THE EFFECT OF TEMPERATURE ON THE INNATE IMMUNE RESPONSE IN THE LUNGS AGAINST RSV

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Abstract
A constant flow of various pathogens enters the respiratory system on daily basis through the involuntary mechanism of breathing. Respiratory viral infections are common yet can be fatal in vulnerable populations. Respiratory syncytial virus (RSV) is one of the first and most common viruses that the human population acquire in the first two years of life. Despite the ability of most infants to recover from a RSV infection, many require hospitalization and, in few cases, die from such an infection. The pattern of seasonality of respiratory viruses also applies to RSV. In this work the temperature dependence of infectivity was studied in Hep-2 cells infected with RSV that had been incubated with bronchoalveolar lavage (BAL) fluid. The results indicate a temperature dependence of infectivity. Inhibition of the viral infectivity was observed at three different temperatures 37 °C, 40°C and 42°C. The inhibition appears to be linked to the appearance of large agglutinates that appear to reduce the infectivity of RSV. Such a study found that viral neutralization is dependent on a temperature-dependent agglutination reaction. The causality of agglutination formation requires further investigation in order to conclusively confirm the immunological component(s) of this reaction, and how temperature is contributing to this reaction.

Keywords: Respiratory syncytial virus, Bronchoalveolar lavage fluid, Hep-2 cells, temperature, immune system, agglutinates.
The recent emergence of Covid-19 virus has highlighted the importance of understanding respiratory viruses, and how the body defends itself against them. RSV does not belong to the family of Coronavirus. Yet, it shares many properties with the members of the coronavirus family such as targeting the pulmonary tract and posing a high risk to older age groups. RSV is a common virus that most often seen in infants between the age of 0-24 months old. Such an infection is capable of developing antibodies that is supposed to last for the rest of the survivor’s life, resulting in a protection against future secondary infections. However, many cases have been reported of secondary infections the in older population (over 65 years old) and among immunocompromised individuals, that can result in the death of the patient. Despite efforts to develop a vaccine against RSV, none has been successful so far.

The seasonality of respiratory viral infections is not a new concept, with many recognizing it without understanding the reason behind it. For example, the influenza virus is known to peak between the months of November and April, whereas RSV appear to peak between November and March. Presumptions and hypothesis of the reason behind the seasonality of respiratory viral infections have led many epidemiologists to construct short- and long-term strategies on how to deal with current Covid-19 pandemic and prepare for possible future second wave(s). A collective investigation of the reasons behind the seasonality of respiratory viruses may help populations and governments to prepare and save lives and cost preventing spread and developing treatments for respiratory viruses, including RSV.

Many studies have shown how the human body adapts to the change of seasons. One of the most important changes is the alterations that occur in the immune system. Hormones such as insulin and melatonin are causally linked to the functionality of the immune system. Melatonin is linked to the anti-inflammatory response, whereas insulin is linked to the response of T cells. Both hormones are affected by seasonal change. The variation in melatonin levels is due to the change in photoperiods, whereas the body switches to insulin-resistance mode in order to store fat to prepare for winter months as a result of brain signals. In addition, human behavior is altered between seasons as most people spend more time indoors during the winter months, creating an ideal environment for virus transmission to everyone living in proximity. External factors such as temperature, humidity and ultraviolet light may also affect the host, as well as the virus itself. Therefore, studying the effect of temperature on the infectivity of RSV in vitro is a reasonable starting point to observe any changes in the infectivity under these temperatures.

In this study, cells were exposed to RSV combined with bronchoalveolar lavage (BAL) fluid. BAL fluid is saline mixed with a sample of the fluid lining the epithelial layer of the pulmonary tract. The BAL fluid was obtained from ten healthy volunteers. BAL fluid should contain the antibodies required to prevent a secondary RSV infection, provided the donors had been infected with RSV. However, at higher temperatures aggregates formation were observed in samples where RSV is mixed with BAL fluid. A possible explanation is that these aggregates may trap the virus, preventing it from reaching the cells and fusing into the cells. In immunology, such mechanism is known as agglutination, where a component of the immune system binds to the pathogen. In Vivo, the agglutinates are eventually engulfed and destroyed by specialized white blood cells. Such variation in the infectivity among the different BAL fluids mixed with RSV may provide a proof that some people are more susceptible to respiratory viral infections than others. Understanding the reason behind such variation requires further studies to identify the different compositions of the BAL fluid.
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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate recognition domains</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-[(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HP</td>
<td>Human plasma</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferons</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>GMHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like receptors</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid - inducible gene I</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
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<tr>
<td>SP-A</td>
<td>Surfactant protein A</td>
</tr>
<tr>
<td>SP-D</td>
<td>Surfactant protein D</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VP-SFM</td>
<td>Virus production serum free media</td>
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Introduction
Respiratory infections can be caused by a multitude of viruses. One such virus is the respiratory syncytial virus (RSV). RSV belongs to the genus Orthopneumovirus, family Pneumoviridae and of the order Mononegavirales (Griffiths, Drews & Marchant, 2017). It has a diameter that ranges from 100 and 300nm (Ezzat et al, 2019). RSV is an enveloped negative sense single stranded RNA virus (Gilman et al, 2019) (Figure 1). RSV triggers the formation of syncytia of nearby cells. Syncytia is a multinucleate cell that is formed when multiple uninuclear cells fuse together, thus the name; Respiratory Syncytial Virus (Tian et al, 2013). RSV is the primary cause of acute lower respiratory tract infections in infant between the age of 0-24 months (Ezzat et al, 2019). It is estimated that almost all children have experienced a RSV infection by the age of two years old, with the severity of the infection varying in relation to the age of the infant (Kawakami et al, 2018). Adults aged ≥65 years and adults with immunocompromised system are at high risk of RSV infections with severe symptoms (Kawakami et al, 2018). It is estimated that annually, 7.2 person per 100,000 are hospitalized as a result of acute lower respiratory infection that is caused by RSV, with 8% of these cases resulting in death (Mazur et al, 2018). Clinical intervention in the case of a RSV infection is via passive prophylaxis with the monoclonal antibody Palivizumab, resulting in reduction in the need for hospitalization (Griffiths et al, 2017). In the northern hemisphere, most RSV infections occur between October and May, with the peak of infections appearing between November and March. In tropical climates, RSV infections peak in months when temperatures are at their lowest and precipitation at the highest (Griffiths et al, 2017).

