Cellular Dynamics and Disease Outcome of Type 3 Streptococcus pneumoniae Clinical Isolates Differ Between Strains

Taylor Rae Plunkett White
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Abstract
Streptococcus pneumoniae is a bacterial pathogen that continues to be a major cause of disease around the world. It is not only the number one cause of bacterial pneumonia but also the cause of about 15% of the deaths of children under 5 around the world. There is a lot of research done on this organism, but with around 100 known serotypes and each one producing a unique capsule, there is still much more to be studied. The Etiology of Pneumonia in the Community (EPIC) study conducted by the CDC observed the burden of hospitalizations caused by pneumonia while determining the organism responsible. One of the most common organisms isolated from both children and adult patients was S. pneumoniae. To look further into these clinical isolates, we chose three different type 3 pneumococcal isolates and observed the immune cellular dynamics as well as capsule production, and saw that even with the same serotype immune responses can differ.

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Cellular Dynamics and Disease Outcome of Type 3 Streptococcus pneumoniae Clinical Isolates Differ Between Strains

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DEDICATION

This work is dedicated to my mom, Tommie. You have always been my biggest supporter and my greatest role model. Your sacrifices, endless dedication, and incredible patience have made me who I am and helped me to accomplish more than I ever thought possible. To my husband, Paul, for pushing me to always be my best and being the best partner in life I could ever ask for. To my grandparents, Rm and Lucy, and Adley and Charlie Mae, you have always made me believe I could achieve anything I set my mind to. The confidence you have instilled in me is the reason I have made it to where I am today. To my stepdad, Scott, you stepped into a role that you didn’t have to and have always treated me as your own. You have always shown such an interest in my work and helped me to always remember the importance and excitement of science. Lastly, to my sweet Audri, becoming your bonus mom is one of my greatest joys in life. You exude love and happiness and always make the hard days better. You are a constant source of light and help me remember how to dream.
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To my friends I have made through this, Tess, McKenna, Madison, and Amy, you guys have made this journey so much more fun and I am forever thankful for your friendships. Your support, guidance, and laughs made those late-night studying sessions all worth it.
ABSTRACT

*Streptococcus pneumoniae* is a bacterial pathogen that continues to be a major cause of disease around the world. It is not only the number one cause of bacterial pneumonia but also the cause of about 15% of the deaths of children under 5 around the world. There is a lot of research done on this organism, but with around 100 known serotypes and each one producing a unique capsule, there is still much more to be studied. The Etiology of Pneumonia in the Community (EPIC) study conducted by the CDC observed the burden of hospitalizations caused by pneumonia while determining the organism responsible. One of the most common organisms isolated from both children and adult patients was *S. pneumoniae*. To look further into these clinical isolates, we chose three different type 3 pneumococcal isolates and observed the immune cellular dynamics as well as capsule production, and saw that even with the same serotype immune responses can differ.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>Alveolar macrophages</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
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<td>CFU</td>
<td>colony forming units</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EPIC</td>
<td>Etiology of Pneumonia in the Community study</td>
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<td>iM</td>
<td>Inflammatory macrophages</td>
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<td>i.n.</td>
<td>Intranasal infection</td>
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<td>IPD</td>
<td>Invasive pneumococcal disease</td>
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<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal Dose, 50%</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>p.i.</td>
<td>Post infection</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
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<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>UTHSC</td>
<td>University of Tennessee Health Science Center</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1. INTRODUCTION

Burden of *Streptococcus pneumoniae*

 Millions of people in the United States are affected by respiratory diseases annually. Included in these and one of the most prevalent is pneumococcal disease caused by *Streptococcus pneumoniae* [1]. *S. pneumoniae* is a Gram-positive, facultative anaerobic bacterium found in the nasal passage of the majority of the population. This bacterium can have a commensal relationship with the host meaning it can be an opportunistic pathogen. Once an individual is infected with *S. pneumoniae* in their upper respiratory tract, it can spread to the lungs, the membranes surrounding the brain and spinal cord, the blood, and the middle ear resulting in pneumococcal disease. Pneumococcal disease can include community-acquired bacterial pneumonia, meningitis, bacteremia, and acute otitis media respectively. *S. pneumoniae* is known as the leading cause of community-acquired bacterial pneumonia and causes around 150,000 hospitalizations each year [2]. The financial burden from pneumococcal hospitalizations is a growing problem in the U.S. as well as across the world with the increasing problem of antibiotic resistance [3,4]. The WHO has also estimated that pneumonia causes about 15% of the death of children under 5 worldwide; with *S. pneumoniae* being the most common cause of bacterial pneumonia [1]. Transmission and disease progression must be understood for this bacterium to aid in preventing the spread and reducing the public health burden of this disease.

