5-1-2020

Pneumococcal resistance to granule-mediated killing by human neutrophils

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By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

May 2020

Pneumococcal resistance to granule-mediated killing by human neutrophils

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Title of Study: Pneumococcal resistance to granule-mediated killing by human neutrophils

Pages in Study 98

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Streptococcus pneumoniae is a significant human pathogen and the leading cause of community-acquired pneumonia and acute otitis media. One of the primary defense mechanisms of the human immune system against pneumococcal infection involves granule-mediated killing of bacterial cells by neutrophils. While this mechanism has previously been shown to kill about half of pneumococci *in vitro*, we hypothesized that some pneumococcal strains have evolved to be more resistant to this granule-mediated killing. Clinical isolates demonstrated a varying range of sensitivity to neutrophil granules. Additionally, we established that the absence of the capsule may affect sensitivity as unencapsulated isolates showed a higher average survival than encapsulated isolates. Finally, pneumococcal surface protease HtrA was found to potentially serve as a protective factor as many knockouts were more sensitive than the wildtypes, recombinant HtrA protected wildtype TIGR4, and a resistant isolate showed higher *htrA* expression levels than sensitive isolates.

ACKNOWLEDGEMENTS

I would like to acknowledge Brock Drumheller, Jessica Surma, and Mary Carr for preliminary work on this project. Jessica and Mary were also quite helpful throughout my work on this project. Kamal Bhalla dedicated time as a high school student to help with this project, as well. Thank you, Jackson Holland, for you have made a significant impact on this work in the last year. I would like to thank fellow graduate students Yoonsung Hu for support with protein work and Daniel Kennedy for much beneficial advice. I would also like to thank Dr. Keun Seok Seo and Dr. Nogi Park of MSU College of Veterinary Medicine for help with my protein. I would like to thank Dr. Jason Rosch of St. Jude Children's Research Hospital for his gracious gift of carriage isolates and Dr. Larry McDaniel of University of Mississippi Medical Center for his gracious gifts of unencapsulated isolates and their mutants. Finally, I would like to thank Dr. Justin Thornton for his wonderful guidance and leadership in my journey to achieving a degree of Master of Science.

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CHAPTER I

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a significant, Gram positive, alpha-hemolytic human pathogen and the leading cause of community-acquired pneumonia and acute otitis media in children worldwide. Many healthy individuals are asymptomatically colonized by pneumococcus intermittently throughout their lives. In fact, roughly 50% of humans are colonized by pneumococcus at any given time (Austrian, 1986). Colonization occurs in the nasopharynx via inhalation of contaminated aerosolized particles, and colonization can occur in children as young as two months (Sisson et al., 2000). Children living in or commonly frequenting densely populated areas or in tight quarters, such as daycares or orphanages, creates an ideal situation for the transmission of this organism (Raymond et al., 2000). Transmission of pneumococcus is not restricted to children, though, as healthy adults can also transmit the bacteria, and it is this cycle of transmission via colonization without causing disease that has made *S. pneumoniae* such a prevalent organism worldwide (Donkor, 2013).

However, colonization of pneumococcus in the susceptible youth, elderly, and immunocompromised can lead to severe invasive disease, including pneumonia, meningitis, and sepsis (Bogaert et al., 2004). Disease typically occurs when the organism disseminates from the nasopharynx into either the blood or lungs, leading to one of the

diseases listed above. A common pathology seen especially with children is when the organism spreads to the middle ear resulting in otitis media.

Adherence to and replication of *Streptococcus pneumoniae* in the alveolar tissue of the lungs leads to pneumonia. Pneumococcal pneumonia is the most common cause of pneumonia in children worldwide (CDC). Pneumonia is complicated by the accumulation of edema fluid in the alveoli in response to bacterial presence and pneumococcal pneumolysin production (Canvin et al., 1995). A number of different bacterial mechanisms and interactions with host immune cells determines whether or not the alveolar spaces are the final location of pneumococcal infection. If the immune response overcomes the bacteria's defense mechanisms, the infection can be cleared in the lungs. If the bacteria persist in the lungs, they can invade the bloodstream causing bacteremia. Replicating pneumococci circulating through the blood causes septicemia. During this circulation, the bacteria can make contact with vascular endothelial cells at the blood-brain barrier and cross the tight junctions into the cerebral spinal fluid (Henriques-Normark and Tuomanen, 2013). This process is initiated via the binding of pneumococcal adhesin choline binding protein A and host laminin receptor (Orihuela et al., 2009). Then, pneumococcal phosphorylcholine interacts with the platelet activating factor receptor to cross the blood-brain barrier (Cundell et al., 1995). After traversing this barrier, pneumococci can enter the meninges and cause the most severe manifestation of pneumococcal disease, meningitis.

A number of virulence factors play key roles in the pathogenesis of pneumococcal pneumonia. One of the primary virulence factors of pneumococcus is an extracellular polysaccharide capsule, which protects against complement and other phagocytic defense mechanisms (Hyams et al., 2010). Pneumococcus expresses over 90 different capsular

serotypes. The capsule is essential in colonization of the nasopharynx as clinically important type 2 and 3 strains require capsule expression for colonization (Magee and Yother, 2001). Furthermore, different capsule types play different roles in the progression from colonization to disease (Kadioglu et al., 2002). The capsule, no matter what type, is the primary effector in pneumococci evading opsonophagocytosis by immune cells (Cross, 1990).

A successful conjugate vaccine eliciting immunity to the polysaccharide capsule is available; however, it is not easily available or affordable to those in developing countries. Despite vaccination efforts, colonization rates have remained constant and around 800,000 children under the age of five die every year due to pneumococcal disease (Bogaert et al., 2004; O'brien et al., 2009).

One of the primary immune defenses against pneumococcal infection is the neutrophil. Neutrophils are granulocytes which can kill invading pathogens via a number of different mechanisms, including phagocytosis, release of granular proteases and antimicrobial peptides, reactive oxygen-mediated killing, and release of neutrophil extracellular traps (NETS) (Nauseef, 2007; Brinkmann et al., 2004). Phagocytosis leads these mechanisms of killing as the primary function of the neutrophil.

Prior to neutrophil killing, however, is the process of neutrophil recruitment and activation. Neutrophils are recruited to combat pneumococcus immediately following colonization and throughout invasive infection (Dallaire et al., 2001; Rossum et al., 2005). They make up the majority of the leukocytes circulating in the blood. When an infection occurs in the tissue, chemokines released by resident macrophages attract neutrophils to the site of infection. Then, neutrophils cross into infected tissues via diapedesis at the post-

capillary venules (Kaufmann et al., 2004). Once inside the tissue, the neutrophil uses an array of pattern recognition receptors (PRRs) to identify and bind to invading pathogens. In the case of pneumococcal infection, the PRR TLR-2 recognizes and binds pneumococcal lipoteichoic acid (Schroder et al., 2003). In addition to this binding, pneumolysin can be recognized and bound by TLR-4 of the neutrophil (Malley et al., 2003). Traditionally, this is where phagocytosis begins to engulf and degrade pathogens. This process is complex and is often aided by opsonizing complement proteins and antibodies. In addition to phagocytosis, though, receptor binding of opsonized material can also lead to the release of neutrophil granule contents (known as degranulation) and the production of reactive oxygen compounds (Kaufmann et al., 2004).

The mode in which neutrophils clear pneumococcal infection was initially not well characterized. However, reactive oxygen-mediated killing was ruled out as neutrophils' primary method of killing pneumococcus, and other catalase negative organisms, as patients with reduced reactive oxygen species were unaffected by pneumococcal colonization (Kaplan et al., 1968). Though the other mechanisms the neutrophil uses to kill pneumococcus often succeed, the pathogen has developed its own defenses to counteract those of the neutrophil. The capsule of pneumococcus prevents phagocytosis. It has also been shown that the negative charge of some capsule types deters nonopsonic killing of pneumococcus by neutrophils (Li et al., 2013). The capsule has also been shown to inhibit the microbicidal functions of NETs (Wartha et al., 2007). Likewise, a couple extracellular vesicle-associated DNases, EndA and TatD, provide *S. pneumoniae* with resistance to NETs (Beiter et al., 2006; Jhelum et al., 2018). However, mechanisms providing protection of the pneumococcus against granule-mediated killing have not been adequately investigated.

The release of toxic granules by the neutrophil conventionally occurs during phagocytosis, when the granules are released into the phagolysosome to degrade the engulfed pathogens. However, granules can also be released into the extracellular matrix during phagocytosis or to aid in breakdown of material too large to be ingested (Weiss, 1989). The primary neutrophil granules, or azurophilic granules, contain toxic enzymes such as cathepsins, defensins, elastase, and myeloperoxidase. Lactoferrin and gelatinase are components of secondary granules, also known as specific granules, which are generally less toxic than azurophilic granules. Standish and Weiser (2009) demonstrated that release of azurophilic granule serine proteases, particularly cathepsin G and elastase, is the neutrophil's primary method of killing pneumococcus.

The sequential stimulation with cytochalasin B and formyl-methionyl-leucyl-phenylalanine (fMLP) was found to artificially degranulate human neutrophils *in vitro* (Bentwood and Henson, 1980). Standish and Weiser utilized this artificial stimulation to extract the azurophilic granules from human neutrophils and subsequently test their abilities to kill *Streptococcus pneumoniae*. They found that this extract significantly killed type 6A pneumococci (2009). Furthermore, they found that this killing by granules was due to protease activity as three different pneumococcal isolates of different capsule types showed significant increases in survival to opsonophagocytosis by neutrophils pre-treated with protease inhibitors (2009). Elastase and cathepsin G, two well-characterized serine proteases found in neutrophil granules, were found in high amounts in the degranulated neutrophil extract. Additionally, Standish and Weiser established concentrations of each of these proteases that significantly killed type 6A pneumococci. This led to their conclusion

that these two serine proteases were the primary effectors of intracellular degradation of pneumococci by human neutrophils (Standish and Weiser, 2009).

Despite a defined mode of neutrophil-mediated killing, *Streptococcus pneumoniae* remains a deadly pathogen affecting millions of people worldwide. Therefore, this organism is able to overcome many neutrophil defenses. However, the mechanism pneumococci utilize to resist this granule-mediated defense by neutrophils has not been defined, nor has the conservation of granule-mediated killing across clinical isolates been determined.

We hypothesized sensitivity to granule-mediated killing would vary across pneumococcal strains and that many pneumococcal isolates express one or more factors which confer resistance to neutrophil granule-mediated killing. This work is the first of its kind to establish a standard sensitivity rate of a panel of clinical isolates, which represent an array of different capsule types, to this mode of killing by neutrophil proteases. Ultimately, we possibly identify a novel component of pneumococcal pathogenesis involved in evading one of the most crucial leukocytes involved in pneumococcal clearance.

CHAPTER II

PNEUMOCOCCAL ISOLATES DISPLAY A VARIABLE RANGE OF SENSITIVITY TO NEUTROPHIL GRANULES

The initial focus of this research was to determine the relative sensitivity of pneumococcal laboratory strains and isolates to neutrophil granules – a finding that had not yet been reported in the literature. To do this, we established a general protocol in which pneumococci were exposed to granules extracted from human neutrophils. This protocol was mostly adapted from the protocol of Standish and Weiser, described above. We then compared the survival of the bacteria exposed to the granules to untreated controls to calculate the percent survival of each strain. This process involved many steps, which are described in detail below.

One of the first facets of pneumococcal sensitivity to neutrophils studied was whether the site of isolation (colonization versus invasive disease) impacted resistance. It possible that invasive pneumococci are able to evade this mechanism of killing better than isolates restricted to nasopharyngeal colonization, which may not have the tools to resist neutrophil killing. To further characterize this aspect of pneumococcal infection, both invasive isolates and colonization isolates were obtained for this sensitivity assay.

Methods

Bacterial Strains and Culture Conditions

Pneumococcal invasive isolates (ABC isolates), isolated from either the blood or cerebral spinal fluid (CSF) of patients with systemic pneumococcal disease, were received from the CDC

ABC isolate bank. Table 2.1 provides data on these invasive isolates. Carriage isolates (SPN isolates), isolated from the nasopharynx of asymptomatic patients, were received from our collaborator, Dr. Jason Rosch at St. Jude Children's Hospital. Table 2.2 provides data on the SPN isolates.

Table 2.1 Data on the invasive (ABC) isolates

Isolate #	Site of Isolation	Patient Age (years)	Patient Survived?	Serotype	Known Antibiotic Resistance
1	Blood	10	Y	23B	None
2	Blood	48	N	12F	None
3	Blood	5	Y	23A	None
4	Blood	4	Y	15A	Tetracycline, Erythromycin
5	Blood	17	Y	19A	Tetracycline
6	Blood	6	Y	06C	None
7	Blood	10	Y	16F	None
8	Blood	37	Y	35B	None
9	Blood	3	Y	06C	Erythromycin
10	Blood	27	Y	18C	None
11	Blood	12	Y	23F	None
12	CSF	25	Y	06B	None
13	Blood	10	Y	19A	None
14	Blood	8	Y	15B	None
15	Blood	37	Y	12F	None
16	Blood	25	N	15C	None
17	Blood	61	Y	19F	None
18	Blood	14	Y	12F	None
19	Blood	37	Y	06C	None
20	Blood	1	Y	15B	None
21	Blood	33	Y	3	None
22	Blood	6	Y	06C	None
23	Blood	9	N	06A	Erythromycin

Table 1 provides known data on all 23 of the invasive (ABC) isolates used provided by the CDC. Data provided includes the tissue the isolates were extracted from (blood or cerebral spinal fluid), the age of the patients the isolates were extracted from (in years), if the patients survived (yes or no), the capsular serotype of the isolates, and what antibiotics the isolates have known resistance to.

Table 2.2 Data on the carriage (SPN) isolates

Isolate #	Site of Isolation	Known Antibiotic Resistance
1	Nasopharynx	Erythromycin
2	Nasopharynx	None
3	Nasopharynx	Streptomycin
4	Nasopharynx	Tetracycline, Erythromycin
5	Nasopharynx	Tetracycline
6	Nasopharynx	None
7	Nasopharynx	None
9	Nasopharynx	None
11	Nasopharynx	None
12	Nasopharynx	None
13	Nasopharynx	None
14	Nasopharynx	Erythromycin
15	Nasopharynx	None
31	Nasopharynx	None

Table 2.2 provides known data on all 14 carriage (SPN) isolates used. Data provided includes the tissue the isolates were extracted from and what antibiotics the isolates have known resistance to.

Each strain was first cultured on tryptic soy agar (TSA) plates supplemented with 5% sheep's blood (blood agar). From the blood agar plates, pneumococcal broth cultures were inoculated using a sterile loop. These cultures were grown in either casitone plus yeast extract (C+Y) medium or Todd Hewitt plus yeast extract (THY) medium in a 37º C water bath without agitation until they reached an OD_{600} of approximately 0.5. Freezer stock cultures were made by supplementing broth cultures with 20% glycerol. Stock cultures were stored at -80ºC. To make aliquots of broth cultures, bacterial cultures at 0.5 OD were centrifuged at 13,000 rpm for ten minutes at 4ºC. The supernatant above bacterial pellets was vacuumed off and pellets were resuspended in freezing media (C+Y or THY containing 20% glycerol). 100 µl aliquots were dispensed in 1.5 ml microcentrifuge tubes and stored at -80ºC for at least 24 hours.

To find the concentration of aliquots, they were thawed on ice, vortexed, then serially diluted in 1x phosphate-buffered saline (PBS) and plated on blood agar plates for quantitation.

Typically, two 10^{-2} dilutions were done followed by two 10^{-1} dilutions, each of which were plated as duplicate 10 µl streaks on blood agar. These plates were incubated overnight in a 5% CO_2 incubator at 37ºC. Colony forming unit (CFU) counts were counted the next day and the average was used to calculate the concentration in CFUs/ml. Most aliquots ranged in concentrations from 2 x 10^8 to 2 x 10^9 CFUs/ml.

Neutrophil Isolation and Degranulation

Human blood was extracted from a healthy donor, and neutrophils were isolated as described by Axis Shield Polymorphprep™ protocol with some minor changes. Five ml of blood was layered slowly over 5 ml of Polymorphprep solution in 15ml conical tubes. Tubes were centrifuged at 500x g for 35 minutes at room temperature. This created a multi-layered solution in the tubes where the plasma layer was on top of mononuclear cell (MC) layer, followed by the polymorphonuclear leukocytes (PMNs), and the majority of the red blood cells on bottom (Figure 2.1). Using a Pasteur pipette, the plasma and MC layers were vacuumed off of the PMN layer. Then, the PMNs were collected and transferred to a new tube. PBS was mixed with the PMNs, and the tubes were centrifuged at 400x g for 10 minutes. This step often did not produce a solid pellet and left many cells still suspended in the supernatant. So, this step was repeated by splitting the solution in half, adding more 1x PBS to the cells to wash them, and centrifuging again at 400x g for 10 minutes. The second wash with PBS usually produced a solid pellet of cells. The supernatant was vacuumed off of the pellets, and the pellets were both resuspended in the same 5 ml of ammonium chloride lysis buffer, or red blood cell lysis buffer, to lyse the remaining red blood cells in a 37ºC water bath for 5 minutes. Cells were then spun again at 400x g for 10 minutes. The supernatant was removed, and the cells were resuspended in 10 ml of RPMI 1640 + L-Glu + HEPES (media). The cells were centrifuged in the media by spinning at

1200 rpm for 5 minutes. Finally, the neutrophils were resuspended in 10 ml of media again to obtain a concentration count. Ten µl was loaded onto a hemocytometer to obtain an average of the quadrant counts. The cells were centrifuged again at 1200 rpm for 5 minutes. They were resuspended in a calculated amount of media to obtain a concentration of 2 x 10^7 cells/ml. This concentration was then rechecked by a hemocytometer count.