Figure 1. Diagram showing the structure of respiratory syncytial virus. Its genomic RNA has 10 genes, which encodes 11 proteins (Ezzat et al, 2019). Glycoprotein G, fusion protein F and protein SH are found on the RSV envelope (Figure 1) with protein F being the most important in the infection process, as the virus appear to remain still infective in the presence of protein F, while in the absence of proteins G and SH (Kahn, Schnell, Buonocore & Rose, 1999)

The seasonality of viral respiratory tract infections is evident in many viruses such as RSV and influenza. Many factors such as temperature, humidity, and ultraviolet radiance affect the survivability of viruses, and subsequently transmission (Khor, Sam, Hooi, Quek & Chan, 2012).
The exact reason why some viral infections are more prominent in the winter months is yet to be identified. However, there appear to be a correlation between respiratory viruses that exhibit seasonality and air temperature (Eccles, 2002). Along with environmental conditions, human behavior can be considered as a factor contributing to the seasonality of some viruses (Tang & Loh, 2014). For example, the population’s tendency to remain indoors in the winter months results in a reduction of physical activity that may influence the host’s immune response to respiratory viral infections (Moriyama, Hugentobler & Iwasaki, 2020). Furthermore, the reduced innate immune response to viruses in the nasal epithelium in cold temperatures leaves many hosts more receptive to contracting RSV infections and transmitting it to other hosts, who are typically confined together in a close proximity (Griffiths et al., 2017).

In winter months, the air quality is marked with relatively low humidity and cold temperatures. Such conditions dry the thin layer of lining the nasal epithelia (D'Amato, M et al., 2018). The dryness affects the viscoelasticity of the mucus layer and immobilizes the cilia in the nasal cavity, resulting in an increased likelihood of respiratory viruses to cause an infection. This is due to weak viral clearance, as well as an increased susceptibility of the viral crossing the epithelial layer (Moriyama, Hugentobler & Iwasaki, 2020).

The immune system is a highly evolved system that comprises all the body’s defense mechanisms. Some of these defenses are physical barriers such as the skin and the acidity of the stomach, other defenses are mediated through the actions of the innate and adaptive parts of the immune system (Parkin & Cohen, 2001). The innate immune system is fast-acting, generic and its main purpose is to kill the pathogen with as little damage to the host as possible (Parkin & Cohen, 2001). On the other hand, the adaptive immune system is slower in comparison to the innate immune system, yet specific (Brodin & Davis, 2017). However, the adaptive immune system is highly developed in its ability to form an immunological memory, hence the development of “immunity” to a particular pathogen (Chaplin, 2010). The immune system adapts to the seasons, and in turn many immune functions are altered starting from the early winter months (Nelson & Demas, 1996). Changes in the lymphatic organ size, hormonal changes and gene expressions are vital in how the body is able to defend itself against pathogens (Nelson & Demas, 1996; Dopico et al., 2014; Dyar et al., 2014).

Elevated levels of the stress hormone cortisol, a steroid hormone released in response to stress has a suppressive effect on the immune system functions (Brodin & Davis, 2017). A seasonal variation has been reported in cortisol levels with highest concentrations being reported in the months February, March and April. While the lowest concentrations recorded are in July and August (Persson et al., 2008). Melatonin, a pineal hormone mediates such changes by directly interfering with the functions of components of the immune system on a cellular level. Cytokines production, haemopoiesis and immune cell interactions are all processes that are stimulated by melatonin (Casba, 2013). Furthermore, melatonin can also influence the immune functions indirectly through other hormones such as cortisol, resulting in altered mechanisms such as the body’s thermoregulation and metabolism (Nelson & Drazen, 1999). Seasonal variation in melatonin levels is linked to the variation of photoperiods lengths among the seasons (Adamsson, Laike & Morita, 2017). Another pivotal hormone that exhibits seasonal variation is insulin. Insulin helps regulate blood sugar levels, as well as functions as a co-stimulatory factor in triggering an immune response (Dyar et al., 2014). In the winter months, insulin resistance is increased. The purpose of such increase is to elevate fat production. The switch into insulin-resistant mode comes as a result of the brain signaling the liver to increase fat production, as a form of preparation for the winter months (Cepeda, Muka, Ikram, Franco & Schoufour, 2018). Furthermore, seasonal gene expression plays a major role in human immunity and physiology.
Antibodies are a key component of the immune system in its fight against viruses. Antibodies are proteins that can recognize the virus prior to the first contact between cells and viruses, with IgG and IgA being the most abundant (Ubol & Halstead, 2010). IgG is most abundant in the blood and extra cellular fluids. In contrast to IgA, which is more abundant in the mucus secretions including the respiratory tract (Maddur et al, 2020). The body’s immunological memory is represented during a secondary infection by the ability of antibodies to recognize a returning virus. Antibodies that have developed through the first infection target the returning virus and neutralize it immediately (Uthman & Gharavi, 2002). A mechanism used by antibodies to neutralize viruses is agglutination (Figure 2), where antibodies attach together and to the virus (Chen et al, 2015). Agglutinates containing a number of virions are easier to be recognized by other lymphatic cells, especially phagocytes. Phagocytes are capable of performing phagocytosis, a process where the virus in engulfed and destroyed within the phagocytes (Ofek & Sharon, 1988). The binding of the antibody to the virus and in turn to the Fc receptors on the phagocytes leads to the recognition of a virus and therefore the initiation of phagocytosis (Madan et al, 1997).

