Influenza virus and related activity in the palatine tonsils after live attenuated influenza vaccination

Master thesis in Pharmacy

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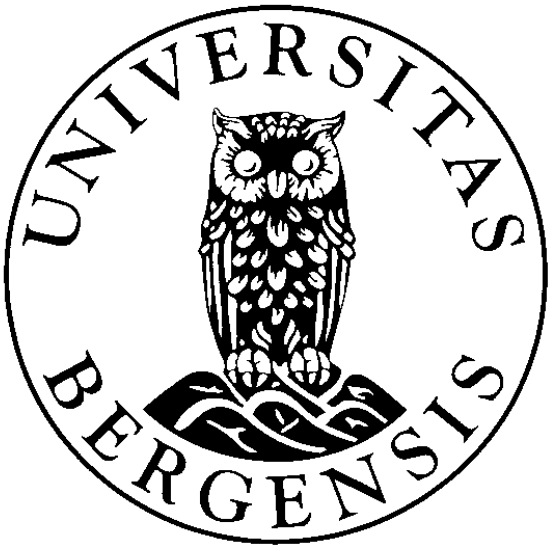

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CONTENT

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ABBREVIATIONS

Ab:	Antibody
Ag:	Antigen
APC:	Antigen presenting Cell
ASC:	Antibody Secreting Cell
BCR:	B cell receptor
CP-value:	Crossing point value
DAMP:	Damage associated molecular pattern molecule
DC:	Dendritic cell
DNA:	Deoxyribonucleic acid
GC:	Germinal Centre
HA:	Haemmagglutinin
HEV:	High Endothelial Venule
HSC:	Haemmatopoietic Stem Cell
ISASC:	Influenza-Specific Antibody Secreting Cell
Ig:	Immunoglobulin
INF:	Interferon
LAIV:	Live attenuated influenza vaccine
LGL:	Large granular lymphocytes
LT:	Lingual tonsil
M1:	Matrixprotein 1
M2:	Matrixprotein 2
MBP:	Maltose binding protein
MHC:	Major histocompatibility complex
NA:	Neuraminidase
NET:	Neutrophil extracellular traps
NK cell:	Natural killer cell
NLR:	NOD like receptor
NP:	Nucleoprotein
NT:	Nasopharyngeal tonsil
PAMP:	Pathogen associated molecular pattern molecule
PCR:	Polymerase Chain Reaction

PRR:	Pattern recognition receptor
PT:	Palatine tonsil
q-PCR:	Quantitative real-time PCR
RNA:	Ribonucleic acid
RNP:	Ribonucleoproteins
RT-PCR:	Reverse Transcription PCR
TCR:	T cell receptor
TIV:	Trivalent inactivated vaccine
TLR:	Toll like receptor
TNF:	Tumor necrosis factor
TT:	Tubal tonsil

SUMMARY

Influenza is a common infection in humans, with annual outbreaks. It can cause severe disease and have great socioeconomic impact. Children are more susceptible to infection than adults, especially when they have a naïve immune system. Two types of influenza vaccines are in the market; the inactivated influenza vaccine and the live attenuated influenza vaccine (LAIV). LAIV is administered intra-nasally and discards the use of needles. It is the preferred vaccine for immune naïve children, with the highest efficacy in children aged from 6 months to 7 years.

The present work focus on the influenza antigen from the LAIV in tonsillar tissues of children aged 3-17 years. In the first part of the work, we assessed whether the influenza virus from LAIV is able to replicate in the tonsillar tissue homogenate of these patients.
In the second part, the influenza-specific antibody secreting cells (ISASC) were counted in tonsillar tissue sections from samples of 20 patients, to assess the differences between vaccinated and unvaccinated subjects.

The methods used were UV-spectrophotometry to assess the quality and quantity of vRNA, reverse transcription q-PCR to determine whether LAIV replication occurred in the tonsils and fluorescent labeling of influenza antigen followed by microscopic detection of the ISASCs to compare the ISASCs between the subjects based on vaccine status.

We found the vRNA quality and quantity to be satisfactory for downstream experiments. The PCR determined that the patient samples were negative for replicating influenza virus, while the positive controls were positive. In the second part we found that all tonsil sections were positive for influenza specific ASCs, and that the largest densities of these were found in the extra follicular zones. The ASCs of the unvaccinated patients were fewer and the number of ASCs in this group of patients were not so scattered. More ASCs were found in the vaccinated patients, and the number of ASCs varied more in this group.

Based on the results, the conclusion is that the LAIV does not replicate in the tonsils. The hypothesis that the dendritic cells transport the virus antigen to the tonsillar tissue have become stronger. The ISASCs were found in all tonsil sections, indicating that none of the patients were immune naïve to the H1N1 antigen.

Nevertheless, differences were found between the vaccinated subjects and the unvaccinated controls. This strongly indicates that the viral antigen from the vaccine have affected the production of antibodies in the tonsil tissues.

INTRODUCTION

1.1: Influenza virus

1.1.1: General

Influenza virus is a common respiratory pathogen in humans and can cause severe respiratory disease. There are three types of influenza viruses, A, B and C. The A and B viruses are associated with clinical influenza illness, while the C virus often gives mild fever in smaller children or subclinical infections in adults.

The Influenza viruses are segmented, single-stranded, negative-sense RNA viruses belonging to the Orthomyxoviridae family. The A and B viruses have 8 viral RNA segments, while the C virus has 7 [1]. The A viruses are further divided into subtypes based on their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA).

1.1.2: Host diversity and reservoir

The natural reservoir for the influenza A viruses is aquatic birds, found in east and central Asia. In birds, the influenza virus is an enteric virus, chronically infecting the birds that continuously shed virus. The combination of constant viral shedding, cold water or ice preserving the virus, and human exposure, keep the virus circulating.

Domestic animals, like pigs, horses, chicken and turkey are a susceptible group for influenza infection because of factors such as high intensity breeding, densely populated milieu and low immunity. The consequence is high mortality.

1.1.3: Virus structure and gene fragment

The influenza virion or infectious particle is between 80-120 nm in size.

The virus is coated by a lipid bilayer, which is derived from the host cell. The A and B viruses contain eight single-stranded genomic RNA segments.

Embedded in the bilayer are the glycoproteins heamagglutinin (HA) and neuraminidase (NA). Their spikes protrude from the viral surface. HA is the major surface antigen of the influenza virus. It mediates the binding between the virus and host cell receptors and fusion of the virus into the host cell. HA is encoded by RNA segment 4 [2]. HA of different virus strains vary in

post-translational modifications (carbohydrate side-chains), that are added to them shortly after their synthesis, and also different amino acid sequences can be found in different HAs.
NA is encoded by RNA segment 6 [2]. It is not evenly scattered over the surface of the virus, but found in patches on the surface. The function of NA is to cleave the Sialic acid at surface of the host cells, aiding the release of newly synthesized viruses. NA also have an important role in the infection phase, digesting Sialic acid and thereby lowering the environmental pH.
The M1 protein is a matrix protein forming the capsid and giving the virus its shape.
The M2 protein is an integral membrane protein. It helps locate the virus to the host cell surface. It is also presumed to act as a proton channel during the HA synthesis, to control the pH of the Golgi apparatus, and to provide acidification of the viral interior during uncoating [3]. The virus also contains a nucleoprotein (NP), which is transported into the nucleus of the infected host cell. The NP is encoded by the RNA segment 5 [2] and contribute to a protective layer of the viral RNA segments [4].

1.1.4: Antigenic drift and shift

NA and HA have so far been classified into 11 and 18 subtypes [5]. These surface antigens are subject to a constant selection pressure and mutations occur frequently.
The viral polymerase complex (PA, PB1 and PB2) do not have any proof-reading capability [6], consequently, mutations are introduced at a rate of 1x10-4 nucleotides per replication cycle. The accumulation of such point mutations is the main cause of antigenic drift, that enable the virus to escape the hosts acquired immunity [7].

Antigenic shift, also called genetic shift, is due to re-assortment of the viral genes in type A viruses [8]. This is thought to occur when a host cell is co-infected with two different influenza viruses, enabling newly synthesized viruses to carry a combination of genetic material from the parental viruses [9]. The newly recombined virus strain may have very different antigenic and pathogenic properties, compared to their parental strains, and are the source of influenza pandemics.

1.1.5: Infection and replication

During annual outbreak, children are more vulnerable to infection. It is assumed that up to 30% of smaller children can be infected with the virus [10]. This number is lower in adults and children with protective immunity.

The incubation period for influenza can vary from 1-5 days, with an average of 2 days [11]. When infected, adults shed larger loads of virus, while children have a longer period of viral shedding [1].

The main route of virus transmission between humans is by virus contained in aerosols and droplets, or by direct contact with virion-contaminated surfaces [12]. The virus infects the epithelium of the upper respiratory tract. The first cycle of replication occurs after about 4-6 hours and very high titres of virus are shed initially.

The life cycle of an influenza virus can be divided into the following six stages: 1) attachment and fusion to the host cell, 2) release of the viral ribonucleoproteins (vRNPs) into the cytosol. 3) transport of the vRNA to the nucleus 4) replication and transcription of the viral genome in the nucleus of the host cell, 5) nuclear export of the viral genome and 6) assembly of the viral proteins, budding and release of the virions [13].

1.2: The innate and adaptive immunity

1.2.1: Overview of the immune system

The immune system is an intricate network of cells and molecules working together to protect the organism against infection. Many types of cells are involved in these processes. These cells belong to the myeloid and lymphoid cell lineages, but they all originate from a common precursor, namely the pluripotent heamatopoetic stem cell (HSC) in the bone marrow. The molecules involved in processes of the immune system are soluble factors such as complement, cytokines, chemokines and antimicrobial peptides.