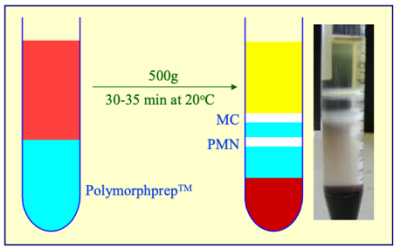

Figure 2.1 Purification of PMNs using Polymorphprep™

MC = mononuclear cells; PMNs = polymorphonuclear leukocytes. This illustration shows the result of centrifuging blood over Polymorphprep solution. Picture on right shows an actual separation in a 15 ml conical tube (taken from Polymorphprep™ Application Sheet).

Isolated neutrophils were degranulated as similarly described by Standish and Weiser. Extracted neutrophils at 2 x 10^7 cells/ml were induced to degranulate by incubating with cytochalasin B (cytB) (5 µg/ml; Sigma-Aldrich) for 5 minutes at 37°C, followed by incubation with fMLP (10^{-7} M; Sigma-Aldrich) for a further 30 minutes. Degranulated neutrophils were centrifuged at 1200 rpm for 5 minutes. Granules were then collected from the supernatant and transferred to a pre-chilled 5 ml microcentrifuge tube to mix the degranulate solution well. From there, 110 µl aliquots were aliquoted into pre-chilled 1.5 ml tubes, which were stored at -80°C for pneumococcal killing assays.

Degranulate Microbicidal Assay

Pneumococcal killing assays were also done as similarly described by Standish and Weiser. Pneumococcal and degranulate aliquots were thawed on ice. Then, pneumococci were diluted in RPMI 1640 + HEPES + L-Glu to 2.5 x 10^6 CFUs/ml, and 10^6 CFU/ml were incubated with 20 µl of degranulate in a 100 µl reaction at 37°C for one hour. Reactions were diluted in ice cold 1x PBS and plated on blood agar. Plates were incubated overnight in 5% CO_2 at 37°C. Colony counts were quantitated the next day to calculate concentrations post-exposure to degranulate, and counts were compared to untreated control counts to determine percent survival. Zero-hour controls were also made, but not subjected to the one-hour incubation, and plated on blood agar to ensure ~10^6 CFUs/ml of bacteria were used each time.

Concentration counts of both control and experimental reactions were loaded into Prism software for graphing and statistical analyses. The percent survival of pneumococci was calculated by dividing the degranulate-exposed reaction concentration by the unexposed concentration and multiplying by 100%. This data was also loaded into Prism, separate of the concentration data. This entire experiment was done in triplicate for each strain to obtain repeatable results. A two-tailed unpaired t-test was performed in Prism on the concentration data between the control and experimental concentration counts for statistical analysis. An alpha value of 0.05 was used, and P-values less than 0.05 designated strains that were significantly killed by granules.

Results

First, the sensitivity of 23 invasive isolates to degranulate killing was assessed. To first look at the concentration counts on all of these strains, we separated the data into four graphs to better analyze the strains individually. In all of these following graphs on concentration data, a

minimum of 10^4 was used for the y-axis since no counts fell below this concentration. Figure 2.2 displays the killing of the first six invasive isolates by showing the concentrations of the exposed concentrations compared to the control unexposed concentrations. Though the concentration counts provide the raw data, graphing the percent survival of each strain is a more succinct format to analyze the sensitivity of all of these isolates. Again, the percent survival was calculated by dividing the experimental concentration by the control concentration and multiplying by 100%. This was done for each replicate for each strain; so, the average percent survival for all 23 invasive isolates is shown in Figure 2.6. Of the first six invasive isolates, ABC2 and ABC5 were killed significantly by degranulate with p-values of 0.0313 and 0.0159, respectively. ABC2 was the most susceptible of these with a survival of 23.4%, and ABC1 was the most resistant demonstrating a survival of 59.8%.

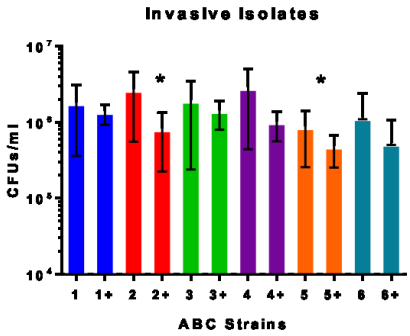

Figure 2.2 Sensitivity to granule-mediated killing of first six invasive isolates

Roughly 10^6 pneumococci per ml were exposed to granules extracted from human neutrophils for one hour at 37ºC. Concentration counts were quantitated and compared to concentration counts of the same concentration of pneumococci, that were not exposed to granules. Data shown are the means ± standard deviation (SD) for control and experimental (+) counts of six invasive isolates – ABC1 (blue), ABC2 (red), ABC3 (green), ABC4 (purple), ABC5 (orange), and ABC6 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

Figure 2.3 shows the concentrations of ABC 7-12 isolates. Of these six isolates only ABC12 was significantly killed by degranulate ($p = 0.0035$). ABC9 was the most susceptible isolate, however, at 12% survival, while ABC10 was the most resistant isolate at 75.7% survival.

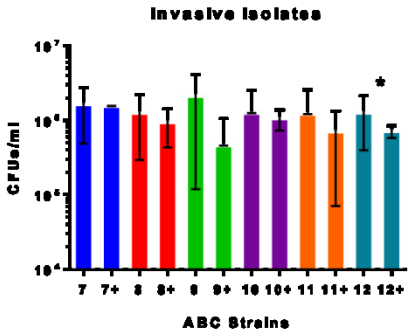

Figure 2.3 Sensitivity to granule-mediated killing of ABC 7-12 isolates

Data shown are the means ± SD for control and experimental counts of six invasive isolates. Shown are ABC7 (blue), ABC8 (red), ABC9 (green), ABC10 (purple), ABC11 (orange), and ABC12 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

The concentrations of ABC 13-18 isolates are displayed in Figure 2.4. The majority of these isolates displayed higher susceptibility rates compared to the first 12 isolates with ABC15 being the most resistant and the only one of these six isolates not being significantly killed ($p = 0.0788$). ABC13 was highly susceptible at 6.6% survival ($p = 0.0014$). ABC14 was also a significantly sensitive invasive isolate at 5.1% survival ($p < 0.0001$). ABC16 displayed a significantly sensitive survival rate of 17% ($p = 0.0011$). ABC17 was the most sensitive of all of the isolates tested at 4.9% survival ($p = 0.026$). Finally, ABC18 exhibited an 8.9% survival rate ($p = 0.0042$).

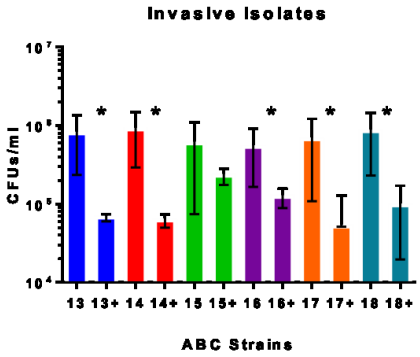

Figure 2.4 Sensitivity to granule-mediated killing of ABC 13-18 isolates

Data shown are the means ± SD for control and experimental counts of six invasive isolates. Shown are ABC13 (blue), ABC14 (red), ABC15 (green), ABC16 (purple), ABC17 (orange), and ABC18 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

For the final invasive isolates, concentration data can be found in Figure 2.5. The majority of these final invasive isolates were also significantly killed by degranulate, with ABC23 being the most resistant invasive isolate at 90.7% survival and the only one of the final five invasive isolates to not be significantly killed by degranulate ($p = 0.1751$). ABC19 displayed a survival rate of 20.1% ($p = 0.0059$). ABC20 was the most sensitive of the final invasive isolates at 16.2% survival ($p = 0.0104$). ABC21 and ABC22 showed similar survival rates of 31.8% ($p = 0.0004$) and 33% ($p = 0.0108$), respectively.

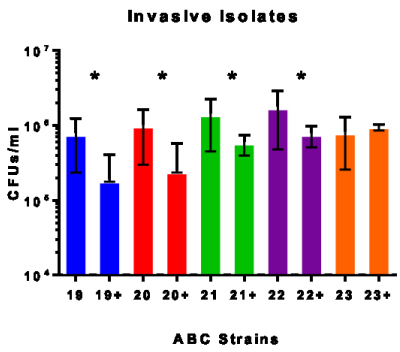

Figure 2.5 Sensitivity to granule-mediated killing of final five invasive isolates

Data shown are the means ± SD for control and experimental counts of the last five invasive isolates. Shown are ABC19 (blue), ABC20 (red), ABC21 (green), ABC22 (purple), and ABC23 (orange). Asterisks (*) indicate strains significantly killed by degranulate.

The average percent survival of all 23 isolates is 35.41% and the median is 33%. Twelve (52.2%) of these isolates had survival rates lower than the average and eleven (47.8%) had rates higher than the average. Twelve (52.2%) of these isolates were significantly killed by degranulate compared to their unexposed controls. ABC 7, 10, and 23 were the most resistant to neutrophil granules, while ABC 13, 14, and 17 were the most susceptible isolates.

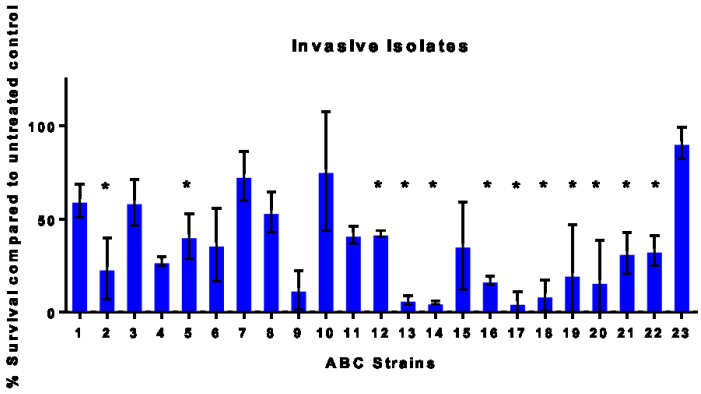

Figure 2.6 Sensitivity of all 23 invasive isolates to degranulate killing by human neutrophils.

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all 23 invasive isolates. Asterisks (*) indicate strains significantly killed by degranulate.

Once the sensitivity of the 23 invasive isolates to degranulate was assessed, we tested the sensitivity of 14 carriage isolates. Like the invasive isolates, the sensitivity was assessed by looking at the concentration counts of exposed bacteria compared to unexposed bacteria and the percent survivals of all 14 isolates (Figure 2.10). Figure 2.7 shows the concentrations of the first six SPN isolates. Of the first six carriage isolates, strains 1 ($p = 0.0108$), 2 ($p = 0.0034$), 3 ($p = 0.0023$), and 6 ($p = 0.0261$) were significantly killed by degranulate. SPN2 was the most sensitive of the first six isolates at 23% survival. SPN5 was the most resistant of all of the clinical isolates and the only one that showed a survival rate above 100% at 127.9% survival.

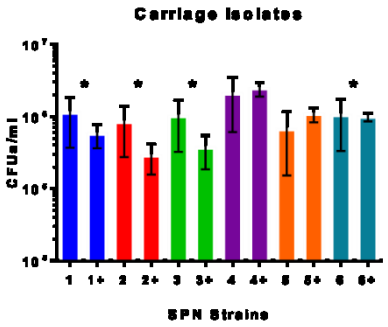

Figure 2.7 Sensitivity to granule-mediated killing of first six carriage isolates

Data shown are the means ± SD for control and experimental counts of six carriage isolates – SPN1 (blue), SPN2 (red), SPN3 (green), SPN4 (purple), SPN5 (orange), and SPN6 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

Next, the degranulate killing assay was done on five more SPN isolates (Figure 2.8). Three of the next five carriage isolates were significantly killed by degranulate – SPN7 at 54.9% survival ($p = 0.0139$), SPN9 at 55.1% survival ($p = 0.0294$), and SPN13 at 83.3% survival ($p = 0.0423$). SPN11 was the most resistant of these five isolates and the second most resistant clinical isolate at 94.8%.

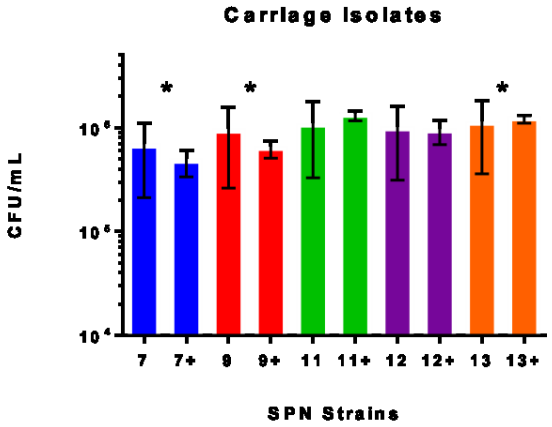

Figure 2.8 Sensitivity to granule-mediated killing of five carriage isolates

Data shown are the means ± SD for control and experimental counts of five carriage isolates. Shown are SPN7 (blue), SPN9 (red), SPN11 (green), SPN12 (purple), and SPN13 (orange). Asterisks (*) indicate strains significantly killed by degranulate.

The final three carriage isolates and their concentration counts after degranulate exposure are displayed in Figure 2.9. SPN15 was the only isolate not significantly killed by degranulate ($p = 0.1512$). However, SPN14 was the most resistant isolate of these three at 68.3% survival, even though it was significantly killed compared to its untreated control ($p = 0.0137$). SPN31 was significantly killed and was the most susceptible of these three at 40.8% ($p = 0.0036$).

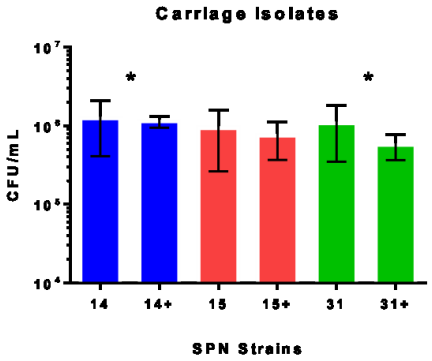

Figure 2.9 Sensitivity to granule-mediated killing of final three carriage isolates

Data shown are the means ± SD for control and experimental counts of three carriage isolates. Shown are SPN14 (blue), SPN15 (red), and SPN31 (green). Asterisks (*) indicate strains significantly killed by degranulate.

The average percent survival of all of the carriage isolates was actually significantly higher than the invasive isolates at 64.55% ($p = 0.0021$) (Figure 2.11). The median of the carriage isolates' survivals was calculated to be 63.55%. Half of the carriage isolates' percent survivals fell under the average and half were above the average. Nine (64.3%) of the 14 carriage isolates were significantly killed by neutrophil degranulate. Collectively, the average survival of all of the clinical isolates tested was 46.4% while the median was 40.77%. Nineteen (51.35%) of the 37 isolates tested were significantly inhibited by granule-mediated killing.

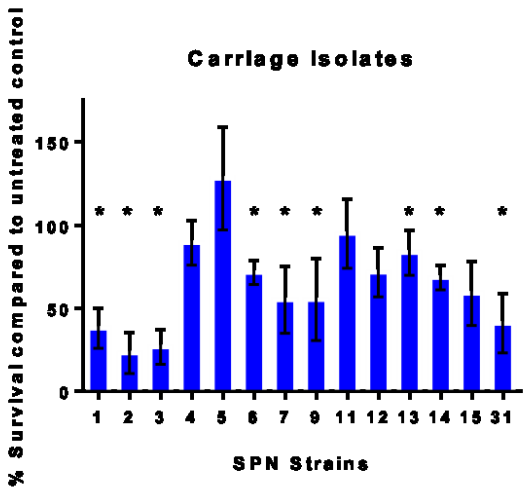

Figure 2.10 Sensitivity of all 14 carriage isolates to granule-mediated killing by human neutrophils

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all 14 carriage isolates. Asterisks (*) indicate strains significantly killed by degranulate.

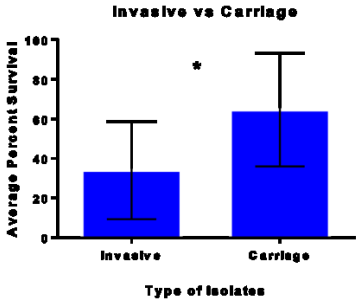

Figure 2.11 Average sensitivity of invasive isolates and carriage isolates

The average sensitivities of invasive isolates and carriage isolates were assessed with an unpaired two-tailed t test. Asterisk (*) indicates that a significant difference was found between the average sensitivities of the two types of isolates.

Discussion

This study was important because it was the first one to be conducted that quantitated the sensitivity of a panel of isolates to granule-mediated killing by human neutrophils. Two major conclusions were drawn from the results of this study. First, resistance to this form of killing is not exclusive only to strains originally isolated from invasive sites. Second, many isolates have acquired a resistance factor or overexpress a virulence factor leading to resistance to degranulate.

As stated earlier, invasiveness of isolates was the first facet of pneumococcal isolates that we wanted to investigate as possibly contributing to resistance. After testing the panel of 23 invasive isolates and finding only a few to display a highly resistant phenotype, we determined that invasive-site isolation was not indicative of resistance against neutrophil granules. This conclusion coupled with the outcome of a 35.41% average survival rate quickly undermined this first theory. Then, we discovered that more carriage isolates displayed the highly resistant phenotype and the average survival rate was 64.55% among carriage isolates, significantly higher than that of the invasive isolates. Therefore, we ruled out the idea that invasiveness was key to completely evading degradation by neutrophils during both colonization and during disease.

While invasiveness was clearly not directly associated with resistance, the presence of many highly resistant isolates led us to believe a factor must be involved. We hypothesized these resistant isolates may have evolved a defense mechanism or are overexpressing a virulence factor with an unknown function of protection from neutrophil granules.