The complement system is a major part of the innate immune response. It is known to protect the mucosal surfaces and is present in the serum at high concentrations. The complement system uses opsonization and agglutination in order neutralize viruses (Jayasekera, Moseman & Carroll, 2007). In the case of opsonization, the envelop of the virus is targeted leading to the destruction of the virus. Since not all viruses are enveloped, the opsonization mechanism may not be always effective against viruses (Chaplin, 2011). However, it uses agglutination via the classical and alternative pathways. The classical pathway of the complement system is activated when C1 binds to the antibody or to a surface with an activating molecule. In contrast, the alternative pathway is activated when C3 binds to a suitable molecule on the surface of a pathogen (Kopf, Abel, Gallimore, Carroll & Bachmann, 2002).

Cells in the respiratory system also secrete other antimicrobial factors (Newton et al, 2016). Lactoferrin is a glycoprotein, secreted by the mucosal epithelia. Lactoferrin binds to the virus preventing it from entering the cells and initiating an infection (Valenti & Antonini, 2005). Cathelicidins fight viruses by disrupting the virus envelop, recruitment of immune cells and enhancing the production of chemokines and cytokines from the local cells (Reinholz, Ruzicka & Schaub, 2012). Surfactant proteins are also a major tool fighting pathogen in the respiratory tract. Alveolar type II cells secrete surfactant protein A (SP-A) and surfactant protein D (SP-D), which are both hydrophilic and function as collectins (Hartshorn, 1996). They act as PRRs and recognize pathogens through their PAMPs. SP-A and SP-D target viruses by forming agglutinates promoting phagocytosis by macrophages and neutrophils (Hartshorn, 2010).

Studies have shown that during the state of injury or disease, the levels of SP-A and SP-D in BAL fluid are reduced, which affects synthesis and release of the pulmonary cytokines (Bry, Lappalainen & Hallman, 1996). The levels of pulmonary SP-A and SP-D increase 24-72 hours following any type of injury to the pulmonary epithelia, such observation has suggested that these surfactants affect the state of inflammation and vice versa (Fehrenbach et al, 1998). According to (Lopez et al, 2013) premature babies that are born with underdeveloped pulmonary surfactant system are more at risk of developing neonatal respiratory distress (NRDS). In such cases

(Dopico et al, 2014). 4000 mRNAs that are responsible for coding proteins in human lymphocytes and fat tissues have seasonal expression profiles (Dopico et al, 2014). This is reflected in variations in the blood’s composition between seasons, such as increased levels of IL-6 receptor, CRP and biomarkers that are regarded as signals for cardiovascular diseases (Dopico et al, 2014).
surfactant is given exogenously in order to initiate breathing until surfactants are produced endogenously (Lopez et al, 2013).

![Diagram showing a representation of the agglutination process where antibodies bind to the virus particles.](image)

Figure 2. Diagram showing a representation of the agglutination process where antibodies bind to the virus particles.

Bronchoalveolar lavage (BAL) fluid is a procedure, where a sample of the fluid lining the epithelial layer of the pulmonary tract is retrieved and examined. BAL fluid is used as a diagnostic tool, as well as a research tool in the understanding of respiratory diseases (Meyer & Raghu, 2011). This technique can provide a snapshot of the cellular and respiratory secretions status in the lungs. The data collected through the analysis of BAL fluid is used to support the findings of biopsy analysis. Such information is pivotal in the diagnosis of diseases affecting the respiratory system (Costabel & Guzman, 2001). Studies have shown that antibodies Immunoglobulin IgG, IgA, IgE and IgM are found in the BAL fluid (Heron et al, 2012). Considering the commonality of RSV, the BAL fluid provided is expected to contain the antibodies against RSV, which is supposed to provide a protection against a secondary infection in the volunteers. There are two agents with immunological properties in the BAL fluid that are abundant, use agglutination as a mechanism of defense and their actions are part of the innate immune response. They are surfactant proteins A&D and antibodies IgG & IgA (Brajer-Luftmann et al, 2019). DPPC is a phospholipid, a major component of the surfactant pulmonary mixture. Dipalmitoylphosphatidylcholine (DPPC) phase transition temperature from gel to liquid is 41.5°C (Albon & Sturtevant, 1978). Temperature affects the surfactant mixture phase, and the function of the proteins (Albon & Sturtevant, 1978). Surfactant proteins decrease the temperature of DPPC’s phase transition to less than 37°C. Only in the liquid phase can surfactants freely spread on the surface of the pulmonary epithelium to form a monolayer (Hills, 1999). The SPs also fastens the DPPC on the interface to prevent the DPPC from being squeezed out when the surface area decreases (Possmayer, Nag, Rodriguez, Qanbar & Schürch, 2001).

**Aim**

The aim of this research is to investigate the effect of temperature as an external factor on the viral infectivity. This may allow for further understanding of the reason behind the seasonality of respiratory viral infections. By using a mixture of GFP-expressing RSV and different samples of
BAL fluid that had been incubated at five different temperatures. These temperatures range from room temperature (22°C) to dangerously high temperature that occurs in patients with fever (42°C). The mixture was added to Hep-2 cells and the percentage of viral GFP expression was measured using FACS.

**Materials and Methods**

**Cell culture**

Hep-2 (human larynx squamous cell carcinoma) cells were used for RSV production and infectivity assays. Hep-2 cells were obtained from Karolinska Institute cell bank, stored at -80°C. At the time of thawing, the cells had been passaged eight times prior to the use in this study. Hep-2 cells were cultured in DMEM media (1X + GlutaMax) (Gibco) containing 5% FBS (Sigma), 1% PenStrep (Penicillin-Streptomycin) (Sigma) and 1% HEPES (Sigma). Cells were seeded in 96-wells plate (standard F plate) (SARSTEDT) with each well containing 13000 cells. The cells undergone splitting every 48 hours with average split ratio at 1:10.

HEP-2 cells were infected once they reached ~75-80% confluency with viruses at MOI-ratio (1:5) of 5. The cells were washed twice with PBS and 100µl of VP-SFM 1X (Gibco) was added to each well, then placed again in the incubator at 37°C.

Following the first observation of the cells via fluorescent microscopy (24 hours post infection), cells were washed once with PBS, and 100µl of fresh DMEM (1X GlutaMax) (Gibco) containing 5% FBS (Sigma), 1% PenStrep (Sigma) and 1% HEPES (Sigma) was added. Following the change of media, the cells were incubated at 37°C until fixation.