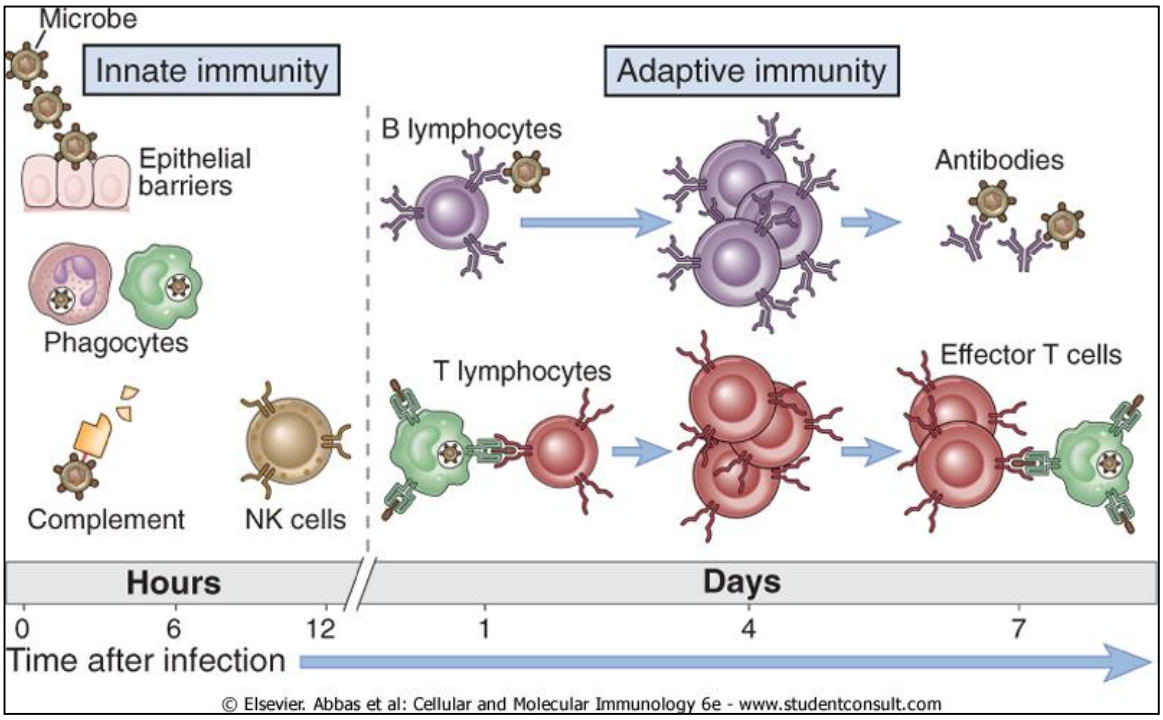

Figure 1. There are two arms of the immune system, the innate and adaptive immunity. Upon exposure to infectious agents these arms collaborate to mount a protective immune response [14].

Some of the molecules and cells that participate in the immune system of an organism are displayed in figure 1. The epithelium is a physical barrier, preventing pathogens from entering the organism. The complement are soluble factors complementing the action of phagocytic cells or antibodies to clear pathogens. Phagocytic cells protect the organism by ingesting dead cells or cells that are about to die and harmful foreign pathogens. Natural killer (NK) cells and B and T lymphocytes are types of white blood cells that in various ways recognize and kill pathogens.

1.2.2: The innate immune system

The innate immune system is often considered the first line of defense. It protects the organism through physical and chemical barriers such as skin and mucosa, and through cells and cellular components such as phagocytic cells, interferons (IFNs), the complement system and other signaling and effector molecules [15].

These cells are immediately activated upon encounter with a pathogen, and the innate system is fully activated after a few hours.

The innate immune system can recognize foreign antigen but have low specificity and low affinity compared to the acquired immunity. Recognition occurs by cell surface or intracellular pattern recognition receptors (PRRs). Toll like receptors (TLRs), NOD like receptors and maltose binding proteins (MBP) are examples of PRRs. The PRRs are conserved in humans and are coded by germline DNA. Its specificity is mainly targeting general molecular patterns found on many microorganisms, e.g. polysaccarides, glycoproteins and bacterial lipids, defined as pathogen associated molecular pattern molecules (PAMPS) or damage associated molecular pattern molecules (DAMPS). These molecules serve as the antigen. The innate immune system is not inducible and does not have memory.

1.2.3: Cells and molecules of the innate immune system

Cytokines are proteins secreted by cells to stimulate themselves or other cells to grow, be activated or differentiated and thereby regulate and determine an immune response. They have both pro- and anti-inflammatory potential. Chemokines are a group of cytokines which are small and can cause movement of a cell by chemotaxis.

A very important class of cells that constitute the innate immune system are called Antigen Presenting Cells (APCs). These cells are capable of presenting antigen through the Major Histocompatibility Complex (MHC) on their surface. The antigen may be presented in a processed form to T cells through their T cell receptors (TCRs). Examles of APCs are dendritic cells (DCs). These cells are professional antigen presenting cells. They are responsible for linking the innate immune system to the adaptive immune system by their ability to be activated by two signals; a foreign antigen and a danger signal. They have PRRs on their surface detecting both PAMPs and DAMPs [16].

Another type of APCs are called macrophages, like DC they also have PRRs on their surface [15]. Macrophages are responsible for phagocytosis of foreign pathogens and of dead or dying cells.

Another important group of cells are called granulocytes. These cells are basophils, eosinophils and neutrophils. They have unique morphological characteristics that can be conspicuously identified by staining.

Granulocytes are produced via granulopoiesis in the bone marrow. Neutrophils are the most abundant type of granulocyte. The nucleus is multilobulated in shape. These sentinel cells survey the blood circulation, and can migrate through tissues to eliminate microorganisms in situ. They can eliminate pathogens by 1) phagocytosis of opsonized microbes, 2) degranulation

and generation of neutrophil extracellular traps (NETs) which are web fibres composed of chromatin and serine proteases capable of trapping and 3) killing extracellular microbes [17].

The basophile nuclei have two lobes, they are the least abundant of the granulocytes. They travel from the bone marrow to the site of infection and release the granule content that consists of histamine, heparin, peroxidase, platelet activating factor and other substances [18]. The eosinophils have two or four lobes which are kidney shaped. They function as professional APCs, as regulators of other immune cells, in destruction of tumor cells and in repairing damaged tissue [19].

Natural killer (NK) cells are another type of effector cell in the innate immune system. They have cytoplasmic granules and spontaneously kill infected cells or tumor cells by lysing them. This is known as cytolysis. Another important function of NKs is their role as regulators of the adaptive immune system. They do so by producing signal molecules known as cytokines that regulate B and T cells. Because NK cells are a subpopulation of lymphocytes that are larger and contain cytoplasmic granules, they are therefore also called Large Granular Lymphocytes (LGL) [20, 21].

Mast cells are also rich in granules. These cells are derived from the myeloid stem cell and inhabit the connective tissue throughout the body. They are responsible for hypersensitivity and allergic reactions as well as inflammatory responses. When they become stimulated by an allergen they release the contents of the granules, such as histamine, heparin and interleukins, into the surrounding tissue. This induces vasodilatation, smooth muscle contraction and the production of mucus [22].

The complement system consists of plasma proteins working together to eradicate pathogens. The eradication of pathogens by the complement system can be initiated via three main pathways namely the classical pathway, the mannose-binding pathway and the alternative pathway. These pathways differ in terms of how the downstream cascades are initiated. Although in summary, each pathway induce pathogen opsonization (a process in which pathogens are labeled for phagocytosis), eradication of the pathogen by the process of cell wall destruction and/or the induction of other inflammatory responses to aid in preventing infection [23].

1.2.4: The adaptive immune system

Lymphocytes, both B and T cells, are the key component of the adaptive immune system. The adaptive immune system is inducible, and may take 5-7 days after infection to become fully activated. The adaptive immune response have memory, enabling the immune system to respond quicker and stronger to subsequent exposure of pathogens. The innate immune system is often involved when activating the adaptive immune system. Cells of the adaptive immune system have specific receptors, namely the B cell (BCR) and T cell (TCR) receptor. The affinity and specificity of theses receptors may increase further through lymphocyte maturation as response to antigen stimulation.

Due to the presence of memory cells, the adaptive immune system can provide long-lasting immunity. The adaptive immunity can be divided into two arms: the humoral immunity (B cells) and the cell-mediated immunity (T cells).

1.2.5: Humoral immunity

The humoral immunity is mediated by B lymphocytes. They mature in the bone marrow and are activated on encounter with foreign antigen. Once they are activated, they can become either immunoglobulin-secreting plasma cells or long-lived memory B cells. Immunoglobulins, or antibodies, are found in two different forms, either as membrane-bound on the surface of the B-lymphocytes where they function as the B-cell receptor (BCR), or soluble antibodies. Both forms can recognize and bind antigens. Although B lymphocytes recognizes antigens, they require T helper cells to become fully activated. They simultaneously bind to an antigen and a T-helper cell (usually T-helper 2 cell). An antigen activated T-helper 2 cell is activated by APCs. Dendritic cells or macrophages activate T-helper 2 cells by presenting peptide sequences on specialized proteins called the Major Histocompatibility Complexes of which there are two; MHC I and MHC II. The former is involved in the activation of Cd8+ T cells while the latter is used to activate Cd4+ T cells.

B cells also have MHC-II on their membrane surface. The T-helper cell delivers an activation signal to the B cell. This occurs through the CD40 ligand (CD40L). Provided with these signals, the B cell will mature, proliferate and undergo isotype switching.

1.2.6: Cell-mediated immunity

Antibody-mediated immunity can only protect against pathogens outside the cells, while the cell-mediated immunity provides protection against intracellular pathogens. Cytotoxic T cells, regulatory T cells, and natural killer cells are all part of the cell-mediated immunity. MHC I complexes are present on the surface of all nucleated cells, and intracellular pathogens are processed: ubiquitinated and cleaved by proteasomes, to peptides and presented on these complexes. They can then be recognized by cytotoxic T cells ($CD8^+$ T cells), which will kill the infected cells [24]. They perform cytolysis on the infected cells by producing cytokines such as IFN-γ and TNF, chemokines and microbicidal molecules [25].

1.2.7: Lymphatic and tonsillar tissue

The human lymphatic system consists of tissues and vessels which fascilitate pathogen-lymphocyte interactions. Pathogens are transported by APCs to the lymph node via the afferent lymphatic vessel. Lymphocytes on the other hand enter via the blood vessels. A schematic representation of the lymph node is provided in figure 2.

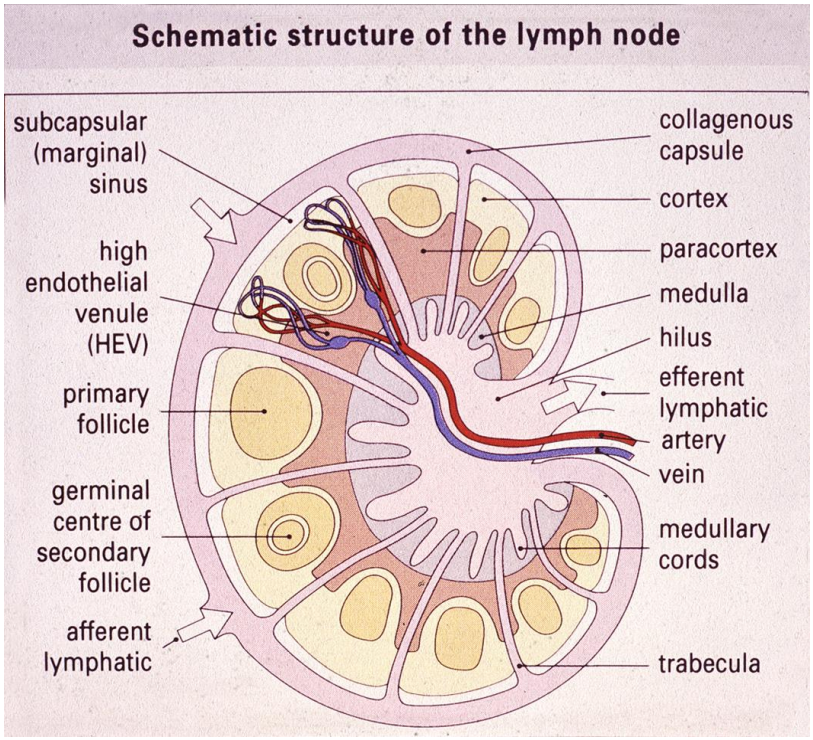

Figure 2. The anatomy of a lymph node [26].