*Streptococcus pneumoniae* Transmission

The transmission of commensal respiratory organisms, specifically *S. pneumoniae* requires encountering respiratory secretions either through direct inhalation of these droplets or through contact with a contaminated surface, while self-inoculation is also possible when a person is a carrier of the bacteria in their upper respiratory tract. Transmission of the disease seems to occur more frequently during the colder months due to more frequent infections of other respiratory diseases as well as more frequent crowding in smaller spaces, but other factors seem to predispose a person to contract pneumococcal disease [5]. These factors include but are not limited to a decreased immune response and function, chronic diseases such as asthma, smoking, alcoholism, or cochlear implants [6].

Polysaccharide Capsule

There are around 100 known serotypes of *Streptococcus* with not all causing disease. Each serotype produces its own unique capsule, which is comprised of polysaccharides. The capsule plays an important role in the evasion of the host immune system, as well as virulence for the organism [7]. Most serotypes have a capsule that is negatively charged which helps with the evasion of the host’s defenses. Upon initial
colonization of pathogens in the nasal passage, mucus is one of the host’s first lines of defense. Mucus is comprised of mostly mucopolysaccharides, which are negatively charged partly because of the sialic acid. Due to this negative charge of the mucus as well as the negative charge of the capsule, there are repelling forces allowing for the escape of pneumococcus [7,8]. Another mechanism for *S. pneumoniae* to evade the host’s immune system is by escaping from phagocytosis from neutrophils and the neutrophil extracellular traps (NETs) they produce [9].

Depending on the serotype of *S. pneumoniae*, the biosynthesis of the capsular polysaccharide can occur through either the synthase-dependent mechanism or the Wzy-dependent pathway. The synthase-dependent pathway is used by types 3 and 37, while the Wzy-dependent pathway is used by all other known types [10]. Serotype 3 uses the Cps3S synthase to initiate synthesis by transferring glucose to a phosphatidylglycerol acceptor and continues the process to extend the polysaccharide [11]. This synthase-dependent mechanism results in the capsule polysaccharide not being covalently linked to the cell wall, unlike other serotypes that use the Wzy-dependent mechanism.

This difference in the biosynthesis of the capsular polysaccharide plays an important role in the effectiveness of the pneumococcal vaccine against *S. pneumoniae* [12]. There are two pneumococcal vaccines in use today, the pneumococcal conjugate vaccine (PCV13) and the pneumococcal polysaccharide vaccine (PPSV23) [20]. *S. pneumoniae* serotype 3 is included in both vaccines, yet it continues to be a major cause of invasive pneumococcal disease [21]. A study by Choi et al., showed the failure in efficacy against pneumococcus type 3 is in part due to the capsule polysaccharide not being covalently linked to the cell wall. If there are anti-CPS antibodies bound to the organism, *S. pneumoniae* type 3 can release its capsule and prevent the antibody-dependent killing of the organism [12]. With only serotypes 3 and 37 using this synthase-dependent mechanism, and type 37 not seen often in human patients [12], it is vital that more is understood about serotype 3 and the diseases it can cause.