**Virus production**

The GFP expressing respiratory syncytial (RSV) was provided by Dr. Xiuming Liang, Karolinska Institute. For the viral culture, HEP-2 cells were seeded in T175 flasks (Corning) in DMEM media (1X + GlutaMax) (Gibco) containing 5% FBS (Sigma), 1% PenStrep (Sigma) and 1% HEPES (Sigma) until they reached ~70–80% confluency. At the day of infection, the cells were washed twice with warm phosphate-buffered saline (PBS) and Virus Production Serum Free Media 1X (VP-SFM) (Gibco) containing 1% PenStrep(Sigma) was added. Cells were infected at MOI of 4 and incubated for 5 to 6 days until ~90% GFP expression was observed via fluorescent microscope. Cells were then scraped, vortexed, sonicated for 10 min, vortexed again, and spun at 1000g for 5 minutes. The supernatant was extracted and transferred to new tubes for concentration.

For the concentration of the RSV stock, the sucrose cushion protocol was followed (Gias, Morgan, & Toms, 2008; Ezzat et al, 2019). The viral concentrated layer was then collected from the interface between the cushion and the supernatant, aliquotted, and frozen at −80°C. The quantification of the virus was done in accordance with the Virus Quantification protocol (Ezzat et al, 2019).

**Bronchoalveolar lavage fluid**

Human Bronchoalveolar lavage (BAL) were provided by Dr. Anders Lindén, Karolinska Institute. The samples were obtained from ten healthy volunteers. All donors had given oral and written informed consent to take part in the bronchoscopy study in accordance with the Helsinki Declaration. The recruitment of volunteers was done at the department of Respiratory Medicine.
and Allergy, Karolinska University hospital, Solna. All the subjects were interviewed and examined in order to ensure the suitability of their participation, and their understanding and approval of the procedure. Bronchoscopy with bronchioalveolar lavage (5x50mL of sterile and PBS) was performed in accordance with clinical routine at Karolinska University Hospital, Solna. No further information was provided regarding the gender and age of the volunteers. As per the information provided by Dr. Anders Lindén, the samples provided were purified of any leukocytes and stored immediately at -80°C.

The BAL fluid samples provided were concentrated using Spin Concentrators MWCO 4ml (Agilent Technologies). The concentrators were centrifuged at 4500g for 30mins at 4°C. The measurement of the protein content was performed according to DC Protein Assay protocol (Bio-RAD).

**RSV & bronchoalveolar lavage fluid mixture**

A master mix was prepared of BAL/PBS and RSV in an Eppendorf tube then divided into individual Eppendorf tubes, which were incubated for 1hour at the designated temperatures 22°C, 32°C, 37°C, 40°C, 42°C. Following the incubation 10µl was added to each well containing the incubated mixture of RSV and BAL fluid. Each sample was done in triplicates. Cells were incubated again for 24hours at 37°C.

**Fluorescence microscopy**

Following the 24 hours incubation period, the cells were viewed using fluorescence microscope (OLYMPUS 1X81). Infected cells were recognized by the appearance of cells in green color when exposed to blue light, due to the virus carrying GFP.

**FACS (Fluorescence activated cell sorting)**

72 hours post infection the cells were fixed. Fixing the cells commenced by washing the cells twice with 0.01M pH 7.4 PBS, then cells were stained using LIVE/DEAD Flexible Far Red Dead Cell Stain Kit (Invitrogen) with 1/2µl per 1ml of PBS. 100µl of the mixture was added to each well and left to incubate for 30 minutes in a dark room at 4°C. Following the 30 minutes incubation, the cells were washed with PBS once, and 50µl of 0.05% Trypsin (Trypsin-EDTA 1X) (Gibco) per well was added, the plate was incubated for 5 minutes at 37°C. 50µl of DMEM media was added to deactivate the trypsin, and the cells were resuspended. The cells were centrifuged at 1000g for 5 minutes at 4°C. The media was removed without disturbing the cells, and the cells were resuspended with 100µl of 4% PFA (VWR Chemicals). Following the addition of 4% PFA, the cells were incubated for 10 minutes in the dark at room temperature. The plate was centrifuged at 1000g for 5 minutes at 4°C. The 4% PFA is removed, and cells were resuspended in 100 µl of PBS and 0.5% FBS (Sigma).

FACS was run using MACS Quant FACS machine. MACSQuantify version 2.11 software will be used to run the collection of samples. Flowjo 10 software was used to extract the cells count and the percentage of GFP expressing cells (Infected cells). FACS provided a quantitative measure of viable infected single Hep-2 cells. Each experiment was performed twice (n=2). Statistical analysis was done using GraphPad Prism, Kruskal Wallis test was used to analyze the samples statistically. Significance levels chosen was 0.05.
Turbidimetric agglutination assay

Using Microtest Plate 96 Well, C (SARSTEDT), two rows were designated. One row for the ten samples of BAL fluid/RSV and PBS/RSV (positive control), and one row for the BAL fluid samples only and PBS only (negative control). 50µl of BAL fluid from each sample was deposited in each well. The experiment was carried out under three different thermal conditions: 1) At room temperature i.e. 22°C. 2) At 37°C 3) At 42°C. The Microplate Spectrophotometer (SpectraMax i3X) had been preheated up to the designated temperatures and the plate was placed in the reader for one hour.

Results

Fluorescence microscopy

Following the first 24 hours post infection, the cells were observed under a fluorescent microscope. The purpose of this observance is to check for any infected cells starting to appear. The infected cells would appear green under the fluorescent microscope’s blue light as a result of GFP-expressing Respiratory syncytial virus (RSV) fusion into the cell. However, unexpectedly black aggregates appeared in some wells containing Bronchoalveolar lavage (BAL) fluid/RSV samples(Figure 3). The appearance of the aggregates (agglutinates) corresponds to temperature in which the BAL fluid/RSV samples were incubated in. For example, in the case of BAL110, the aggregates are seen in the wells that contain the BAL fluid/RSV that was incubated at 37°C (Figure 3).

