n (%)	13/33 (39.39 %)	2/7 (28.57 %)	p = 0.691
	K. pneumoniae moderate and strong biofilm-producing strains, n	0	0	–

Table 4.2 continued

Patients' microbiological data		RT Group	PTA Group	p value
Associations between variables by study groups	Gram-positive microbe and biofilm-producing strain	p = 0.001	p = 0.001	–
	Gram-negative microbe and biofilm-producing strain	p = 0.227	p > 0.999	–
	Candida spp. and biofilm-producing strain	p > 0.999	p = 0.215	–
	Comorbidities and biofilm-producing strain	p = 0.759	p = 0.540	–
	Episodes of tonsillitis and biofilm-producing strain	p = 0.313	p = 0.738	–
	PTA in medical history and biofilm-producing strain	p = 0.091	p = 0.640	–

4.1.4 Antibacterial Susceptibility

S. aureus strains were sensitive to cefoxitin, ceftriaxone, ampicillin/sulbactam, amoxicillin/clavulanic acid, amikacin, erythromycin, clindamycin and chloramphenicol, intermediate resistant to ciprofloxacin, resistant to benzylpenicillin and ampicillin. *S. aureus* 1 strain was identified as methicillin-resistant *S. aureus* (MRSA) as it was resistant to cefoxitin. *S. aureus* strains that were resistant to benzylpenicillin, ampicillin and at least one other antibiotic were shown in Table 4.3 and 4.4 together with their biofilm-formation ability. Resistant strains were mostly non-biofilm-forming strains or weak biofilm producers. None of the *K. pneumoniae* strains was an extended-spectrum beta-lactamase producer.

Table 4.3

Antibiotic-susceptibility and biofilm-production ability of *S. aureus* strains by patient group (Klagisa, Racenis, Broks, Balode, et al., 2022)

Patient group	Biofilm-production ability	Antibiotic resistance to BP and AMP	Absence of antibiotic resistance or antibiotic resistance to only one antibiotic	p value
RT group	0 or 1	14	6	p = 0.590
	2 or 3	9	4	p > 0.999
PTA group	0 or 1	4	1	p > 0.999
	2 or 3	1	1	p > 0.999
PTA + RT group	0 or 1	18	7	p = 0.590
	2 or 3	10	5	p > 0.999

BP, benzylpenicillin; AMP, ampicillin. Each antibiotic resistance was determined separately. Biofilm-production ability levels are marked as follow 0 – non-producers of biofilm; 1 – weak biofilm producers; 2 – moderate biofilm producers; 3 – strong biofilm producers.

Table 4.4

Antibiotic-susceptibility and biofilm-production ability of *S. aureus* strains (Klagisa, Racenis, Broks, Balode, et al., 2022)

Antibiotics	***S. aureus* strains (n = 33) of RT patients**			***S. aureus* strains (n = 7) of PTA patients**		
	Resistant	**Biofilm 0 or 1**	**Biofilm 2 or 3**	**Resistant**	**Biofilm 0 or 1**	**Biofilm 2 or 3**
BP, AMP, CIP*	20/33	12/20	8/20	5/7	4/5	1/5
BP, AMP, CIP*, CD*	1/33	1	–	–	–	–
CIP*	9/33	5/9	4/9	2/7	1/2	1/2
BP, AMP, CIP*, E	1/33	–	1	–	–	–

Table 4.4. continued

Antibiotics	*S. aureus* strains (n = 33) of RT patients			*S. aureus* strains (n = 7) of PTA patients		
	Resistant	Biofilm 0 or 1	Biofilm 2 or 3	Resistant	Biofilm 0 or 1	Biofilm 2 or 3
CIP*, E	1/33	1	–	–	–	–
FOX, CRO, BP, AMP, AMS, AUG, CIP*	1**/33	1	–	–	–	–

FOX, cefoxitin; CRO, ceftriaxone; P, benzylpenicillin; AMP, ampicillin; AMS, ampicillin/sulbactam; AUG, amoxicillin/clavulanic acid; CIP, ciprofloxacin; E, erythromycin; CD, clindamycin. *, intermediate resistance; **, MRSA; Each antibiotic resistance was determined separately. Biofilm-production ability levels are marked as follow 0 – non-producers of biofilm; 1 – weak biofilm producers; 2 – moderate biofilm producers; 3 – strong biofilm producers.

Summary: *S. aureus* was more frequently isolated in RT and PTA groups, i.e. 33.3 % in RT patients and 24.14 % in PTA patients. *K. pneumoniae* was isolated relatively less often – in 10.1 % and 13.4 % cases, respectively. Biofilm-producing strains reached 37.4 % in RT and 27.6 % in PTA groups.

S. aureus in RT and PTA groups is susceptible to empiric antibacterial therapy, with expressed susceptibility to amoxicillin with clavulanic acid and clindamycin. *S. aureus* 1 strain was identified as MRSA.

4.2 Evaluation of pathogenic microorganisms on the surface of the tonsils and in the crypts of the palatine tonsils in patients with RT

The study is described in the manuscript of Renāta Klagiša, Juta Kroiča and Ligija Ķīse *S. aureus and K. pneumoniae on the Surface and within Core of Tonsils in Adults with Recurrent Tonsillitis. Medicina* 2021, 57(10), 1002; https://doi.org/10.3390/medicina57101002

This study included 25 adult patients diagnosed with RT who underwent tonsillectomy at PSCUH between August 2020 and September 2020. The patients enrolled in the study were 4 men and 21 women aged from 20 to 71 years, with a median age of 31. There were 17 patients from Riga, 3 patients from Jelgava, and 5 patients from other Latvian cities (Engure, Bauska, Salaspils, Limbaži and Tukums) (Klagisa et al., 2021b).

S. aureus was isolated in brush samples in 12 cases and in punch biopsy samples in 9 cases. *Fisher's* exact test demonstrated a statistically significant association between isolation of *S. aureus* from brush and punch biopsy samples ($p = 0.004$). *K. pneumoniae* was isolated in brush samples in 4 cases and in punch biopsy samples in 5 cases. *Fisher's* exact test has demonstrated a statistically significant association between isolation of *K. pneumoniae* from brush and punch biopsy samples ($p < 0.001$). *McNemar* test used to compare the accuracy of the two methods did not show any statistically significant differences (Klagisa et al., 2021b).

S. aureus isolated from punch biopsy samples were tested for biofilm-producing ability (see Table 4.5). Of the 9 *S. aureus* isolates, 5 ones had biofilm-producing ability, 2 isolates were strong biofilm producers, 3 were weak biofilm producers, and 4 of the 9 *S. aureus* isolates did not produce any biofilm at all.

Of the 5 *K. pneumoniae* isolates, 4 had weak biofilm-producing ability and 1 of the 5 *K. pneumoniae* isolates was a non-biofilm producer (see Table 4.5) (Klagisa et al., 2021b).

Table 4.5

Characterisation of biofilm production and antibacterial susceptibility of *S. aureus* and *K. pneumoniae* strains obtained by punch biopsy during tonsillectomy (Klagisa et al., 2021b)

Strains	Biofilms		Antibacterial Susceptibility			
	Biofilm-producing strains (n)	Biofilm non-producing strains (n)	BP	AMP	CIP	E
Sa	5 (9)	4 (9)	R 7 (9)	R 7 (9)	I 9 (9)	0 (9)
Kp	4 (5)	1 (5)	R 5 (5)	R 5 (5)	0 (5)	R 5 (5)

Sa, *S. aureus;* Kp, *K. pneumoniae*; BP, benzylpenicillin; AMP, ampicillin; CIP, ciprofloxacin; E, erythromycin, R, resistant; I, sensitive at elevated concentrations.

S. aureus and *K. pneumoniae* isolates identified in punch biopsy samples were tested for antibacterial susceptibility. Of the 9 *S. aureus* isolates, 7 isolates were resistant to benzylpenicillin and ampicillin; 5 out of 7 isolates were biofilm producers. All *S. aureus* isolates were sensitive to ampicillin/sulbactam, amoxicillin/clavulanic acid, amikacin, erythromycin, clindamycin, chloramphenicol and, intermediate resistant to ciprofloxacin. All *K. pneumoniae* isolates were resistant to benzylpenicillin, ampicillin and erythromycin, but sensitive to ampicillin/sulbactam, amoxicillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, cefotaxime, ceftriaxone, meropenem, imipenem, amikacin, and ciprofloxacin (Klagisa et al., 2021b).

There was no statistically significant association between RT recurrence rate and comorbidities (*Fisher's* exact test, $p = 0.542$); RT recurrence rate and the presence of *S. aureus* in punch biopsy samples (*Fisher's* exact test, $p = 0.260$), or the presence of *K. pneumoniae* in punch biopsy samples (*Fisher's* exact test, $p > 0.999$); RT recurrence rate and biofilm production ability of *S. aureus* (*Fisher's* exact test, $p = 0.238$), or biofilm production ability of *K. pneumoniae* (*Fisher's* exact test, $p = 0.617$); RT recurrence rate and antibacterial resistance of *S. aureus* (*Fisher's* exact test, $p = 0.294$), or

antibacterial resistance of *K. pneumoniae* (*Fisher's* exact test, $p > 0.999$) (Klagisa et al., 2021b).

The palatine tonsils after tonsillectomy was sent for histopathological examination. In all patients, the histopathological examination confirmed chronic non-specific tonsillitis (Klagisa et al., 2021b).

Summary: The material obtained from the surface of the palatine tonsils is less reliable since it is highly contaminated with the microbiota of the oral cavity. The most common RT agents, i.e. *S. aureus* and *K. pneumoniae*, were isolated from both the surface of the tonsils and the tonsil crypts. Gram-positive (5 of 9) and Gram-negative (4 of 5) bacteria isolated from crypts were biofilm-producing strains.

4.3 Assessment of biofilm production, antibacterial susceptibility of pathogenic microorganisms obtained from the crypts of the palatine tonsils of healthy individuals

The study is described in the manuscript of Renāta Vīksne, Kārlis Rācenis, Renārs Broks, Arta Olga Balode, Ligija Ķīse and Juta Kroiča *In Vitro Assessment of Biofilm Production, Antibacterial Resistance of Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Obtained from Tonsillar Crypts of Healthy Adults. Microorganisms.* 2023; 11(2):258. https://doi.org/10.3390/microorganisms11020258

4.3.1 Patient data

The study group included 52 females (57 %) and 39 males (43 %) aged 19–29 years old (mean value 21.2 ± 1.41 years, median value 21 years) (Viksne et al., 2023).

4.3.2 Diversity of isolated microorganisms

Of the 91 participant samples, a positive cultivation result (at least 1 pathogen or potential pathogen) was found in 54 participant samples (59.3 %) (see Table 4.6) (Viksne et al., 2023).

Table 4.6

Microorganisms isolated from palatine tonsillar crypts of 91 healthy individual (Viksne et al., 2023)

Combinations of Isolated Strains	Count (n)
Normal oral microbiota	37
S. aureus + normal oral microbiota	20
S. aureus	16
S. aureus + *A. junii*	2
S. aureus + *K. pneumoniae*	1
S. aureus + *Candida* spp. + *S. viridans*	1
S. aureus + *K. pneumoniae* + *S. liquefaciens* + normal oral microbiota	1
K. pneumoniae	5
P. aeruginosa	2
A. pittii	2
A. johnsonii	1
S. liquefaciens	1
S. dysgalactiae	1
A. ewofii + normal oral microbiota	1

The cultivation results were negative, i.e. only normal oral microbiota was present in 37 participant samples (40.7 %) (see Table 4.6). The most frequently isolated pathogenic bacterium was *S. aureus*, isolated as the sole microorganism or together with other potentially pathogenic microorganisms in 41 participant samples (45 %) (see Table 4.6). Gram-positive bacteria predominated, but at least 1 Gram-negative bacterium was found in 16 samples (17.6 %). Among the Gram-negative bacteria, *K. pneumoniae* was the most common one; it was isolated in 7 samples (Viksne et al., 2023).