Lymph nodes are the site of lymphocyte formation and of lymph filtering (figure 2). The afferent and efferent lymphatic vessels are passageways through the lymph node. They are lined by endothelial cells and have a thin layer of smooth muscle. High endothelial venules consist of plump endothelial cells which makes the venules appear thick. Blood vessels transverse each HEV, fascilitating direct delivery of lymphocytes. The germinal centers are sites that can appear within secondary lymphoid follicles where B cells can proliferate, differentiate and mutate antibody genes following contact with an antigen.
Lymph nodes exist throughout the body. There are other organs that are very similar to lymph nodes. These are tonsils in the pharynx, peyers` patches in the small intestine and the appendix.

Tonsils are secondary lymphoid organs containing lymphoid cells. They are not encapsulated like lymph nodes and spleen and they do not contain an afferent lymphatic. They consist of many lymphoid follicles [27].
The palatine tonsils are part of the Waldeyer`s ring, which consists of the adenoid (or nasopharyngeal tonsil (NT)), the paired tubal tonsils (TT), the paired palatine tonsils (PT) and the lingual tonsil (LT) [27]. The ring is located as shown in figure 3.

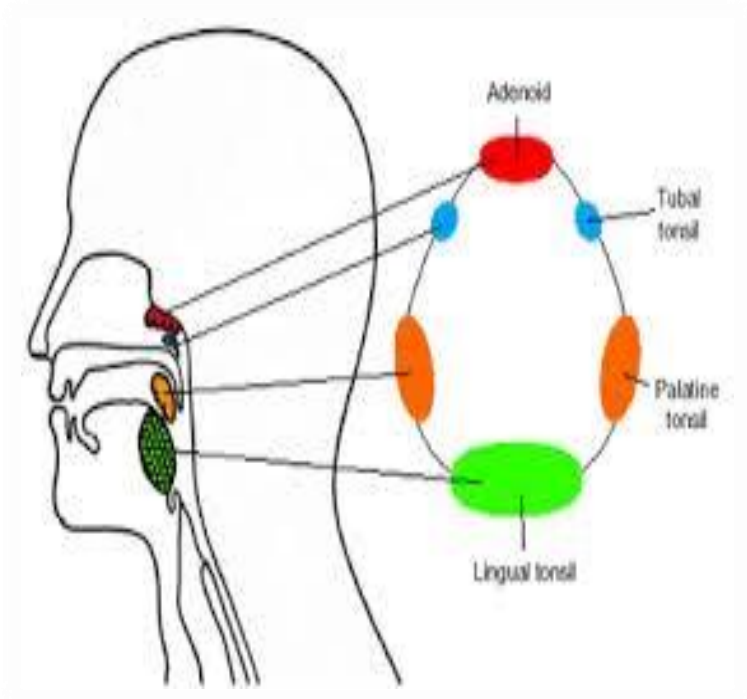

Figure 3. Waldeyer`s ring with its different tonsils. The figure displays the nasopharyngeal tonsil, or adenoid, (NT), the paired tubal tonsils (TT), the paired palatine tonsils (PT) and the lingual tonsil (LT) [27].

The palatine tonsils vary in size. They are the first lymphoid tissue to meet inhaled or ingested foreign microorganisms [28]. Each tonsil consists of a network of crypts that become denser toward the center of the tonsil. In these crypts the antigens can encounter the dendritic cells and be incorporated in, and presented on, their surface [29].
The B cells enter the tonsils through the High Endothelial Venules (HEVs). As they encounter antigens in the crypts, they are directed to the follicles.

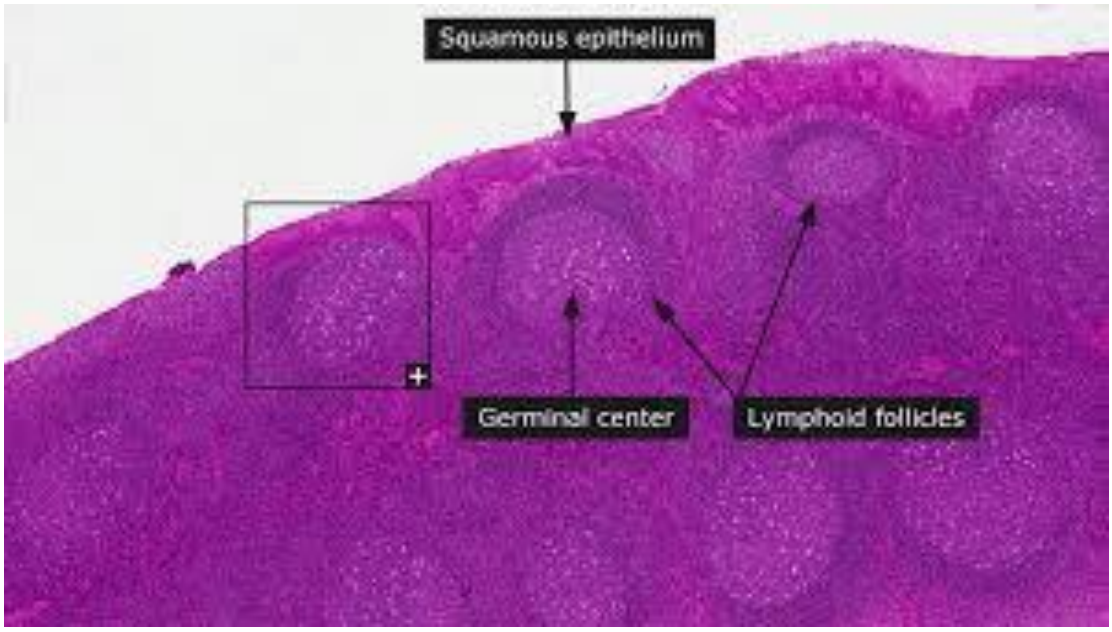

Figure 4. Section of tonsils displaying the squamous epithelium, and lymphoid follicles with and without germinal centers [30].

Germinal centres

Germinal centres (GCs) are sites within secondary lymphoid organs. They develop in the follicles as a response to antigens and are the sites of the B cell proliferation and differentiation that occur as an immune response is taking place [31]. The formation of GCs occurs when B cells rapidly proliferate as an antigen response, expanding from the centres of the follicles. A GC will form after approximately six days during a primary immunization [32]. They are therefore relevant to this study because they can provide information about whether there has been a primary immunization to the antigen provided in the study, and whether this has occurred more recently than six days prior to the tonsillectomy. The light zone appears as the B cells become activated and are selected based on their affinity for the antigen (affinity-based selection). In this way, antibodies with high affinity for the antigen are produced and the B

cells become antigen-specific. Both plasma- and memory- B cells are induced in the GC. T-follicular helper cells help antigen-specific B cells in differentiation and antibody development in the GCs [33,34]. As plasma- and memory B cells, they migrate out of the GC, into the extrafollicular zones where they secrete antibodies [35].

1.3: Vaccination

A vaccine is a formulation that mimics a pathogen and trigger the adaptive immune system without causing serious illness, so that the immune system can recognize the pathogen at a later time and respond appropriately. Vaccination prevents people from getting sick with influenza. Vaccination is to date, the most cost effective measure of preventing influenzarelated hospitalization and death particularly among risk groups e.g. immunosuppressed patients, elderly patients and children.

Vaccines can contain antigens from viruses or bacteria, whole viruses, virus fragments or live attenuated viruses. LAIV is formulated as a droplet suspension and is administered intranasally. It can be trivalent or quatrivalent depending on whether it covers one or two of circulating B strain viruses. In addition to the B strain viruses there are two influenza A strains in circulation each year; H1N1 and H3N2. The natural route of entry for the influenza virus is through the nose, where it replicates on the mucosal membrane. This is the same route of entry for the LAIV virus, which have limited replication in the nasal mucosa. This provides local immunity to the nasal mucosa, in addition to systemic immunity, and it mimics the influenza virus entry into the body, which should give a more similar type of immune response to the influenza virus than the conventional inactivated vaccine [36, 37].

The inactivated vaccine is safe and provides protection from influenza, albeit with limited efficacy in children that has had no or very little exposure to the virus. Interestingly, children respond better to LAIV than to the conventional influenza vaccine [38], and particularly among children in risk groups seems to benefit from this [39,40]. The trivalent, inactivated vaccine (TIV) produces a larger amount of serum antibodies (measured as titre HI), while LAIV produces higher levels of mucosal antibodies IgA. These findings indicate that TIV provides a stronger stimulation of the systemic immune system, while LAIV provides higher stimulation to the local immune system [41].

LAIV is now the preferred type of vaccination in young children above 6 months.

Studies performed on adult subjects show a relatively higher response for the conventional inactivated vaccine [37]. This difference between the age groups is thought to be caused by

older people having encountered more influenza viruses in their life time so their immune system is better adapted to influenza than the immuno naïve children. The highest efficacy of LAIV has been found in children aged 6 months to 7 years. In this demographic, LAIV provides a consistently higher level of protection than the inactivated vaccine [42]. Twenty percent (20%) of children are infected with influenza each year, they have a high burden of the disease and are the main source of spread of the virus [43]. This makes them a good target for vaccination.

Some of the positive aspects of LAIV include the relatively few and mild side-effects, the intranasal formulation negates the need for qualified health personnel, and has relatively better efficacy in terms of protection for up to a year [37].

1.4: Aim of the project

Previous studies on the same cohort have shown that LAIV influences B and T lymphocytes in tonsil tissues [44]. The aim of the current study is to follow up these findings and try to understand the mechanism behind them.

This project has two main goals:

1) To disseminate the process of how the influenza antigen is reaching the tonsillar tissue. This can occur via transport by dendritic cells from the site of vaccination, or by the virus traveling unaided directly to the tonsils, or a combination of these processes.

2) To enumerate the number of influenza specific antibody secreting cells (ISASC) in the tonsils following vaccination.

By answering these two questions, we will achieve a better knowledge of how the immune system responds to the vaccine, or more specifically, influenza specific antigens present in the LAIV.

2: Materials and methods

2.1: Materials and study layout

2.1.1: Study design

This project is part of a cohort study called "Immune response to live attenuated influenza vaccination". The vaccination and tonsillectomy of children took place from October 2012 to January 2013. Subjects were recruited during their pre-tonsillectomy consultation at the otorhinolaryngology unit at the Haukeland University Hospital in Bergen, Norway. They were given a detailed explanation of the study. Both parents and children over 12 years gave written nand informed consent prior to participating in the study. The main material used in this study are the tonsils obtained from the patients.

2.1.2: Patients

The study cohort consists of 58 children aged 3-17 years. They were divided in four groups according to their scheduled time for tonsillectomy.

Controls: not vaccinated

Group 1: Tonsillectomised 2-4 days post vaccination

Group 2: Tonsillectomised 5-9 days post vaccination

Group 3: Tonsillectomised 10 + days post vaccination

4 of the children were excluded, and 16 of the children were controls. 38 children were vaccinated, 20 boys and 18 girls.