Figure 3. Visualization of samples BAL110 and BAL117 after 24 hours of infection using fluorescence microscope (OLYMPUS 1X81). The images of BAL110 37°C 24h shows agglutinates forming at 37°C. The images of BAL117 shows agglutinates forming at 40°C and in larger scale at 42°C.

Figure 3 shows pictures taken 24hours following the incubation of two BAL fluid samples BAL110 and BAL117 for 1 hour at 37°C and 42°C respectively in the presence of RSV. The two samples chosen to be included in Figure 3 were chosen only as examples to show agglutinates formation at different temperatures (pictures of all samples are included in the appendix). The formation of
these agglutinates appears to be temperature dependent. Furthermore, the formation of these agglutinates appears to influence infectivity.

**FACS Analysis**

Cell fixation and FACS were done 72 hours post infection. Following the first observance via fluorescent microscopy and change of media 24 hours post infection, the cells were incubated again for further 48 hours at 37°C. Once the 72 hours setpoint passed, the cells were fixed with 4% PFA, and undergone FACS analysis. FACS analysis allowed for the counting of infected cells. Based on this number, the percentage of viral GFP expression was used a measurement of infectivity in all the samples as shown in figure 4. Negative control and positive control samples were included in each assay. Untreated cells served as negative control, whereas RSV/PBS served as a positive control. Both RSV/PBS samples and BAL fluid/RSV samples were incubated for 1 hour at one of these temperatures 22°C, 32°C, 37°C, 40°C, 42°C prior to the infection assays taking place. The percentage of viral GFP expression in BAL fluid/RSV samples was compared to the same percentage in positive controls for the same incubation temperature in order to determine statistical significance.

Figure 4 shows the percentage of viral GFP expression in all BAL fluid samples at five different temperatures: 22°C (A), 32°C(B), 37°C(C), 40°C(D) and 42°C(E). Each experiment was repeated twice with each sample being done in triplicates (n=2). Kruskal Wallis test was performed to show the significance level (*p<0.05) (**p<0.01) (***p<0.001) (****p<0.0001). The percentage of viral GFP expression in BAL fluid/RSV samples was compared to the same percentage in positive controls for the same incubation temperature in order to determine statistical significance.

Figure 4 shows the effect of five different temperatures 22°C, 32°C, 37°C, 40°C, 42°C in which the mixture of BAL fluid/RSV and RSV/PBS samples were incubated in for 1 hour. A variation of the percentage of viral GFP expression appears among these different temperatures. At 22°C, only sample BAL119/RSV shows a significant reduction in the percentage of viral GFP expression in comparison to sample RSV/PBS (positive control) (Figure 4/A). At 32°C, samples BAL119/RSV and BAL120/RSV show a significant reduction in the percentage of viral GFP expression in comparison to sample RSV/PBS (Figure 4/B). At 37°C, samples BAL110/RSV, BAL113/RSV,
BAL119/RSV, BAL120/RSV and BAL121/RSV show a significant reduction in the percentage of viral GFP expression in comparison to sample RSV/PBS (Figure 4/C). At 40°C, sample BAL116/RSV shows a significant reduction in the percentage of viral GFP expression in comparison to sample RSV/PBS (Figure 4/D). At 42°C, samples BAL114/RSV, BAL115/RSV, BAL117/RSV and BAL118/RSV show a significant reduction in the percentage of viral GFP expression in comparison to sample RSV/PBS (Figure 4/E).

**Turbidimetric agglutination assay**

Turbidimetric assay relies on the changes in the light scattering as agglutinates are formed (Generalova, Buryakov, Lukin & Zubov, 2000). This assay was pivotal to the study in order to narrow down the time setpoint of agglutinates' formation. Thus, providing a reference setpoint for possible future trials of the same technique on animals, where the exposure to higher temperatures for prolonged periods of time can be damaging to the subjects. In addition, Turbidimetric agglutination assay shows changes in all the samples at once under one temperature. The experiment was carried out under three different thermal conditions: 1) At room temperature i.e. 22°C. 2) At 37°C. 3) At 42°C (Figure 5). The set point of agglutinates' formation and peak times for all the samples at the three aforementioned temperatures is shown in figure 6.

![Image of Turbidimetric Agglutination Assay](image_url)

**Figure 5.** Image shows the samples of BAL fluid/RSV and PBS/RSV (Row A), and PBS only and BAL fluid only (Row B). Samples were incubated in Microplate Spectrophotometer at three different temperatures RT (22°C), 37°C and 42°C (picture shown above) for 1 hour.

Figure 5 shows a visible change in the color of the mixture in the wells in row A, where the mixtures of all BAL fluid samples/RSV were incubated at 42°C for 1 hour (Figure 5/A). No visible change was seen in the well where RSV was incubated in the presence of PBS only (Figure 5/A). Similarly, all the wells in row B containing the BAL fluid samples without RSV and the well containing PBS only do not indicate a change in turbidity (Figure 5/B).
At RT, all the samples with the exception of BAL110 /RSV and BAL120/RSV show almost no change in turbidity during the 60 minutes incubation period. At 37°C, the change in turbidity in all the samples varied with no distinct pattern emerging from this assay. At 42°C, all the BAL fluid/RSV samples have shown a change in turbidity in the form of a sharp increase in turbidity at 5 min and a peak around 20-30 min. With the exception of BAL117/RSV sample, all the other samples reach the highest level of turbidity under this temperature between the 20-30 minutes mark.

Overall, the turbidimetric assay (Figure 6), has shown similarities and differences in how the BAL fluid/RSV samples react to the three designated temperatures. At RT, all the samples with the exception of BAL110 /RSV and BAL120/RSV show almost no change in turbidity during the 60 minutes incubation period. At 37°C, the change in turbidity in all the samples varied with no distinct pattern emerging from this assay. At 42°C, all the BAL fluid/RSV samples have shown a change in turbidity in the form of a sharp increase at the 5 minutes mark. With the exception of BAL117/RSV sample, all the other samples reach the highest level of turbidity under this temperature between the 20-30 minutes mark.