4.3.3 Biofilms

Biofilm-production ability was observed in 41 *S. aureus* strains and 15 Gram-negative bacteria strains. *S. aureus* mainly presented biofilm-producing strains: 25 of 41 (61 %) *S. aureus* strains were moderate or strong biofilm producers, and 14 of 41 (34.1 %) *S. aureus* strains were weak biofilm producers, while 2 of 41 (4.9 %) strains did not produce biofilms (see Figure 4.3) (Viksne et al., 2023).

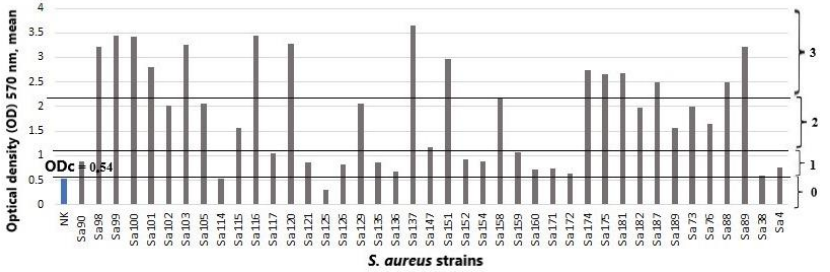

Figure 4.3 **Biofilm-production ability of 41 *S. aureus* strains** (Viksne et al., 2023)

Bars represent the mean OD values. TSB with 1 % glucose is a negative control (NC). The number represents the participant; Sa – *Staphylococcus aureus*. The cut-off value (ODc) and biofilm-production-capacity: 0 – non-producers of biofilm; 1 – weak biofilm producers; 2 – moderate biofilm producers; 3 – strong biofilm producers.

Among the Gram-negative bacteria, 6 out of 15 (40 %) bacteria were moderate to strong biofilm producers, and 6 out of 15 (40 %) strains were weak biofilm producers, while 3 out of 15 (20 %) strains did not produce biofilms (see Figure 4.4) (Viksne et al., 2023).

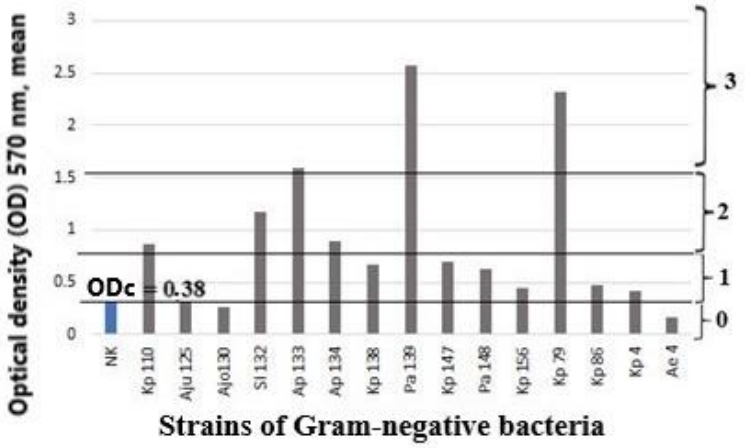

Figure 4.4. **Biofilm-production ability of 15 Gram-negative bacteria strains** (Viksne et al., 2023)

Bars represent the mean OD values (measured at 570 nm wavelength). LB medium was used as a negative control (NC). The number represents the participant; the letters indicate the strain: Kp – *Klebsiella pneumoniae*; Aju – *Acinetobacter junii*; Ajo – *Acinetobacter johnsoni*; Sl – *Serratia liquefaciens*; Ap – *Acinetobacter pitti*; Pa – *Pseudomonas aeruginosa*; Ae – *Acinetobacter ewoffi.* The cut-off value (ODc) and biofilm-production-capacity levels are marked with horizontal lines: 0 – non-producers of biofilm; 1 – weak biofilm producers; 2 – moderate biofilm producers; 3 – strong biofilm producers.

A summary of the microbiological data of the study participants is shown in Table 4.7. There was a statistically significant correlation between the presence of the Gram-positive bacteria and the biofilm-formation phenotype. If a Gram-positive microbe is present, a biofilm-forming phenotype is more likely to be present as well (*Pearson* χ^2 test, $p < 0.001$) (see Table 4.7) (Viksne et al., 2023).

Table 4.7

Summary of microbiological data of the study participants
(Viksne et al., 2023)

Microbiological data		**Result**	**p value**
Isolation rate	Only normal oral microbiota, n (%)	37/91 (40.7 %)	–
	Gram-positive strains, n	43	–
	Gram-negative strains, n	17	–
Biofilms, mean OD	*S. aureus* biofilms, mean OD	1.89	–
	Gram-negative microbe biofilms, mean OD	0.95	–
Biofilm-producing strains	Biofilm-producing strains, n	51	–
	S. aureus biofilm-producing strains, n	39	–
	Gram-negative microbe biofilm-producing strains, n	12	–
	Strong and moderate biofilm-producing strains, n	31	–
Associations between variables	Gram-positive microbe and biofilm-producing strain	–	$p < 0.001$
	Gram-negative microbe and biofilm-producing strain	–	$p = 0.808$

4.3.4 Antibacterial Susceptibility

The tested *S. aureus* strains were sensitive to cefoxitin, ceftriaxone, ampicillin/sulbactam, amoxicillin/clavulanic acid, norfloxacin, amikacin, clindamycin, chloramphenicol, but resistant to benzylpenicillin and ampicillin in 75.6 % and erythromycin in 14.6 % of cases (see Table 4.8). Of all the *S. aureus* strains, 1 strain was determined as MRSA, as it was resistant to cefoxitin (Viksne et al., 2023).

Table 4.8

Antibacterial resistance among *S. aureus* strains isolated from healthy individuals (Viksne et al., 2023)

Strains (n)		Antibacterial resistance (%)					
		FOX, CRO	**BP, AMP**	**AMS, AUG, NOR, AK**	**E**	**CD**	**C**
S. aureus	41	2.4	75.6	2.4	14.6	0	4.9

FOX, cefoxitin; CRO, ceftriaxone; BP, benzylpenicillin; AMP, ampicillin; AMS, ampicillin–sulbactam; AUG, amoxicillin–clavulanic acid; NOR, norfloxacin; AK, amikacin; E, erythromycin; CD, clindamycin; C, chloramphenicol.

Gram-negative bacteria were sensitive to all tested antibiotics (see Table 4.9). Only 1 *Acinetobacter junii* strain was resistant to amikacin. None of the *K. pneumoniae* strains was an extended-spectrum beta-lactamase (ESBL) producer. No statistically significant correlations were found between antibacterial susceptibility and biofilm-production ability (Viksne et al., 2023).

Table 4.9

Antibacterial resistance among *K. pneumoniae, P. aeruginosa, Serratia liquefaciens* and *Acinetobacter spp.* isolated from healthy individuals (Viksne et al., 2023)

Strains (n)		Antibacterial resistance (%)											
		AUG	**TZP**	**CTX**	**CAZ**	**ETP**	**IMP**	**MEM**	**CIP**	**GM**	**SXT**	**AK**	**LEV**
Kp	7	0	0	0	0	0	0	0	0	0	0	–	–
Pa	2	–	0	–	0	–	0	0	0	–	–	0	–
A. spp.	5	–	–	–	–	–	0	–	0	0	0	20	0
Sl	1	0	0	0	0	0	0	0	0	0	0	–	–

Kp, *Klebsiella pneumoniae*; Pa, *Pseudomonas aeruginosa*; A, *Acinetobacter*; Sl, *Serratia liquefaciens*; AUG, Amoxicillin–clavulanic acid; TZP, Piperacillin – Tazobactam; CTX, Cefotaxime; CAZ, Ceftazidime; ETP, Ertapenem; IMP, Imipenem; MEM, Meropenem; CIP, Ciprofloxacin; GM, Gentamicin; SXT, Trimethoprim/sulfamethoxazole; AK, Amikacin; LEV, Levofloxacin.

Summary: Palatine tonsils of healthy people are colonised mainly by Gram-positive microorganisms, i.e. *S. aureus* in 45 % of cases. Gram-negative microorganisms also colonise tonsils, for example *K. pneumoniae* 7 %, *Acinetobacter* spp. 5.5 %. These agents may be in the aetiology of an opportunistic infection. It has been shown that 95 % of the identified *S. aureus* strains are biofilm producers. Biofilms represent a naturally produced form of pathogenic bacteria colonising healthy human tissues. Gram-negative bacteria formed biofilms much less frequently. All bacteria isolated from the palatine tonsils of healthy individuals were sensitive to antibiotics.

4.4 Evaluation of *Staphylococcus aureus* colonisation in patients undergoing tonsillectomy for RT

The study is described in the manuscript of Renāta Klagiša, Kārlis Rācenis, Renārs Broks, Ligija Ķīse and Juta Kroiča Evaluation of Staphylococcus aureus Colonisation in Adult Patients Undergoing Tonsillectomy for Recurrent Tonsillitis. Pathogens 2022, 11, 427. https://doi.org/10.3390/pathogens11040427

The study included 16 patients, of whom 8 were females and 8 were males, aged 21 to 50 years old, with a mean age of 29 years (± 7.23), 7 patients lived in Riga, the rest of the patients lived in other Latvian cities (Klagisa, Racenis, Broks, Kise, et al., 2022).

The bacterium most frequently isolated from tonsillar crypts was *S. aureus*, which was the only microorganism isolated in 6 patients, and one isolated together with normal oral microbiota or other potentially pathogenic microorganisms in 10 patients. There were 16 *S. aureus* strains isolated and tested for biofilm-production ability, and 15 of the 16 were biofilm producers. In addition, 1 of the strains was a strong biofilm producer, 5 of 16 strains were

moderate and 9 of 16 were weak biofilm producers, and 1 *S. aureus* strain did not produce any biofilm (Klagisa, Racenis, Broks, Kise, et al., 2022).

One year after tonsillectomy, 8 *S. aureus* strains were isolated from 6 of 16 patients – from the throat culture in 3 of 16 patients, from the nasal vestibule in 4 of 16 patients, and from armpit samples in 1 of 16 patients. Of the throat samples, 1 of 3 strains was a firm biofilm producer and 2 of 3 *S. aureus* strains were weak biofilm producers. Of the nasal vestibule samples, 1 of 4 strains was a moderate biofilm producer, 1 of 4 strains was a weak biofilm producer, and 2 of 4 *S. aureus* strains did not form any biofilm. From the armpit samples, 1 *S. aureus* strain did not form any biofilm. Only 1 patient had *S. aureus* in throat, nasal, and armpit samples and it was a weak biofilm producer (Klagisa, Racenis, Broks, Kise, et al., 2022).

S. aureus was only isolated during tonsillectomy in 10 patients. In these cases, *S. aureus* was the agent causing RT. After tonsillectomy, *S. aureus* was present in the throat or nasal vestibule, but *S. aureus* strains were found to have different biofilm production capacities in 4 patients. In these cases, patients were predisposed to *S. aureus* colonisation. *S. aureus* with the same biofilm production capacity was isolated from the palatine tonsils during tonsillectomy and 1 year after tonsillectomy from the throat, nasal vestibule and armpit of 2 patients. In these cases, *S. aureus* was part of the patients' microbiota (Klagisa, Racenis, Broks, Kise, et al., 2022).