Immediately, the capsule types of all of these isolates were investigated to determine whether the capsule type or another characteristic of the capsule may be at play. We utilized data provided by the CDC on the capsule types of all of the invasive isolates we used. Unfortunately,

we did not have this data on the carriage isolates. One interesting finding was that our most resistant invasive isolate ABC23 was of the capsule type 6A, the same capsule type Standish and Weiser saw significant killing by degranulate. On the other hand, we did not see significant killing and recovered 91% of the cells of this isolate after exposure to degranulate. In our panel of 23 invasive isolates, four capsule types were represented by multiple isolates. Capsule type 06C was represented by ABC6, ABC19, and ABC22. The average survival of these three isolates were strictly tight around 33%. However, we saw a few discrepancies in the other isolates that represented the same capsule types. ABC2, ABC15, and ABC18 represented capsule type 12F. The average percent survival of these were 23.4%, 35.6%, and 8.9%, respectively. These percent survivals varied substantially more than the 06C isolates. Likewise, two isolates of capsule type 19A varied greatly in their percent survival. ABC5 had a 40.7% survival rate, whereas ABC13 only had a 6.6% survival rate. Finally, two type 15B isolates, ABC14 and ABC20, had a small difference in their sensitivities to degranulate – 5.1% and 16.2% survival, respectively. This disparity was not as substantial as the ones among type 12F and 19A isolates, but it also wasn't as close as the 06C isolates. These differences led us to believe capsule type, alone, does not determine sensitivity to granule-mediated killing by neutrophils.

Another aspect of the capsule type that we investigated was whether resistance was profound among capsule types infamous for causing invasive disease. Of the capsule types notorious for invasive disease, especially in children, types 6A, 6B, and 19F were represented in our panel of invasive isolates. Again, our type 6A isolate, ABC23, was highly resistant. However, our type 6B isolate, ABC12, showed moderate sensitivity at 42.1%. Furthermore, our type 19F isolate, ABC17, displayed heightened susceptibility at 4.9% survival. This is further evidence that the capsule type does not factor extensively in the sensitivity to degranulate. We

did not, however, completely discount the capsule in involvement to susceptibility or resistance. Rather, we investigated if the presence of the capsule affected the sensitivity of pneumococci to granule killing.

CHAPTER III

ABSENCE OF PNEUMOCOCCAL CAPSULE MAY AFFECT SENSITIVITY TO GRANULE-MEDIATED KILLING BY NEUTROPHILS

Analysis of the invasive isolates revealed that the capsule type alone was not predictive of susceptibility to granules. However, this evidence required further investigation since the capsule's contribution to pneumococcal protection is multifactorial. For instance, the negative surface charge of most pneumococci plays an extensive role in the bacteria's abilities to evade host defenses including the also negatively charged mucus and neutrophils (Nelson et al., 2007; Li et al., 2013). Additionally, the absolute requirement for capsule as a sine qua non virulence factor has recently been called into question.

Non-encapsulated *Streptococcus pneumoniae* (NESp) isolates have been steadily increasing in the population; and they are being maintained in the population, even at low levels, despite lacking this key virulence factor (Ing et al., 2012; Park et al., 2014). These NESp isolates have occurred due to either mutated capsule polysaccharide synthesis (*cps*) genes leading to no capsule production (Hathaway et al., 2004), or other genes substituted in place of the *cps* genes (Keller et al., 2016). In the cases of the latter, three genes have so far been found to replace those of *cps* locus: *aliC*, *aliD*, and pneumococcal surface protein K (pspK). Three different null capsule clades (NCCs) divide this group of NESp isolates based on which gene(s) can be found at the *cps* locus. NCC1 isolates are $pspK^{+}$, but $aliC^{-}$ and $aliD^{-}$. NCC2 isolates are $aliC^{+}$ and $aliD^{+}$, but $pspK^{-}$. And NCC3 isolates are only $aliD^{+}$.

We investigated the granule-sensitivity of many unencapsulated isolates, including isolates of the three different NCCs, to determine if the absence of the capsule impacted resistance to granule-mediated killing, and if the specific gene in place of the *cps* locus determined susceptibility to neutrophil granules.

Methods

Bacterial Strains and Culture Conditions

Non-encapsulated *Streptococcus pneumoniae* isolates and their mutants were graciously gifted to us by Larry McDaniel at the University of Mississippi Medical Center. Table 3.1 provides information on all of the NESp isolates and their mutants. All NESp isolates were carriage isolates isolated from the nasopharynx of colonized individuals. Mutants of MNZ41 include an *aliC* and *aliD* double knockout mutant (JLB01) and *cbpAC* knockout (JLB10). Two mutants of MNZ67 were received – a *pspK* knockout (LEK05) and a *potD* knockout (PIP01). Finally, LEK08 is an *aliC* and *aliD* double knockout of MNZ85. Wildtype TIGR 4 and a capsule knock-out mutant of TIGR4 (T4R) were also used in this study and displayed in Table 3.1. Culture conditions were carried out in the same manner as with the ABC and SPN isolates. One interesting aspect of the NESp isolates to be noted was that flocculent growth was observed throughout cultures in C+Y. This type of growth differed from the uniform growth of the encapsulated isolates, and it most likely was an occurrence of the absence of the capsule. However, vigorous pipetting was used to disperse bacteria during growth phase. This helped speed up the exponential growth phase. Another distinctive aspect of NESp isolate growth was the small size of their colonies, compared to encapsulated isolates.

Table 3.1 Data on NESp isolates, their mutants, TIGR4, and its capsule knockout mutant T4R

Strain Name	Type of Strain	Site of Isolation (only for isolates)	Parental Strain and Gene Deleted (only for mutants)	Known Antibiotic Resistance
MNZ11	Isolate	Nasopharynx	N/A	None
MNZ41	Isolate	Nasopharynx	N/A	None
MNZ67	Isolate	Nasopharynx	N/A	None
MNZ85	Isolate	Nasopharynx	N/A	None
NCC1A	Isolate	Nasopharynx	N/A	None
NCC1B	Isolate	Nasopharynx	N/A	None
NCC1D	Isolate	Nasopharynx	N/A	None
NCC1E	Isolate	Nasopharynx	N/A	None
NCC1F	Isolate	Nasopharynx	N/A	None
NCC1G	Isolate	Nasopharynx	N/A	None
NCC1H	Isolate	Nasopharynx	N/A	None
NCC2A	Isolate	Nasopharynx	N/A	None
NCC2B	Isolate	Nasopharynx	N/A	None

Table 3.1 (continued)

Strain Name	Type of Strain	Site of Isolation (only for isolates)	Parental Strain and Gene Deleted (only for mutants)	Known Antibiotic Resistance
NCC2C	Isolate	Nasopharynx	N/A	None
NCC2D	Isolate	Nasopharynx	N/A	None
NCC2E	Isolate	Nasopharynx	N/A	None
NCC2F	Isolate	Nasopharynx	N/A	None
NCC3A	Isolate	Nasopharynx	N/A	None
NCC3B	Isolate	Nasopharynx	N/A	None
JLB01	Mutant	N/A	MNZ41; *aliC* and *aliD*	None
JLB10	Mutant	N/A	MNZ41; *cbpAC*	None
LEK05	Mutant	N/A	MNZ67; *pspK*	None
LEK08	Mutant	N/A	MNZ85; *aliC* and *aliD*	None
PIP01	Mutant	N/A	MNZ67; *potD*	None
TIGR4	Laboratory Strain	N/A	N/A	None
T4R	Mutant	N/A	TIGR4; *cps* genes	Chloramphenicol

Table 3.1 provides data on all 19 NESp isolates used, five of their mutants, TIGR4, and T4R. Data provided for each strain includes type of strain (isolate, mutant, or laboratory strain), the site of isolation (only for isolates), the parental strain and gene deleted only in mutants, and what antibiotics the strains have known resistance to.

Degranulate Microbicidal Assay

Killing assays were performed as described above for invasive and encapsulated carriage isolates (Chapter II). Again, CFU counts were quantitated to determine concentrations post-

exposure to degranulate and compared to untreated controls. Concentration data along with percent survival of each strain were graphed and analyzed using Prism software. Two-tailed t-tests were performed on control and exposed concentrations to determine if strains were significantly killed. An alpha value of 0.05 was deemed significant. The sensitivities of mutants of some of the NESp isolates were also tested and the differences in survivals compared to wildtypes assessed via a t-test. Additionally, a t-test was carried out on the survival of T4 compared to T4R to determine significance.

Results

First, the sensitivity of four NESp isolates to degranulate was assessed. Figure 3.1 displays the sensitivity of the four unencapsulated isolates, while the percent survival of the NESp isolates is shown in Figure 3.2. Three of the four isolates exhibited the highly resistant phenotype with MNZ85 displaying the highest resistance at 146.6% survival. One of these isolates, MNZ67 was significantly killed by degranulate with only 33.7% survival ($p = 0.0113$). The average percent survival of these four isolates was 109% while the median was 110.9%.

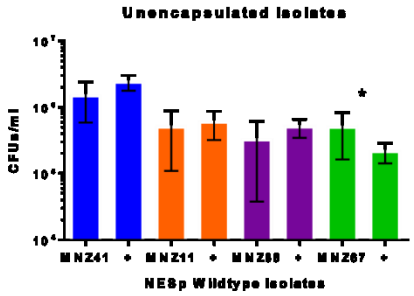

Figure 3.1 Sensitivity to granule-mediated killing of first four unencapsulated isolates

Roughly 10^6 pneumococci per ml were exposed to granules extracted from human neutrophils for one hour at 37ºC. Concentration counts were quantitated and compared to concentration counts of the same concentration of pneumococci, that were not exposed to granules. Data shown are the means ± standard deviation (SD) for control and experimental (+) counts of four unencapsulated isolates – MNZ41 (blue), MNZ11 (orange), MNZ85 (purple), and MNZ67 (green).

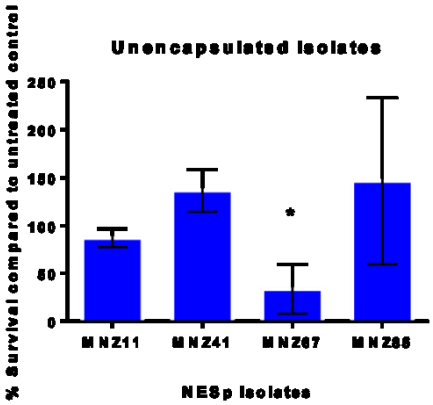

Figure 3.2 Sensitivity of the first 4 NESp isolates to granule-mediated killing

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of ten unencapsulated isolates. Asterisk (*) indicates the one strain significantly killed by degranulate.

After analyzing this data on the first four unencapsulated isolates and finding that 75% of them displayed the extremely resistant phenotype, it was theorized that the absence of the capsule may actually reduce pneumococcal susceptibility to granule-mediated killing by neutrophils. We speculated that without a capsule, these pneumococci may be upregulating or displaying surface proteins with the ability to combat the granules. However, we needed to test the sensitivity of a larger panel of NESp isolates and then compare the comprehensive data to the encapsulated isolates before this can be determined. In order to further characterize the sensitivity of NESp isolates, we were granted 15 NCC isolates from Larry McDaniel.

The 15 NCC isolates underwent exposure to degranulate via our microbicidal assay. Exposed concentrations were again compared to control unexposed concentrations to characterize percent survival. Percent survival of these 15 isolates is displayed in Figure 3.6. Figure 3.3 shows the concentration data of the first five NCC isolates. Only the most resistant of

these isolates NCC1D was not significantly killed by degranulate at 97.8% survival (p = 0.7728). NCC1A was the most sensitive of these isolates at 46.2% survival (p = 0.0021). The other three isolates displayed intermediate sensitivity – NCC1B at 53.5% (p = 0.0467), NCC1E at 73.6% survival (p = 0.0159), and NCC1F at 77.4% survival (p = 0.0036) – but were all significantly inhibited by granules.

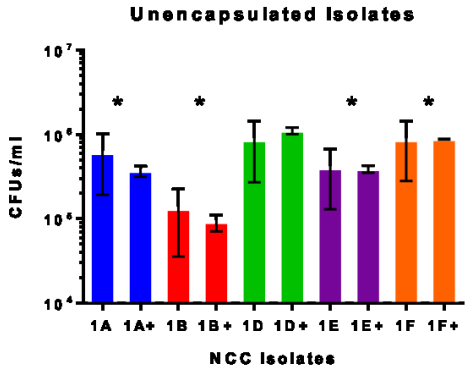

Figure 3.3 Sensitivity to granule-mediated killing of first five NCC isolates

Data shown are the means ± SD for control and experimental counts of the first five NCC isolates. Shown are NCC1A (blue), NCC1B (red), NCC1D (green), NCC1E (purple), and NCC1F (orange). Asterisks (*) indicate strains significantly killed by degranulate.

Five more NCC isolates were exposed to degranulate and their concentrations are indicated in Figure 3.4. NCC1H was the most sensitive of these isolates at 10.8% survival (p = 0.0009) while NCC2C was the most resistant at 55.6% survival, even though it was also significantly killed (p = 0.0277). NCC2B was also significantly killed at 21.2% survival (p = 0.0002).

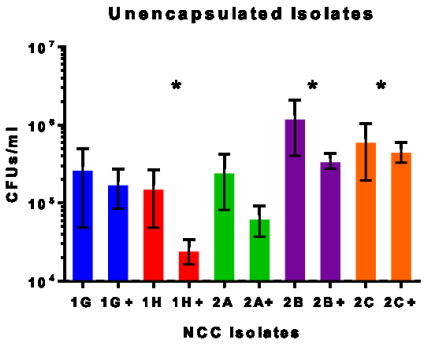

Figure 3.4 Sensitivity to granule-mediated killing of five more NCC isolates

Data shown are the means ± SD for control and experimental counts of five NCC isolates. Shown are NCC1G (blue), NCC1H (red), NCC2A (green), NCC2B (purple), and NCC2C (orange). Asterisks (*) indicate strains significantly killed by degranulate.

The final five NCC isolates and their concentration data from degranulate killing assays are shown in Figure 3.5. NCC2F was the most sensitive and the only isolate of this panel significantly killed by degranulate at 38.2% survival ($p = 0.0017$). NCC3B was the most resistant of these isolates at 93.7% survival.

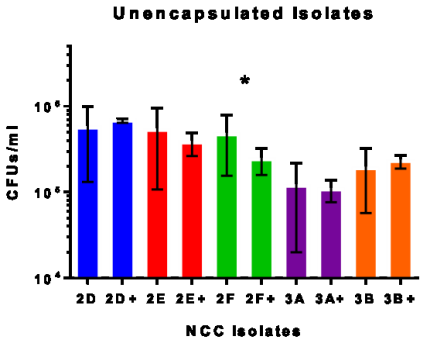

Figure 3.5 Sensitivity to granule-mediated killing of last five NCC isolates

Data shown are the means ± SD for control and experimental counts of five NCC isolates. Shown are NCC2D (blue), NCC2E (red), NCC2F (green), NCC3A (purple), and NCC3B (orange). Asterisk (*) indicates the strain significantly killed by degranulate.

The average percent survival among the NCC isolates was 55% and the median was 55.6% survival. Eight (53.3%) of the 15 NCC isolates were significantly killed by degranulate. NCC1D was the most resistant NCC isolate at 97.8% survival. NCC1H was the most susceptible NCC isolate at 10.8% survival. These data correlated more with the results from the encapsulated isolates than the first panel of NESp isolates.

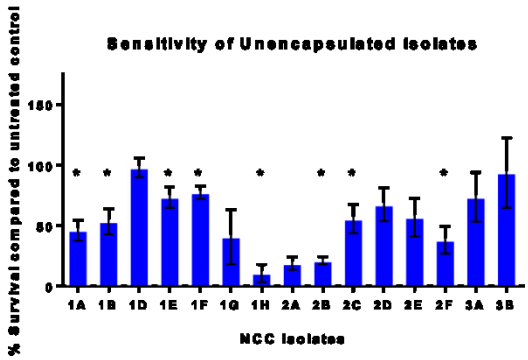

Figure 3.6 Percent survivals of all NCC isolates to granule-mediated killing by neutrophils

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all 15 NCC isolates. Asterisks (*) indicate strains significantly killed by degranulate.

After analyzing the NCC isolates, it was more apparent that the first panel of unencapsulated isolates were improbably more resistant than the rest of the isolates tested, given that it was a much smaller panel. The average of all of the unencapsulated isolates was calculated to be 64.7%, and the median was calculated to be 57%. The survival of all of the unencapsulated isolates was slightly higher than that of the carriage isolates but significantly higher than that of the combined survival of the invasive and carriage encapsulated isolates, which averaged only 46.4% survival ($p = 0.048$) (Figure 3.7). Collectively, nine (47.4%) of the 19 unencapsulated

isolates were significantly killed by neutrophil degranulate. However, this percentage of significantly killed NESp isolates was slightly lower than that of the percentage of significantly killed encapsulated isolates (51.35%).

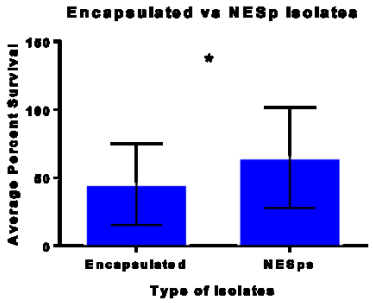

Figure 3.7 Average sensitivity of encapsulated isolates and unencapsulated isolates

The average sensitivities of encapsulated isolates and unencapsulated isolates were assessed with an unpaired two-tailed t test. Asterisk (*) indicates that a significant difference was found between the average sensitivities of the two types of isolates.

In addition to all of the unencapsulated isolates' sensitivities to the degranulate microbicidal assay, we investigated the killing of a wildtype pneumococcal strain TIGR4 and its capsule knock-out mutant T4R to access the effect the absence of the capsule has on degranulate sensitivity. The concentrations of these two strains post-exposure to degranulate and their untreated controls are shown in Figure 3.8, and the percent survivals are displayed in Figure 3.9. These concentrations indicated that TIGR4 was significantly killed with an average survival rate of 40.6% ($p < 0.0001$). T4R also exhibited significant susceptibility and an almost equal survival rate to TIGR4 at 40% ($p = 0.0006$). It was evident from the concentration data that there was no difference between survival of these two strains. This was confirmed by a t-test ($p = .9416$).