**Discussion**

All cells treated with Bronchoalveolar lavage (BAL) fluid/ Respiratory syncytial virus (RSV) showed a reduced percentage of viral GFP expression at different incubation temperatures (Figure 4). The setup of the infection assay limits the variants to temperature. Since all the BAL fluid/RSV samples were prepared in a master mix with MOI of 5 under the same conditions, it can be concluded that the variation in the viral GFP expression is due to the effect of the designated temperatures on the mixture of BAL fluid/RSV. Following the incubation of the BAL fluid/RSV at 22°C, 32°C, 37°C, 40°C, 42°C, aggregates (agglutinates) are formed (Figure 3) at 37°C, 40°C, 42°C. These agglutinates are temperature dependent. Agglutinates formation appears to influence infectivity, causing a reduction in the percentage of viral GFP expression (Figure 4). In Turbidimetric assay, the change in turbidity indicates the formation of agglutinates. An increase in turbidity at 5 mins and a peak around 20-30 min is shown (Figure 6).
Temperature has a profound effect on the body's systems. Subsequently, the immune system reactions are affected by a change of temperature. Normal temperature in humans ranges between 37.2-38.3°C. Temperature above this range is labeled as fever. When fever hits 41-42°C, a serious concern of brain damage and systems failure is raised (Laupland, 2009). The elevation of body temperature through fever enhances the immune cells while impairing the replication mechanism of microorganisms (Blomqvist & Engblom, 2018). The thermal mapping of the humans airways shows a differentiation of the temperature in the airways and an interchangeable relationship with the external environment. When breathing room temperature air, the average temperature in the subsegmental bronchi ranges from 32.0 +/- 0.05 degrees°C. Upon an increase in ventilation, the temperature progressively decreases along the airways to a range from 29.2 +/- 0.05 to 33.9 +/- 0.8 degrees°C. During breathing cold air, the temperature in the proximal and distal airways ranges from 20.5 +/- 0.6 and 31.6 +/- 1.2 degrees°C (McFadden et al, 1985). Hence, the five chosen temperatures in this study were designated as a representation of the temperatures set points mentioned above.

The change in turbidity seen in figure 5 & 6 is a signal of the formation of agglutinates in the samples containing BAL fluid/RSV. As per figure 6 the agglutination formation occurs at 37°C, 40°C, and 42°C. However, in the turbidity assay, two samples of BAL110/RSV and BAL120/RSV demonstrate a change in morbidity at room temperature during the 1 hour incubation. Considering the commonality of RSV, and the absence of any information about the medical history of the volunteers, it was assumed that all the volunteers have had RSV infection as infants. However, if the opposite is to be assumed, it is possible to consider that the volunteers who provided samples BAL110 and BAL120, were the only volunteers who have had RSV infection as infants and their anti RSV antibodies caused the a binding to the virus, which resulted in the change of turbidity i.e. agglutination. This would suggest that the process of neutralizing a virus during a second infection is not temperature dependent. However, this finding contradicts the results shown in figure 4, where the same samples did not show any inhibition at 22°C and 32°C. In such cases, the repetition of the experiment is required in order to verify the findings and understand the reason behind the change of turbidity in these two samples.

A possible reason for the variation in the response to temperature among the BAL fluid/RSV samples can be due to a variation in the lipids concentration of the BAL fluid. The composition of the surfactant membrane is 40% Dipalmitoylphosphatidylcholine (DPPC), 40% Phosphatidylcholine (PC), 10% surfactant proteins, 10% neutral lipids (cholesterol) (Agudelo, Samaha & Garcia-Arcos, 2020). The lipid-protein composition influences the functions of the surfactant (Parra, & Pérez-Gil, 2015). In the case of DPPC, which is the main constituent of the surfactant membrane, the phase transition temperature from gel to liquid is 41.5°C (Wright, 2004). This may suggest that the lipids in the BAL fluid affect the adsorption kinetics of the immunological components in the BAL fluid. Such components as antibodies, surfactant proteins A & D and the complement system require the motility provided by the surfactant membrane to attach to the virus. The different temperatures used 37°C, which the temperature of the human body, 40°C representing a common temperature in fever and 42°C, a temperature that is associated to dangerously high fever. All these temperatures may influence the phase of the DPPC, and subsequently altering its state. Considering that the surfactant membrane of the lungs is composed of 90% lipids (Agudelo, Samaha & Garcia-Arcos, 2020), It is possible that the biophysics are playing a key role in the process. The liquid state of DPPC is assisting the pulmonary antibodies or surfactant proteins to reach the virus. The higher the lipid content in the BAL fluid samples, the higher temperatures required for the lipids to move from the gel phase to the liquid phase. In
conclusion, the analysis of the lipidomics profile of each sample may differ, thus affecting the functionality of the immune components in the BAL fluid. Studies have shown that lipids are capable of interacting with TLRs and causing its activation (Agudelo, Samaha & Garcia-Arcos, 2020). Palmitoyl-oleoyl-phosphatidylglycerol and phosphatidylinositol (PI) can antagonize TLRs activation. Such activation plays a key role in the virulence of RSV, thus highlighting the role of lipids in the fight against respiratory viral infections and the kickstarting the inflammatory cascade (Agudelo, Samaha & Garcia-Arcos, 2020). The variation in the lipid's contents among the BAL fluid samples may explain the variation in percentage of GFP viral expression.

Heat is known to cause the denaturation of proteins. The denaturation of proteins in cells can be fatal to the cells leading to apoptosis. As temperature affects the protein folding, proteins lose their functionality when they are denatured (Roche & Royer 2018). The reason behind protein denaturing is the breaking of the hydrogen bonds, which play a key role in protein folding (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016). A consequence of protein denaturation is protein aggregation. Protein aggregation occurs when mis-folded proteins accumulate and bind together. Temperature is an environmental stressor that may cause proteins to lose its 3-D structure, i.e. unfold and subsequently aggregate (Brodkorb et al, 2016). This is done by the breaking of non-covalent interactions among the amino acids that form that particular protein. (Tyedmers, Mogk & Bukau, 2010). Studies have shown that in the case of Cryoglobulinemia, antibodies, which are proteins become insoluble at lower temperatures. This suggests a direct relationship between temperature and antibodies (Bazerbachi et al, 2017).