2.1.3: The vaccine

The intranasal live attenuated influenza vaccine formulation contains the 2012-2013 seasonal influenza virus strains (see table 4), and is marketed under the name Fluenz (Astra Zeneca, UK) in Europe (FluMist in the USA). Fluenz is produced by Astra Zeneca, UK. The vaccine contained 107.0 fluorescent focus units (FFU) of live attenuated influenza virus of each of the

strains mentioned in table 4. The vaccine was administered intranasally as a 0.1 ml spray dose in each nostril. Children 3-9 years old were immunized with 2 doses at an interval of 4 weeks, and children over 10 years old were immunized with a single dose of the vaccine. After each dose of vaccine the local or systemic side-effects and influenza symptoms occurring after vaccination were self-reported and registered in the study database. Examples of influenza-like symptoms include nasal congestion, rhinorrhea, upper airway symptoms, malaise, fever, reduced appetite, and headache.

2.1.4: Inclusion and exclusion criteria

A physical examination was performed and medical history was recorded by the recruiting doctor. Generally healthy subject between 3-17 years, scheduled for tonsillectomy were chosen for the study. The children were also included if they had mild to moderate asthma (clinically stable with daily use of inhalers) and if they were already immunized with an influenza vaccine. Individuals with the following conditions or symptoms were excluded from the study. These include patients with serious chronic medical conditions, serious asthma, recent influenza, fever, pregnancy, use of acetyl salisylic acid (ASA) or immunosuppressive therapy, allergy to the vaccine components or earlier complications to vaccination, or children under governmental custody.

2.1.5: Approval

The study is approved by the Regional Ethical Committee of Western-Norway and the Norwegian Medicines Agency [37]

2.1.6: Sample collection

The children were vaccinated between 2-20 days prior to their scheduled tonsillectomy. For this project, tonsil RNA from 20 patients were previously extracted and prepared. Three of them were non-vaccinated patients. After the tonsillectomy, the tonsils were homogenized and added to PAX gene tubes (table1), before downstream analysis was performed.

2.2: Total RNA extractions from tonsillar tissue

2.2.1: Sample extraction and storage conditions

Total RNA from tonsil tissues were initially extracted and prepared for PCR analysis and gene expression profiling. For the purpose of this study, PCR analysis was performed to investigate the presence of viral DNA in the tonsils which is indicative of viral replication.
For gene expression analysis, palatine tonsil tissue was submerged in PAXgene Blood RNA Tube reagent (table 1). The proprietory reagent in the tubes stabilizes intracellular RNA by inhibiting ribonuclease (RNase) activity. The tubes were stored at room temperature for ≥ 2 hours and then at -20℃ until total RNA isolation experiments were performed.

2.2.2: Total RNA Isolation

Preparations: The entire total RNA isolation procedure was performed at room temperature (20-30˚C). The frozen samples were thawed at room ambience for approximately 2 hour prior to processing.
Ethanol was added to the washing buffer concentrate. Inverted gently and repetitively until a homogenous washing buffer solution was attained. RNaseI solution was prepared by adding 550 µl of diluents to the lyophilized DNase I in the glass vial and mixed by inverting it gently. Subsequently, the DNase I solution was aliquoted into 1.5 ml microcentrifuge tubes and kept at 4℃ until the genomic DNA step of the experiment. 700µl of buffer solution was added to 100µl of DNase I solution, to make the DNase I incubation mix. The contents were subsequently mixed into a homogenous mixture and centrifuged for 1-2 seconds. The shaker incubator was programmed to 5℃ before proceeding.

2.2.3: Tonsillar tissue re-suspension

The tubes with pellets containing the thawed tissue mixture were vortexed. 3 ml of the suspension was transferred to a new PAXgene Blood RNA tube (BRT) and centrifuged at 4000g for 10 minutes. After discarding the supernatant, the pellet was resuspended in 4 ml of RNAse-free water and centrifuged at 4000g for 10 minutes. The subsequent discarding of the supernatants was performed carefully to avoid disrupting the pellet. Following this, the pellet was dissolved in 350µl of buffer and vortexed for approximately 1 minute until a homogenous lysate was formed. The lysate was then transferred into the labeled 1.5 ml tubes. 300µl of biding buffer solution and 40µl proteinase K were added to the lysate to denature the proteins. The mixture was briefly vortexed and then placed in a shaker-incubator for 10 minutes at 1400

rpm in 55 °C. They were then placed on ice-bath. The lysates were placed on PAXgene Shredder Spin Columns and centrifuged for 3 minutes at 18000g. The fibrate was transferred to new 1.5 ml tubes, and 350µl of 96% ethanol was added to the tubes. The tubes were then vortexed and centrifuged briefly. The solution from these tubes was transferred onto membranes in the PAXgene RNA Spin Columns. The columns were centrifuged for 1 minute at 1800 g. Washing buffer was added to the membrane and the membrane columns were centrifuged for 1 minute at 18000 g. 80µl of DNase I incubation mix were placed on the columns and incubation time was 15 minutes at room temperature. The columns were then washed again with 350µl washing buffer at the same speed and time. 500µl washing buffer and ethanol solution was pipetted directly onto the membrane and centrifuged for the same time and at the same speed as above. After the centrifugations, the processing tubes were discarded and replaced. The membrane was again washed with the washing buffer and centrifuged for 1 minute at 18000g. The RNA was placed in the shaker-incubator for 5 minutes at 65 ℃. The total RNA was obtained from the samples and the yield was about 80µl per sample.

2.2.4: Chemicals and kits

The chemicals and kits are listed in table 1.

Tabel 1.Overview of chemicals and kits.

Name	Number	Country	Supplier	Use
PAXgene BloodRNA tubes (100)	762165	Hornbrecht Germany	PreAnalytix GmbH	Tonsil tissue storage
PAXgene BloodRNA kit (50)	762174	Hornbrecht Germany	PreAnalytix GmbH	Total RNA Isolation
PAXgene tissue RNAkit	765134	Hornbrecht Germany	PreAnalytix GmbH	Total RNA Isolation
RNeasy Midikit	75142	Hilden Germany	Quiagen GmbH	Total RNA Isolation

2.3: Detection of influenza genes in total RNA samples

2.3.1: RNA purification

The concentration was recorded and so were the 260/280 nm and 260/230 nm ratios [45]

The concentration of RNA is calculated by the given formula from

Beer-Lamberts law: C = (A*E) / B. Where A = absorbance at 260nm, E = extincion coefficient (40 ng-cm/ul for RNA), B= pathlength of 0.02 cm and C = concentration of RNA.

2.3.2: Polymerase chain reaction (PCR) overview

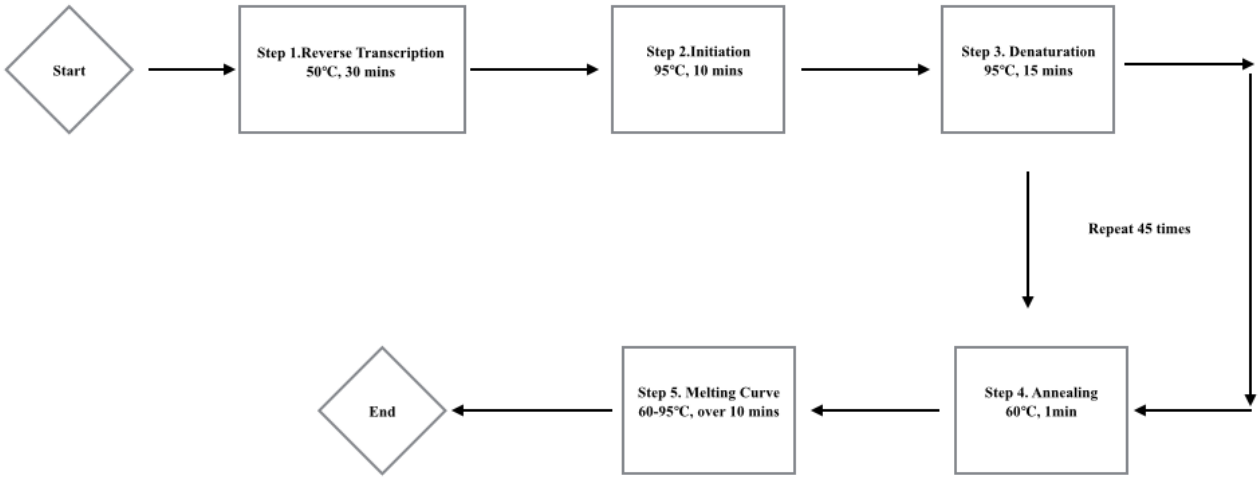

Figure 5. Flow chart over the PCR steps.

The PCR cycle is depicted here in 5 different steps (figure 5), with each step comprising a certain temperature.

In the first step of the PCR cycle, the total RNA is used as a template for complementary DNA (cDNA) production. This is achieved by reverse transcription which was performed for 30 minutes at 50℃.

The second step, the initiation step, was run at 95 ℃ for 10 minutes to activate the Taq polymerases.

During the denaturation step, step 3, the reaction was held at 95℃ for 15 seconds. This causes the DNA double strands to split up to single strands by disrupting the hydrogen bonds between the complementary bases.

The fourth step is the annealing step. Here the reaction temperature was lowered to 60˚C for 1 minute, which is the recommended temperature for annealing of the primers to the target sequence. Annealing is mediated by stable hydrogen bonds which forms when the primer sequence closely matches the target sequence. Sequentially to this, the polymerase binds to the primer-target sequence ready to start elongating the primer sequence along the target DNA to form a complementary strand. Steps 3 to 4 were repeated 45 times (PCR- cycle). The fifth program was a melting curve where the temperature raises steadily from 60 ℃-95℃ in 10 minutes.

2.3.3: Performing quantitative PCR (q-PCR)

The primers and probes were thawed at room temperature for approximately 5 minutes and vortexed briefly. The PCR-components were then prepared in master mixes (see table 2).

Table 2. Mastermix for PCR.

Components	Amounts (µl)
Nuclease-Free Water	88
2 X Reaction Mix	200
Forward Primer	8
Reverse Primer	8
Probe	8
Superscript III RT/plat. Taq Mix	8
Total	320

Four master mixes were prepared, one for each virus type (table 3).

Table 3. Primers and probes for the different virus strains applied to the experiment.

Virus	Type	Function
1	Influenza A	*Fprimer, Rprimer, Probe
2	Influenza H1	Fprimer, Rprimer, Probe
3	Influenza H3	Fprimer, Rprimer, Probe
4	Influenza B	Fprimer, Rprimer, Probe

*Fprimer = Forward primer, Rprimer = Reverse primer.

Twenty µl (20) of mastermix was applied to 8 wells (vertically on a 96 well plate) for each mastermix. Then 5 µl either of negative control (RNase-free water), positive controls (Human Seasonal Influenza Positive control and A/H5N1 Positive control) or RNA-samples from the tonsillar extractions of different patients, was pipetted into the wells with the different mastermixes on the PCR-plate. The plate was centrifuged at 1500 g for 2 minutes at 4˚C.