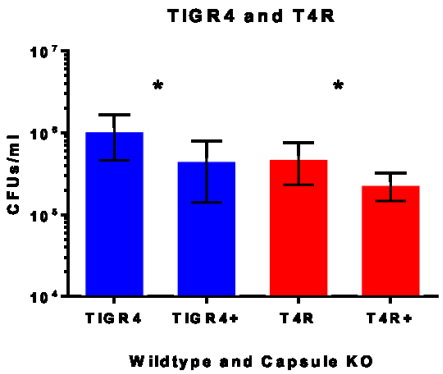

Figure 3.8 Sensitivity to granule-mediated killing of TIGR4 and T4R

Data shown are the means ± standard deviation (SD) for control and experimental counts of TIGR4 (blue) and its capsule knock-out mutant T4R (red). Asterisks (*) indicate strains significantly killed by degranulate.

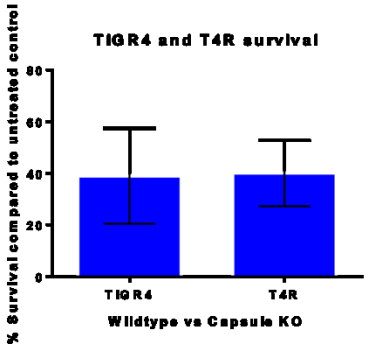

Figure 3.9 Percent survival of TIGR4 and T4R to granule-mediated killing by neutrophils

Percent survival of each strain was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all TIGR4 and T4R.

Next, we investigated the role of the genes in place of the *cps* locus and if any of them affected sensitivity of unencapsulated pneumococci to granule-mediated killing. The mutants of

NESp isolates were exposed to neutrophil degranulate, and their concentration data is shown in Figure 3.10. Most of these mutants were extremely resistant except for PIP10 which was significantly killed at 36% survival ($p = 0.0008$). JLB 10 was the most resistant mutant at 158.7% survival.

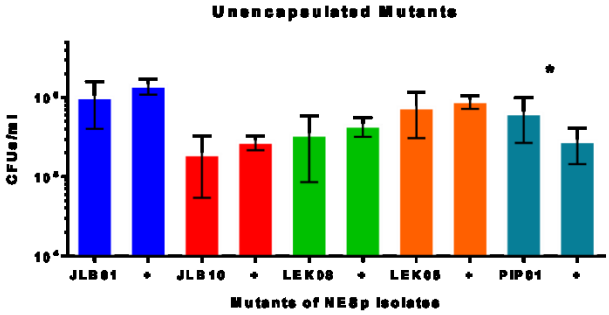

Figure 3.10 Sensitivity to granule-mediated killing of six mutants of unencapsulated isolates

Data shown are the means ± SD for control and experimental counts of six mutants of NESp isolates. Shown are JLB01 (blue), JLB10 (red), LEK08 (green), LEK05 (orange), and PIP01 (teal). Asterisk (*) indicates the strain significantly killed by degranulate.

Figure 3.11 displays the percent survival of all of these mutants to their wildtype NESp isolates. The only significant difference between wildtype and mutant survival was found between the wildtype MNZ67 and its *pspK* knockout LEK05 ($p = 0.0025$). LEK05 was found to be significantly more resistant than its wildtype. The survival of the *potD* knockout of MNZ67, on the other hand, was not found to be significantly different than the wildtype. The *aliC* and *aliD* double mutants of MNZ41 and MNZ85 were both found to be more sensitive than the wildtypes, but not significantly.

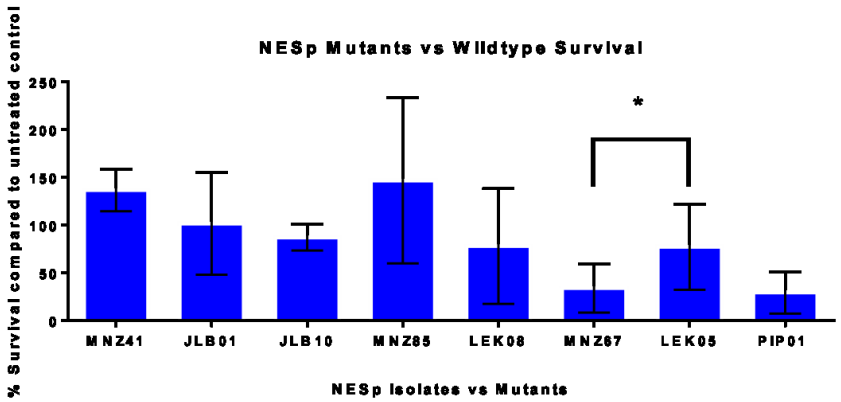

Figure 3.11 Percent survivals of all NESp wildtypes versus their mutants

Data shown are the means ± SD for the percent survival of four NESp isolates and their mutants. Asterisk (*) indicates the only significant difference in percent survivals of wildtype and mutant.

The factor of which gene was in place of the *cps* locus was also assessed in the NCC isolates. NCC1 isolates averaged a percent survival of 57.2%. NCC2 isolates averaged a survival of 43%. NCC3 isolates averaged a survival of 83.6%. While the two NCC3 isolates were more resistant than the other two types of isolates, a one-way ANOVA test determined that a significant difference was not found among the three different clades of isolates (Figure 3.12).

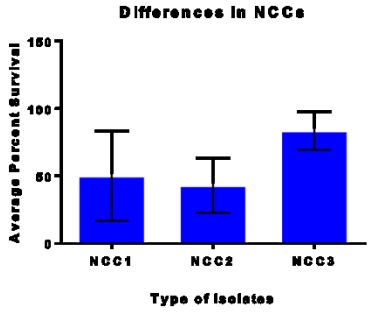

Figure 3.12 Average survival of NCC1, NCC2, and NCC3 isolates

The average sensitivities of all three clades of NCC isolates were assessed with one-way ANOVA. No significant difference was found among the three clades of isolates.

Discussion

This study was important as it further elaborates on the sensitivity of pneumococcal isolates to granule-mediated killing by human neutrophils. This is also the first study to investigate the sensitivity of a panel of unencapsulated isolates to granule-mediated killing. The conversation of the rising prevalence of NESp isolates is beginning to escalate as more NESp isolates are isolated. Keller et al. (2016) predicted that these increases can be attributed to the introduction of the pneumococcal conjugate vaccines, which have led to positive selection of unencapsulated pneumococci, and a rise in awareness and better techniques for typing collected samples. In the conversations of rising NESp isolate prevalence are multiple studies on the resistance of these isolates to antibiotics (Sa-Leao et al., 2008, Sánchez-Tatay et al., 2008). However, resistance of unencapsulated *Streptococcus pneumoniae* to granule-mediated inhibition by human neutrophils is not been assessed prior to our studies.

We concluded in this study that NESp isolates have a minor significant advantage over encapsulated isolates when it comes to resistance to this method of killing by neutrophils. Furthermore, focusing more on the null capsule clades, it appears there is not a major difference among the three NCC groups. The NCC1 (*pspK*$^+$) and NCC2 (*aliC*$^+$ and *aliD*$^+$) isolates varied greatly in their range of sensitivities to degranulate. Interestingly, both of our NCC3 (only *aliD*$^+$) isolates were of the highly resistant phenotype. Testing of more NCC3 isolates in future studies would better characterize if the substitution of only *aliD* at the *cps* locus has an effect on susceptibility. From our data on NCC1 and NCC2 isolates, it appears *pspK* and *aliC* do not have effects on sensitivity, alone. However, the combination of any of these three genes substituted at the *cps* locus could be further expanded on in future studies. Conflicting results were reported from data on the mutants of NESp isolates, though, compared to the NCC isolates. The *ΔpspK*

mutant was significantly more resistant than its wildtypes. This *pspK* data contradicts the data on NCC isolates. The only *pspK*$^{+}$ isolates (NCC1) were slightly more resistant than NCC2 isolates but more sensitive than NCC3 isolates. Again, testing of more NCC3 isolates could help establish the role of *pspK* in susceptibility to granule-mediated killing by neutrophils.

Data on the survival of TIGR4 and its capsule knock-out mutant T4R provide evidence that, at least for type 4, the capsule is not the sole factor involved in resistance to neutrophil granules. This information combined with the capsule type information elaborated on in Chapter II failed to strengthen an argument for capsule type having a significant impact on resistance of pneumococci to granule-mediated killing by human neutrophils. However, the minor significant difference between the survival of all NESp isolates and all encapsulated isolates provides evidence to the contrary. Even within strains of the same capsule type, individual strains could possess modifications to the capsule or differences in levels of capsule expression. As well, strains of the same null capsule clade could have variable expression of surface factors involved in resistance to granules. These are areas worthy of future investigation before capsule can be completely ruled out as a contributing factor to resistance.

The increased survival shown by NESp isolates compared to encapsulated isolates may provide evidence for a previously mentioned theory. We speculated that some pneumococcal isolates may be upregulating or better-displaying components on their cell-surfaces due to the differences in capsule type, amount, or presence. Therefore, investigation of known surface virulence factors, to possibly identify a surface protein with an uncharacterized function in protection from toxic neutrophil granules, is warranted.

CHAPTER IV

POTENTIAL CONTRIBUTION OF PNEUMOCOCCAL CELL WALL-ASSOCIATED PROTEASE HTRA AS A DEFENSE MECHANISM AGAINST NEUTROPHIL GRANULE-MEDIATED KILLING

Our data on the sensitivity of the invasive and colonization isolates to granule-mediated killing indicated that isolation of strains from invasive sites alone did not directly correlate to granule resistance and that some strains express a factor that contributes to resistance. Additionally, the capsule type alone does not appear to have a significant effect on susceptibility of *Streptococcus pneumoniae* to neutrophil granular inhibition. Therefore, our third objective was to identify a possible protective factor by reviewing the literature on already known virulence factors.

Cell wall-localized aminopeptidase N (PepN) was reported as a target receptor for neutrophil elastase (Nganje et al., 2019). By studying PepN in *Streptococcus thermophilus*, Chavagnat et al. (1999) estimated PepN to cleave small peptides from the N-terminus. More recently, though, *S. pneumoniae* PepN has been shown to inhibit production of IFNγ and TNF-α and release of cytolytic granules (Blevins et al., 2017). PepN belongs to the family of proteins called non-classical surface proteins (NCSPs). While PepN appears to be a sensitivity factor in this mechanism of neutrophil granule killing, we sought to determine whether other factors may also contribute to resistance.

Another protein in the NCSP family of proteins is a protease called HtrA (high-temperature requirement A). HtrA is a heat shock-induced, serine protease that was first defined in *E. coli*, in which it is known as DegP or DO protease (Strauch et al., 1989). DegP was found to be localized in the periplasm, where it functions in the degradation of misfolded proteins due to elevated temperatures (Strauch et al., 1989; Seol et al., 1991). While protease activity is the primary function of this protein at high temperatures, HtrA also functions as a chaperone protein at lower temperatures (Spiess et al., 1999). In pneumococcus, HtrA is localized to the cell wall (Sebert et al., 2002), and it primarily functions in the digestion of denatured proteins and competence stimulating peptide (CSP) (Sebert et al., 2005; Cassone et al., 2012). In the latter case, the proteolytic function is used to repress competence when the frequency of errors in the folding of proteins is low (Stevens et al., 2011). HtrA and its homologues are pretty conserved across bacterial species and mostly function in the responses to environmental stresses.

Because HtrA is vital to maintaining the normal metabolic functions of pneumococcus, it has been characterized as a potential virulence factor. Pneumococcal HtrA is required for full virulence in both pneumonia and bacteremia models, as D39 and TIGR4 wildtype strains were significantly more lethal than HtrA-defecient mutants (Ibrahim et al., 2004). Similarly, fitness of pneumococci during colonization appears to be dependent on HtrA as a *ΔhtrA* mutant showed decreased fitness (Sebert et al., 2002).

Given this information on the roles of HtrA as a virulence factor in response to environmental stresses, we theorized that HtrA may play a role in the response to neutrophil granule toxicity. If this is the case, the sensitivity of pneumococcal isolates may depend on the concentrations of the protease the bacteria secrete in order to counter neutrophil serine proteases. It has already been shown that HtrA helps some Gram-negative bacteria escape phagocytes

(Baumler et al., 1994; Elzer et al., 1996; Li el al., 1996). Therefore, this serine protease may be the key to pneumococcal survival against the human neutrophil granular defense.

We hypothesized that pneumococcal HtrA protects the bacteria from the neutrophil's granular proteases. To test this theory, we made HtrA knockouts of both laboratory strains and clinical isolates and tested the sensitivity of these strains to neutrophil granules. Additionally, we expressed recombinant HtrA in an *E. coli* expression strain and subjected TIGR4 to both degranulate and rHtrA. Finally, we performed quantitative real-time PCR on both highly resistant and sensitive isolates to assess the levels of HtrA expression.

Methods

Construction of *ΔhtrA* mutants

The HtrA gene was deleted from wildtype strains and clinical isolates by overlap extension PCR. In short, we amplified DNA flanking the *htrA* gene from the strains or isolates using HtrA forward and reverse primers and conducted SOE and filling PCR to replace the gene with an erythromycin (Erm) resistance gene cassette. Pneumococci were transformed with the SOEing PCR product using standard methods. Briefly, cultures were grown to 0.6 OD_{600} in competence media (C+Y supplemented with 0.4% BSA and 20% glucose) prior to being back diluted (1:50) into competence media plus competence stimulating peptide 2 (CSP2). These reactions incubated in a water bath at 37°C for 13 minutes before addition of the HtrA KO SOEing product (1µg). These reactions incubated a further two hours before being plated on 0.5 mg/ml Erm blood agar plates and grown overnight to select for *ΔhtrA* mutants. Transformants were screened with Erm forward and reverse primers and grown in C+Y to an OD of 0.5, then frozen in glycerol. Table 4.1 provides data on all of the primers used in this study. Table 4.2 provides data on the strains and mutants used in this study.

Table 4.1 Primers

Primer Name	Primer Sequence
ERM Forward	GGA AAT AAG ACT TAG AAG CAA AC
ERM Reverse	CCA AAT TTA CAA AAG CGA CTC
HtrA Forward	AAT TCA AAC ATA TGG AGG CAA A
HtrA Reverse	CTG TTG ATG AAG CAA TCT CTT TGT
HtrA 2	CCA TGA TTC TAC ACT AAC AC
HtrA 5	CAC CAT AAC TCA ACT AAC TCA AAA AAG
HtrA RT Forward	TCG GAA GAT GGA CAA GCT ATT T
HtrA RT Reverse	CCG ATA ACC TGC CCT TGA ATA
HtrA-KO 1	GAA GGC GAA TGC TCT ATC CA
HtrA-KO 2	GTT TGC TTC TAA GTC TTA TTT CCC ATA TTT GCC TCC ATA TG
HtrA-KO 3	GAG TCG CTT TTG TAA ATT TGG TTG ACA TCT ATG TAA AGA AAG C
HtrA-KO 4	AGC CTT ATT TCA GGC TGT TG
Gyrase Forward	ATG AAC TCT TGG CTC TGA TTG
Gyrase Reverse	CAA CTC TGT ACG GCG CTT AT

Table 4.1 provides data on all of the primers used in this study. The data includes the primer name and sequence.

Table 4.2 Data on strains and mutants used in HtrA knockout experiments

Strain Name	Type of Strain	Parental Strain and Gene Deleted (only for mutants)	Known Antibiotic Resistance
T4R	Laboratory Strain and Mutant	TIGR4; *cps* genes	Chloramphenicol
JAT97	Mutant	T4R; *htrA*	Chloramphenicol, Erythromycin
TIGR4	Laboratory Strain	N/A	None
JHJ1	Mutant	TIGR4; *htrA*	Erythromycin
JAT103	Mutant	TIGR4; *htrA*	Erythromycin
EF3030	Laboratory Strain	N/A	None
JHJ2	Mutant	EF3030; *htrA*	Erythromycin
ABC7	Isolate	N/A	None
JAT111	Mutant	ABC7; *htrA*	Erythromycin
ABC10	Isolate	N/A	None
JAT110	Mutant	ABC10; *htrA*	Erythromycin

Table 4.2 provides data on all of the laboratory strains, isolates, and mutants used in the HtrA knockout studies. Data provided on each strain includes the type of strain (laboratory strain, isolate, or mutant), the parental strain and gene deleted only for mutants, and what antibiotics the strain have known resistance to.

HtrA-deficient mutants and their wildtypes were tested for sensitivity to neutrophil degranulate. These sensitivities were compared via the t-test to assess if *ΔhtrA* mutants were significantly more susceptible to degranulate than the parental strains.

Construction of an *E. coli* expression strain and purification of HtrA

BL-21 *E. coli* cells were used to construct an HtrA expression strain. The cells were incubated with pHtrA plasmid for 15 minutes on ice, then heat-shocked in a water bath for one minute. 400 µl of LB broth were added to the cells. The cells were then incubated on ice for five minutes followed by 90 minutes of incubation in a shaking incubator at 37°C. Then, 100 µl of transformed cells was plated on LB agar supplemented with 100 µg/ml Ampicillin and incubated overnight at 37°C. Colonies were patched onto another LB Amp 100 plate for a master plate and were also screened via PCR using HtrA forward primer 5 and reverse primer 2. Two positive colonies were inoculated into LB broth supplemented with 100 µg/ml Ampicillin from the master plate and allowed to grow to an OD_{600} of 0.6. Freezer stock cultures were made from this culture and stored at -80°C. These stock cultures were labeled "HtrA100-1" and "HtrA100-2".