The agglutination process observed in this study in the presence of the BAL fluid and RSV can be due to the actions of either the complement system, surfactant proteins A and D, or antibodies IgG and IgA, individually or collectively. The complement system is capable of neutralizing viruses through different mechanism. Virolysis occurs when the complement produces a hole in the virus envelop. Such mechanism has been observed in the neutralization of herpesviruses, coronaviruses and retroviruses by the complement system (Agrawal, Nawadkar, Ojha, Kumar & Sahu, 2017). Studies have shown that non-enveloped viruses with C3b can trigger antiviral signaling, where the virus undergo a rapid proteasomal degradation (Tam, Bidgood, McEwan & James, 2014). Another mechanism used by the complement system is aggregation. The opsonization of virus surface with the complement results in the aggregation of the virus and subsequently phagocytosis. Studies on this mechanism have shown that the complement system was able to neutralize polyoma virus and influenza virus via (Agrawal, Nawadkar, Ojha, Kumar & Sahu, 2017). In the case of RSV, the complement system has shown it is ability to neutralize RSV. The complement system opsonizes RSV prior to entering the cell, leaving the virus inactive on the host cell membrane of endosome plasma membrane. This study, which was done in vitro shows that the complement cascade taking place prior to the virus fusing into the host’s cell. In comparison, the complement intracellular signaling was absent after the infection has occurred (Mellors, Tipton, Longet & Carroll, 2020). Furthermore, temperature appears to be related to the activity of the complement system. Research has shown that when the body temperature drops below 37°C, an increase in the antibody-initiated complement activation is observed (Shah et al, 2014). Hence, considering the variation of temperatures in this study, the complement system may neutralize RSV through the process of aggregation, thus the reduction of the percentage of GFP viral expression.

Surfactant proteins are present in the BAL fluid, and form a part of the innate immune response against pathogens in the lungs. The role of surfactant proteins in innate immunity in the
respiratory tract is limited to SP-A and SP-D (Aramini et al, 2019). Both surfactants recognize the sugars on the surface of a pathogen, either opsonize the virus if enveloped, or form agglutinates, which in both cases result in the uptake of the compound by phagocytes (Albon & Sturtevant, 1978). SP-A is a collectin that forms part of the innate immune system. SP-A has a collagen-like domains (Nathan et al, 2016). Previous studies have shown that SP-A appears to be able to mark pathogens for phagocytosis, which is performed by the alveolar macrophages. Previous studies done on mice and rats has shown that mice that lack SP-A are more prone to infections when compared to mice with normal levels of SP-A (LeVine et al, 1997). The immunological properties produced by SP-A appear to be temperature and concentration dependent (Sáenz et al, 2007).

SP-D is also a collectin that is primarily found in the lungs. SP-D’s main role is defending the lungs against pathogens, especially viruses (Van Eijk et al, 2019). SP-D structure is of a particular importance as it forms a triple helical parallel coil, which helps the protein form a trimer (Kovacs et al, 2002). It belongs to the family of lectins. They contain collagen containing C-type lectin (calcium dependent), which are called collectins. Collectins play a role in the control of immune and inflammatory responses (Van Eijk et al, 2019). The structure of collectins consists of triple -helical collagen region and carbohydrate recognition domain (CRD) (Guo et al, 2008). SP-D is a monomer, three of the same type of monomers associate to form a homotrimer (Kishor et al, 2006). SP-D has a complex quaternary structure in which monomers (43 kDa) are assembled into tetramers of trimers thus forming dodecamers. Dodecamers are further assembled into large multimeric structures (Watson, Phipps, Clark, Skylaris & Madsen, 2019). The oligomerization of SP-D results in the burial of the tail domains while the head domains are exposed. Oligomerization is dependent upon the cysteine residues at positions 15 and 20 within the N-terminal tail region (Guo et al, 2008; Watson et al, 2019).

In literature, SP-A and SP-D have been described as PRRs that belong to the collectin family (Serré et al, 2019). Studies have shown that deficiencies of SP-A and SP-D in transgenic mice led to a defective response in inhibiting viruses in vivo (Crouch & Wright, 2001). Studies in vitro on influenza A virus have shown that SP-A and SP-D hemagglutination activity of influenza A virus, causing a reduction in the infectivity of the virus (Hsieh, De Luna, White & Hartshorn, 2018). SP-D inhibits the hemagglutination activity by binding to the high mannose oligosaccharides. These oligosaccharides lie on the top of the cell attachment site referred to as the sialic acid binding site pocket. SP-D causes a massive aggregation of influenza A virus (Hartshorn et al, 1996). The aggregation of viral particles is calcium dependent and correlates with SP-D state of multimerization. In the state of dodecamers, and trimeric CRDs, only minimal agglutination is seen in comparison to the agglutination that occurs when the SP-D is multimerized (Hartshorn et al, 1996). The agglutinates formed as a result of SP-D binding to the viral particles promotes phagocytosis by phagocytes. Thus, paralyzing the virus and preventing it from binding to the cells and subsequently causing an infection (Hartshorn et al, 1994). BAL fluid is found to inhibit influenza A virus in vitro through the inhibition of hemagglutinin activity and the formation of agglutinates (Hartshorn et al, 1994).

In the case of RSV, studies have shown that SP-A uses calcium-dependent mechanism to bind to the F glycoprotein of RSV resulting in the loss of infectivity of RSV (Ghildyal et al, 1997). Whereas SP-D binds to the G protein if RSV by the CRD-dependent mechanism resulting in inhibition of the RSV infectivity (Hickling et al, 1999). A paper by (Vos, Rijtema & Blanco, 1996) reported that infants with pneumonia caused by RSV infection showed an alleviation of their symptoms as a result of using treatments when surfactants proteins were given (Vos, Rijtema & Blanco, 1996).
Another study found that surfactant proteins in BAL of infants with RSV disease are decreased leading to the development of acute respiratory distress syndrome (Kerr & Paton, 1999). The immunological role of SP-A in the defense against RSV is supported in research especially that SP-A knock out mice have shown an increased susceptibility to RSV infection (LeVine et al., 1999). In addition, mice that are deficient of SP-A show an increase in the secretion of proinflammatory cytokines and poor viral clearance (LeVine et al, 1999). In a similar manner, mice that are deficient of SP-D show poor viral clearance and an increase in the lung inflammation as a result of an increase in the release of proinflammatory cytokines (Sorensen, 2018). In summary, considering the vital role SP-A and SP-D play in the pulmonary immune response against viruses, their abundance in the BAL fluid, and their ability to form agglutinates that can be taken up by phagocytes. It is likely that the appearance of the agglutinates is the result of the SP-A and SP-D binding to the virus with the purpose of neutralizing the virus.