The positive controls are
1: Human Seasonal Influenza (H.S.I.) and
2: Influenza A/ H5N1 (A/H5N1).

PCR was run to analyze each sample, the positive and negative controls were applied. The second and third experiments were programmed and prepared the same way as the first, the only difference being that more samples were examined in the first PCR run where the total amount in each well was 25µl instead of 19µl in the following experiments, to save material. After the PCR-run, the wells that contained positive sequences for the viruses examined, had been amplified. The amplification is detected by the Light Cycler PCR machine during the amplification, and is depicted as sigmoidal curves. They have crossing point-values (Cp-values). A Cp is the PCR-cycle where the fluorescence from amplification exceeds the background fluorescence [46]. The Cp value is inversely correlated with the number of target sequences. The viral strains shown in table 4 were the strains that were used in the 2012/2013 LAIV influenza vaccine.

Table 4. Viral nomenclature.

Strain Name	Antigen	Strain/ Isolate	Year	Origin
A/California/07/2009 (H1N1) pdm09-like strain	A	7	2009	California
A/Victoria/361/2011 (H3N2)-like strain	A	7	2011	Victoria USA
B/Massachussettes/2/2012 – like strain	B	2	2012	Massachusettes

2.4: Detection of influenza specific antibody secreting cells in tonsillar tissue

2.4.1: Labeling of influenza antigen with a fluorescent dye.

Protocol for conjugating virus antigens with Alexa Fluor488:

The conjugated antigen was a reassortant, whole, attenuated virus (X-179A) from the H1N1 strain. A/California/07/2009pdm swine flu strain had donated heamagglutinin, neuraminidase and PB1. The other viral proteins; PPB2, PA, MP, NP and NS was donated from A/PuertoRico/8/1934 (H1N1) strain. This virus contains all the antigens from the 2012/2013 influenza season.

In this experiment Alexa Fluor488 dye was used as the fluorescent colour. Alexa 488 emits a green colour when excited by blue fluorescent light. The green colour is visible in the microscope when the microscope radiates the tissue with blue light. Alexa was conjugated to the antigen from the virus H1N1. Antibodies to the virus specific antigen will be detected as green spots.

Influenza whole virus antigens were labeled with alexa 488 as described in the Alexa Fluor Protein Labeling Kit that comes with the Alexa Fluor Protein Labeling package.

A row of 20 tubes with different amounts of antigen and dye that has been homogeneously mixed was obtained from running the column. The tubes were tested on the NanoDrop^{tm} to measure the amount of proteins in each tube. The eluation buffer was used as a blank and IgG was put as the sample type. The chosen program was "Proteins and Labels". Alexa Fluor 488 was elected as dye. The baseline was automatically set to 340 nm. This is very close to zero absorbance, and all spectra are referenced off this zero.

For protein measurements a 2 µl sample is required. Nanodrop measured the absorbance of the Alexa 488 dye as dye 1 ($\lambda 1$), and the absorbance at the user-selected wavelength (set to 280nm) as dye 3 ($\lambda 3$). Proteins are visible at 280 nm. The ratio between dye 1 and dye 3 ($\lambda 1/\lambda 3$) was also calculated. The concentration of proteins in the sample was calculated, by putting the absorbance at 280 nm minus the absorbance at 340nm (zero absorbance), in mg/ml. This measures the µM of the Alexa dye [47]. The influenza specific antigen was conjugated with the colour using a gel-filtration column. The reaction mixture was collected in different fractions. The concentration peak was between fraction 12 and 14, with the highest concentration at fraction 13. This fraction was therefore elected to stain the tonsillar tissues.

2.4.2: Immune fluorescent staining of influenza specific B cells in tonsillar tissue.

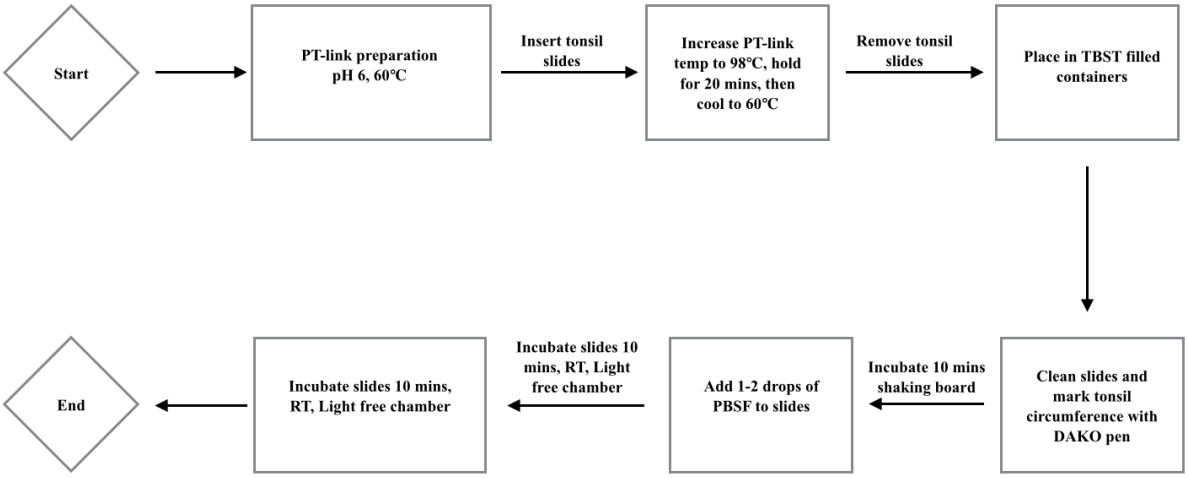

Figure 6. Flowchart depicting preparation for antigen conjugation.

The tonsil tissue slides were inserted into a conditioned PT-link instrument to remove the paraffin, obtain cell permeability and reduce background staining by washing away superfluous antigens and antibodies. They were subsequently rehydrated before the antigen was retrieved. TBST is a mixture of Tris-buffered saline and Tween 20. TBST, which consists of 500ml 0.87% NaCl, 550ml 10*TBS and 5.5 ml Tween 20 has a pH of 7.6. Tween is a detergent and can be used to make cell membranes permeable to for example antigens. Note, that the sections must not be too wet nor risk drying out. The boundaries of the tonsils were marked with a DAKO pen. The marking provides a barrier to liquids such as antibody solutions or chromogenic substrates applied to the sections, thus helping to obtain more

uniform immunohistochemical staining [48].

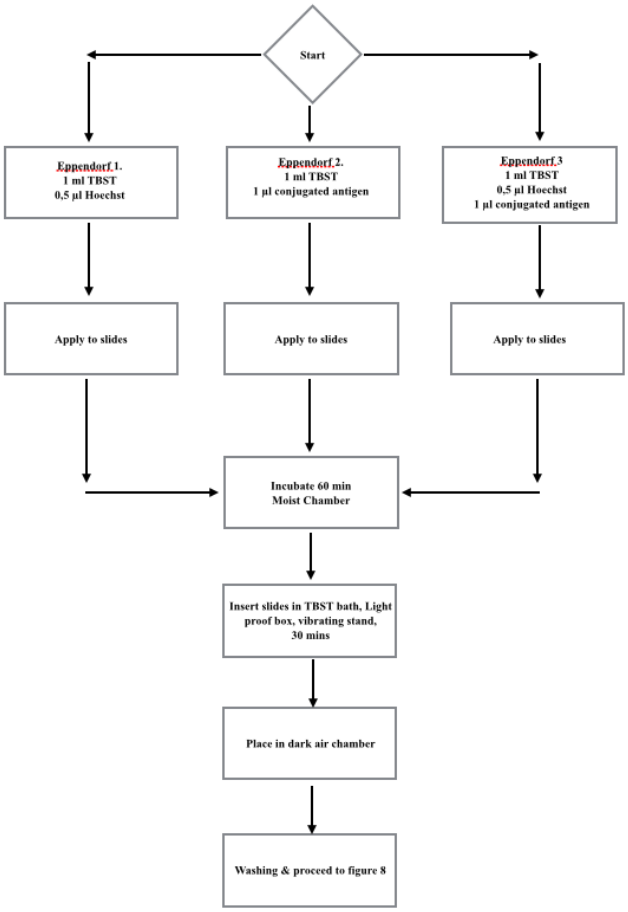

Figure 7. Flowchart depicting the antigen conjugation process.

One to two drops of the protein block serum-free (PBSF) were applied is to prevent non-specific background staining.

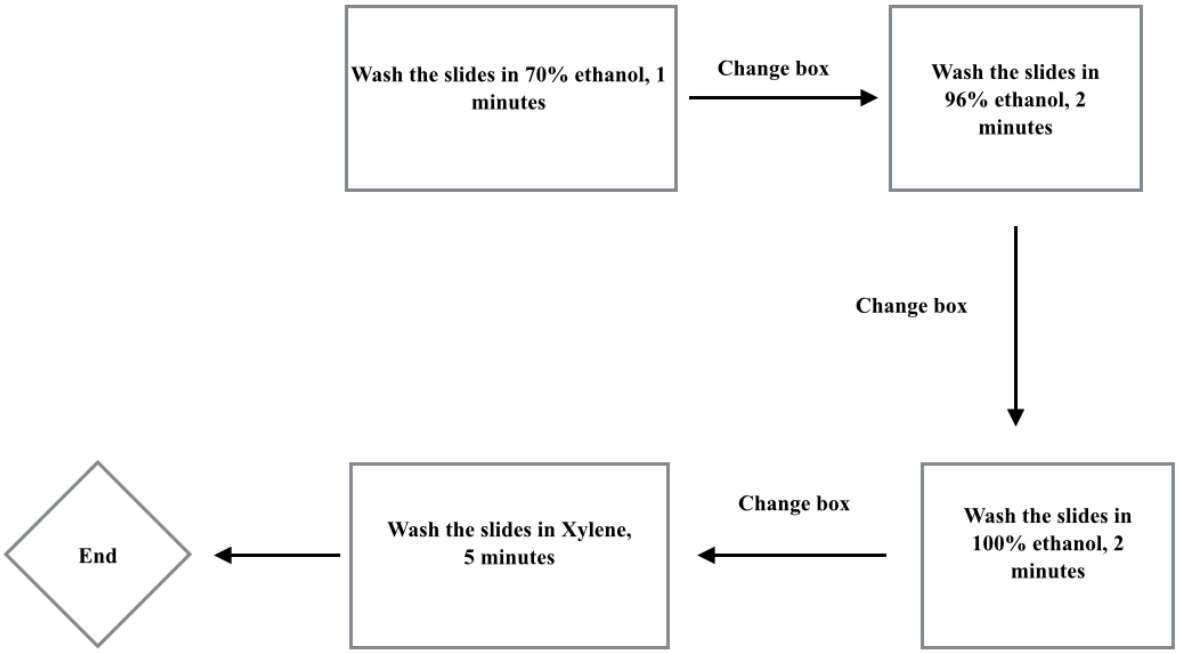

Figure 8. Flowchart of preparing the sections before covering them with Eukitt.