To express HtrA, HtrA100-1 or HtrA100-2 was inoculated from the freezer stock into ten ml of 100 µg/ml Ampicillin LB broth, grown over night in a shaking incubator, back-diluted into 250 ml of LB Amp 100 broth, allowed to grow to 0.6 OD_{600}, then induced with 1µM IPTG and allowed to grow overnight. Induced culture was pelleted in a Scorvall centrifuge at 5,000x g for five minutes. The supernatant was decanted, and the pellet was resuspended in 30 ml of 1x PBS for a wash – centrifuged at 5,000x g and supernatant decanted. The pellet was then resuspended in 15 ml of bacterial protein extraction reagent (B-PER). The culture was then sonicated twice before lysing with DNase and lysozyme. After rotating end-over-end at room temperature for 15

minutes to lyse, the culture was spun again, this time at 13,000 rpm for five minutes. Lysate was collected and stored at 4°C until use in the nickel affinity chromatography experiment to purify the rHtrA samples.

A volume of 3 ml of nickel resin was used in the chromatography to purify HtrA. The nickel was washed in ultra-pure water before being charged by 16 ml of 1x charge buffer. Then, 10 ml of B-PER was used to equilibrate the nickel. The sample was then run over the nickel and the flow through collected. Two washes with 1x 60mM imidazole wash buffer were run over the nickel column to wash contaminating proteins out of the nickel. Elution buffer (1x) was used to elute the protein, and half ml elutions were collected. SDS-PAGE was conducted on elutions, sample flow through, and wash flow through. The nickel column was stripped with 1x strip buffer before being regenerated for further use. A buffer exchange was conducted on elutions with clean protein to suspend recombinant HtrA in HtrA storage buffer (25mM Tris-HCl, 300mM NaCl, 10% Glycerol).

HtrA Protection Assay

This assay was done similar to the standard degranulate microbicidal assay described in Chapter II. 10^6 pneumococci/ml (checked by a zero-hour control) were incubated for one hour in RPMI alone or supplemented with 20 µls of degranulate, 20 µl of degranulate plus 20 µl of 8 mg/ml rHtrA (in HtrA storage buffer), degranulate plus 8 mg/ml BSA, rHtrA, or BSA. Reactions were diluted in 1x PBS and plated on 5% sheep's BAPs, which were incubated overnight in 5% CO_2 at 37°C. And colony counts were quantitated to assess the concentrations post-exposure to degranulate and level of protection from HtrA.

Quantitating levels of HtrA expression in resistant and sensitive isolates

To quantitate the levels of HtrA expression in isolates, RNA extractions and cDNA synthesis were performed. Isolates were streaked on BAPs and grown overnight. From the overnight cultures, C+Y broth cultures were inoculated and allowed to grow to 0.4 OD. Cultures were then centrifuged and the supernatant removed. Pellets were resuspended in 400 µl of 65°C hot acid phenol chloroform (HAPC). This reaction incubated at 65°C for five minutes before addition of 400 µl of 65°C NAES buffer. This reaction shook for one minute, incubated at 65°C for five minutes, then incubated on ice for two minutes. Then, the reactions were centrifuged for two minutes at 13,000 rpm. The top aqueous layer was removed and collected in a new tube and the HAPC and NAES treatment was repeated. After the second spin, the aqueous layers were transferred to 5 ml microcentrifuge tubes. A tenth volume of DEPC-treated 3 M NaAC was added to each tube followed by 2.5 volumes of 100% ethanol (EtOH). These reactions were allowed to precipitate at -80°C overnight.

The next day, reactions were thawed on ice and centrifuged at 15,000x g for 30 minutes. Pellets were washed in one ml of 70% EtOH. After a 10-minute spin at 15,000x g, EtOH was removed and the pellets air dried in a biosafety cabinet. Then, the RNA was isolated using the Qiagen RNeasy kit. Pellets were resuspended in 100 µl of RNase-free water before addition of 350 µl of RLT buffer plus beta mercaptoethanol (β-ME) and 250 µl of 100% EtOH. Samples were transferred to RNeasy mini spin columns in collection tubes and centrifuged at 10,000x g for 30 seconds. Then, 350 µl of RW1 buffer was added to the column, and the column was spun again. DNase I incubation mix (10 µl DNase stock solution plus 70 µl RDD buffer per sample) was prepared and all 80 µl was added to the columns, which were incubated at RT for 15

minutes. Then, 350 µl of RW1 buffer was again added to the columns, and the columns were centrifuged. Two washes were done with 500 µl of RPE buffer before the columns were moved to a 1.5 ml collection tube. 30 µl of RNase-free water was added to the tubes to elute the RNA, tubes were centrifuged, and the RNA samples were stored at -80°C overnight.

Samples were thawed on ice and the RNA quantitated by a Qubit Fluorometer 2.0. RNA broad range and DNA high sensitivity working solutions were prepared and distributed into Qubit assay tubes. RNA and DNA standard tubes were prepared by adding 10 µl of the respective standard to 190 µl of working solution. Assay tubes were prepared by adding 5 µl of RNA samples to 195 µl of either RNA or DNA working solution. Standards were quantitated prior to the assay tubes. Finally, assay tubes were quantitated, and a stock concentration was calculated by the fluorometer.

To reduce residual contaminating DNA, samples underwent further RNA cleanup and DNase treatment using the two-step rigorous DNase treatment protocol of the Ambion TURBO DNA-*free*™ kit by Invitrogen. A tenth volume of 10x TURBO DNase™ Buffer was added to RNA samples. Then, 1.5 µl of TURBO DNase™ enzyme was added to the samples, which incubated in a 37°C water bath for 30 minutes. Another 1.5 µl of TURBO DNase™ enzyme was added to each sample, and samples were incubated an additional 30 minutes. Next, 0.2 volumes of resuspended DNase Inactivation Reagent were added to each sample, which were then incubated at RT for 5 minutes. Finally, samples were centrifuged at 10,000x g for 90 seconds and most of the supernatants above the pellets was collected and transferred to new tubes. The new RNA samples were brought to a total volume of 100 µl with RNase-free water and the Qiagen RNeasy RNA cleanup protocol was again used to further purify the RNA. Samples were

again quantitated by the Qubit Fluorometer 2.0. Second readings usually indicated significantly less DNA.

cDNA synthesis using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR was performed on the RNA samples. For each sample, 1 µg of RNA template, 4 µl of 5x reaction mix, 2 µl of Maxima enzyme mix, and enough nuclease-free water to fill to 20 µl was mixed in a sterile PCR tube. A no-RT reaction was done as well using the RNA template of the strain with the most contaminating DNA in the RNA samples, and this sample underwent the same treatment as the others but with no Maxima enzyme mix. In a thermocycler, the samples were incubated at 25°C for 10 minutes followed by incubation at 50°C for 15 minutes. Reactions were terminated at 85°C for 5 minutes.

qPCR was performed on 50 ng of cDNA synthesized from cleaned RNA samples. Luminaris Color HiGreen High Rox SYBR Green was used to perform qPCR on the four samples targeting both HtrA and the housekeeping gene of Gyrase (*gyrA*). Each sample was run in triplicate and in 20 µl reactions in a 96-well plate. Negative controls consisted of no-RT samples as well as no template controls. Reactions targeting *gyrA* and *htrA* used 0.6 µl of RT forward and reverse primers. Holding stage was conducted at 95°C for 10 minutes. 40 cycles of cycling stage were conducted at 95°C for 15 seconds followed by 60°C for one minute. Melt curve stage was conducted once with the same parameters as cycling stage.

The delta cycle threshold (Δct) of at least two qPCR runs of three isolates was calculated by subtracting the mean ct of *gyrA* from the mean ct of *htrA*. The ΔΔct of two sensitive isolates was calculated by subtracting the absolute value of the highly resistance isolate Δct from the Δct

of the sensitive isolates. The fold change in expression of each sensitive isolate was calculated by taking the power of 2 to the -ΔΔct.

Results

Construction of *ΔhtrA* mutants and their sensitivities to degranulate killing

HtrA knockout mutants were constructed from an array of wildtype pneumococci and resistant pneumococcal isolates by overlap extension PCR followed by transformation of SOEing PCR products. Preliminary work involved the deletion of *htrA* from the unencapsulated lab strain T4R. JAT97 was the resulting mutant and the gel electrophoresis to confirm this knock out can be seen in Figure 4.1. Survival counts are shown in Figure 4.2 while percent survivals are displayed in Figure 4.3. When assessed in the standard microbicidal killing assay, T4R was significantly killed at 40.93% ($p = .0003$). JAT97 was also significantly killed at 9.9% ($p < 0.0001$). JAT97 was significantly more sensitive to neutrophil granules than its wildtype T4R ($p = 0.0372$).

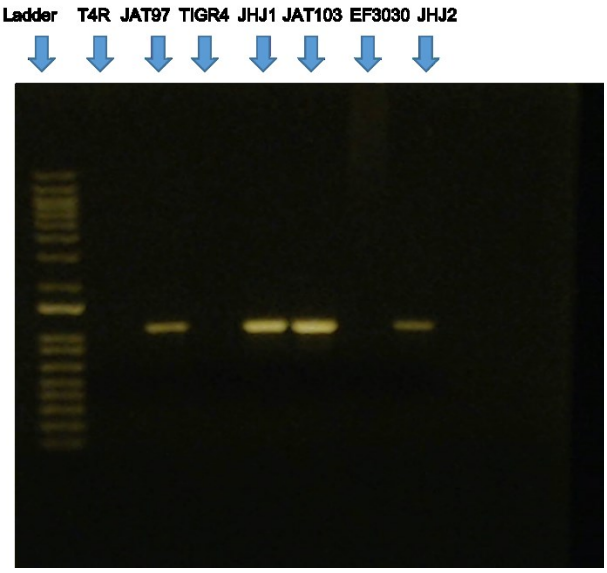

Figure 4.1 Electrophoresis gel of wildtype pneumococcal strains and their *htrA* knockouts

Ex Taq PCR screening was conducted on wildtype and mutant pneumococci using Erm forward and reverse primers. Bands indicate presence of Erm cassette in place of *htrA*.

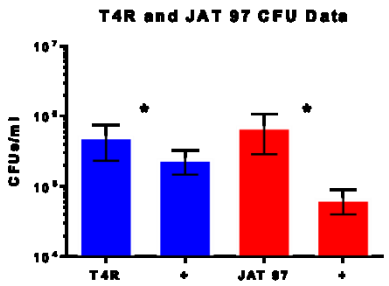

Figure 4.2 Survival of T4R and its *htrA*-deficient mutant JAT97 post-exposure to granules

Roughly 10^6 pneumococci per ml were exposed to granules extracted from human neutrophils for one hour at 37ºC. Data shown are the means ± standard deviation (SD) for control and experimental counts of T4R (blue) and JAT97 (red). Asterisks (*) indicate strains significantly killed by degranulate.

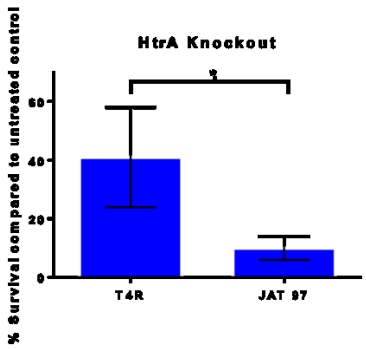

Figure 4.3 Percent survival of T4R and JAT97 to granule-mediated killing by neutrophils

Data shown are the means ± SD for the percent survival of T4R and JAT97. Asterisk (*) indicates that JAT97 was significantly more susceptible to degranulate killing than the wildtype T4R.

After the demonstration of increased susceptibility by the *ΔhtrA* mutant of T4R, we wanted to ensure that this significant change in survival due to HtrA was not exclusive to just one wildtype *Streptococcus pneumoniae* strain. Therefore, we deleted *htrA* from the chromosomes of two other wildtype strains, TIGR4 and EF3030. Two mutants of TIGR4 were created and were named JHJ1 and JAT103, and the gel electrophoresis screens to confirm the knockouts can be seen in Figure 4.1. Additionally, *htrA* was deleted from the genome of EF3030 to create JHJ2, also confirmed in Figure 4.1. Survival counts for these wildtype and knockout mutants are shown in Figure 4.4 while percent survivals are displayed in Figure 4.5. When assessed in the microbicidal killing assay compared to its *htrA*-deficient mutants, TIGR4 displayed a survival rate of 17.3% ($p < 0.0001$). However, neither of the TIGR4 mutants were killed like the wildtype. In fact, they were significantly more resistant than the wildtype. JHJ1 exhibited a survival rate of 97.8%, significantly more resistant than TIGR4 ($p = 0.0078$). JAT103 demonstrated an average survival of 119.84%, also significantly more resistant than the wildtype

TIGR4 (p = 0.0015). EF3030 was significantly killed at 59% survival (p = 0.029). JHJ2 was also significantly killed, displaying a survival rate of 3.57% (p = 0.0003). Interestingly, the EF3030 *ΔhtrA* mutant, JHJ2, was killed significantly more than the parental EF3030 (p = 0.0214), similar to results seen with JAT97 and indicating that HtrA may be protective in this strain.

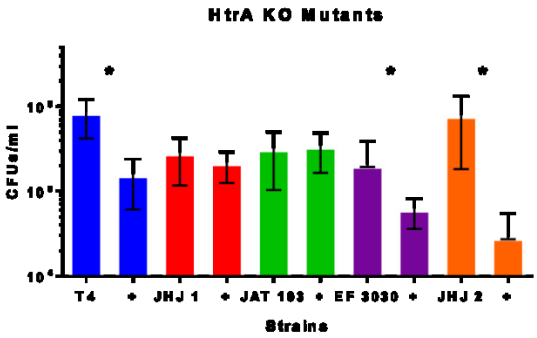

Figure 4.4 Survival of wildtype pneumococci and their *htrA*-deficient mutants to granules

Data shown are the means ± standard deviation (SD) for control and experimental counts of two wildtype pneumococcal strains and their *ΔhtrA* mutants. Shown are T4 (blue), JHJ1 (red), JAT103 (green), EF3030 (purple), and JHJ2 (orange). Asterisks (*) indicate strains significantly killed by degranulate.

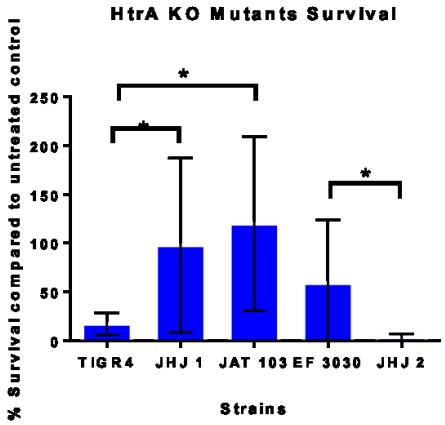

Figure 4.5 Sensitivity of wildtype pneumococci and their *htrA*-deficient mutants

Data shown are the means ± SD for the percent survival of TIGR4, JHJ1, JAT103, EF3030, and JHJ2. Asterisks (*) indicate significant differences in wildtype and mutant survivals.

The final study on sensitivities of wildtype versus *ΔhtrA* mutants to degranulate was done with two of the most resistant invasive isolates. A knockout of ABC7 was created and named JAT111, and a mutant of ABC10 was made and named JAT110. The confirmations of these knockouts can be found in Figure 4.6. The survival counts are shown in Figure 4.7 while the percent survivals are displayed in Figure 4.8. In this study, ABC7 exhibited an average survival of 112%. JAT111 showed a survival of 66.94%. JAT111 is significantly more sensitive to degranulate than ABC 7 ($p = 0.0408$). ABC10 demonstrated an average survival rate of 71.46%. JAT110 was significantly killed at 65.7% ($p = 0.0113$). However, the difference in percent survival between ABC10 and JAT110 was not significant ($p = 0.676$).

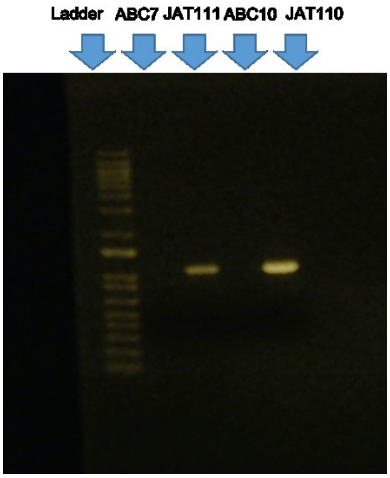

Figure 4.6 Electrophoresis gel of wildtype pneumococcal isolates and their *htrA* knockouts

Ex Taq PCR screening was conducted on wildtype and mutant pneumococci using Erm forward and reverse primers. Bands indicate presence of Erm cassette in place of *htrA*.

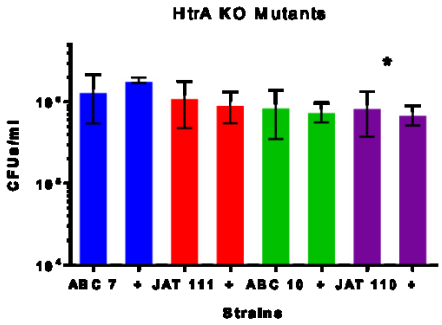

Figure 4.7 Concentrations of resistant invasive isolates and their *htrA*-deficient mutants post-exposure to degranulate

Data shown are the means ± standard deviation (SD) for control and experimental counts of two wildtype pneumococcal invasive isolates and their *ΔhtrA* mutants. Shown are ABC7 (blue), JAT111 (red), ABC10 (green), and JAT110 (purple). Asterisk (*) indicates the strain significantly killed by degranulate.

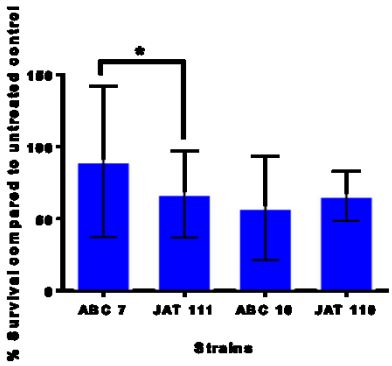

Figure 4.8 Sensitivity of wildtype invasive isolates and their *htrA*-deficient mutants

Data shown are the means ± SD for the percent survival of ABC7, JAT111, ABC10, and JAT110. Asterisk (*) indicates that a significant difference of survival was found between wildtype and mutant.