**EPIC Study**

In January 2010, the CDC began the Etiology of Pneumonia in the Community (EPIC) study which tried to determine what the burden of hospitalizations due to pneumonia was in children and adults as well as determine which viruses and bacteria were associated with these hospitalizations. There were eight hospitals enrolled in this study with three being pediatric hospitals and five being adult hospitals. Specimens such as blood, urine, nasopharyngeal, pleural fluid, and sputum were collected to test for different bacterial organisms or viral particles. One of the most common pathogens detected in the samples collected was *S. pneumoniae* in 5% of the adult patients and 4% of the children; of these isolates collected several of them were serotype 3 pneumococcus [13,14]. Three of these serotype 3 strains were chosen to determine the host’s immune cell dynamics as well as the lung and blood bacterial burdens due to the complications that can occur with this serotype, as well as to determine any differences that may occur within the same serotype of *S. pneumoniae* that have been isolated from different
environments. These isolates are labeled as P110585, P210828, P310795 and were isolated from a sputum sample of a 56-year-old patient, a sputum sample of a 23-month-old patient, and a pleural fluid sample from a 22-month-old patient respectively. Each of these patients was virally coinfected at the time of isolation as well. P110585 was coinfected with parainfluenza virus type 3 (PIV3), P210828 was coinfected with influenza B, and P310795 was coinfected with respiratory syncytial virus (RSV). The lab strain A66.1 was used as a known comparison.
CHAPTER 2. MATERIALS AND METHODS

Mice

Female BALB/c 6-8 week old mice were obtained from Charles River Laboratories. Mice were housed in groups of five in 38.2 cm x 19.4 cm x 13.0 cm solid-bottom polysulfone individually ventilated cages. Rooms for housing mice were maintained on a 12:12-hour light: dark cycle at 22 ± 2°C with 50% humidity in the biosafety level two facility at the University of Tennessee Health Science Center Regional Biocontainment Center (Memphis, TN). Prior to inclusion in the experiments, mice were allowed to acclimate to the animal facility for at least seven days. Envigo irradiated rodent diet (#2918) and autoclaved water were available ad libitum. All experiments were performed under an approved protocol and in accordance with the guidelines set forth by the Animal Care and Use Committee at UTHSC.

Infection Experiments

All experiments were done using type 3 pneumococcal clinical isolates obtained from hospitalized patients labeled either P110585, P210828, or P310795. The bacterial doses were determined by previous LD50 experiments. The doses were chosen as a uniformly lethal dose (referred to as “high”) and a 50 percent lethal dose (referred to as LD50). Frozen stocks of the inoculum were diluted in sterile PBS and administered intranasally to groups of 5 mice. Mice were lightly anesthetized with 2.5% inhaled isoflurane (Baxter, Deerfield, IL) in a total volume of 100 µL (50 µL per nostril). Mice were inoculated with a designated dose at day 0. Weight loss, appearance, and behavior were monitored to assess illness and mortality. Mice were euthanized if they lost 30% of their starting body weight or became septic based on appearance and behavior.

Lung and Blood Harvesting and Processing

Mice were euthanized by 33% isoflurane inhalation. Lungs were aseptically harvested, washed in PBS, and digested with collagenase (1 mg/ml, Sigma C0130) and physical homogenization against a 40 µm cell strainer for immunology. Lung digest supernatants were used to determine bacterial titers; bacterial titers were also measured in peripheral blood. This was achieved by plating either the lung supernatant or blood in dilutions from neat to 10^-6 on blood agar with 20% neomycin and incubating for 48 hours at 37°C. Following red blood cell lysis, lung cells were washed in staining buffer (PBS, 5mM EDTA, 10mM HEPES, and 0.5% bovine serum albumin), counted with trypan blue exclusion using a Cell Countess System (Invitrogen, Grand Island, NY), and prepared for flow cytometric analysis as described below.
Flow Cytometry

Flow cytometry (ZE5 Cell Analyzer, Bio-Rad, Hercules, CA (UTHSC)) was performed on the cell pellets after incubation with 200 ul of 1:200 Fc block at 4°C for 20 minutes, followed by viability (Biolegend, Zombie Violet Fixable Viability Kit) and surface marker staining with anti-mouse antibodies. For total neutrophil, macrophage, CD4+ T cell, and CD8+ T cell quantification, antibodies Ly6G (1A8, PeCy7), F4/80 (BM8, PE), CD11c (N418, FITC), CD11b (M1/70, A700), CD3e (145-2C11, BV786), CD4 (RM4-5, V500), CD8a (53-6.7, BV605), CD49b (DX5, APC-e780) were used. The data were analyzed using FlowJo 10.8.0 (Tree Star, Ashland, OR). Data were cleaned using the flowAI application followed by gating for viable cells from a forward scatter/side scatter plot, singlet inclusion, and viability dye exclusion. Neutrophils (Ly6G^hi), macrophage populations (CD11c^{hi}F4/80^{hi}), more specific alveolar macrophages (AMΦ) (CD11c^{hi}F4/80^{hi}CD11b^-), inflammatory macrophages (iMΦ) (CD11c^{hi}F4/80^{hi}CD11b^+), CD4 T Cells (CD3^+CD8^-CD4^-CD49b^-), and CD8 T Cells (CD3^+CD8^+CD4^-CD49b^-) were gated.