The sections were obtained from the xylene bath with tweezers and placed on paper. One drop of Eukitt was pipetted over the tonsil tissue sections and a thin glass cover was put over the section.

The tonsil tissue sections are very sensitive to light after they are stained with immune fluorescence. Contact with light will make the fluorescence fade, therefore it is important to keep the stained tonsil tissue sections away from contact with light. They were stored in a paper booklet.

The microscope is from Leica, and the objective used had a 20X enlargement. The microscope was connected to a computer and a camera could transfer the microscope image to the computer and the image could be captured on the computer. Three images from each tonsil sheet were captured. The cell specific signals on each image were counted. For each tonsil sheet the mean of the signals were calculated.

Table 5. Chemicals and kits used for the experiments

Name	**LOT/Catalogue nr**	**Country**	**Supplier**	**Use**
Invitrogen Superscript III One-Step RT-PCR Platinum Taq HiFi	LOT: 1565687	USA	Life technologies	Reverse transcription
Influenza Virus Real Time RT-PCR Assay	LOT: 58893016	USA	bei Resources	PCR
PreAnalytiX RNase-free Water	LOT: 145026459	Germany	QIAGEN GmbH	Dilution
Ambion Nuclease-Free Water	AM9938$1308045	USA	Life technologies	Dilution
Alexa Fluor 488 Protein Labeling Kit	LOT:	USA	Invitrogen	Detection of antibodies

3. Results

3.1: Detection of Influenza vRNA in tonsillar tissue

3.1.1: vRNA isolation

Total RNA was extracted from tonsillar tissue homogenate from 20 patients. The quality and quantity was assessed. The quantity of the RNA samples was measured by reading the absorbance at 260 nm in a nanodrop instrument. We found that the RNA concentrations varied from 42 – 179 ng/µl, with an average of 99 ng/µl.

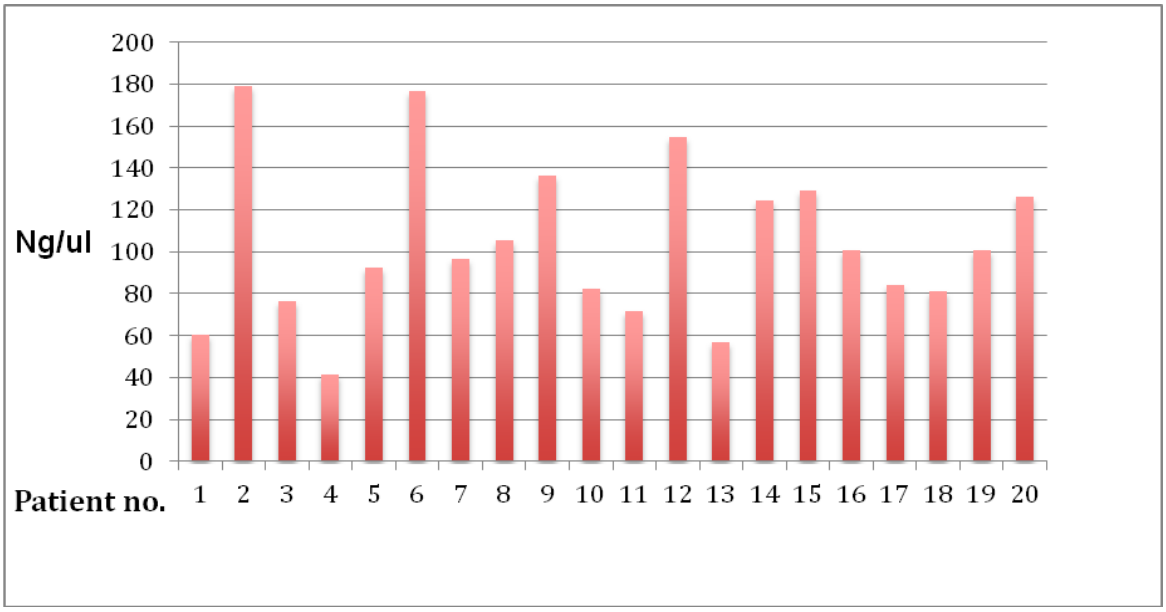

Figure 9. RNA concentration (ng/µl) per patient. The figure shows the RNA concentration in the samples extracted from tonsillar tissue. The Y-axis on the figure represents the RNA concentration (nanogram per microloiter) obtained for each patient (X-axis).

The quality of the RNA samples was determined by measuring the absorbance at 260 and 280 nm and calculating the ratio. An A260/A280 ratio of 1.8-2.2 indicate pure RNA from proteins [49, 50]. High absorbance at 280 nm indicate contamination of protein, so a ratio below 1.8 would indicate protein contamination in the samples [51]. The quality of the purified RNA is displayed in figure 10, with a mean ratio of 2.16. This indicates that the RNA is pure [52].

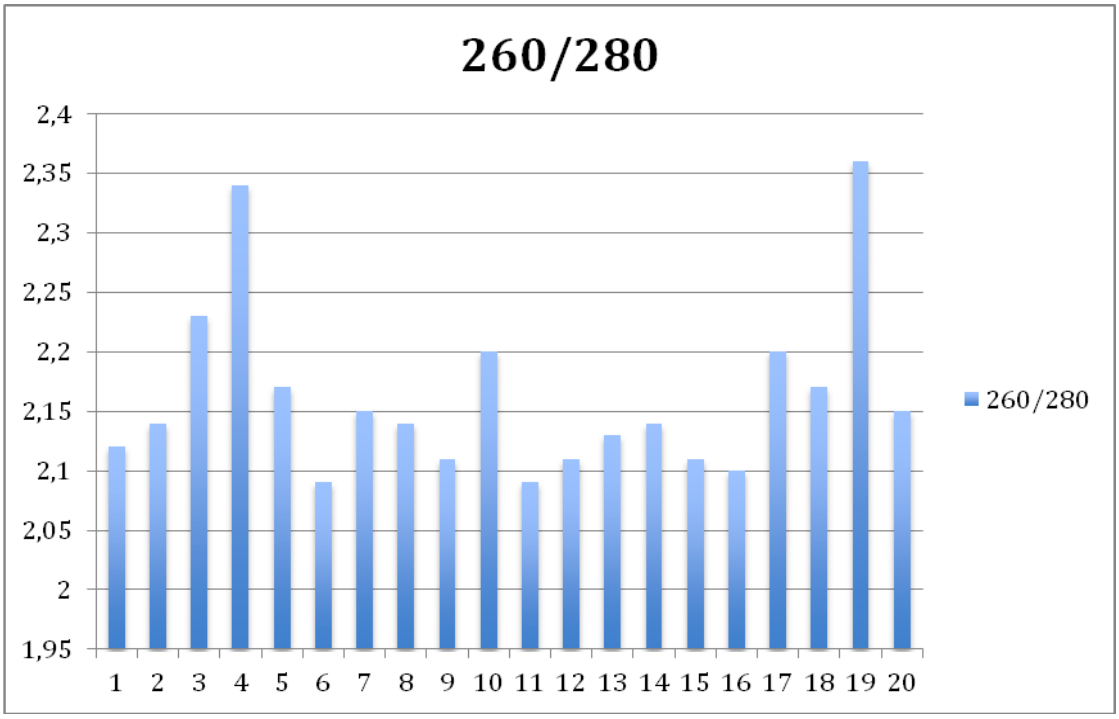

Figure 10. Absorbance ratio assessing RNA purity**.** The figure shows the purity of the RNA extractions from 20 patient samples. The Y-axis displays the A260/A280 ratios from 20 patient samples that are distributed along the X-axis.

3.1.2: Reverse transcription and quantitative PCR

We performed RT and qPCR on tonsillar RNA to investigate the presence of influenza specific RNA. We found the positive control samples to be positive, but were not able to detect any vRNA in the patient samples. (Figure 11 and table 6).

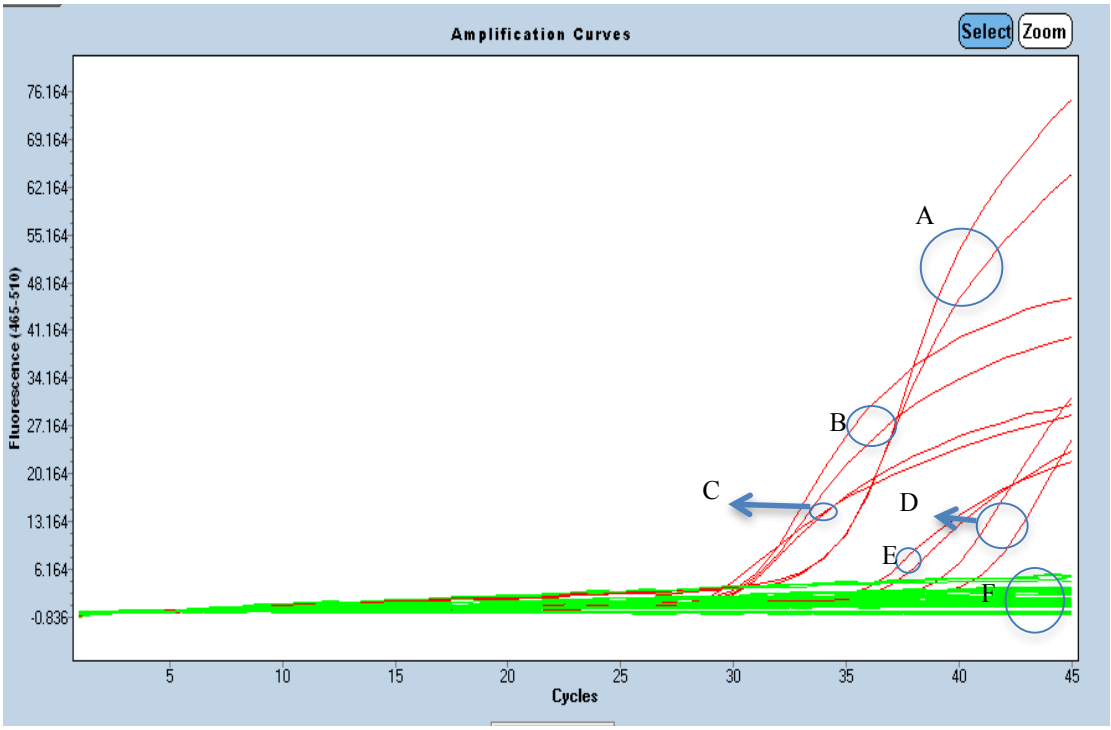

Figure 11. Reverse-transcription real-time PCR of influenza gene sequences in RNA samples extracted from tonsillar tissue (green) and positive controls (red). The y-axis shows the fluorescent signal during the 45 PCR cycles indicated on the x-axis. A): Human Seasonal Influenza (H.S.I) positive control in the influenza B mastermix.

B): A/H5N1 positive control in the influenza A mastermix. C): H.S.I positive control in the influenza A mastermix. D): H.S.I positive control in the influenza A H1N1 mastemix. E): H.S.I positive control in the influenza A H3N2 mastermix. F): RNA samples extracted from patients, in all mastermixes.