E. coli expression strains and purification of HtrA

The pHtrA plasmid previously cloned into *E. coli* (TOP-10) was screened with primers HtrA 2 and HtrA 5 to confirm the presence of the gene within the plasmid. The *htrA* gene was, in fact, present within the plasmid, and the plasmid was used to transform BL-21 cells. After transformation of the plasmid into *E. coli* (BL21), twelve colonies were selected and patched onto another LB plate, and we screened the first nine colonies patched for pHtrA. Figure 4.9 displays the gel electrophoresis of the screened transformants. The first two transformants were positive and were selected to grow and freeze for purification of HtrA. These two colonies were labelled “HtrA 100-1” and “HtrA 100-2”.

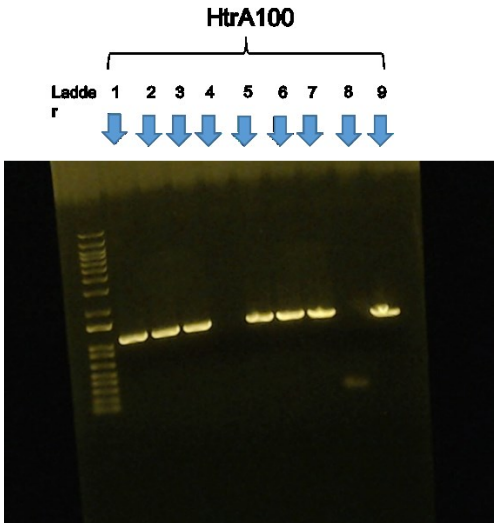

Figure 4.9 Gel electrophoresis of nine transformants from the transformation of pHtrA into BL-21 *E. coli*

Nine transformants were screened with HtrA primers 2 and 5 and ran on a .8% agarose gel. All transformants screened except for numbers 4 and 8 were positive for the HtrA plasmid. The first two were selected to grow for purification of recombinant HtrA.

HtrA Protection

To determine if HtrA contributed to protection against neutrophil granules, *S. pneumoniae* TIGR4 was exposed to degranulate in the presence or absence of rHtrA (8 mgs/ml). BSA was used as a protein control in this assay to ensure that the neutrophil granules were not simply overloaded with protein. The bacterial survival counts from this assay can be seen in Figure 4.10 and the percent survivals in Figure 4.11. A zero-hour control was plated on blood, and concentration counts revealed the correct concentration of bacteria was used. However, the one-hour TIGR4 control displayed a lower concentration. Following one-hour incubation with degranulate, TIGR4 CFUs decreased 97.9% ($p = 0.0311$). This was abrogated when TIGR4 was exposed to degranulate in the presence of recombinant HtrA, actually resulting in a significant

increase in bacterial growth of 1,048% ($p = 0.0438$) over TIGR4 incubated for one hour in the absence of degranulate. The survival of HtrA-protected TIGR4 was also significantly higher than that of unprotected TIGR4 exposed to granules ($p = 0.0004$). This effect did not seem to be HtrA-specific since addition of BSA also substantially protected TIGR4 against degranulate, though it did not result in the increase in growth seen with HtrA supplemented samples. TIGR4 was exposed to either HtrA or BSA alone to see if the proteins hindered TIGR4 growth, but no such hinderance was found (data not shown).

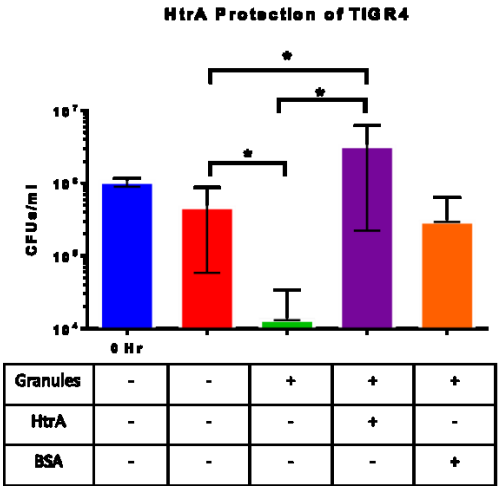

Figure 4.10 Survival of TIGR4 exposed to granules and rHtrA or BSA

10^6 pneumococci per ml were exposed to granules extracted from human neutrophils and either HtrA or BSA for one hour at 37ºC. Data shown are the means ± standard deviation (SD) for a zero-hour control (blue), a one-hour control (red), a degranulate-exposed (green), and degranulate-exposed and HtrA-protected (purple), and a degranulate-exposed and BSA-protected (orange) TIGR4. Asterisks (*) indicate significant differences in CFUs.

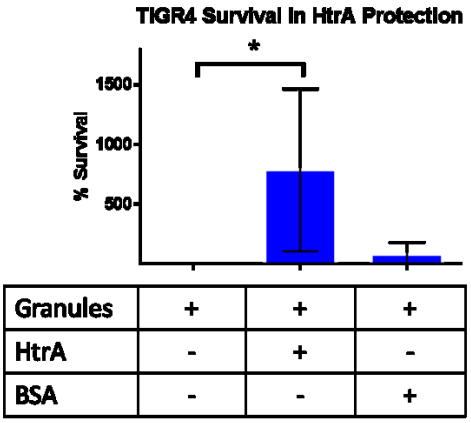

Figure 4.11 Percent survival of TIGR4 exposed to granules and HtrA or BSA

Data shown are the means ± SD for the percent survival of TIGR4 to degranulate and TIGR4 exposed to degranulate and HtrA or BSA. Asterisk (*) indicates a significant difference in survival.

HtrA Expression

To evaluate the possible role of HtrA in resistance of isolates, *htrA* expression was compared in two highly granule-susceptible isolates (ABC13 and ABC14) to expression in a highly resistant isolate (ABC7). The fold changes of the two sensitive isolates compared to ABC7 are shown in Figure 4.12. ABC13 displayed only 29.4% the level of *htrA* expression ABC7 did, while ABC14 displayed 57.2% the level of *htrA* expression as ABC7.

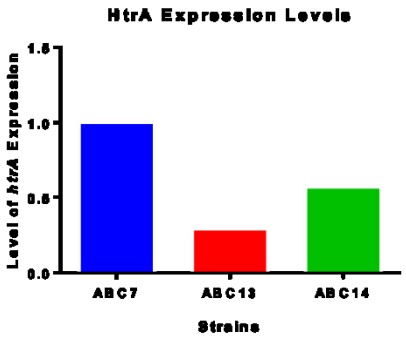

Figure 4.12 Expression of ABC13 and ABC14 compared to ABC7

qPCR was conducted on ABC7, ABC13, and ABC14 targeting *htrA*. ΔΔct was calculated for the isolates 13 and 14 compared to ABC7 using mean cts of multiple runs. Shown are expression levels of ABC13 (red) and ABC14 (green) related to ABC7 (blue).

Discussion

Much of our data indicates that HtrA may have a significant role in the resistance of *Streptococcus pneumoniae* to granule-mediated defense by human neutrophils. The evidence to this claim is found in the significant decrease in survival to degranulate by half of the *ΔhtrA* mutants compared to the wildtypes, the increase of TIGR4 survival when protected by recombinant HtrA purified from *E. coli*, and the lower expression levels of *htrA* in two extremely susceptible isolates compared to a highly resistant isolate.

The experiments involving the HtrA-deficient mutants were encouraging as half of the mutants were significantly more susceptible to degranulate than the wildtypes. Neither of the TIGR4 mutants were more sensitive than the wildtype. In fact, they were both significantly more resistant. On the other hand, the T4R mutant was significantly more susceptible than the wildtype. But T4R itself is an unencapsulated mutant of TIGR4. This is quite interesting as neither the loss of the capsule nor HtrA, alone, decreased the sensitivity of TIGR4. But the loss

of both the capsule and HtrA extensively decreased sensitivity. The significant decrease in survival when HtrA was deleted from EF3030 was promising, however, as it provided evidence that another pneumococcal strain benefited from HtrA expression in the presence of neutrophil granules. The testing of the two resistant invasive isolates and their mutants was also intriguing. ABC7's mutant was not significantly killed by degranulate compared to its control that was not exposed to degranulate. But the survival of JAT111 was significantly lower than that of its wildtype. On the other hand, ABC10's mutant was significantly killed by degranulate compared to its untreated control; but, the difference in survivals between JAT110 and ABC10 was not significant. This provided conflicting results when taken as a whole and indicates strain-specific contribution. ABC7 did appear to be much more resistant in the three trials with its mutant compared to its testing with the panel of invasive isolates. Conversely, ABC10 appeared to be quite more sensitive. These studies should be improved on in the future where more isolates of different rates of susceptibility are tested, and tighter standard deviations are observed.

The HtrA protection data on TIGR4 is promising in that we see such substantial protection. However, there are a few drawbacks to this assay. First, the purification of the protein was not very clean as there were contaminating proteins in the elutions. While HtrA is the major protein found in the elutions, it is concerning to see the presence of other proteins. Without a clean purification of HtrA, one could speculate that protection may also be due in part by the other proteins, or simply, the presence of other proteins distracts the proteases of the neutrophil granules from targeting the bacteria. The latter case is something we speculated might be seen when supplementing HtrA into the protection reactions. This is why we also ran protection reactions with BSA. It was concerning to see such protection by the BSA. However, TIGR4

survival from this protection was not as substantial as the protection seen by HtrA, nor was it significant compared to degranulate alone exposed TIGR4 like the HtrA-protected TIGR4.

Another drawback in this assay was the fact that TIGR4 did not acclimate well in the RPMI medium used for killing and protection assays. This was evident in the difference between the zero-hour and one-hour controls. However, this difference between zero-hour controls and one-hour controls was also seen across many of the wildtype strains and isolates tested and resulted in a decrease in CFU after one-hour incubation. This medium was used, though, as it was the best medium for extracting the azurophilic granules from the human neutrophils. Besides, the medium didn't seem to be a major factor as such a wide range of sensitivity was observed across all of the strains tested, even with an incubation of only one hour.

To investigate if the granule-mediated killing or HtrA protection was time-sensitive, we did run two-hour and three-hour reactions in the protection assay (data not shown). However, controls and exposed TIGR4 were decreasing to nearly none by hour 2, and HtrA-protected TIGR4 remained in the 10^6 to 10^7 CFUs/ml range. We could not gather reliable data on the survival if even the controls were beginning to die nearly 100% after multiple hours. Therefore, the one-hour reactions were considered sufficient for the experiment.

We are currently working on expressing and purifying clean HtrA. Small-scale and large-scale inductions on new clones are being performed to clarify the presence of the protein in *E. coli* lysates. If data from these experiments does not show tremendous levels of HtrA expression, a new clone with a different signal sequence may need to be used – possibly using a *S. aureus* vector rather than *E. coli*.

Lower levels of *htrA* expression in two extremely sensitive isolates compared to a highly resistant isolate complemented the HtrA knockout data and HtrA protection results. However, a

small sample size limits this analysis. An improvement to this analysis is currently underway. The ΔΔcts of the two sensitive isolates, the resistant isolate used in this study, and another resistant isolate will be calculated by comparing the cts of all of these isolates to that of a control TIGR4 ct to garner a better baseline comparison. Significantly higher levels of expression compared to TIGR4 in both resistant isolates than sensitive isolates would provide much better evidence in the genetic role of *htrA* in pneumococcal resistance to granule-mediated killing by human neutrophils.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

With increasing rates of antibiotic resistance among pneumococcal isolates, alternative treatments must be explored. Likewise, the ability of pneumococci to switch capsule serotypes calls for the investigation of new vaccines with conserved protein targets. In fact, it has been shown that the human antibody response to invasive pneumococcal disease is naturally dependent on protein antigens rather than capsular antigens (Wilson et al., 2017). Therefore, our lab aims to identify novel components of pneumococcal infection and to develop innovative approaches to treating and bolstering immunity to this significant pathogen. The central hypothesis of the research presented in this thesis was that some pneumococci have developed a natural resistance to granule-mediated killing by neutrophils.

We concluded from the testing of sensitivity of a large panel of isolates that many strains have developed the highly resistant phenotype and can survive neutrophil proteases. This phenotype is not exclusive to isolates isolated from invasive sites. In fact, more isolates isolated in the carriage state in the nasopharynx possessed the resistant phenotype. This finding may be more reasonable given that the bacteria are much more easily transmissible during colonization than during invasion. Moreover, the neutrophil defense has already begun during pneumococcal colonization. We speculate that is for these reasons, the carriage isolates displayed a significantly more resistant average survival than the invasive isolates.

The capsule type alone was disregarded as having a substantial role in resistance as many isolates of the same capsule type displayed highly varying sensitivity rates. As well, the isolates representing the common capsule types seen in invasion and in the conjugate vaccines also displayed varying sensitivity. The absence of the capsule, however, was theorized to possibly give pneumococci an advantage to the neutrophil's granule defense as the first panel of NESp isolates were mostly of the highly resistant phenotype. Testing of a larger panel showed many NESp isolates to also possess the susceptible phenotype. Still, the average sensitivity rate among NESp isolates was significantly higher than that of the encapsulated isolates (carriage and invasive isolates from the first study). On the other hand, the capsule knockout lab strain T4R did not show a different sensitivity to its wildtype TIGR4. We concluded from this data that the absence of the capsule may affect the sensitivity of pneumococci to granule-mediated killing by neutrophils. More capsule knockout mutants of different capsule types need to be constructed and tested to fully assess the role the presence of the capsule plays in sensitivity.

As discussed in Chapter III, the unencapsulated isolates possess different genes in substitution for the capsule locus. However, data on the survival of strains that represent the three different null capsule clades indicate that these differences are not significant. As well, the survival of most of the knockout mutants of NESp isolates were not considerably different than their wildtypes. Only a *pspK* knockout was significantly more resistant than its wildtype. Furthermore, the survival of *pspK*$^-$ NCC isolates were not significantly different than the *pspK*$^+$ isolates. Again, more isolates representing the NCC3 clade need to be tested along with further investigation into the *pspK* gene and its role in survival to fully understand how unencapsulated pneumococci are possibly more resistant than encapsulated pneumococci.

The data on the unencapsulated isolates indicates that resistance to neutrophil granules is a multifactorial result. Because we saw significantly more resistance in the NESp isolates than the encapsulated isolates, we theorized that NESp isolates acquired or overexpress another factor that may contribute to resistance without their capsules. We thought this factor must be a secreted factor or a cell-surface factor for it to provide resistance to granules extracellularly. We hypothesized that the protease high temperature requirement A (HtrA) may be the non-capsule factor contributing to resistance as it has been shown to be vital to pneumococcal virulence in response to external stresses. We found that in all but one parental strain, *htrA* knockouts were more susceptible to neutrophil granules than their wildtypes.

We also found that recombinant HtrA protected TIGR4 from exposure to granules. However, this study was concerning for a few reasons. The HtrA eluted from *E. coli* expression strain lysates was not pure – it was contaminated by other proteins – nor was it concentrated. The BSA assay to measure the concentration of protein could not differentiate the amount of HtrA from the contaminating proteins. Therefore, the protection seen by our rHtrA may not be trustworthy. Additionally, protection was seen by exogenous protein control BSA. At the same concentration of BSA protein as the unclean HtrA, protection was not significant like protection from HtrA nor was it as substantial. This could mean that we were simply overloading the granules' proteases with proteins and distracting them from targeting the cells. Another artifact to this study was that the HtrA protease may have been dividing chains of pneumococci. This separation of cells, which would lead to more CFUs on the blood agar plates, could actually be the reason we saw such a significant increase of "survival" seen from HtrA-protected TIGR4 compared to control unexposed TIGR4. This study most certainly is being improved upon to better elucidate the physical role of HtrA in resistance to neutrophil granules. When clean rHtrA

samples are able to be used, another aspect of this study to be examined would be to pretreat the HtrA with protease inhibitors before applying them to protect bacteria from the granules. Abrogating protection via this method would imply that protease activity is needed by the bacteria to combat the proteolytic activity of the neutrophil granules.

To complement data on the sensitivities of the *htrA* knockout mutants, the expression levels of *htrA* were compared in sensitive isolates to a highly resistant isolate via qPCR. The results of this study indicated that the level of the resistant isolate's *htrA* expression was over three times and nearly twice the expression levels of the two granule-susceptible isolates. This study used a small sample size, and we plan on expanding the study to compare multiple isolates of both phenotypes to a control expression in TIGR4. As it stands, however, this study provides additional evidence to the substantial genetic role htrA has in resistance to granule-mediated killing.

Understanding how *Streptococcus pneumoniae* evades the innate defenses of the human immune system has already made enormous advances in modern medicine. However, morbidity and mortality from pneumococcal disease continues to remain a major problem worldwide. Therefore, further research to develop better strategies to combat this deadly pathogen is vital. Our research has better illustrated the rate of sensitivity to granule-mediated killing by neutrophils seen across pneumococcal isolates. We have also identified two factors of a multifactorial system used by a complex organism that may contribute to the resistance of this organism to our most abundant innate immune cell. Developing strategies to abrogate this resistance would enhance innate immunity to this important pathogen. Optimistically, any approaches to abrogating pneumococcal resistance may benefit the combat to other important human pathogens.

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APPENDIX A

TABLES

Table A.1 Data on the invasive (ABC) isolates

Isolate #	Site of Isolation	Patient Age (years)	Patient Survived?	Serotype	Known Antibiotic Resistance
1	Blood	10	Y	23B	None
2	Blood	48	N	12F	None
3	Blood	5	Y	23A	None
4	Blood	4	Y	15A	Tetracycline, Erythromycin
5	Blood	17	Y	19A	Tetracycline
6	Blood	6	Y	06C	None
7	Blood	10	Y	16F	None
8	Blood	37	Y	35B	None
9	Blood	3	Y	06C	Erythromycin
10	Blood	27	Y	18C	None
11	Blood	12	Y	23F	None
12	CSF	25	Y	06B	None
13	Blood	10	Y	19A	None
14	Blood	8	Y	15B	None
15	Blood	37	Y	12F	None
16	Blood	25	N	15C	None
17	Blood	61	Y	19F	None
18	Blood	14	Y	12F	None
19	Blood	37	Y	06C	None
20	Blood	1	Y	15B	None
21	Blood	33	Y	3	None
22	Blood	6	Y	06C	None
23	Blood	9	N	06A	Erythromycin

Table 2.1 provides known data on all 23 of the invasive (ABC) isolates used provided by the CDC. Data provided includes the tissue the isolates were extracted from (blood or cerebral spinal fluid), the age of the patients the isolates were extracted from (in years), if the patients survived (yes or no), the capsular serotype of the isolates, and what antibiotics the isolates have known resistance to.