Capsule Quantification

Capsule quantification for each *S. pneumoniae* patient isolate (P110585, P210828, P310795) as well as the type 3 laboratory strain A66.1 was measured using ELISA and read at 405nm. Wells of a 96 well plate were coated in bacteria that had been grown to a specified OD_{620} for there to be 1e6 bacteria per well and stored at 4°C overnight. Plates were incubated with a blocking buffer for 2 hours. Serially diluted anti-capsule type 3 rabbit serum was incubated on the bacterial cells for 1 hour at RT and washed using a wash buffer. The plate was then incubated with an anti-rabbit IgG, washed, and finally incubated with yellow ELISA substrate. The plates were read at OD_{405}.

Statistical Analysis

Significant differences in lung and blood bacterial loads and immune cells were determined using multiple Mann-Whitney tests on linear values to compare the means of a group of data and determine how different they may be (GraphPad Prism 9.3.0). The *P* values ≤0.05 were considered significant.
CHAPTER 3. RESULTS

Growth Dynamics

To begin understanding the characteristics of the chosen isolates, survival studies were performed using varying doses of the bacterial strains. Balb/c mice were intranasally infected with the chosen dose of the assigned S. pneumoniae isolate. The mice were monitored for 8 days post-infection. The lethal dose 50% (LD$_{50}$) for each strain was determined when 50% of the survival groups succumbed to the infection and a lethal dose was determined when all mice in the group succumbed to the infection. The LD$_{50}$ for P110585, P210828, and P310795 were determined to be 1e3, 1e4, and 1e4, respectively, while the high (uniformly lethal) dose was determined to be 1e4, 1e5, and 1e5, respectively (Figure 3-1A). For each of the high doses, the mice lost weight at similar rates with each losing approximately 16% if infected with P110585, 20% if infected with P210828, and 18% if infected with P310795 (Figure 3-1B). There was minimal weight loss observed for the LD$_{50}$ of each isolate (Figure 3-1B).

Bacterial Burdens

To determine the bacterial burden of each clinical isolate throughout the infection at differing doses, lung supernatants were plated on blood agar with 20% neomycin and incubated at 37°C for 48 hours. The mice that had been given the LD$_{50}$ did not grow to high titers in the lung and most mice were able to clear the infection by 48 hours (Figure 3-2A). The bacterial burden in the lungs of the high dose infected animals was high and most animals were unable to clear the infection with P210828 having the most bacterial burden at 24 hours post bacterial infection, but mice were able to clear some of the infection by 48 hours except for the lab strain A66.1 infected animals, which had titers that grew by 48 hours (Figure 3-2B). The number of bacteria in the blood differed for each strain and dose with most mice not having any bacteria in the blood for the LD$_{50}$ except for P110585. This strain was able to invade the blood by 24 hours with even higher titers at 48 hours (Figure 3-2C). The high doses of each strain had bacteria in the blood with each being at similar titers at 24 hours, but both P110585 ($P=0.007937$) and the lab strain A66.1 ($P=0.015873$, $P=0.007937$) grew to significantly higher titers as compared to strains P210828 and P310795 by 48 hours (Figure 3-2D).