Figure 4.5 represents biofilm formation of 11 *S. aureus* strains in a 96-well microplate. The crystal violet absorbance is proportional to the adhesion cells and biofilm concentration.

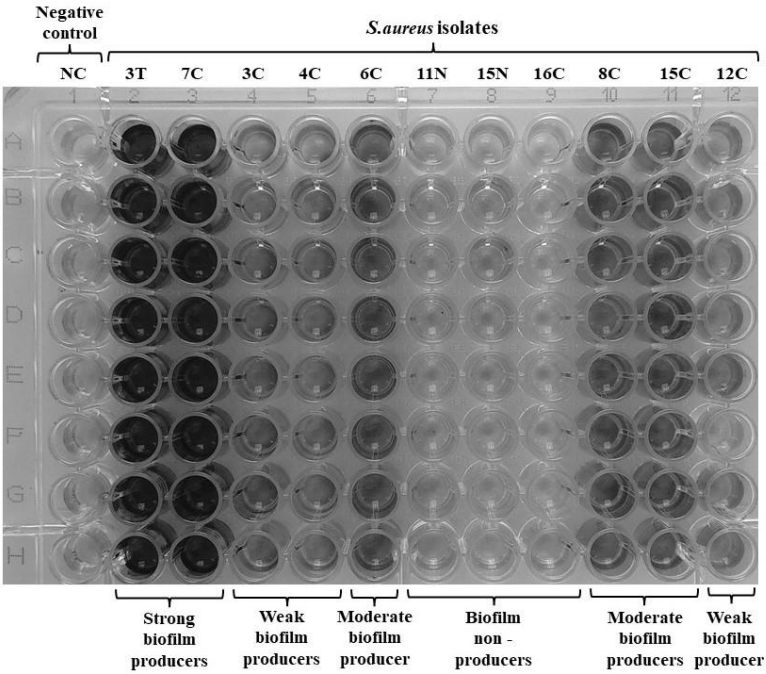

Figure 4.5 **Biofilm formation of 11 *S. aureus* strains in 96-well microplate** (Klagisa, Racenis, Broks, Kise, et al., 2022)

The plate contains 11 strains and a negative control (sterile broth) with 8 wells for each strain. Staining was carried out with crystal violet dye, which distinguishes a strong (3T, 7C), moderate (6C, 8C, 15C), weak (3C, 4C, 12C) degree of biofilm production and non-producers of biofilm (11N, 15N, 16C).

The crystal violet dye attached to the cells forming biofilms on microplates was quantified. The OD of the bacterial biofilm was measured at a wavelength of 570 nm with a microplate spectrophotometer. The OD value of each strain was expressed as a number. All mean OD values of isolated strain biofilms are summarised in Figure 4.6.

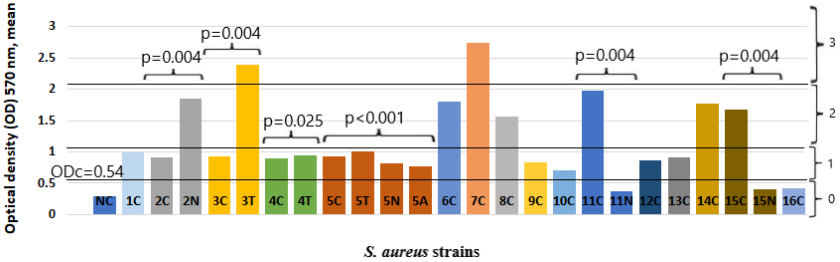

Figure 4.6 **Biofilm-production ability of 24 *S. aureus* strains** (Klagisa, Racenis, Broks, Kise, et al., 2022)

Bars represent the mean OD values (measured at 570 nm wavelength). TSB with 1 % glucose represents a negative control (NC). The cut-off value (ODc) and biofilm-production-capacity levels are marked with horizontal lines: 0 – non-producers of biofilm; 1 – weak biofilm producers; 2 – moderate biofilm producers; 3 – strong biofilm producers. Differences between biofilm-forming abilities of *S. aureus* strains were analysed using *Mann-Whitney* U test for the analysis of 2 strains and *Kruskal-Wallis* test for the analysis of 4 isolates, and p-values were provided as a result. *S. aureus* strain code is the number designating the patient, but the letter means the carriage site (C – tonsillar crypts; T – throat; N – nasal cavity: A – armpits).

Resistance to benzylpenicillin and ampicillin had 14 isolates. As MRSA was identified 1 isolate as it was resistant to cefoxitin. All isolates were intermediate resistant to ciprofloxacin, while 1 isolate was also intermediate resistant to clindamycin (see Table 4.10) (Klagisa, Racenis, Broks, Kise, et al., 2022).

Table 4.10

Antibiotic resistance among *S. aureus* isolated from RT patients
(Klagisa, Racenis, Broks, Kise, et al., 2022)

		Antibiotics										
		FOX	**CRO**	**BP**	**AMP**	**AMS**	**AUG**	**CIP**	**AK**	**E**	**CD**	**C**
***S. aureus* strains**	**1C**	S	S	R	R	S	S	I	S	S	S	S
	2C	S	S	S	S	S	S	I	S	S	S	S
	2N	S	S	R	R	S	S	I	S	S	S	S
	3C	S	S	R	R	S	S	I	S	S	S	S
	3T	S	S	S	S	S	S	I	S	S	S	S
	4C	R	R	R	R	R	R	I	S	S	S	S
	4T	S	S	R	R	S	S	I	S	S	S	S
	5C	S	S	R	R	S	S	I	S	S	S	S
	5T	S	S	S	S	S	S	I	S	S	S	S
	5N	S	S	S	S	S	S	I	S	S	S	S
	5A	S	S	S	S	S	S	I	S	S	S	S
	6C	S	S	R	R	S	S	I	S	S	S	S
	7C	S	S	R	R	S	S	I	S	S	S	S
	8C	S	S	R	R	S	S	I	S	S	S	S
	9C	S	S	R	R	S	S	I	S	S	S	S
	10C	S	S	R	R	S	S	I	S	S	S	S
	11C	S	S	S	S	S	S	I	S	S	S	S
	11N	S	S	S	S	S	S	I	S	S	S	S
	12C	S	S	R	R	S	S	I	S	S	S	S
	13C	S	S	R	R	S	S	I	S	S	I	S
	14C	S	S	S	S	S	S	I	S	S	S	S
	15C	S	S	R	R	S	S	I	S	S	S	S
	15N	S	S	R	R	S	S	I	S	S	S	S
	16C	S	S	S	S	S	S	I	S	S	S	S

FOX, cefoxitin; CRO, ceftriaxone; BP, benzylpenicillin; AMP, ampicillin; AMS, ampicillin–sulbactam; AUG, amoxicillin–clavulanic acid; CIP, ciprofloxacin; AK, amikacin; E, erythromycin; CD, clindamycin; C, chloramphenicol. J, susceptible; R, resistant; I, intermediate resistant.

Summary: *S. aureus* isolated from tonsillar crypts had a higher biofilm-production capacity compared to isolates from other body sites. One year after tonsillectomy, *S. aureus* was not found in 10 of 16 patients. Tonsillectomy significantly reduces carriage of *S. aureus* and is an effective RT treatment.

5 Discussion

The mechanisms of the pathogenesis of RT are widely studied all over the world, research is being conducted in the field of both microbiology and immunology in search of objective biomarkers to identify patients with RT. Current RT diagnostic methods are based on the clinical picture only and routine microbiological testing does not reflect the situation in the tonsils. Currently, the main decisive factor when considering indications for tonsillectomy is the frequency of episodes of tonsillitis during the previous 3 years (*Paradise* criteria). Due to the lack of objective biomarkers in clinical practice, difficulties arise, for example, it takes 1–3 years to make a diagnosis of RT and decide on the need for tonsillectomy, and in scientific studies, for example, the inclusion criteria for RT patients in a study are based on anamnesis data, records in the corresponding medical documentation.

After analysing the literature, we concluded that there are several studies comparing the data of RT patients and healthy individuals, but no significant differences were found as a result, and some extensive microbiological studies of PTA were carried out as well, but without a comparison group. Therefore, 3 groups were studied in the Doctoral Thesis: RT, PTA patients and healthy individuals.

The pathogenesis of RT is not entirely clear, however, bacteria with antibiotic resistance mechanisms are known to be involved. In our study, tonsil samples from RT and PTA patients were obtained by punch biopsy needle, avoiding the normal oral microbiota, and bacteriologically tested for objective markers.

Bacteriological examination from highly colonised areas is quite challenging, with positive cultures in 60 (60.6 %) RT patients, 24 (82.8 %) PTA patients, and 54 (59.3 %) healthy individuals. There was a wide variety of microorganisms in the studied groups, Gram-positive microorganisms

predominated, and the most frequently isolated pathogenic bacterium was *S. aureus*; only in the PTA group was a high frequency of *Candida* spp. Broad antibacterial resistance of *S. aureus* isolates was not found in any of the studied groups, however, a pronounced ability to form biofilms was observed for *S. aureus* isolates from the tonsils of healthy individuals. It leads to the conclusion that mucosal biofilms are a natural existence form of bacteria and do not indicate tonsillopathy, unlike biofilm studies associated with medical prostheses or foreign bodies.

Antibacterial testing was performed according to EUCAST standards, the isolates were tested for many antibiotics to characterise the antibacterial resistance of the isolates, despite the fact that in clinical practice such antibiotics are not used in RT therapy, for example, in the case of *S. aureus*, cefoxitin to characterise resistance to methicillin, and Gram-negative bacteria in the case of meropenem to decide on further testing for carbapenemases.

5.1 Analysis of microorganism colonisation, biofilm production, and antibacterial susceptibility in RT and PTA patients

RT and PTA are diseases with different clinical presentation, course of the disease, outcome and prognosis. Both diseases have common features; firstly, they are often found in the practice of otorhinolaryngologists, secondly, bacteria are most often their aetiological factor, and thirdly, if antibiotic therapy is ineffective, both diseases can be successfully treated surgically. A successful treatment plan for both infectious diseases may be related to their similar aetiology (Zautner et al., 2010; Klagisa, Racenis, Broks, Balode, et al., 2022).

Streptococcus pyogenes (*S. pyogenes*) is the most common cause of acute bacterial tonsillitis in immunocompetent adults. It is assumed that acute tonsillitis has 1 aetiological factor, but the aetiology of RT is multifactorial in the presence of 2 and several types of bacteria (Babaiwa, 2013; Katkowska

et al., 2017). In our study, it was noted that patients with RT had a high diversity of microorganisms, polycultures prevailed. This fact has also been observed in other diseases, for example, in gingivitis (Jorgensen et al., 2015; Klagisa, Racenis, Broks, Balode, et al., 2022).