Immunoglobulin (IgG) represents about 75% of the serum antibodies, making it the most abundant antibody in the blood (Vidarsson, Dekkers & Rispens, 2014). IgG is capable of binding to the pathogen and forming aggregates (agglutinates), which allows phagocytes to recognize the pathogen and subsequently eliminate the pathogen (Mallery et al, 2010). Considering the commonality of RSV infection, it is assumed that the ten samples used contain the antibodies for the virus, which can protect the body in case of secondary infection. Studies looking into antibody binding to the influenza A virus shows agglutination by antibody-like polymers, by recognizing an epitope area on the surface of the antigen (Mariuzza, Phillips & Poljak, 1987). Studies using IgG on influenza virus have found that IgG was able to inhibit an infection by an influenza virus strain that the body had been exposed to previously (Palladino, Mozdzanowska, Washko & Gerhard, 1995).

Immunoglobulin (IgA) or as referred to SlgA, is an antibody with crucial immunological role in the body's mucous membranes. IgA is further divided into two subclasses, where IgA1 in blood circulation and IgA2 found in the mucosal membranes (Bonner, Almogren, Furtado, Kerr & Perkins, 2009). Previous research by (Yel, 2009) found that deficiency of IgA is one of the most common immune deficiencies. (Yel, 2009) adds that IgA deficiency is asymptomatic, with in individuals suffering from repetitive respiratory and gastrointestinal infections. Such deficiency is most commonly due to the defect in the maturation of B cells resulting in an inability to produce IgA (Yel, 2010). SlgA has the ability of recognizing Polyvalent antigens on the surfaces of viruses, binding to the viruses and forming agglutinates, such mechanism is seen in the gastrointestinal tract, which suggests the same mechanism is shown in the respiratory tract (Mantis & Forbes, 2010).

The relationship between antibodies and temperature is a new domain that requires further investigation. However, in the case of Autoimmune Hemolytic Anemia (AIHA), a rare disorder where the erythrocytes are destroyed faster than being made. AIHA can be classified into cold or warm AIHA (Peckman, 2015). Such classification comes from the temperature, which autoantibodies bind to red blood cells (Anderson, Winter, Jorge & Dourado, 2020). In warm AIHA, the destruction of red blood cells (hemolysis) uses IgG as an intermediary. IgG antibodies bind to the Rh-type antigen at 37°C, leading to the phagocytosis of red blood cells in the spleen (Anderson, Winter, Jorge & Dourado, 2020). Conversely, IgM, another type of antibodies mediates the destruction of red blood cells at temperatures less than 37°C in the case of cold AIHA. Furthermore, Cold agglutinin syndrome is used to describe when the immune system attacks red blood cells (Hopkins & Walters, 2013). Such attack is triggered by cold temperatures, as the
antibodies are directly impacted by the low body temperature and subsequently attach themselves to the red blood cells instead of viruses and lead to the killing of red blood cells (Hopkins & Walters, 2013). Thus, a possible direct effect of temperature on the function of antibodies is likely to occur.

**Ethical aspects, gender perspectives, and impact on the society**

Since RSV is a virus that infects the respiratory tract, and this study focuses on further understanding the infectivity of RSV in humans, the use of BAL fluid samples from humans is paramount for this study. All donors had given oral and written informed consent to take part in the bronchoscopy study in accordance with the Helsinki Declaration. No information was provided regarding the age, sex, and the medical histories of the donors. The recruitment of volunteers was done at the department of Respiratory Medicine and Allergy, Karolinska University hospital, Solna. All the subjects were interviewed and examined in order to ensure the suitability of their participation, and their understanding and approval of the procedure. Bronchoscopy with bronchioalveolar lavage (5x50mL of sterile and PBS) was performed in accordance with clinical routine at Karolinska University Hospital, Solna.

The use of human bronchoalveolar lavage fluid samples for this study was approved by the Regional Committee for Ethical Review in Stockholm, (D. No 2016/1985-32).

In the case of RSV infections, higher incidence rates have been observed in males. In addition, studies show that males between the age of 6 months to 6 years old are more likely to require hospitalization than females of the same age group (Segal, Crighton, Moineddin, Mamdani, Upshur, 2005). Observations of differences in the pulmonary tract anatomy between men and women, as well as differences in the antiviral immunity are clearly related to more severe cases of RSV infections (Muenchhoff & Goulder, 2014).

The findings from this research can be hugely and equally beneficial to individuals and governments. The commonality of RSV infections among infants has been a major concern to parents and governments. Parents are faced with the inevitable event that the infant is going to contract the virus at some stage in the first two years of his/her life. In some cases these infections can be severe and life-threatening, especially among infants and premature babies and people in risk groups. Governments are faced with the cost of intensive care that is to be provided to infants and vulnerable groups as a result of an RSV infection. Understanding the reason behind the seasonality of RSV infections can help plan, prepare, and possibly prevent future infections.

**Future perspectives**

This study has highlighted an immune response mechanism that is temperature dependent. This research can be further developed by looking into the plasma of these volunteers and repeating the experiment with plasma instead of BAL fluid. Such an experiment may help identify the reason behind the formation of these agglutinates. Furthermore, a proteomics and lipidomics analysis of these samples pre and post heating in order to identify any alterations as a result of the incubation in that particular temperature is beneficial in order to determine the differences among the ten BAL fluid samples. Understanding a variation in the proteins or lipids may hold the key for understanding why some aggregates appeared in five samples at 37°C and in others at 42°C.
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Appendix