Immune Cell Dynamics

To observe the immune cells, flow cytometry was used by staining for cell surface markers. The gating scheme is shown in Figure 3-3. All clinical isolates at the LD$_{50}$ had a slight increase in neutrophils by 48 hours. The high dose of the clinical isolates had a significant increase in neutrophils by 24 hours for P210828 compared to P110585 ($P=0.007937$), which was not a significant difference between the LD$_{50}$ and the high dose.
Figure 3-1. Survival and Weight Loss of Animals Infected with *S. pneumoniae* Clinical Isolates

(A) Survival curves for both LD$_{50}$ and high doses of all type 3 pneumococcal clinical isolates. (B) Weight loss curves of both LD$_{50}$ and high doses of all type 3 pneumococcal clinical isolates.
Figure 3-2.  Bacterial Burdens Differ with Strain and Dose

(A) Lung bacteria for the LD$_{50}$ of each clinical isolate. (B) Lung bacteria for the high dose of each clinical isolate as well as the lab strain A66.1. (C) Blood bacteria for the LD$_{50}$ of each clinical isolate. (D) Blood bacteria for the high dose of each clinical isolate as well as the lab strain A66.1 (*$P\leq 0.05$, **$P\leq 0.01$).
Figure 3-3. Flow Cytometry Gating Scheme

Flow cytometry gating scheme for immune cells. Cells were gated on a forward scatter (FSC-A) and side scatter (SSC-A), singlets were then gated, and lastly living cells using a zombie violet viability dye. Neutrophils (Ly6G\textsuperscript{hi}) were excluded, followed by macrophages (Ly6G\textsuperscript{neg}CD11c\textsuperscript{hi}F4/80\textsuperscript{hi}), more specific alveolar macrophages (CD11c\textsuperscript{hi}F4/80\textsuperscript{hi}CD11b\textsuperscript{-}), and inflammatory macrophages (CD11c\textsuperscript{hi}F4/80\textsuperscript{hi}CD11b\textsuperscript{+}), CD4 T Cells (CD3\textsuperscript{+}CD8\textsuperscript{-}CD4\textsuperscript{+}CD49b\textsuperscript{-}), and CD8 T Cells (CD3\textsuperscript{+}CD8\textsuperscript{+}CD4\textsuperscript{-}CD49b\textsuperscript{-}).
at either time point (Figure 3-4A-B). The alveolar macrophage (AM) populations did not change over time with the LD$_{50}$ for any of the clinical isolates besides P310795, which increased over time. The high dose of clinical isolates as well as the lab strain A66.1 had decreasing AM populations except for patient isolate P110585 which still maintained the AMs over time similar to the LD$_{50}$ (Figure 3-4C-D). The inflammatory macrophage (iM) population did not have any significant changes over time with the LD$_{50}$ for any of the clinical isolates but did trend slightly upwards. The high dose of clinical isolates did have significant increases in the iM population for P110585 and P210828 with significant differences seen at time points between strains as well. Clinical isolate P310795 did not have significant differences between time points but did have differences between doses with the high dose having significantly more iM cells at both time points (Figure 3-4E-F). Although not significant, there was a negative correlation between number of neutrophils per lung and lung bacteria burdens for high dose strains P110585 and A66.1 ($P = 0.9651$, $r = -0.01876$ and $P = 0.9114$, $r = -0.04268$). There was a positive correlation between number of neutrophils per lung and lung bacteria burdens for high dose strains P210828 and P310795, with P210828 had a significant positive correlation ($P = 0.0472$, $r = 0.6483$ and $P = 0.6212$, $r = 0.1768$). Comparing the high dose for all strains with the alveolar macrophages seen per lung, though not significant there was a slight positive correlation seen with strains P110585 and A66.1 ($P = 0.8991$, $r = 0.04961$and $P = 0.9604$, $r = 0.01813$), and there was also a positive correlation seen with strains P210828 and P310795 ($P = 0.3996$, $r = 0.3000$ and $P = 0.7428$, $r = 0.1193$). The inflammatory macrophage cells per lung and high dose bacterial burden for strains P110585 and A66.1 were not significantly positively correlated ($P = 0.3577$, $r = 0.3487$ and $P = 0.6192$, $r = 0.1945$ and $P = 0.1936$, $r = -0.4485$).