In our study, the isolation frequency of *Streptococcus* spp. was low; 17 strains of streptococci were isolated in the case of RT, and 3 strains of streptococci in the case of PTA. *S. pyogenes* strains were isolated with other microorganisms in 4 patients with RT, but not in any patient with PTA. Also, in other studies, a low (1.7–5 %) frequency of streptococci isolation in patients with RT has been found, it has been proven that they are more often found on the surface of the palatine tonsils than in the tonsils (Syryło et al., 2007; Alasil et al., 2013). The role of *S. pyogenes* in the pathogenesis of RT has been overestimated or has been decreased over the years (Lindroos, 2000; Zautner et al., 2010; Kostić et al., 2022). In RT and PTA patients, the use of rapid antigen detection test for streptococci and the determination of antistreptolysin O (ASO) are not useful due to the low percentage of *S. pyogenes*. The frequency of microorganism isolation depends on the method of obtaining the sample. In a study conducted by *Zautner et al*. (2010) the tonsillar cell suspensions were analysed and it was found that *S. aureus* prevailed in patients with RT (57.7 %), and *S. pyogenes* prevailed in patients with PTA (20.2 %) (Zautner et al., 2010). In a study conducted by *Vaikjärv et al.* (2016) it was found that in the case of PTA abscess, biopsies of palatine tonsil niches represent better material for microbiological analysis than PTA pus material as they reveal more bacteria in one culture (Vaikjärv et al., 2016). *Streptococcus* spp. was the most common pathogen found in both tonsillar fossa biopsy samples and pus samples, while *Staphylococcus* spp. was the most common pathogen in tonsillar fossa biopsy samples, but no staphylococci were found in pus samples (Vaikjärv et al., 2016). In our study, we have chosen tonsillar crypt biopsies so that the obtained material

would be informative and the RT and PTA groups would be mutually comparable (Klagisa, Racenis, Broks, Balode, et al., 2022).

In our study, *Streptococcus anginosus (S. anginosus)* was only found in RT patients but not in PTA patients. Another study has demonstrated that bacteria of the *S. anginosus* group were more common in patients with recurrent PTA than in patients with cured PTA. The authors of the study declare that the presence of bacteria of the *S. anginosus* group is associated with the PTA renewal (Wikstén et al., 2017; Klagisa, Racenis, Broks, Balode, et al., 2022).

Our study has revealed high *Candida* spp. frequency. In RT patients *Candida* spp. was isolated in 8.08 % (n = 8) cases, but in PTA patients in 48.23 % (n = 14) cases. In other studies, *Candida* spp. has demonstrated lower isolation frequency. In a study conducted by *Katkowska et al.* (2017) *Candida* spp. was found in tonsillar tissue samples of RT patients in 2.5 % of cases, in tonsillar surface samples in 8.3 % of cases, in throat samples prior to tonsillectomy in 9.3 % of cases (Katkowska et al., 2017). In a study conducted by *Zautner et al.* (2010) it was reported, that in the tonsillar tissues of RT patients, *Candida* spp. was found in 12.8 % of cases, but in PTA patients – in 4.9 % of cases (Zautner et al., 2010). *Slouka et al.* (2020) reported that *Candida* spp. was detected in PTA pus aspirates in 2.3 % of cases (Slouka et al., 2020). The results of our study resemble those of *Jokinen et al.* (1976), where in 147 patients with chronic tonsillitis, *Candida albicans (C. albicans)* was found in 41.4 % of cases, while in healthy individuals the frequency of *C. albicans* was 51.5 % (Jokinen et al., 1976). Due to such a high isolation frequency, the pathogenicity of the fungi was also evaluated by histological examination of the samples. The histological evaluation did not reveal signs of fungal pathogenicity, as fungi were found in the tonsillar crypts only, without granulomatous inflammation seen around them (Jokinen et al., 1976). Another study assessed the palatine tonsil mycobiomes between individuals with and without human

immunodeficiency virus (HIV) (Fukui et al., 2018). The palatine tonsil mycobiome does not differ significantly between individuals with HIV and individuals without HIV infection (Fukui et al., 2018). Based on the results of our study, we can conclude that *Candida* spp. could be a microbiological indicator for the PTA group. *Candida* spp. may be an indicator of a chronic course of the process when, firstly, the underlying problem, which may be bacterial or structural, should be addressed and, secondly, antifungal therapy should be considered, only if the underlying problem does not respond to antibacterial therapy (Klagisa, Racenis, Broks, Balode, et al., 2022).

Gram-negative microorganisms were not playing a significant role in this study. *K. pneumoniae* is known to be a potent biofilm producer (Wang et al., 2020), but in our study, *K. pneumoniae* was not only rarely detected, but also showed low biofilm-producing ability. None of the isolated *K. pneumoniae* strains was a potent biofilm producer. It is known that *Haemophilus influenzae (H. influenzae)* can be isolated from tonsils, but in our study, *H. influenzae* was isolated in only 2 cases in the RT patient group. There was no statistically significant relationship found between the presence of Gram-negative microorganisms and biofilm-producing strains in the tonsils of either patients with RT or patients with PTA (Klagisa, Racenis, Broks, Balode, et al., 2022).

In patients with PTA, group A *Streptococcus* spp. was more often isolated in winter and spring than in summer (Klug, 2017). Seasonal differences were not found in our study. Inflammation indicators such as white blood cells (WBCs), CRP were statistically significantly increased in PTA patients compared to RT patients. PTA patients were included in the study at the stage of acute inflammation, explaining the high WBCs and CRP values(Klagisa, Racenis, Broks, Balode, et al., 2022).

S. aureus strains have shown resistance to benzylpenicillin and ampicillin, which has also been observed in other studies. For instance, in a study conducted by *Katkowska et al.* (2017), *S. aureus* isolates of RT patients were resistant to penicillin in 79 % of cases and to ampicillin in 63.2 % of cases, but 1 S. aureus isolate was MRSA (Katkowska et al., 2017). In our study, we have not observed a relationship between antibacterial resistance and intensity of biofilm production. Although several studies have suggested that increased biofilm production is associated with greater antibacterial resistance, *Ma et al.* (2019) have demonstrated in their study that increased biofilm production leads to the opposite effect, a decrease in antibacterial tolerance (Ma et al., 2019). The data of our study do not support the hypothesis of the predominance of biofilm-producing strains in the PTA patients' samples. In contrast, of the 7 *S. aureus* isolates, 2 were weak biofilm producers and 5 were non-biofilm producers in the PTA patients' group. These results suggest that *S. aureus* isolated may be a wild-type strains rather than an endogenous infection agents (Klagisa, Racenis, Broks, Balode, et al., 2022).

Biofilms are characterised as a community of bacteria surrounded by a matrix of self-produced extracellular polymeric substances (Forson et al., 2020). Research is being conducted to understand the interaction between microorganisms in biofilms. Interaction between *C. albicans* and *Staphylococcus spp.* appears to be mutually synergistic and is increasingly being reported (Schnurr et al., 2021). Research data demonstrate that fungal cells are able to modulate the activity of antibiotics, and bacteria can influence antifungal activity in mixed fungal-bacterial biofilms (Adam et al., 2002). Prostaglandin E2 secreted by *C. albicans* promotes the growth of *S. aureus* in mixed biofilms (Krause et al., 2015). Chronic inflammation of the palatine tonsils can be explained by the protective interaction of the bacterial community and a large variety of microorganisms (Klagisa, Racenis, Broks, Balode, et al., 2022).

5.2 Evaluation of pathogenic microorganisms on the surface of the tonsils and in the crypts of the palatine tonsils in patients with RT

The incidence of surface and core isolates of palatine tonsil samples, the reliability of the results of the obtained cultures have been analysed in many studies (Brook et al., 1980; Lindroos, 2000; Khadilkar & Ankle, 2016; Sarkar et al., 2017; Dickinson et al., 2020). Accurate identification of the causative agent of tonsillitis allows to improve the choice of antibiotic therapy and avoid elective tonsillectomy (Sarkar et al., 2017). In the study, much attention was paid to the analysis of *S. aureus*, since it was the most frequently isolated pathogen, and other studies have also proven that *S. aureus* plays a significant role in the pathogenesis of RT, since *S. aureus* is able to remain both in mucosal biofilms, and exist intracellularly (Zautner et al., 2010; Zautner, 2012; Stępińska et al., 2014; Cavalcanti et al., 2019). Studies have confirmed the ability of *K. pneumoniae* to form persistent biofilms. *K. pneumoniae* biofilms facilitate colonisation of respiratory tract, nasopharynx, and tonsils (Alasil et al., 2013; Wang et al., 2020). *Sarkar et al.* (2017) in their study have found that *S. aureus*, group A β-hemolytic streptococci and *Klebsiella* spp. are the most common isolates in both tonsil surface samples and tonsil biopsy samples, with a higher presence of these microorganisms in the tonsils (Sarkar et al., 2017). In our study, it has been demonstrated that *S. aureus* was more common in brush samples obtained from tonsil crypts, and *K. pneumoniae* was more common in tonsillar samples obtained with a punch biopsy needle (Klagisa et al., 2021b).

5.3 Assessment of biofilm production, antibacterial susceptibility of pathogenic microorganisms obtained from the crypts of the palatine tonsils of healthy individuals

The oral cavity and pharynx have many niches for bacterial colonisation. Since infection of the palatine tonsils is more likely to arise from bacteria within the crypts or parenchyma of the palatine tonsils than from bacteria on the surface, we have investigated the microbiota of the crypts of the palatine tonsils as the most important cause of tonsillopathy (Khadilkar & Ankle, 2016; De Martin et al., 2021). In our study of healthy individuals, we have isolated and analysed important pharyngeal and respiratory pathogens – *S. aureus*, *K. pneumoniae*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter* spp. (Hamilos, 2019; Zaatout, 2021; Viksne et al., 2023).

The primary ecological niche of *Staphylococcus* spp. is the nostrils; however, the oral cavity is an important reservoir of these bacteria, and exclusive oral colonisation has been observed in some adult patients (Kearney et al., 2020). In a study by *Albrich & Harbarth* (2008), colonisation of extranasal areas was associated with persistent carriage of *S. aureus* (Albrich & Harbarth, 2008). In the study conducted by *Hanson et al.* (2018) in the USA, it was reported that 6.2 % of adults carried *S. aureus* only in the nasal vestibule, 18.6 % of adults only in the oropharynx, and 19.8 % had it in both sites (Hanson et al., 2018). It has been reported that the number of oral carriers of *S. aureus* reaches from 17 to 48 % in student populations (Smith et al., 2003; Blomqvist et al., 2015). Among healthy Swedish dental students, the prevalence of *S. aureus* was 44.6 %; MRSA was not found among them (Blomqvist et al., 2015). Healthcare workers were found to have MRSA in 23.7 % cases (Albrich & Harbarth, 2008). Studies by other authors mention that the prevalence of MRSA among healthy carriers ranges from 1.5 to 26 % (Petti & Polimeni, 2011; Roberts et al., 2011; Laheij et al., 2012). In healthy subjects in our study, *S. aureus* was the most common

isolated pathogen; it was isolated in 45 % of cases, and MRSA in 1.1 % of cases, which is consistent with the results of previous studies (Viksne et al., 2023).

In a study conducted by *Jeong et al.* (2007), *K. pneumoniae* was isolated from the tonsillar core samples of RT patients in 6.7 % of cases, and in the case of palatine tonsil hypertrophy patients – in 1.5 % of cases (Jeong et al., 2007). Our study has demonstrated that *K. pneumoniae* was also present in the tonsillar crypt samples of healthy individuals in 7.7 % of cases (Viksne et al., 2023).

Several studies have analysed the role of extracellular or intracellular *P. aeruginosa* in the origins of periodontal or lung disease (Mirzaei et al., 2020; Li et al., 2021). Other studies have reported a prevalence of *P. aeruginosa* in 1.4–3.8 % of tonsillar samples of RT patients (Loganathan et al., 2006; Jeong et al., 2007; Al Ahmary et al., 2012) and a prevalence of *P. aeruginosa* in 0.9 % of tonsillar samples of tonsillar hypertrophy patients (Jeong et al., 2007). Our study showed that *P. aeruginosa* was present in 2.2 % of cases in tonsillar crypt samples of healthy individuals. *Acinetobacter baumannii* was not detected among the *Acinetobacter* isolates. The diversity and prevalence of pathogen isolation from palatine tonsil samples may vary depending on the sampling method; for example, smears from the surface of the tonsils may be less informative than material obtained from the crypts of the tonsils (Klagisa et al., 2021b; Viksne et al., 2023).