Tabel 6. The Cp-values of the positive controls from Human Seasonal Influenza and A/H5N1.

	Positive control∗	Control A/H5N1
H3N2	35.28	
	36.17	
H1N1	39.60	
	37.97	
A	29.26	30.06
	31.04	30.18
B	34.01	
	34.53	

∗The Human Seasonal Influenza virus sample contains all seasonal influenza viruses; H1, H3 and B strains.

All patient samples had Cp-values >45, and are deemed influenza negative.

3.2: Quantification in tonsillar tissue sections

3.2.1: Fluorescent labeling of influenza antigen

We conjugated whole influenza virus proteins with a fluorescent dye (Alexa 488), enabling to stain the virus by fluorescence microscopy. This was done using a column which yielded 20 fractions containing conjugated antigens. Antigens are proteins and they emit UV-light at 288nm [51]. After the labeling procedure we ran a column filtration to remove unbound fluorescent dye. The samples were collected and analyzed. The absorbance values at 280 and 488 nm of the fractions were measured using the nanodrop instrument. There was a peak between fractions 12-14, with fraction 13 having the highest concentration of antigen. This fraction was elected to stain the tonsillar tissues.

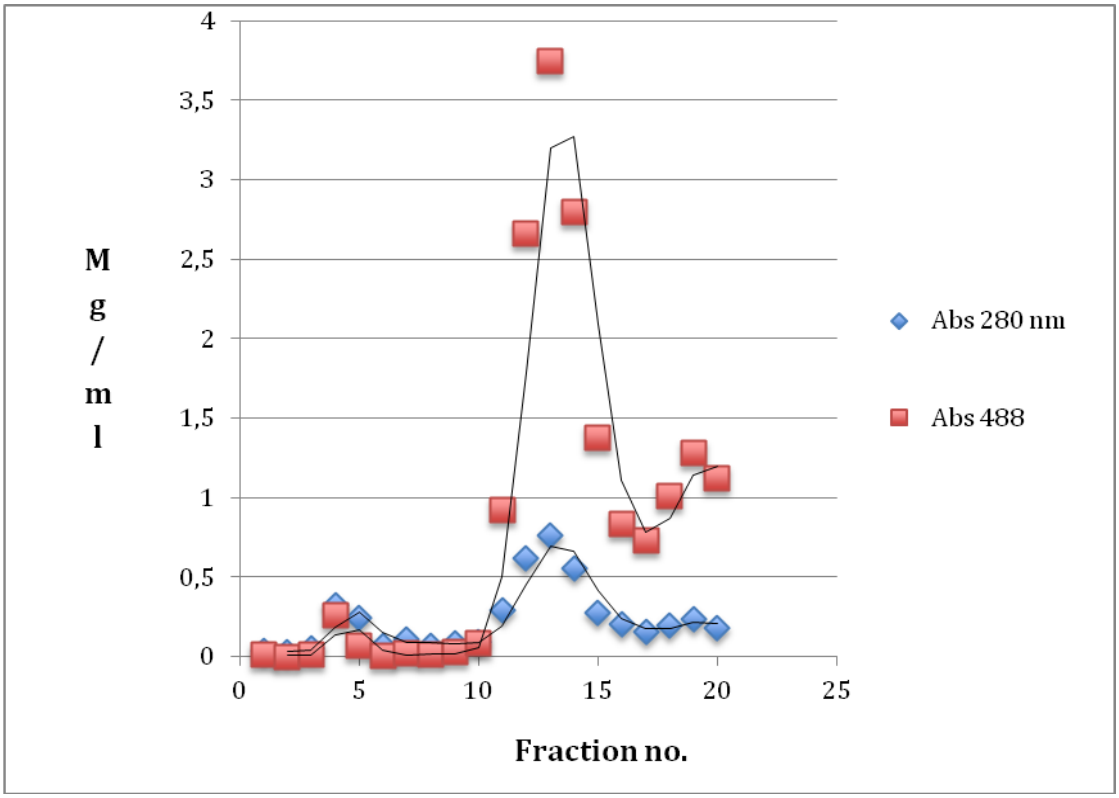

Figure 12. Alexa 488 labeled influenza antigen. After labeling influenza antigen with Alexa 488, the labeling reaction was applied to a gel-filtration column, and fractions from 1-5 ml was collected. The absorbance at 280 and 488 nm was measured in all fractions, by nanodrop as described in materials and methods.

The peak fraction was fraction 13 (figure 12), and was used in the immune fluorescent staining. The antigen stained sections were evaluated under microscopy. Three sections from each tonsillar tissue were photographed and the antigen secreting cells (ASC) in each image were counted. The magnification was 20X, and the picture size was 0.36mm^2. 3 pictures * 0.36 mm^2 = 1.08mm^2. Each ASC signal was divided by 1.08mm^2 to give the metres in square mm (mm^2).

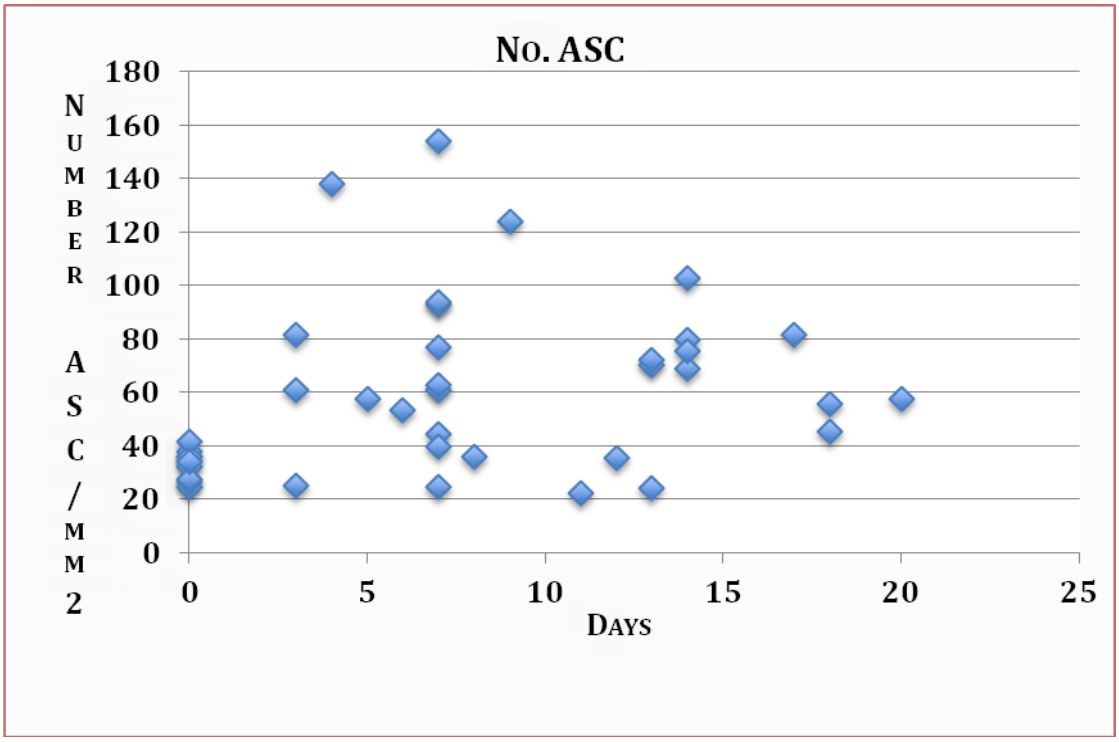

Figure 13. Number of influenza H1N1 specific antibody secreting cells in tonsillar tissue sections. Each dot indicate one patient, with number of ASC/mm^2 on the y-axis and days post vaccination on the x-axis.

Table 7. Patients, days and mean ASC.

Days	Patients	Mean No. ASC
0	9	35.6
3	3	60.4
4	1	35.3
5	1	62
6	1	57.7
7	9	78
8	1	39
9	1	134
11	1	24
12	1	38.3
13	3	53.3
14	3	88.3
17	1	88
18	2	54.7
20	1	62

The table displays what days post vaccination the tonsillectomy was performed, how many patients` tonsillar tissue sheets were counted on that day and the mean no. ASC calculated for the patients tonsillectomised on the same day post vaccination.

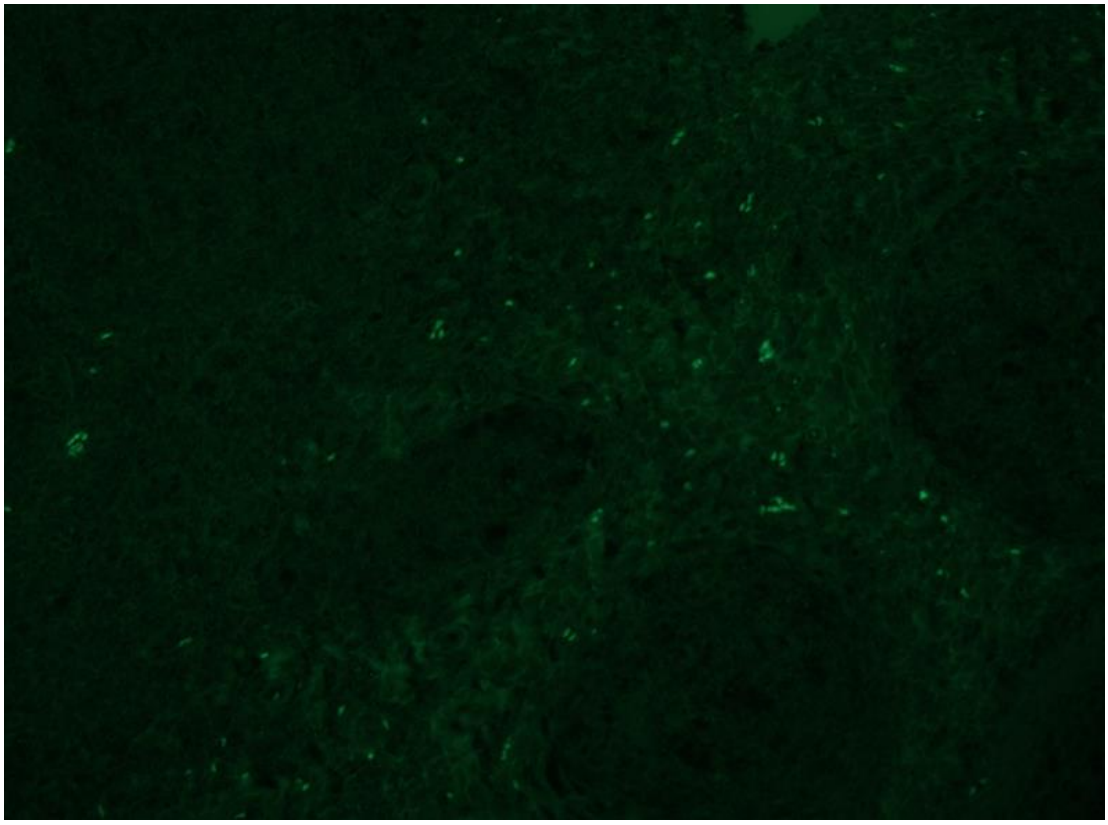

Figure 14. A microscopy image of tonsillar tissue stained with influenza virus antigens conjugated with Alexa 488. The size of the image is 0.36mm².