**Capsule Quantification**

Because the polysaccharide capsule of *S. pneumoniae* plays such a vital role in evasion of the host immune system as well as virulence, the capsule of each clinical isolate, as well as the lab strain A66.1, was quantified using an ELISA. To determine the growth curve of each isolate, each strain was grown in a nutrient-rich media and the OD$_{620}$ was measured over time. The lab strain A66.1 reached its exponential phase faster than the clinical isolates with P110585 being the slowest to reach the exponential phase (Figure 3-5A). The bacterial strains were grown to the determined OD$_{620}$ from the previous growth curve (Figure 3-5B) that would allow for 1e6 CFU to be plated per well in a 96 well plate. The bacterial stocks grown in nutrient-rich media were diluted out and a back titer was performed to make sure there was 1e6 CFU plated per well on each plate. The bacteria were coated with blocking buffer, serially diluted anti-capsule type 3 rabbit serum, anti-rabbit IgG, and yellow ELISA substrate. The plates were then read at OD$_{405}$. Strains P110585 and the lab strain A66.1 both contain more capsular polysaccharides than strains P210828 and P310795 (Figure 3-5C). The increase in the optical density corresponded to the increase in the capsule that was present over a serum concentration. At each serum concentration, each strain can be compared to another. More replicates
Figure 3-4. Host Immune Cell Populations Differ Between Strains

(A) Clinical isolate LD<sub>50</sub> neutrophil populations at 24 and 48 hours p.i. P110585 versus P210828 at 24 hours (*P=0.031746) (B) Clinical isolate and lab strain A66.1 high dose neutrophil populations at 24 and 48 hours p.i. P210828 versus P110585 at 24 hours (P=0.007937) and 48 hours (P=0.015873), A66.1 versus P110585 at 24 hours (P=0.007937) (C) Clinical isolate LD<sub>50</sub> alveolar macrophage populations at 24 and 48 hours p.i. P310795 versus P210828 at 24 hours (P=0.007937) and 48 hours (P=0.031746) (D) Clinical isolate and lab strain A66.1 high dose alveolar macrophage populations at 24 and 48 hours p.i. P110585 versus P210828 at 48 hours (P=0.007937), P110585 versus P310795 at 24 hours and 48 hours (P=0.015873), P110585 versus A66.1 at 24 hours (P=0.039683) and 48 hours (P=0.007937), P210828 versus P310795 at 24 hours (P=0.007937), P310795 versus A66.1 at 24 hours (P=0.039683) (E) Clinical isolate LD<sub>50</sub> inflammatory macrophage populations at 24 and 48 hours p.i. P110585 versus P210828 at 24 hours (P=0.023810), P210828 versus P310795 at 24 hours (P=0.015873) (F) Clinical isolate and lab strain A66.1 high dose alveolar macrophage populations at 24 and 48 hours p.i. P110585 versus P310795 at 24 hours (P=0.015873) and 48 hours (P=0.007937), P110585 versus A66.1 at 48 hours (P=0.031746), P210828 versus P310795 at 48 hours (P=0.007937), P210828 versus A66.1 at 48 hours (P=0.007937).
Figure 3-5.  Quantification of the Polysaccharide Capsule for All Strains

(A) Growth curve of all clinical isolates and lab strain A66.1 at OD<sub>620</sub> versus time (min).
(B) OD<sub>620</sub> versus bacterial CFU of all clinical isolates and lab strain A66.1 (C) The serum concentration versus OD<sub>405</sub> of the plated clinical isolates and lab strain A66.1. Increasing OD corresponds to the amount of polysaccharide capsule present for each isolate.

need to be performed to calculate if there is a significant difference between the capsule production of each strain.
CHAPTER 4. DISCUSSION

The goal of this project was to observe any differences that may be present between different strains of the same serotype of *Streptococcus pneumoniae* clinical isolates. This began by determining the dosage dynamics for each of the strains through survival studies. The initial results showed that the clinical isolate P110585 was more infectious than the other two isolates and required a lower infectious dose to be lethal. The lethal dose, 50% was determined to be 1e3 CFU with a uniformly lethal dose being 1e4 CFU (Figure 3-1A). This difference in lethality between the strains leads to questions of why there is a difference between the strains when they are all the same serotype. To begin figuring out what these differences may be, the blood and lung supes of infected Balb/c mice were plated on blood agar to determine the bacterial burden of each strain. The bacterial burdens in the lungs were not significantly different between strains at either dose but grew to higher titers with the high dose for isolates P210828 and P310795 (Figure 3-2A-B). The higher titers correspond with an influx of neutrophils as well as a depletion of alveolar macrophages in the lungs of mice that had been infected with P210828, P310795, and lab strain A66.1 (Figure 3-4).