The crypts of the palatine tonsils are a suitable site for biofilm production. The crypts of the palatine tonsils are capable of accumulating debris, and its mineralisation leads to the formation of tonsilloliths (Ferguson et al., 2014). Tonsilloliths are characterised by dynamic biofilms similar to dental biofilms (Stoodley et al., 2009). Our study has shown that in healthy subjects, 61 % of *S. aureus* and 40 % of Gram-negative bacteria strains have moderate to strong biofilm-producing abilities. Our study has confirmed that biofilm-production is part of the normal bacterial lifestyle and that biofilms can exist in the palatine

tonsils of healthy individuals (Viksne et al., 2023). In a study by *Penesyan et al.* (2021), biofilm is described as the main mode of microbial life; biofilms perform an important function, providing microbes with a protective environment in which genotypic and phenotypic diversity is created (Penesyan et al., 2021). The properties of the biofilm can differ between diseased and healthy people. *Chervinets et al.* (2021) reported that the oral microbiota of patients with periodontitis had a greater ability to attach to mucosal cells than of healthy individuals, and an increased ability to produce biofilms and exhibit pathogenic properties was also observed (Chervinets et al., 2021).

Localisation of the etiological agent in biofilms may contribute to antibiotic resistance. Antibiotic resistance is a major problem in relation to *S. aureus*, especially MRSA. An increasing prevalence of MRSA has been reported among healthy carriers, up to 21 % in nasal samples of dental students (Roberts et al., 2011). Less well known is the prevalence of MRSA in the oral cavity; subgingival areas and the surface of the tongue were examined, and as a result MRSA was not detected (Koukos et al., 2015). Our study has revealed one (1.1 %) MRSA isolate in palatine tonsil samples. Healthy individuals had a high number of *S. aureus* isolates with resistance to benzylpenicillin and ampicillin; however, none of the isolates were resistant to clindamycin (Viksne et al., 2023). The obtained data on antibacterial resistance of *S. aureus* in our study are consistent with the results of the study conducted by *Katkowska et al.* (2017) (Katkowska et al., 2017). Clindamycin is widely used in dentistry, and many clinics have replaced penicillin (oxacillin and methicillin); clindamycin is also prescribed in cases of allergy to beta-lactams (Blomqvist et al., 2015).

It has been hypothesised that infectious strains have a different virulence arsenal than those that colonise healthy individuals (Blomqvist et al., 2015). However, studies have not demonstrated, for instance, that *S. aureus* strains isolated from infected oral cavity and non-infected oral cavity represent different

subgroups of phenotypic and genotypic characteristics (Blomqvist et al., 2015). Thus, it was concluded that classical opportunistic infections develop as a result of unbalanced host-microorganism interactions and that the infectious disease persists as long as the immunodeficiency condition exists (Blomqvist et al., 2015).

We would like to highlight some of the strengths of this study. We used brushes as an alternative, non-invasive method to collect palatine tonsil samples, avoiding the need to use a traumatic method to obtain tonsillar samples. Thus, we were able to include healthy people without signs of palatine tonsil disease in our study (Viksne et al., 2023).

5.4 Evaluation of Staphylococcus aureus colonisation in adult patients who were undergoing tonsillectomy for RT

The dominant ecological niche for *S. aureus* colonisation is the vestibule of the nose; other areas of the body that can be colonised are the armpits, groin and throat (Peacock et al., 2001). Reducing *S. aureus* carriage in the nasal vestibule with topical antibacterial agents also reduces *S. aureus* carriage elsewhere (Reagan, 1991). *S. aureus* is able to easily re-colonise the nasopharynx, pharynx, and other areas of the body several months after the completion of antibiotic therapy (Mody et al., 2003; Coates et al., 2009). *S. aureus* carriers have a higher infection rate than non-*S. aureus* carriers. Carriers are usually infected with the same strain they were colonised with (Lister & Horswill, 2014). In our study, the presence of *S. aureus* in the pharynx, armpits, and vestibule of the nose was assessed one year after tonsillectomy in RT patients (Klagisa, Racenis, Broks, Kise, et al., 2022).

Carriage of *S. aureus* is influenced by both bacterial and host factors. The main risk factors for staphylococcal infection are age, comorbidities or immunodeficiency, genetic factors, direct contact with a healthcare facility, or

hospitalisation (Chmielowiec-Korzeniowska et al., 2020). The patients enrolled in this study were young adults (mean age 29 years old) without risk factors for *S. aureus* such as HIV infection, insulin dependent diabetes mellitus, ongoing peritoneal or haemodialysis, intravenous drug use (Chang et al., 2021; Ding et al., 2021; Peacock et al., 2001; Wu et al., 2021; Klagisa, Racenis, Broks, Kise, et al., 2022). Also, in other studies analysing patients with tonsillectomy, the mean age was 28 years old (Witsell et al., 2008; Senska et al., 2015).

The study focused on evaluating the biofilm produced by isolated *S. aureus* strains. *S. aureus* strains isolated from tonsillar crypts showed more pronounced biofilms compared to *S. aureus* strains isolated from other parts of the body. Biofilm-producing *S. aureus* strains were primarily isolated from tonsillar crypts and were susceptible to most of the antibiotics tested. *S. aureus* strain was identified as MRSA as it was resistant to cefoxitin. MRSA was a weak biofilm-producing isolate. MRSA was isolated from a 25-year-old woman without comorbidities who underwent RT and had 5 episodes of tonsillitis during the last 3 years. MRSA was no longer detected after tonsillectomy (Klagisa, Racenis, Broks, Kise, et al., 2022). MRSA in RT patients has also been found in other studies. In a study conducted by *Katkowska et al.* (2017, 2020) in Poland, the MRSA strain isolated from palatine tonsils was found in 1 of 118 adults and 2 of 73 children who underwent tonsillectomy (Katkowska et al., 2017, 2020). The relationship between biofilm production and antibiotic resistance in MRSA and methicillin-susceptible *S. aureus* (MSSA) is unclear (Senobar Tahaei et al., 2021). Environmental factors such as temperature, pH, glucose levels, type of media, etc. influence the production of bacterial biofilms. Thus, these factors should be considered in biofilm studies. For the results of different studies to be mutually comparable, the environmental factors and method of biofilm analysis must be the same or very similar (Liu et al., 2020). It is important to analyse *S. aureus* colonisation to understand what infectious

diseases are caused by *S. aureus*. The study evaluated patients with RT and the results of microbiological examination one year after tonsillectomy. In our study one year after tonsillectomy, *S. aureus* was not detected in 10 patients, so we can conclude that tonsillectomy prevents bacterial colonisation for one year (Klagisa, Racenis, Broks, Kise, et al., 2022).

5.5 Strengths and weaknesses of the study

The surface and crypts of human palatine tonsils contain a wide variety of pathogens in high concentrations. RT episodes repeat despite effective conservative therapy during the acute period, and may also be complicated by PTA. The strengths of the study are that samples of palatine tonsils from patients with RT and PTA for microbiological analysis were obtained during tonsillectomy with a biopsy needle, excluding the influence of the oral microbiota. Samples from healthy individuals were obtained with a brush from the crypts of the palatine tonsils to avoid invasive manipulation. Differences in sampling methods limited intercomparison between the 3 study groups. It should be noted that healthy individuals were statistically significantly younger compared to patients with RT, PTA, whose average age was 21 and 32 years old, respectively. There are no data in the literature on differences in the microbiota in adults in this age range. For the first time, bacterial factors have been identified that could help explain repeated episodes of exacerbations of tonsillitis and, in some cases, the development of a severe complication, i.e. PTA. The study provides for a follow-up period of 1 year for RT patients undergoing surgical treatment. The study assessed the impact of tonsillectomy on microbiota presence and carriage of *S. aureus*. The size of the patient group was influenced by several factors, for example, the limited ability of postoperative patients to come from different cities in Latvia for a return visit to Riga. The sampling amount of PTA patients was influenced by various treatments for PTA, patients

who underwent abscess opening, drainage and outpatient treatment for PTA were not included in the study.

Bacteria were identified using VITEK-2 Compact device or a MALDI-TOF MS system. The bacterial biofilm production in the laboratory is influenced by various factors, such as temperature, pH, glucose level, and type of nutrient medium. The crystal violet method adapted from *Stepanovic et al.* was used in the study as it is simple, specific, economically efficient, as well as is often used to quantify staphylococcal biofilms (Stepanović et al., 2007). It is important to note that this method can be easily repeated by other researchers and the results of studies can be compared. In future studies, it is recommended to analyse the genetic factors of bacteria that play an important role in biofilm production. Such information will make it possible to more accurately determine the role of bacteria in the clinical course of RT and to choose the therapy.

5.6 Concluding paragraph of the study

For the first time, a biopsy needle was used to obtain a sample of palatine tonsil crypts in order to exclude the presence of bacteria from the surface microbiota of the oral cavity. The punch biopsy needle was patented.

During the study, bacteria colonising the crypts of the palatine tonsils of RT and PTA patients were isolated and identified, and their characteristics were evaluated, which could influence the recurrence of exacerbations of tonsillitis and the occurrence of complications. We assessed the ability of bacteria to produce biofilms and the relationship of this ability with antibacterial resistance, which affects the choice of RT and PTA therapy.

It has been proven that, contrary to existing ideas, *S. pyogenes* is not the predominant causative agent of RT and PTA, and therefore the need for rapid antigen tests for streptococci and detection of ASO in these patients should be reconsidered. The most common causative agent of RT in the study population

was *S. aureus* (33.3 %, n = 33/99). *Candida* spp. was detected in the material of patients with PTA in 48.28 % of cases (n = 14/29). The presence of *Candida* spp. can possibly serve as an indicator of PTA development. The hypothesis of the paper was also confirmed, i.e. that in the case of RT and PTA, the microorganisms present in the crypts of the palatine tonsils have a pronounced ability to produce biofilms, which ensures the presence of a chronic infection with possible reactivation. Antibacterial resistance studies have demonstrated that the antibiotics recommended for the treatment of RT and PTA, benzylpenicillin and ampicillin, were ineffective due to bacteria resistance. The aetiological agents of RT and PTA were sensitive to amoxicillin with clavulanic acid and clindamycin.

For the first time, an extensive study of the palatine tonsil microbiota of healthy individuals was carried out. *S. aureus* was isolated from the palatine tonsils of healthy individuals in 45 % of cases. At the same time, in 95 % of cases (n = 39/41) these were biofilm-producing isolates. In healthy individuals, no association was observed between biofilm production capacity and antibacterial resistance. Both *S. aureus* strains and other more commonly isolated bacteria – *K. pneumoniae* and *P. aeruginosa* are resistant to the background empirical antibiotic therapy, with special sensitivity to amoxicillin with clavulanic acid and clindamycin.

For the first time, the efficacy of surgical and antibacterial treatment was evaluated one year after tonsillectomy. Swabs from the throat, nasal cavity and armpits have shown that *S. aureus* was isolated less frequently. Detection of *S. aureus* and its biofilm-production ability is a way to identify *S. aureus* carriers.