4: Discussion

This study was performed to assess the status of influenza virus in tonsils. Also, the expression levels of ASCs between subjects who have been vaccinated and the subjects who were not vaccinated prior to tonsillectomy.

4.1: RNA Isolation

We assessed the quality and quantity of vRNA in the tonsillar tissue homogenates from 20 subjects, using the UV spectrophotometry method. The nucleic acid concentration was calculated by using the Beer-Lambert law, where the reading at 260 nm is used along with an extinction coefficient for RNA which is 40µg/ml, and the RNA purity is assessed by using the A260/A280 ratio. RNA absorb maximally at 260 nm, and proteins have an absorption maximum at 280 nm. The Nanodrop™ spectrophotometer (Thermo Fisher, USA) is capable of analyzing small samples of 1µl which saves valuable sample material and avoids the use of a cuvette. Because of the small sample size required, samples does not need to be diluted before measurement and one measurement can be taken in less than 30 seconds.

RNA is very susceptible to degradation because of the abundancy of RNases in the environment. While the RNA purity is determined by measuring the ratio at 260/280 nm. A ratio of 1.8–2.2 is considered as a sample of pure RNA. Because the ratio can be affected by pH, a ratio of over 1.8 is generally satisfactory [52, 53]. High vRNA quality and quantity is essential for reliable downstream analyses and for attaining accurate results. Our results show that the concentrations of vRNA from the 20 subjects measured, were between 42-179 ng/µl. From figure 10 we can see that all of the fractions had a ratio of over 2.0 and over half have a ratio of above 2.1. It was therefore deemed that the vRNA was of good enough quality and quantity to be subjected to the PCR experiment.

4.2: Reverse transcription and quantitative PCR

Reverse transcription qPCR was performed on the samples in order to identify if the influenza virus was present in the samples. The PCR was run for 20 samples and the positive controls: Human Seasonal Influenza (HSI) and A/H5N1 virus. Both the positive and the negative control samples gave the expected results, showing that the method was working properly. However, we were not able to detect any positive vRNA in the patient samples. The positive control sample sequences had amplified during the PCR because the primers and probes used in the

experiment had specific sequences for the positive controls. This decreases the possibility of false negative results as the primers work on the materials that have been used in the experiment. This does not however exclude the possibilities of false-negative results. False-negative results can for example occur by glove-powder inhibiting the various PCR-steps. False-positive results can occur by contamination of amplified sequences [54]. The results indicate that the LAIV virus does not replicate as far down into the body as the tonsils.

The LAIV is cold adapted to a temperature of 25 ℃. This is the optimal temperature for replication of the virus. This ability will decrease as the temperature rises [55]. The temperature in the upper respiratory tract is about 32-33˚C, which is lower than the rest of the body [56].

There have previously been detected dynamic changes in gene expression levels in the tonsils after vaccination. This gives strong indication that the vaccine has reached the tonsils [44].

This present work aim to assess the mechanism behind this.

The initial hypothesis was that this could have happened in one or more of three ways. One option is that the virus travels directly to the tonsils after vaccination and replicate there, the other option is that the virus antigen is collected in tonsillar crypts and the third option is that the virus or virus antigen is brought to the tonsils from other areas.

The direct route of the virus, or viral antigens, to the tonsils were deemed to be less likely because no replicating vRNA was detected in the patient samples.

The crypts are invaginations surrounded by patches of epithelial cells, where replicating virus usually are collected [57, 58]. We couldn’t find any replicating virus which indicates that this was an unlikely pathway for the virus to reach the tonsils. The third way seems the more probable way. Along this route the virus antigen is transported to the tonsils from other areas in the upper airways. Dendritic cells, macrophages and B cells are all professional antigen presenting cells (APCs). APCs reside in the upper airways and sample antigen from inhaled air and migrate to lymph nodes where they accumulate and present antigen to naïve T cells [59]. The main hypothesis in the current study is that the DCs have captured the antigens and transported them to the tonsils. This mechanism for the antigen to enter the tonsils was deemed the most probable mechanism, and it does not require the virus to actively replicate on the tonsils. APCs can transport the virus antigens to the lymphocytes in the tonsils and present them there. APCs circulate through the environment of the body and capture antigens along the way. They incorporate them and process them either into proteins to be presented via humeral immunity (helper T cells), or into peptides to be presented via cell-mediated immunity (cytotoxic T cells). APCs present proteins through MHC class II molecules, to helper T cells

($CD4^+$ T cells). These T cells can help the B cells recognize the antigen, and start the humoral immune response. This is the common route for extracellular antigen, whereas viruses invade cells and virus antigens are found inside the cells. The cell-mediated arm of immunity responds to intracellular pathogens in that the antigens are processed to peptides and presented on MHC class I molecules. These MHC molecules exist on all nucleated cells, and present the peptide to $CD8^+$ T cells. MHC class I molecules are also found on dendritic cells, as the only APCs, which can cross-present antigens in both the humoral and cell-mediated arm of immunity. DCs are found in tissues that are in contact with the outside environment, such as the nasal lining. Once they become activated by an antigen, they move to the nearest lymphatic tissue. In this case the tonsils. Here they activate antigen specific T cells. DCs are the only APCs to activate naive T cells [60]. They are also the only APCs that efficiently present viruses that are no longer active. They do not require actively replicating viruses to present an antigen peptide to CTLs. The presentation of a peptide is enough to mediate a cytolytic T cell response. In this way, they are considered the most efficient candidate for presenting the vaccine virus antigen to the tonsillar tissues. Of all the APCs, they are the most potent in eliciting cytotoxic T cell responses [61]. A previous study has discovered that as long as a virus is incorporated to DCs it can provide the same $CD8^+$ cytolytic T cell response in the lymphatic tissue as a live virus, even when the studied virus is no longer replicating [62]. Because of the abilities of the DCs mentioned above, they are the most probable APCs to present the virus antigens to the tonsils. The fluorescent labeling of influenza antigen shows that T and B lymphocytes have responded to the influenza virus.

4.3: Fluorescent labeling of influenza antigen

In the final part of the present project the H1N1 virus antigen was labeled with a fluorescent marker. The fluorescently labeled antigen was purified by gel filtration. The fraction that contained the highest and purest antigen was collected and used to stain influenza antibody secreting cells in the tonsil sections of 20 different subjects. The number of cells that produces specific antibodies for the H1N1 virus (ASCs) were counted. The standard and most commonly

used method for measuring humoral immune responses is the hemagglutination inhibition assay (HAI). However it is impossible to accurately assess the proven protective immunity elicited by LAIV [63]. Other methods to measure the humoral immune responses include quantifying serum antibodies and ASCs. All of these three methods have been found to correlate with the administered dose of LAIV. Results from other studies have identified the antigen-specific ASC frequency as a good marker of LAIV –induced B cell immune response [64]. It is not a completely accurate method either because it involves counting the ASCs and in this current project to choose the a few different parts of the tonsil sections to count. The largest mean ASC count (134) was observed on day 9 post vaccination (table 7). This number is higher than the other days, day 14 and 17 share the second highest mean count (88.3 and 88 respectively). The antigen-specific memory B cells and plasma cells appear within one week of the first encounter with the pathogen [65], so a high ASC count around 9 days is not unexpected. Yet the ASC numbers for different days post vaccination are scattered and does not give a clear trend.

We found all tonsil samples to contain influenza specific ASC. There were no negative sections. We examined the tissues to find the locations of the ASC signals, and the location of largest densities of signals. We found that the largest densities of signals were located outside the germinal centres, in the extra follicular zones. This is where one would expect antibodies to be when they have migrated out of the GCs, after the clonal expansion. In some of the tissues the ASC signals were also found in the germinal centres but they were only a few single signals scattered sparsely around in the GCs. This was found in all the tissues examined. There were also ASC signals found along the crypt linings. They were found at all the crypts. Pre-existing antibodies for the H1N1 virus in all the examined tissues could be due to the exposure of the swine flu pandemic of the year 2009, because the virus strain is the same and immunity to this pathogen is high in children and adolescents [66]. The children in the current study were between 3-17 years, and they were all born before the emergence of the pandemic influenza infection. The findings in the tonsil sections were as expected because the tonsils are the first lymph substance to come across pathogens that have entered through the nasal and oral pathways. In addition, many children and adolescents have been vaccinated against this influenza strain. They are very likely to come across influenza virus as they enter through these sites. These findings can be used in further studies where for example tonsil tissue sections from all of the subjects included in the tonsillectomy study were analyzed. One can then look further into the medical history of each patient and have more specific criteria to base the

findings on. The total number of children in the current study was 38, therefor it is possible to go through the medical history for all of them, as long as the history is kept in full. It is also possible to perform this kind of vaccination on children scheduled for tonsillectomy again at a later time. In that case some of the children would be too young to have experienced the H1N1 pandemic and other infections that can be common pathogens in children scheduled for tonsillectomy at the same time. Ultimately the research on these subjects could lead to more types of vaccines being developed that use the nasal route to enter the body and be more effective than the vaccines that are delivered into the upper arm. The antigens in vaccines are likely to give the main immune response in the nearest lymph node to the site of vaccine entry. A probable explanation is that the immune response to the influenza virus is better when it mimics the natural infection pathway of the virus which is through the upper respiratory tract. The virus is live, attenuated and this further makes the vaccine more alike the natural infection because it can replicate in the nasal cavity like in natural infection with the virus.

There are a lot more information of interest to be obtained from the subjects of the study, and the current project revealed a limited information about how the LAIV vaccine provides an immune response with the focus being on the first lymphatic substance encountered by the antigens. There are also evidence that LAIV can provide cross-protection against heterosubtypic influenza strains in mice and ferrets [67], thereby making also this an interesting further field of evaluation of the vaccine in humans. This would be of importance if another pandemic occurs to which there are no prepared vaccines.

5. Conclusion

Based on the current study, LAIV does not seem to replicate in the tonsils. The reason for this could be that the temperature is too high in the tonsils. The likely pathway for the virus or the viral antigens to enter the tonsils is via DCs. The DCs can "carry" the antigen to the tonsillar cells where they can bind to antigen specific B cells. These B cells produce antibodies that are specific for the antigen (ASC). In the tonsillar tissue of the patients in the current study the density of ASCs were observed in the extra follicular zones and some were also found in the crypts. In the tissues of the subjects who had been vaccinated before their tonsillectomy, there was discovered more, and a wider range, of ASCs than the tissue of patients who had not been vaccinated. This indicates that the tonsils of the vaccinated patients have encountered the viral antigen from the vaccine. There were some detected antigen in the unvaccinated patients and this may originate from earlier encounters of influenza. The pandemic of 2009 occurred a couple of years prior to the current study where H1N1 was the circulating influenza strain. This could be the probable antigen source to the ASCs discovered in the unvaccinated subjects.

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