In the lungs, the first line of defense for the host is the alveolar macrophages that are resident phagocytes and aid in the clearance of any foreign organisms that the host may encounter. Upon the clearance of the bacteria, there is alveolar macrophage depletion or dysfunction/apoptosis [15,16]. This depletion/dysfunction and apoptosis of the alveolar macrophages can be seen with each of these bacterial infections and differ between the strains at the high dose (Figure 3-4D). When the bacterial infection cannot be controlled, the alveolar macrophages have the ability to not only phagocytose the bacterial cells but also to recruit PMN cells to the infection site. These PMN cells include neutrophils which assist with the inflammatory environment to aid in the clearance of the bacteria as well as phagocytosing the bacteria cells [17]. Another cell found at the site of an infection is the inflammatory macrophage which can begin inflammation in the lung as well as sustain it and recruit other inflammatory cells [18]. The increase in inflammatory macrophages in the lung is also seen with the higher doses of clinical isolates as compared to the LD$_{50}$ of each isolate at both time points (Figure 3-4E-F).

When looking at the blood bacteria of the infected mice clinical isolate P110585 and lab strain A66.1 both have high titers of bacteria in the blood by 48 hours and even the LD$_{50}$ of P110585 has high titers of bacteria in the blood at 48 hours (Figure 3-2C-D). This could be partly due to the amount of capsule each strain contains. As previously discussed, the polysaccharide capsule found on the outside of most pneumococcal serotypes aids in the virulence of the bacterium by helping to evade the host’s immune system, and the ability of type 3 pneumococcus to shed its capsule can then allow for further infection leading to invasive pneumococcal disease (IPD). It has been shown that pneumococcal strains with greater amounts of capsular polysaccharides are more virulent and are associated more with invasive disease, whereas strains with less capsular polysaccharides allow for more epithelial adherence and are associated more with lower respiratory tract infections [19]. The results from the polysaccharide capsule


quantification experiment correspond with these data because the clinical isolate P110585 and lab strain A66.1 both contained more capsule than the other two clinical isolates and these two strains were able to invade the blood more frequently and at higher titers than the other two strains (Figure 3-5C) (Figure 3-2D). The amount of capsule each isolate produced should be repeated in the future to determine if there is a significant difference is between the pneumococcal isolates. Taken all together the bacterial burdens, host innate immune responses, and capsule production for each pneumococcal type 3 strain can differ and cause different disease outcomes and lethality. Patient isolate P110585 was more similar to the lab strain A66.1 in all aspects from lung immunological response to capsule production and patient isolates P210828 and P310795 were more similar.

Streptococcus pneumoniae serotype 3 is part of the clonal class 180 (CC180) and recently has been shown to contain several distinct lineages. CC180 can be divided into Clade I-α, Clade I-β, and Clade II. Clade II has become prevalent in North America and is associated with increased antibiotic resistance [21]. This could be an explanation for why these type 3 clinical isolates behave differently. Some questions that arise from these results are: did the environment these strains were isolated from play a major role in these differences? Does the factor of coinfection with a virus aid in the virulence or structure of the different strains? Future studies to continue to observe and try to understand what these differences are and how to address them could be comparing the genetic sequences of each. Are there mutations that could lead to increased lethality? Are these isolates in different clades? Understanding more about these clinical isolates would further the field of clinical medicine as well as vaccine development.
LIST OF REFERENCES


Taylor Rae Plunkett White was born in 1995 in Muscle Shoals, AL to Tommie Plunkett. She attended Muscle Shoals High School and graduated in 2013. She received her Bachelor of Science degree in Microbial, Cellular, and Molecular Biology with a concentration in Microbiology at Auburn University in Auburn, AL in May of 2017. After graduation, she worked as a microbiologist for 2 years and then decided to enroll in a graduate program. She began the Integrated Biomedical Sciences Program at the University of Tennessee Health Science Center in Memphis, TN in the fall of 2019 and joined the lab of Dr. Amber M. Smith in spring of 2020. Taylor anticipates graduating from the College of Graduate Health Sciences with a Master of Science degree in Biomedical Sciences and a concentration in Microbiology, Immunology, and Biochemistry in April 2022.