Conclusions

1. In the crypts of the palatine tonsils of RT patients, a wide variety of microorganisms is observed. *S. aureus* is the most common pathogen isolated from tonsil crypts of RT patients, with an expressed ability to produced biofilms. The extent of *K. pneumoniae* biofilm production was insignificant. Bacterial strains isolated from the crypts of the palatine tonsils of PTA patients rarely produced biofilms. *S. aureus* in RT and PTA groups is susceptible to empiric antibacterial therapy, with expressed susceptibility to amoxicillin with clavulanic acid and clindamycin.
2. Surface samples of palatine tonsils of RT patients have a higher bacterial diversity compared to crypt biopsy material. Biopsy of palatine tonsillar crypts with a biopsy needle more accurately reflects RT etiologic agents, as in this case the presence of oral microbiota is excluded. *S. aureus*, isolated from the crypts of the palatine tonsils, is a biofilm-producing strain.
3. The palatine tonsils of healthy individuals are colonised by *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp. and other bacteria. *S. aureus* represent biofilm-producing strains. Tonsillar crypts are an important reservoir of biofilm-producing pathogenic bacteria that may play a role in the aetiology of opportunistic tonsillitis.
4. *S. aureus* isolated from tonsillar crypts was a biofilm-producing strain compared to *S. aureus* isolated from the nasal cavity and armpits. One year after tonsillectomy, the presence of *S. aureus* decreased in RT patients. Tonsillectomy is an effective treatment method that prevents bacterial colonisation.

Publications

Scientific publications in international databases (5):

1. **Viksne, R.**, Polikarpova, K., Jenbajeva, K. 2023. Evaluation of Tonsillectomy Patients and Factors Related to Immediate Tonsillectomy. *Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences*. (Under review.)

2. **Viksne, R.**, Racenis, K., Broks, R., Balode, A. O., Kise, L., Kroica, J. 2023. In Vitro Assessment of Biofilm Production, Antibacterial Resistance of Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Obtained from Tonsillar Crypts of Healthy Adults. *Microorganisms*. 11, 258. https://doi.org/10.3390/microorganisms11020258

3. **Klagisa, R.**, Racenis, K., Broks, R., Balode, A.O., Kise, L., Kroica, J. 2022. Analysis of Microorganism Colonisation, Biofilm Production, and Antibacterial Susceptibility in Recurrent Tonsillitis and Peritonsillar Abscess Patients. Int. J. Mol. Sci. 23, 10273. https://doi.org/10.3390/ijms231810273

4. **Klagisa, R.**, Racenis, K., Broks, R., Kise, L., Kroica, J. 2022. Evaluation of Staphylococcus aureus Colonisation in Adult Patients Undergoing Tonsillectomy for Recurrent Tonsillitis. *Pathogens*. 11, 427. https://doi.org/10.3390/ pathogens11040427

5. **Klagisa, R.**, Kroica, J., Kise, L. 2021. S. aureus and K. pneumoniae on the Surface and within Core of Tonsils in Adults with Recurrent Tonsillitis. *Medicina*. 57, 1002. https:// doi.org/10.3390/medicina57101002

Patent (1):

1. **Klagisa, R.**, Kroica, J., Kise, L. 2021. Punch Biopsy Needle in *"Izgudrojumi, Preču Zīmes un Dizainparaugi".* Patent Office of the Republic of Latvia. Riga, Latvia, 5. 315. ISSN 2255-9655. Latvia Patent Aplication No.: LVP2020000055

Presentations at international scientific conferences with oral papers or theses (7):

1. **Viksne, R.**, Polikarpova, K., Jenbajeva, K. *Evaluation of Tonsillectomy Patients and Factors Related to Immediate Tonsillectomy.* VIII Baltic ENT Congress. Viļņa, Lietuva, 8–10 June, 2023. (Oral presentation).

2. **Viksne, R.**, Racenis, K., Broks, R., Kroica, J. *Bacterial Colonisation and the Role of Bacterial Biofilms in the Upper Respiratory Tract.* Riga Stradiņš University International Conference on Medical and Health Care Science "Knowledge for Use in Practice". Rīga, Latvija, 29–31 March, 2023. (Oral presentation).

3. **Klagisa, R.**, Kroica, J., Kise, L., Sumeraga, G., Asare, L. *The Associations Between Bacteria, Fungi and Biofilm Production in Patients with Recurrent Tonsillitis and Healthy Controls.* Riga Stradiņš University International Conference on Medical and Health Care Science "Knowledge for Use in Practice". Rīga, Latvija. 24–26 March, 2021. (Oral presentation).

4. **Klagisa, R.**, Kroica, J., Kise, L., Sumeraga, G., Asare, L. *Staphylococcus aureus Colonisation in Patients with Recurrent Tonsillitis.* Riga Stradiņš University International Conference on Medical and Health Care Science "Knowledge for Use in Practice". Rīga, Latvija. 24–26 March, 2021. (Poster presentation).

5. **Klagisa, R.**, Balode, A. O., Broks, R., Kroica, J., Kise, L. *Assessment of Biofilm Production by Pathogenic Bacteria Isolated from Tonsillar Crypts of Patients with Chronic Tonsillitis.* Riga Stradiņš University International Conference on Medical and Health Care Science "Knowledge for Use in Practice". Rīga, Latvija. 1– 3 April, 2019. (Poster presentation. Award for best poster presentation).

6. **Klagisa, R.**, Balode, A. O., Broks, R., Kroica, J., Kise, L. *Microbiological Characteristics of Pathogenic Bacteria Isolated from Tonsillar Crypts of Patients with Chronic Tonsillitis*. Daugavpils Universitātes 61. starptautiskā zinātniskā konference. Daugavpils, Latvija. 11–12 April, 2019. (Oral presentation).

7. **Klagisa, R.**, Balode, A. O., Broks, R., Kroica, J., Kise, L. *Microorganisms identified in tonsillar crypts of patients with chronic tonsillitis*. 5th Congress of European ORL – HNS. Brisele, Beļģija. 29 June – 03 July, 2019. (Poster presentation).

References

1. Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. 2005. Defining the Normal Bacterial Flora of the Oral Cavity. *Journal of Clinical Microbiology*. 43(11), 5721–5732. https://doi.org/10.1128/JCM.43.11.5721-5732.2005

2. Adam, B., Baillie, G. S., & Douglas, L. J. 2002. Mixed species biofilms of Candida albicans and Staphylococcus epidermidis. *Journal of Medical Microbiology*. 51(4), 344–349. https://doi.org/10.1099/0022-1317-51-4-344

3. Al Ahmary, M. S., Al Mastour, A. S., & Ghnnam, W. M. 2012. The Microbiology of Tonsils in Khamis Civil Hospital, Saudi Arabia. *ISRN Otolaryngology*. 1–3. https://doi.org/10.5402/2012/813581

4. Alasil, S. M., Omar, R., Ismail, S., Yusof, M. Y., Dhabaan, G. N., & Abdulla, M. A. 2013. Evidence of Bacterial Biofilms among Infected and Hypertrophied Tonsils in Correlation with the Microbiology, Histopathology, and Clinical Symptoms of Tonsillar Diseases. *International Journal of Otolaryngology*. 1–11. https://doi.org/10.1155/2013/408238

5. Albrich, W. C., & Harbarth, S. 2008. Health-care workers: Source, vector, or victim of MRSA? *The Lancet Infectious Diseases*. 8(5), 289–301. https://doi.org/10.1016/S1473-3099(08)70097-5

6. Anonymous. 2020. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 10.0. 34–38, 84–87. Available online: https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/ (accessed on 12 February 2022)

7. Archer, N. K., Mazaitis, M. J., Costerton, J. W., Leid, J. G., Powers, M. E., & Shirtliff, M. E. 2011. Staphylococcus aureus biofilms. *Virulence*. 2(5), 445–459. https://doi.org/10.4161/viru.2.5.17724

8. Babaiwa, U.F., Onyeagwara, N.C., Akerele, J.O. 2013. Bacterial tonsillar microbiota and antibiogram in recurrent tonsillitis. *Biomedical Research*. 24 (3), 298-302.

9. bin Abu Bakar, M., McKimm, J., Haque, S. Z., Majumder, A. A., & Haque, M. 2018. Chronic tonsillitis and biofilms: A brief overview of treatment modalities. *Journal of Inflammation Research.* 11, 329–337. https://doi.org/10.2147/JIR.S162486

10. Blomqvist, S., Leonhardt, Å., Arirachakaran, P., Carlen, A., & Dahlén, G. 2015. Phenotype, genotype, and antibiotic susceptibility of Swedish and Thai oral isolates of Staphylococcus aureus. *Journal of Oral Microbiology*. 7(1), 26250. https://doi.org/10.3402/jom.v7.26250

11. Brook, I., & Foote, P. A. 2006. Isolation of methicillin resistant Staphylococcus aureus from the surface and core of tonsils in children. *International Journal of Pediatric Otorhinolaryngology.* 70(12), 2099–2102. https://doi.org/10.1016/j.ijporl.2006.08.004

12. Brook, I., Yocum, P., & Shah, K. 1980. Surface vs Core-Tonsillar Aerobic and Anaerobic Flora in Recurrent Tonsillitis. *JAMA*. 244(15), 1696–1698. https://doi.org/10.1001/jama.1980.03310150032024

13. Buname, G., Kiwale, G. A., Mushi, M. F., Silago, V., Rambau, P., & Mshana, S. E. 2021. Bacteria Patterns on Tonsillar Surface and Tonsillar Core Tissue among Patients Scheduled for Tonsillectomy at Bugando Medical Centre, Mwanza, Tanzania. *Pathogens*. 10(12), 1560. https://doi.org/10.3390/pathogens10121560

14. Burton, M. J., Glasziou, P. P., Chong, L. Y., & Venekamp, R. P. 2014. Tonsillectomy or adenotonsillectomy versus non-surgical treatment for chronic/recurrent acute tonsillitis. *The Cochrane Database of Systematic Reviews.* 11, CD001802. https://doi.org/10.1002/14651858.CD001802.pub3

15. Cavalcanti, V. P., Camargo, L. A. de, Moura, F. S., Fernandes, E. J. de M., Lamaro-Cardoso, J., Braga, C. A. da S. B., & André, M. C. P. 2019. Staphylococcus aureus in tonsils of patients with recurrent tonsillitis: Prevalence, susceptibility profile, and genotypic characterisation. *The Brazilian Journal of Infectious Diseases.* 23(1), 8–14. https://doi.org/10.1016/j.bjid.2018.12.003

16. Chang, G.-H., Lu, A., Yang, Y.-H., Liu, C.-Y., Chang, P.-J., Lee, C.-P., Tsai, Y.-T., Hsu, C.-M., Wu, C.-Y., Shih, W.-T., & Tsai, M.-S. 2021. High Risk of Peritonsillar Abscess in End-Stage Renal Disease Patients: A Nationwide Real-World Cohort Study. *International Journal of Environmental Research and Public Health.* 18(13), 6775. https://doi.org/10.3390/ijerph18136775

17. Chervinets, V. M., Chervinets, Yu. V., Leont'eva, A. V., Kozlova, E. A., Stulov, N. M., Belyaev, V. S., Grigoryants, E. O., & Mironov, A. Yu. 2021. The microbiome of oral cavity patients with periodontitis, adhesive and biofilm forming properties. *Russian Clinical Laboratory Diagnostics*. 66(1), 45–51. https://doi.org/10.18821/0869-2084-2021-66-1-45-51

18. Chmielowiec-Korzeniowska, A., Tymczyna, L., Wlazło, Ł., Nowakowicz-Dębek, B., & Trawińska, B. 2020. Staphylococcus aureus carriage state in healthy adult population and phenotypic and genotypic properties of isolated strains. *Postepy Dermatologii I Alergologii*, 37(2), 184–189. https://doi.org/10.5114/ada.2020.94837