Table A.2 Data on the carriage (SPN) isolates

Isolate #	Site of Isolation	Known Antibiotic Resistance
1	Nasopharynx	Erythromycin
2	Nasopharynx	None
3	Nasopharynx	Streptomycin
4	Nasopharynx	Tetracycline, Erythromycin
5	Nasopharynx	Tetracycline
6	Nasopharynx	None
7	Nasopharynx	None
9	Nasopharynx	None
11	Nasopharynx	None
12	Nasopharynx	None
13	Nasopharynx	None
14	Nasopharynx	Erythromycin
15	Nasopharynx	None
31	Nasopharynx	None

Table 2.2 provides known data on all 14 carriage (SPN) isolates used. Data provided includes the tissue the isolates were extracted from and what antibiotics the isolates have known resistance to.

Table A.3 Data on NESp isolates, their mutants, TIGR4, and its capsule knockout mutant T4R

Strain Name	Type of Strain	Site of Isolation (only for isolates)	Parental Strain and Gene Deleted (only for mutants)	Known Antibiotic Resistance
MNZ11	Isolate	Nasopharynx	N/A	None
MNZ41	Isolate	Nasopharynx	N/A	None
MNZ67	Isolate	Nasopharynx	N/A	None
MNZ85	Isolate	Nasopharynx	N/A	None
NCC1A	Isolate	Nasopharynx	N/A	None
NCC1B	Isolate	Nasopharynx	N/A	None
NCC1D	Isolate	Nasopharynx	N/A	None
NCC1E	Isolate	Nasopharynx	N/A	None
NCC1F	Isolate	Nasopharynx	N/A	None
NCC1G	Isolate	Nasopharynx	N/A	None
NCC1H	Isolate	Nasopharynx	N/A	None
NCC2A	Isolate	Nasopharynx	N/A	None
NCC2B	Isolate	Nasopharynx	N/A	None

Table A.3 (continued)

Strain Name	Type of Strain	Site of Isolation (only for isolates)	Parental Strain and Gene Deleted (only for mutants)	Known Antibiotic Resistance
NCC2C	Isolate	Nasopharynx	N/A	None
NCC2D	Isolate	Nasopharynx	N/A	None
NCC2E	Isolate	Nasopharynx	N/A	None
NCC2F	Isolate	Nasopharynx	N/A	None
NCC3A	Isolate	Nasopharynx	N/A	None
NCC3B	Isolate	Nasopharynx	N/A	None
JLB01	Mutant	N/A	MNZ41; *aliC* and *aliD*	None
JLB10	Mutant	N/A	MNZ41; *cbpAC*	None
LEK05	Mutant	N/A	MNZ67; *pspK*	None
LEK08	Mutant	N/A	MNZ85; *aliC* and *aliD*	None
PIP01	Mutant	N/A	MNZ67; *potD*	None
TIGR4	Laboratory Strain	N/A	N/A	None
T4R	Mutant	N/A	TIGR4; *cps* genes	Chloramphenicol

Table 3.1 provides data on all 19 NESp isolates used, five of their mutants, TIGR4, and T4R. Data provided for each strain includes type of strain (isolate, mutant, or laboratory strain), the site of isolation (only for isolates), the parental strain and gene deleted only in mutants, and what antibiotics the strains have known resistance to.

Table A.4 Primers

Primer Name	Primer Sequence
ERM Forward	GGA AAT AAG ACT TAG AAG CAA AC
ERM Reverse	CCA AAT TTA CAA AAG CGA CTC
HtrA Forward	AAT TCA AAC ATA TGG AGG CAA A
HtrA Reverse	CTG TTG ATG AAG CAA TCT CTT TGT
HtrA 2	CCA TGA TTC TAC ACT AAC AC
HtrA 5	CAC CAT AAC TCA ACT AAC TCA AAA AAG
HtrA RT Forward	TCG GAA GAT GGA CAA GCT ATT T
HtrA RT Reverse	CCG ATA ACC TGC CCT TGA ATA
HtrA-KO 1	GAA GGC GAA TGC TCT ATC CA
HtrA-KO 2	GTT TGC TTC TAA GTC TTA TTT CCC ATA TTT GCC TCC ATA TG
HtrA-KO 3	GAG TCG CTT TTG TAA ATT TGG TTG ACA TCT ATG TAA AGA AAG C
HtrA-KO 4	AGC CTT ATT TCA GGC TGT TG
Gyrase Forward	ATG AAC TCT TGG CTC TGA TTG
Gyrase Reverse	CAA CTC TGT ACG GCG CTT AT

Table 4.1 provides data on all of the primers used in this study. The data includes the primer name and sequence.

Table A.5 Data on strains and mutants used in HtrA knockout experiments

Strain Name	Type of Strain	Parental Strain and Gene Deleted (only for mutants)	Known Antibiotic Resistance
T4R	Laboratory Strain and Mutant	TIGR4; *cps* genes	Chloramphenicol
JAT97	Mutant	T4R; *htrA*	Chloramphenicol, Erythromycin
TIGR4	Laboratory Strain	N/A	None
JHJ1	Mutant	TIGR4; *htrA*	Erythromycin
JAT103	Mutant	TIGR4; *htrA*	Erythromycin
EF3030	Laboratory Strain	N/A	None
JHJ2	Mutant	EF3030; *htrA*	Erythromycin
ABC7	Isolate	N/A	None
JAT111	Mutant	ABC7; *htrA*	Erythromycin
ABC10	Isolate	N/A	None
JAT110	Mutant	ABC10; *htrA*	Erythromycin

Table 4.2 provides data on all of the laboratory strains, isolates, and mutants used in the HtrA knockout studies. Data provided on each strain includes the type of strain (laboratory strain, isolate, or mutant), the parental strain and gene deleted only for mutants, and what antibiotics the strain have known resistance to.

APPENDIX B

FIGURES

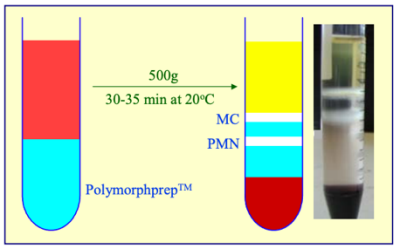

Figure B.1 Purification of PMNs using Polymorphprep™

MC = mononuclear cells; PMNs = polymorphonuclear leukocytes. This illustration shows the result of centrifuging blood over Polymorphprep solution. Picture on right shows an actual separation in a 15 ml conical tube (taken from Polymorphprep™ Application Sheet).

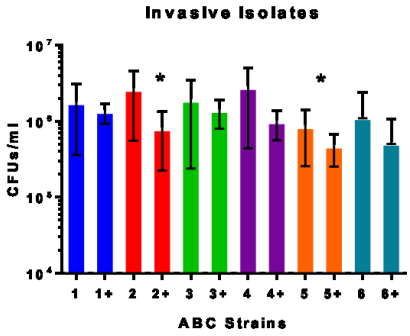

Figure B.2 Sensitivity to granule-mediated killing of first six invasive isolates

Roughly 10^6 pneumococci per mL were exposed to granules extracted from human neutrophils for one hour at 37ºC. Concentration counts were quantitated and compared to concentration counts of the same concentration of pneumococci, that were not exposed to granules. Data shown are the means ± standard deviation (SD) for control and experimental counts of six invasive isolates – ABC1 (blue), ABC2 (red), ABC3 (green), ABC4 (purple), ABC5 (orange), and ABC6 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

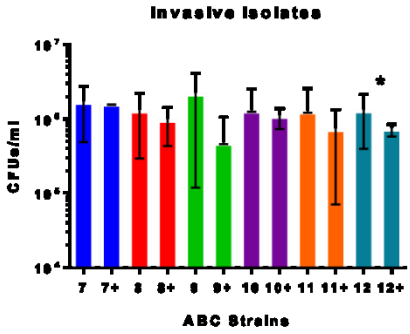

Figure B.3 Sensitivity to granule-mediated killing of ABC 7-12 isolates

Data shown are the means ± SD for control and experimental counts of six invasive isolates. Shown are ABC7 (blue), ABC8 (red), ABC9 (green), ABC10 (purple), ABC11 (orange), and ABC12 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

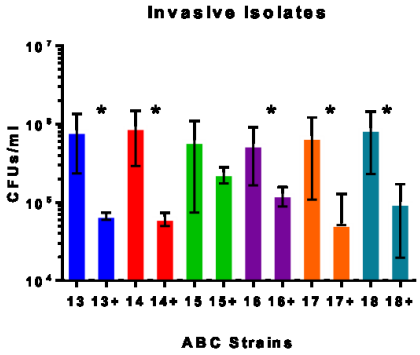

Figure B.4 Sensitivity to granule-mediated killing of ABC 13-18 isolates

Data shown are the means ± SD for control and experimental counts of six invasive isolates. Shown are ABC13 (blue), ABC14 (red), ABC15 (green), ABC16 (purple), ABC17 (orange), and ABC18 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

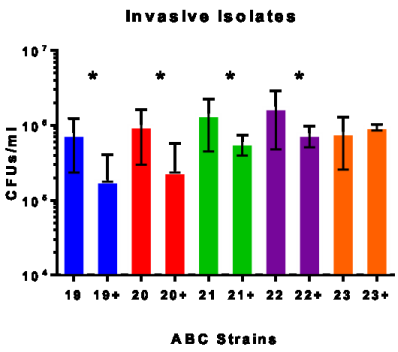

Figure B.5 Sensitivity to granule-mediated killing of final five invasive isolates

Data shown are the means ± SD for control and experimental counts of the last five invasive isolates. Shown are ABC19 (blue), ABC20 (red), ABC21 (green), ABC22 (purple), and ABC23 (orange). Asterisks (*) indicate strains significantly killed by degranulate.

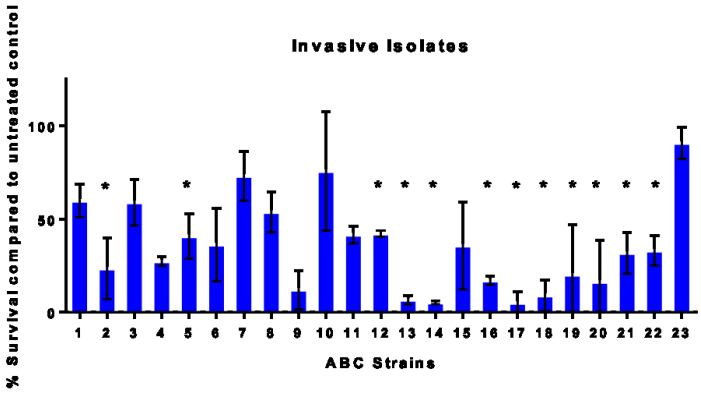

Figure B.6 Sensitivity of all 23 invasive isolates to degranulate killing by human neutrophils.

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all 23 invasive isolates. Asterisks (*) indicate strains significantly killed by degranulate.

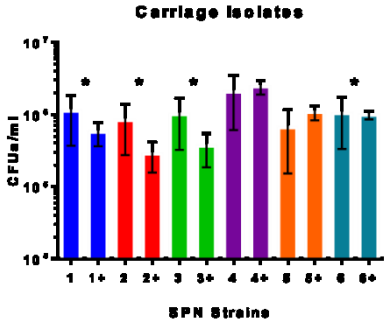

Figure B.7 Sensitivity to granule-mediated killing of first six carriage isolates

Data shown are the means ± SD for control and experimental counts of six carriage isolates – SPN1 (blue), SPN2 (red), SPN3 (green), SPN4 (purple), SPN5 (orange), and SPN6 (teal). Asterisks (*) indicate strains significantly killed by degranulate.

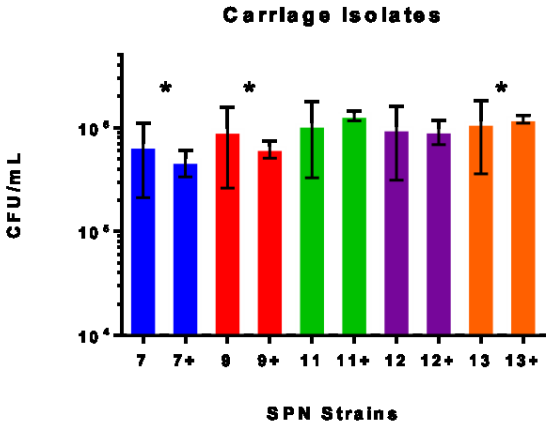

Figure B.8 Sensitivity to granule-mediated killing of five carriage isolates

Data shown are the means ± SD for control and experimental counts of five carriage isolates. Shown are SPN7 (blue), SPN9 (red), SPN11 (green), SPN12 (purple), and SPN13 (orange). Asterisks (*) indicate strains significantly killed by degranulate.

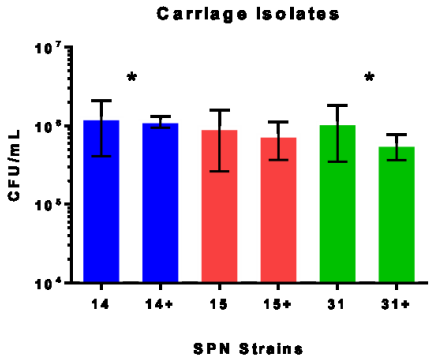

Figure B.9 Sensitivity to granule-mediated killing of final three carriage isolates

Data shown are the means ± SD for control and experimental counts of three carriage isolates. Shown are SPN14 (blue), SPN15 (red), and SPN31 (green). Asterisks (*) indicate strains significantly killed by degranulate.

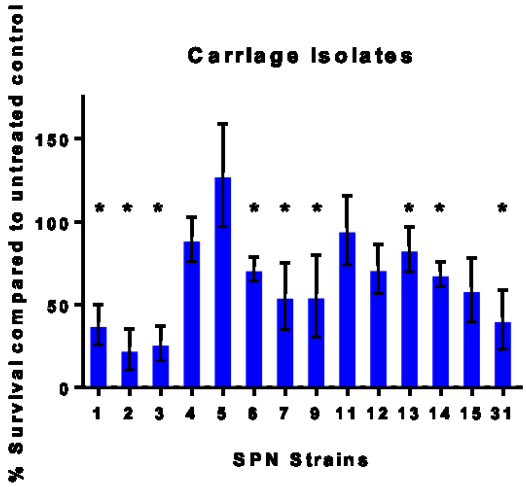

Figure B.10 Sensitivity of all 14 carriage isolates to granule-mediated killing by human neutrophils

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all 14 carriage isolates. Asterisks (*) indicate strains significantly killed by degranulate.

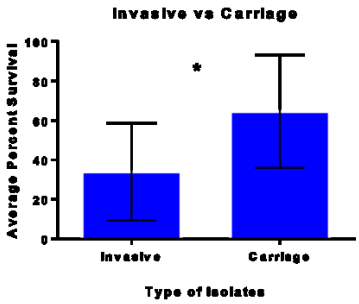

Figure B.11 Average sensitivity of invasive isolates and carriage isolates

The average sensitivities of invasive isolates and carriage isolates were assessed with an unpaired two-tailed t test. Asterisk (*) indicates that a significant difference was found between the average sensitivities of the two types of isolates.

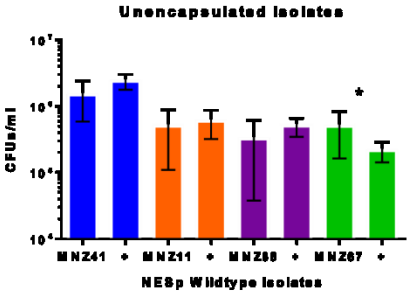

Figure B.12 Sensitivity to granule-mediated killing of first four unencapsulated isolates

Roughly 10^6 pneumococci per ml were exposed to granules extracted from human neutrophils for one hour at 37ºC. Concentration counts were quantitated and compared to concentration counts of the same concentration of pneumococci, that were not exposed to granules. Data shown are the means ± standard deviation (SD) for control and experimental counts of four unencapsulated isolates – MNZ41 (blue), MNZ11 (orange), MNZ85 (purple), and MNZ67 (green).

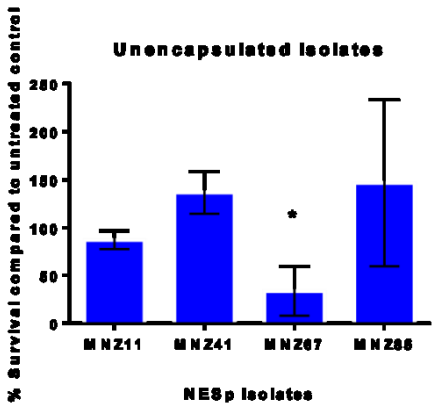

Figure B.13 Sensitivity of the first 4 NESp isolates to granule-mediated killing

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of ten unencapsulated isolates. Asterisk (*) indicates the one strain significantly killed by degranulate.

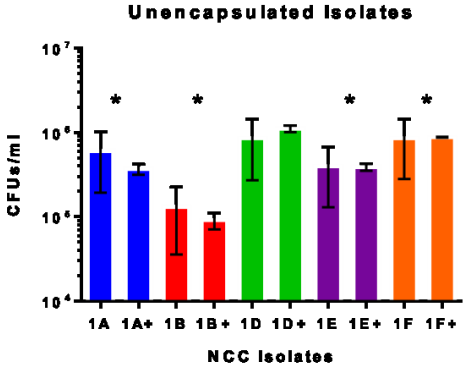

Figure B.14 Sensitivity to granule-mediated killing of first five NCC isolates

Data shown are the means ± SD for control and experimental counts of the first five NCC isolates. Shown are NCC1A (blue), NCC1B (red), NCC1D (green), NCC1E (purple), and NCC1F (orange). Asterisks (*) indicate strains significantly killed by degranulate.