19. Coates, T., Bax, R., & Coates, A. 2009. Nasal decolonisation of Staphylococcus aureus with mupirocin: Strengths, weaknesses and future prospects. *Journal of Antimicrobial Chemotherapy*. 64(1), 9–15. https://doi.org/10.1093/jac/dkp159

20. Cornaglia, G.; Courcol, R.; Herrmann, J.L. 2012. *European manual of clinical microbiology.* Société Franaise Microbiol. 262, 145–152

21. De Martin, A., Lütge, M., Stanossek, Y., Engetschwiler, C., Cupovic, J., Brown, K., Demmer, I., Broglie, M. A., Geuking, M. B., Jochum, W., McCoy, K. D., Stoeckli, S. J., & Ludewig, B. 2021. Distinct microbial communities colonize tonsillar squamous cell carcinoma. *Oncoimmunology.* 10(1), 1945202. https://doi.org/10.1080/2162402X.2021.1945202

22. Dickinson, A., Kankaanpää, H., Silén, S., Meri, S., Haapaniemi, A., Ylikoski, J., & Mäkitie, A. 2020. Tonsillar surface swab bacterial culture results differ from those of the tonsillar core in recurrent tonsillitis. *The Laryngoscope*. 130(12). https://doi.org/10.1002/lary.28403

23. Ding, M.-C., Tsai, M.-S., Yang, Y.-H., Liu, C.-Y., Tsai, Y.-T., Hsu, C.-M., Wu, C.-Y., Chang, P.-J., Lin, K.-M., & Chang, G.-H. 2021. Patients with comorbid rheumatoid arthritis are predisposed to peritonsillar abscess: Real-world evidence. *European Archives of Oto-Rhino-Laryngology*. 278(10), 4035–4042. https://doi.org/10.1007/s00405-021-06638-3

24. Ferguson, M., Aydin, M., & Mickel, J. 2014. Halitosis and the Tonsils: A Review of Management. *Otolaryngology–Head and Neck Surgery*. 151(4), 567–574. https://doi.org/10.1177/0194599814544881

25. Forson, A. M., van der Mei, H. C., & Sjollema, J. 2020. Impact of solid surface hydrophobicity and micrococcal nuclease production on Staphylococcus aureus Newman biofilms. *Scientific Reports.* 10(1), 12093. https://doi.org/10.1038/s41598-020-69084-x

26. Fukui, Y., Aoki, K., Ishii, Y., & Tateda, K. 2018. The palatine tonsil bacteriome, but not the mycobiome, is altered in HIV infection. *BMC Microbiology*. 18(1), 127. https://doi.org/10.1186/s12866-018-1274-9

27. Hamilos, D. L. 2019. Biofilm Formations in Pediatric Respiratory Tract Infection: Part 1: Biofilm Structure, Role of Innate Immunity in Protection Against and Response to Biofilm, Methods of Biofilm Detection, Pediatric Respiratory Tract Diseases Associated with Mucosal Biofilm Formation. *Current Infectious Disease Reports*. 21(2), 6. https://doi.org/10.1007/s11908-019-0658-9

28. Hanson, B. M., Kates, A. E., O'Malley, S. M., Mills, E., Herwaldt, L. A., Torner, J. C., Dawson, J. D., Farina, S. A., Klostermann, C., Wu, J. Y., Quick, M. K., Forshey, B. M., & Smith, T. C. 2018. Staphylococcus aureus in the nose and throat of Iowan families. *Epidemiology and Infection.* 146(14), 1777–1784. https://doi.org/10.1017/S0950268818001644

29. Haq, S. N., Ayub, Z., & Ahmed, A. 2017. Tonsillar Surface Micro Flora: Does it Truly Represent Pathological Tonsillar Flora?. *Journal of the College of Physicians and Surgeons-Pakistan : JCPSP*. 27(1), 23–25.

30. Ivaska, L. E., Hanif, T., Ahmad, F., Tan, G., Altunbulakli, C., Mikola, E., Silvoniemi, A., Puhakka, T., Akdis, C. A., Toppila-Salmi, S., & Jartti, T. 2020. Tonsillar microbial diversity, abundance, and interrelations in atopic and non-atopic individuals. *Allergy*. 75(8), 2133–2135. https://doi.org/10.1111/all.14306

31. Jeong, J. H., Lee, D. W., Ryu, R. A., Lee, Y. S., Lee, S. H., Kang, J. O., & Tae, K. 2007. Bacteriologic Comparison of Tonsil Core in Recurrent Tonsillitis and Tonsillar Hypertrophy. *The Laryngoscope.* 117(12), 2146–2151. https://doi.org/10.1097/MLG.0b013e31814543c8

32. Jokinen, K., Pajarre, S., Palva, A., & Sipilä, P. 1976. Mycotic flora in tonsils and adenoids: A microbiolgical and histological eveluation. *The Journal of Laryngology & Otology*. 90(10), 945–952. https://doi.org/10.1017/S0022215100082955

33. Jorgensen J. H. Pfaller M. A. Carroll K. C. & American Society for Microbiology. 2015. *Manual of clinical microbiology* (11th ed.). *ASM Press*.

34. Katkowska, M., Garbacz, K., Kopala, W., Schubert, J., & Bania, J. 2020. Genetic diversity and antimicrobial resistance of Staphylococcus aureus from recurrent tonsillitis in children. *APMIS*. 128(3), 211–219. https://doi.org/10.1111/apm.13007

35. Katkowska, M., Garbacz, K., & Stromkowski, J. 2017. Staphylococcus aureus isolated from tonsillectomized adult patients with recurrent tonsillitis. *APMIS*. 125(1), 46–51. https://doi.org/10.1111/apm.12628

36. Kearney, A., Kinnevey, P., Shore, A., Earls, M., Poovelikunnel, T. T., Brennan, G., Humphreys, H., & Coleman, D. C. 2020. The oral cavity revealed as a significant reservoir of Staphylococcus aureus in an acute hospital by extensive patient, healthcare worker and environmental sampling. *Journal of Hospital Infection.* 105(3), 389–396. https://doi.org/10.1016/j.jhin.2020.03.004

37. Khadilkar, M. N., & Ankle, N. R. 2016. Anaerobic Bacteriological Microbiota in Surface and Core of Tonsils in Chronic Tonsillitis. *Journal of Clinical and Diagnostic Research: JCDR.* 10(11), MC01–MC03. https://doi.org/10.7860/JCDR/2016/22124.8819

38. Klagisa, R., Kroica, J., & Kise, L. 2021a. Punch Biopsy Needle. *The Patent Office of the Republic of Latvia, Izgudrojumi, Preču Zīmes un Dizainparaugi.* 5, 315.

39. Klagisa, R., Kroica, J., & Kise, L. 2021b. S. aureus and K. pneumoniae on the Surface and within Core of Tonsils in Adults with Recurrent Tonsillitis. *Medicina*. 57(10), 1002. https://doi.org/10.3390/medicina57101002

40. Klagisa, R., Racenis, K., Broks, R., Balode, A. O., Kise, L., & Kroica, J. (2022). Analysis of Microorganism Colonisation, Biofilm Production, and Antibacterial Susceptibility in Recurrent Tonsillitis and Peritonsillar Abscess Patients. *International Journal of Molecular Sciences.* 23(18), 10273. https://doi.org/10.3390/ijms231810273

41. Klagisa, R., Racenis, K., Broks, R., Kise, L., & Kroiča, J. (2022). Evaluation of Staphylococcus aureus Colonisation in Adult Patients Undergoing Tonsillectomy for Recurrent Tonsillitis. *Pathogens*. 11(4), 427. https://doi.org/10.3390/pathogens11040427

42. Klug, T. E. 2017. Peritonsillar abscess: Clinical aspects of microbiology, risk factors, and the association with parapharyngeal abscess. *Danish Medical Journal*. 64(3).

43. Kostić, M., Ivanov, M., Babić, S. S., Tepavčević, Z., Radanović, O., Soković, M., & Ćirić, A. 2022. Analysis of Tonsil Tissues from Patients Diagnosed with Chronic Tonsillitis – Microbiological Profile, Biofilm-Forming Capacity and Histology. *Antibiotics*. 11(12), 1747. https://doi.org/10.3390/antibiotics11121747

44. Koukos, G., Sakellari, D., Arsenakis, M., Tsalikis, L., Slini, T., & Konstantinidis, A. 2015. Prevalence of Staphylococcus aureus and methicillin resistant Staphylococcus aureus (MRSA) in the oral cavity. *Archives of Oral Biology.* 60(9), 1410–1415. https://doi.org/10.1016/j.archoralbio.2015.06.009

45. Krause, J., Geginat, G., & Tammer, I. 2015. Prostaglandin E2 from Candida albicans Stimulates the Growth of Staphylococcus aureus in Mixed Biofilms. *PLOS ONE*. 10(8), e0135404. https://doi.org/10.1371/journal.pone.0135404

46. Laheij, A. M. G. A., Kistler, J. O., Belibasakis, G. N., Välimaa, H., de Soet, J. J., & European Oral Microbiology Workshop (EOMW). 2012. Healthcare-associated viral and bacterial infections in dentistry. *Journal of Oral Microbiology*. 4(1), 17659. https://doi.org/10.3402/jom.v4i0.17659

47. Li, Q., Wang, H., Tan, L., Zhang, S., Lin, L., Tang, X., & Pan, Y. 2021. Oral Pathogen Fusobacterium nucleatum Coaggregates With Pseudomonas aeruginosa to Modulate the Inflammatory Cytotoxicity of Pulmonary Epithelial Cells. *Frontiers in Cellular and Infection Microbiology*. 11, 643913. https://doi.org/10.3389/fcimb.2021.643913

48. Lindroos, R. 2000. Bacteriology of the tonsil core in recurrent tonsillitis and tonsillar hyperplasia – A short review. *Acta Oto-Laryngologica. Supplementum*. 543, 206–208. https://doi.org/10.1080/000164800454404

49. Lister, J. L., & Horswill, A. R. 2014. Staphylococcus aureus biofilms: Recent developments in biofilm dispersal. *Frontiers in Cellular and Infection Microbiology.* 4, 178. https://doi.org/10.3389/fcimb.2014.00178

50. Liu, Y., Zhang, J., & Ji, Y. 2020. Environmental factors modulate biofilm formation by Staphylococcus aureus. *Science Progress*. 103(1), 003685041989865. https://doi.org/10.1177/0036850419898659

51. Loganathan, A., Arumainathan, U., & Raman, R. 2006. Comparative study of bacteriology in recurrent tonsillitis among children and adults. *Singapore Med J*. https://pubmed.ncbi.nlm.nih.gov/16572236/

52. Ma, D., Mandell, J. B., Donegan, N. P., Cheung, A. L., Ma, W., Rothenberger, S., Shanks, R. M. Q., Richardson, A. R., & Urish, K. L. 2019. The Toxin-Antitoxin MazEF Drives Staphylococcus aureus Biofilm Formation, Antibiotic Tolerance, and Chronic Infection. *MBio*. 10(6), e01658-19. https://doi.org/10.1128/mBio.01658-19

53. Mirzaei, R., Mohammadzadeh, R., Sholeh, M., Karampoor, S., Abdi, M., Dogan, E., Moghadam, M. S., Kazemi, S., Jalalifar, S., Dalir, A., Yousefimashouf, R., Mirzaei, E., Khodavirdipour, A., & Alikhani, M. Y. 2020. The importance of intracellular bacterial biofilm in infectious diseases. *Microbial Pathogenesis*. 147, 104393. https://doi.org/10.1016/j.micpath.2020.104393

54. Mody, L., Kauffman, C. A., McNeil, S. A., Galecki, A. T., & Bradley, S. F. 2003. Mupirocin-Based Decolonisation of Staphylococcus aureus Carriers in Residents of 2 Long-Term Care Facilities: A Randomized, Double-Blind, Placebo-Controlled Trial. *Clinical Infectious Diseases.* 37(11), 1467–1474. https://doi.org/ 10.1086/379325

55. Moormeier, D. E., & Bayles, K. W. 2017. Staphylococcus aureus Biofilm: A Complex Developmental Organism. *Molecular Microbiology*. 104(3), 365–376. https://doi.org/10.1111/mmi.13634

56. Paradise, J. L., Bluestone, C. D., Bachman, R. Z., Colborn, D. K., Bernard, B. S., Taylor, F. H., Rogers, K. D., Schwarzbach, R. H., Stool, S. E., Friday, G. A., Smith, I. H., & Saez, C. A. 1984. Efficacy of Tonsillectomy for Recurrent Throat Infection in Severely Affected Children: Results of Parallel Randomized and Nonrandomized Clinical Trials. *New England Journal of Medicine.* 310(11), 674–683. https://doi.org/10.1056/NEJM198403153101102

57. Peacock, S. J., de Silva, I., & Lowy, F. D. 2001. What determines nasal carriage of Staphylococcus aureus? *Trends in Microbiology*. 9(12), 605–610. https://doi.org/ 10.1016/S0966-842X(01)02254-5

58. Penesyan, A., Paulsen, I. T., Kjelleberg, S., & Gillings, M. R. 2021. Three faces of biofilms: A microbial lifestyle, a nascent multicellular organism, and an incubator for diversity. *Npj Biofilms Microbiomes*. https://doi.org/10.1038/s41522-021-00251-2

59. Peter Sell, Rubeor, A., & Fort, G. 2023. Peritonsillar Abscess. *Ferri's Clinical Advisor 2023*. 1189-1190. www-clinicalkey-com.

60. Petti, S., & Polimeni, A. 2011. Risk of Methicillin-Resistant Staphylococcus aureus Transmission in the Dental Healthcare Setting: A Narrative Review. *Infection Control & Hospital Epidemiology.* 32(11), 1109–1115. https://doi.org/ 10.1086/662184

61. Pichichero, M., & Casey, J. 2007. Systematic review of factors contributing to penicillin treatment failure in Streptococcus pyogenes pharyngitis. *Otolaryngol Head Neck Surg*. 137(6), 851–857. https://doi.org/doi: 10.1016/ j.otohns.2007.07.033.

62. Reagan, D. R. 1991. Elimination of Coincident Staphylococcus aureus Nasal and Hand Carriage with Intranasal Application of Mupirocin Calcium Ointment. *Annals of Internal Medicine*. 114(2), 101. https://doi.org/10.7326/0003-4819-114-2-101

63. Reisner, A., Krogfelt, K. A., Klein, B. M., Zechner, E. L., & Molin, S. 2006. In Vitro Biofilm Formation of Commensal and Pathogenic Escherichia coli Strains: Impact of Environmental and Genetic Factors. *Journal of Bacteriology*. 188(10), 3572–3581. https://doi.org/10.1128/JB.188.10.3572-3581.2006

64. Roberts, M. C., Soge, O. O., Horst, J. A., Ly, K. A., & Milgrom, P. 2011. Methicillin-resistant Staphylococcus aureus from dental school clinic surfaces and students. *American Journal of Infection Control*. 39(8), 628–632. https://doi.org/10.1016/j.ajic.2010.11.007

65. Sarkar, S., Sil, A., Sarkar, S., & Sikder, B. 2017. A Comparison of Tonsillar Surface Swabbing, Fine-Needle Aspiration Core Sampling, and Dissected Tonsillar Core Biopsy Culture in Children with Recurrent Tonsillitis. *Ear, Nose & Throat Journal*. 96(6), E29–E32. https://doi.org/10.1177/014556131709600606

66. Schnurr, E., Paqué, P. N., Attin, T., Nanni, P., Grossmann, J., Holtfreter, S., Bröker, B. M., Kohler, C., Diep, B. A., Ribeiro, A. de A., & Thurnheer, T. 2021. Staphylococcus aureus Interferes with Streptococci Spatial Distribution and with Protein Expression of Species within a Polymicrobial Oral Biofilm. *Antibiotics*. 10(2), 116. https://doi.org/10.3390/antibiotics10020116

67. Senobar Tahaei, S. A., Stájer, A., Barrak, I., Ostorházi, E., Szabó, D., & Gajdács, M. 2021. Correlation Between Biofilm-Formation and the Antibiotic Resistant Phenotype in Staphylococcus aureus Isolates: A Laboratory-Based Study in Hungary and a Review of the Literature. *Infection and Drug Resistance*. 14, 1155–1168. https://doi.org/10.2147/IDR.S303992

68. Senska, G., Atay, H., Pütter, C., & Dost, P. 2015. Long-Term Results from Tonsillectomy in Adults. *Deutsches Ärzteblatt International*. 112(50), 849–855. https://doi.org/10.3238/arztebl.2015.0849

69. Slouka, D., Hanakova, J., Kostlivy, T., Skopek, P., Kubec, V., Babuska, V., Pecen, L., Topolcan, O., & Kucera, R. 2020. Epidemiological and Microbiological Aspects of the Peritonsillar Abscess. *International Journal of Environmental Research and Public Health*. 17(11), 4020. https://doi.org/10.3390/ijerph17114020

70. Smith, A. J., Robertson, D., Tang, M. K., Jackson, M. S., MacKenzie, D., & Bagg, J. 2003. Staphylococcus aureus in the oral cavity: A three-year retrospective analysis of clinical laboratory data. *British Dental Journal*. 195(12), 701–703. https://doi.org/10.1038/sj.bdj.4810832

71. Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. 2007. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*. 115(8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

72. Stępińska, M., Olszewska-Sosińska, O., Lau-Dworak, M., Zielnik-Jurkiewicz, B., & Trafny, E. A. 2014. Identification of intracellular bacteria in adenoid and tonsil tissue specimens: The efficiency of culture versus fluorescent in situ hybridisation (FISH). *Current Microbiology.* 68(1), 21–29. https://doi.org/10.1007/s00284-013-0436-0

73. Stoodley, P., deBeer, D., Longwell, M., Nistico, L., Hall-Stoodley, L., Wenig, B., & Krespi, Y. P. 2009. Tonsillolith: Not Just a Stone but a Living Biofilm. *Otolaryngology–Head and Neck Surgery*. 141(3), 316–321. https://doi.org/10.1016/j.otohns.2009.05.019

74. Sykes, E. A., Wu, V., Beyea, M. M., Simpson, M. T. W., & Beyea, J. A. 2020. Pharyngitis: Approach to diagnosis and treatment. *Canadian Family Physician Medecin De Famille Canadien.* 66(4), 251–257.

75. Syryło, A., Wojdas, A., & Jurkiewicz, D. 2007. Bacterial flora of the tonsillar surface versus tonsillar core in chronic tonsillitis. *Otolaryngologia Polska*. 61(4), 598–601. https://doi.org/10.1016/S0030-6657(07)70493-6

76. Tzelnick, S., Hilly, O., Vinker, S., Bachar, G., & Mizrachi, A. 2020. Long-term outcomes of tonsillectomy for recurrent tonsillitis in adults. *The Laryngoscope*. 130(2), 328–331. https://doi.org/10.1002/lary.27928

77. Vaikjärv, R., Kasenõmm, P., Jaanimäe, L., Kivisild, A., Rööp, T., Sepp, E., & Mändar, R. 2016. Microbiology of peritonsillar abscess in the South Estonian population. *Microbial Ecology in Health & Disease.* 27(0). https://doi.org/10.3402/mehd.v27.27787

78. Viksne, R., Racenis, K., Broks, R., Balode, A. O., Kise, L., & Kroica, J. (2023). In Vitro Assessment of Biofilm Production, Antibacterial Resistance of Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Obtained from Tonsillar Crypts of Healthy Adults. *Microorganisms.* 11(2), 258. https://doi.org/10.3390/microorganisms11020258

79. Wang, G., Zhao, G., Chao, X., Xie, L., & Wang, H. 2020. The Characteristic of Virulence, Biofilm and Antibiotic Resistance of Klebsiella pneumoniae. *International Journal of Environmental Research and Public Health.* 17(17). https://doi.org/10.3390/ijerph17176278

80. Wikstén, J., Kaltiainen, E., Pitkäranta, A., & Blomgren, K. 2017. Renewal of peritonsillar abscess: Impact of the bacterial species of the infection and clinical features of the patient-A prospective comparative aetiological study. *Clinical Otolaryngology*. 42(6), 1358–1362. https://doi.org/10.1111/coa.12893

81. Windfuhr, J. P., Toepfner, N., Steffen, G., Waldfahrer, F., & Berner, R. 2016a. Clinical practice guideline: Tonsillitis I. Diagnostics and nonsurgical management. *European Archives of Oto-Rhino-Laryngology.* 273(4), 973–987. https://doi.org/10.1007/s00405-015-3872-6

82. Windfuhr, J. P., Toepfner, N., Steffen, G., Waldfahrer, F., & Berner, R. 2016b. Clinical practice guideline: Tonsillitis II. Surgical management. *European Archives of Oto-Rhino-Laryngology*. 273(4), 989–1009. https://doi.org/10.1007/s00405-016-3904-x

83. Witsell, D. L., Orvidas, L. J., Stewart, M. G., Hannley, M. T., Weaver, E. M., Yueh, B., Smith, T. L., & Goldstein, N. A. 2008. Quality of life after tonsillectomy in adults with recurrent or chronic tonsillitis. *Otolaryngology–Head and Neck Surgery*. 138(1_suppl), S1–S8. https://doi.org/10.1016/j.otohns.2007.08.015

84. Woo, J. H., Kim, S. T., Kang, I. G., Lee, J. H., Cha, H. E., & Kim, D. Y. 2012. Comparison of tonsillar biofilms between patients with recurrent tonsillitis and a control group. *Acta Oto-Laryngologica*. 132(10), 1115–1120. https://doi.org/10.3109/00016489.2012.689859

85. Wu, C.-L., Tsai, M.-S., Lee, T.-J., Wang, Y.-T., Liu, C.-Y., Yang, Y.-H., Tsai, Y.-T., Hsu, C.-M., Wu, C.-Y., Chang, P.-J., & Chang, G.-H. 2021. Type 2 Diabetes Mellitus Increases Peritonsillar Abscess Susceptibility: Real-World Evidence. *Clinical and Experimental Otorhinolaryngology*. 14(3), 347–354. https://doi.org/10.21053/ceo.2020.02257

86. Zaatout, N. 2021. Presence of non-oral bacteria in the oral cavity. *Arch. Microbiol.* 203, 2747–2760. https://doi.org/10.1007/s00203-021-02300-y

87. Zautner, A. E. 2012. Adenotonsillar Disease. *Recent Patents on Inflammation & Allergy Drug Discovery*. 6(2), 121–129.

88. Zautner, A. E., Krause, M., Stropahl, G., Holtfreter, S., Frickmann, H., Maletzki, C., Kreikemeyer, B., Pau, H. W., & Podbielski, A. 2010. Intracellular Persisting Staphylococcus aureus Is the Major Pathogen in Recurrent Tonsillitis. *PLoS ONE*. 5(3), e9452. https://doi.org/10.1371/journal.pone.0009452

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