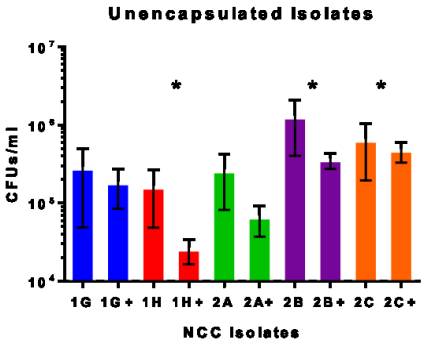

Figure B.15 Sensitivity to granule-mediated killing of five more NCC isolates

Data shown are the means ± SD for control and experimental counts of five NCC isolates. Shown are NCC1G (blue), NCC1H (red), NCC2A (green), NCC2B (purple), and NCC2C (orange). Asterisks (*) indicate strains significantly killed by degranulate.

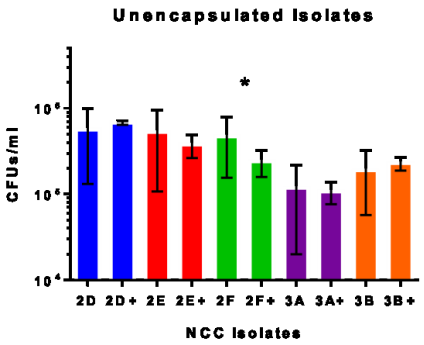

Figure B.16 Sensitivity to granule-mediated killing of last five NCC isolates

Data shown are the means ± SD for control and experimental counts of five NCC isolates. Shown are NCC2D (blue), NCC2E (red), NCC2F (green), NCC3A (purple), and NCC3B (orange). Asterisk (*) indicates the strain significantly killed by degranulate.

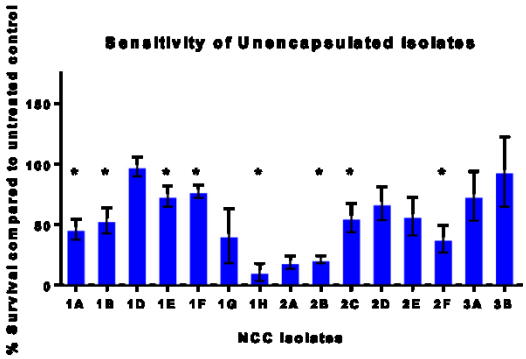

Figure B.17 Percent survivals of all NCC isolates to granule-mediated killing by neutrophils

Percent survival of each isolate was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all 15 NCC isolates. Asterisks (*) indicate strains significantly killed by degranulate.

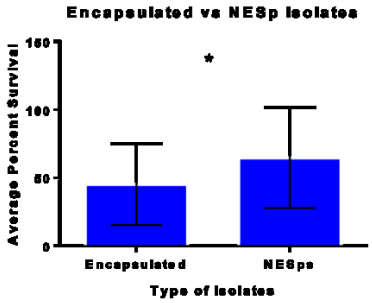

Figure B.18 Average sensitivity of encapsulated isolates and unencapsulated isolates

The average sensitivities of encapsulated isolates and unencapsulated isolates were assessed with an unpaired two-tailed t test. Asterisk (*) indicates that a significant difference was found between the average sensitivities of the two types of isolates.

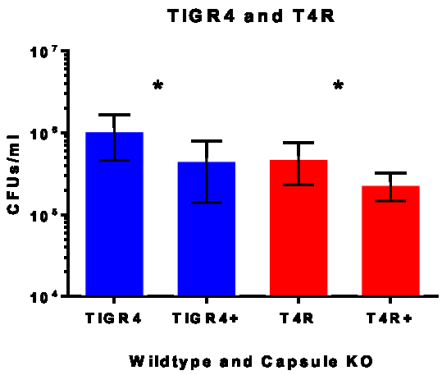

Figure B.19 Sensitivity to granule-mediated killing of TIGR4 and T4R

Data shown are the means ± standard deviation (SD) for control and experimental counts of TIGR4 (blue) and its capsule knock-out mutant T4R (red). Asterisks (*) indicate strains significantly killed by degranulate.

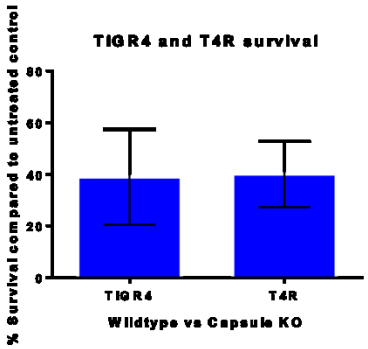

Figure B.20 Percent survival of TIGR4 and T4R to granule-mediated killing by neutrophils

Percent survival of each strain was calculated by dividing the concentration counts of experimental reactions by control reactions and multiplying by 100%. Data shown are the means ± SD for the percent survival of all TIGR4 and T4R.

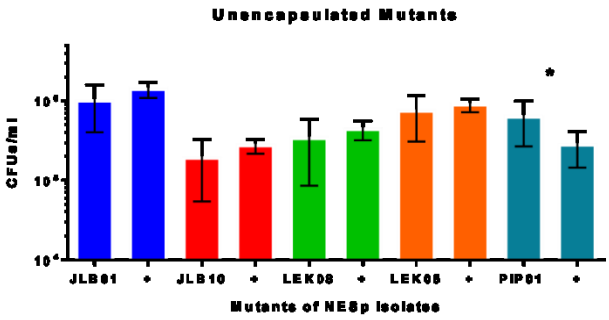

Figure B.21 Sensitivity to granule-mediated killing of six mutants of unencapsulated isolates

Data shown are the means ± SD for control and experimental counts of six mutants of NESp isolates. Shown are JLB01 (blue), JLB10 (red), LEK08 (green), LEK05 (orange), and PIP01 (teal). Asterisk (*) indicates the strain significantly killed by degranulate.

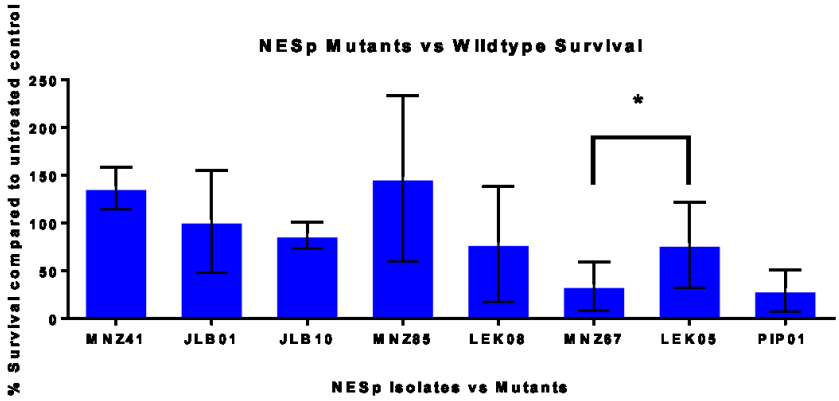

Figure B.22 Percent survivals of all NESp wildtypes versus their mutants

Data shown are the means ± SD for the percent survival of four NESp isolates and their mutants. Asterisk (*) indicates the only significant difference in percent survivals of wildtype and mutant.

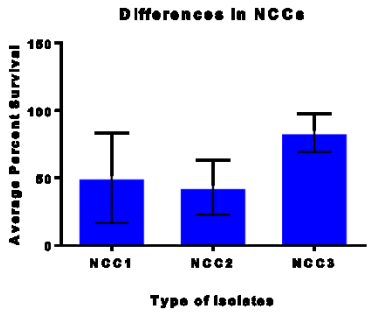

Figure B.23 Average survival of NCC1, NCC2, and NCC3 isolates

The average sensitivities of all three clades of NCC isolates were assessed with one-way ANOVA. No significant difference was found among the three clades of isolates.

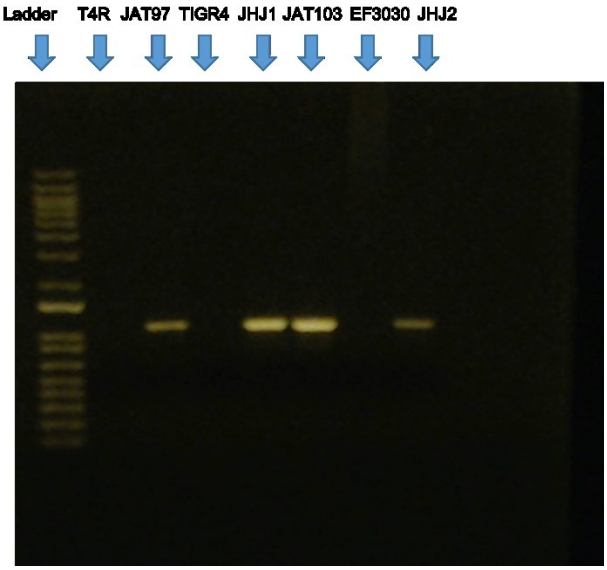

Figure B.24 Electrophoresis gel of wildtype pneumococcal strains and their *htrA* knockouts

Ex Taq PCR screening was conducted on wildtype and mutant pneumococci using Erm forward and reverse primers. Bands indicate presence of Erm cassette in place of *htrA*.

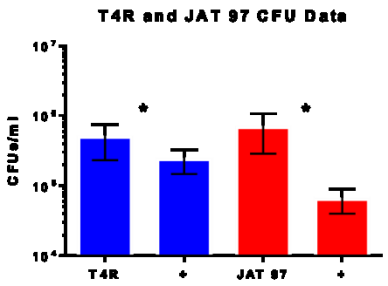

Figure B.25 Survival of T4R and its *htrA*-deficient mutant JAT97 post-exposure to granules

Roughly 10^6 pneumococci per ml were exposed to granules extracted from human neutrophils for one hour at 37ºC. Data shown are the means ± standard deviation (SD) for control and experimental counts of T4R (blue) and JAT97 (red). Asterisks (*) indicate strains significantly killed by degranulate.

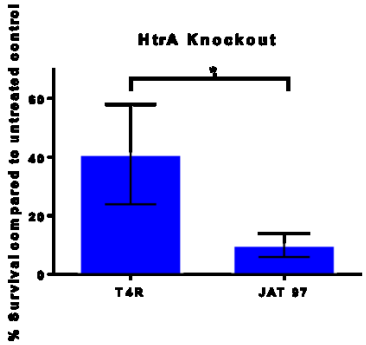

Figure B.26 Percent survival of T4R and JAT97 to granule-mediated killing by neutrophils

Data shown are the means ± SD for the percent survival of T4R and JAT97. Asterisk (*) indicates that JAT97 was significantly more susceptible to degranulate killing than the wildtype T4R.

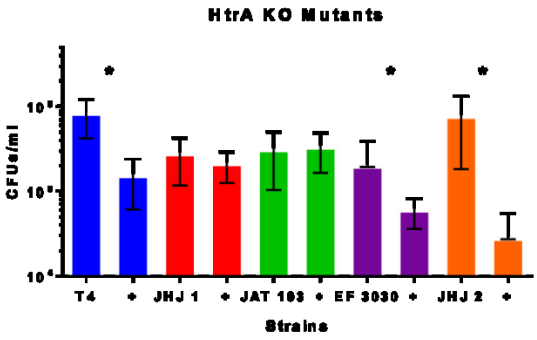

Figure B.27 Survival of wildtype pneumococci and their *htrA*-deficient mutants to granules

Data shown are the means ± standard deviation (SD) for control and experimental counts of two wildtype pneumococcal strains and their *ΔhtrA* mutants. Shown are T4 (blue), JHJ1 (red), JAT103 (green), EF3030 (purple), and JHJ2 (orange). Asterisks (*) indicate strains significantly killed by degranulate.

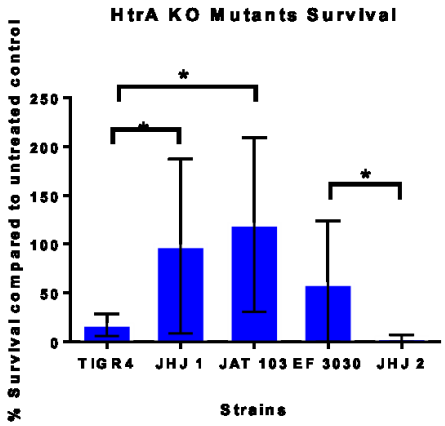

Figure B.28 Sensitivity of wildtype pneumococci and their *htrA*-deficient mutants

Data shown are the means ± SD for the percent survival of TIGR4, JHJ1, JAT103, EF3030, and JHJ2. Asterisks (*) indicate significant differences in wildtype and mutant survivals.

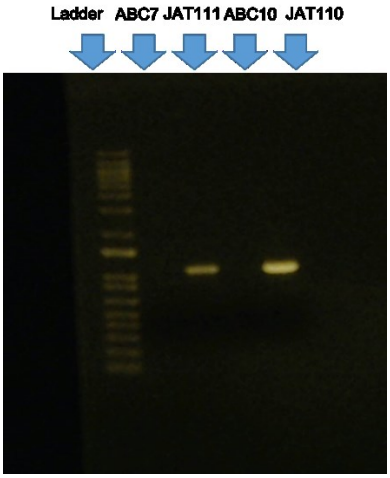

Figure B.29 Electrophoresis gel of wildtype pneumococcal isolates and their *htrA* knockouts

Ex Taq PCR screening was conducted on wildtype and mutant pneumococci using Erm forward and reverse primers. Bands indicate presence of Erm cassette in place of *htrA*.

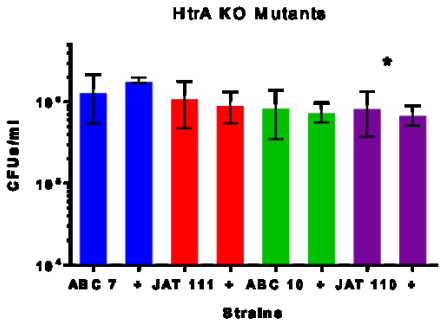

Figure B.30 Concentrations of resistant invasive isolates and their *htrA*-deficient mutants post-exposure to degranulate

Data shown are the means ± standard deviation (SD) for control and experimental counts of two wildtype pneumococcal invasive isolates and their *ΔhtrA* mutants. Shown are ABC7 (blue), JAT111 (red), ABC10 (green), and JAT110 (purple). Asterisk (*) indicates the strain significantly killed by degranulate.

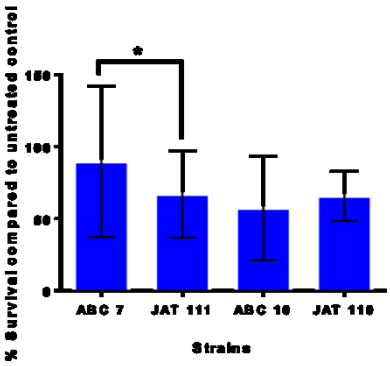

Figure B.31 Sensitivity of wildtype invasive isolates and their *htrA*-deficient mutants

Data shown are the means ± SD for the percent survival of ABC7, JAT111, ABC10, and JAT110. Asterisk (*) indicates that a significant difference of survival was found between wildtype and mutant.

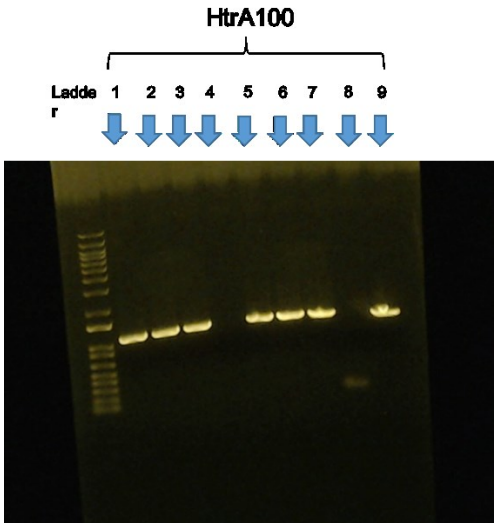

Figure B.32 Gel electrophoresis of nine transformants from the transformation of pHtrA into BL-21 *E. coli*

Nine transformants were screened with HtrA primers 2 and 5 and ran on a .8% agarose gel. All transformants screened except for numbers 4 and 8 were positive for the HtrA plasmid. The first two were selected to grow for purification of recombinant HtrA.

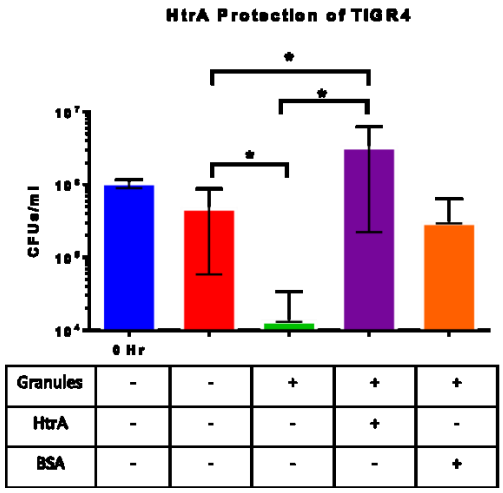

Figure B.33 Survival of TIGR4 exposed to granules and rHtrA or BSA

10^6 pneumococci per ml were exposed to granules extracted from human neutrophils and either HtrA or BSA for one hour at 37ºC. Data shown are the means ± standard deviation (SD) for a zero-hour control (blue), a one-hour control (red), a degranulate-exposed (green), and degranulate-exposed and HtrA-protected (purple), and a degranulate-exposed and BSA-protected (orange) TIGR4. Asterisks (*) indicate significant differences in CFUs.

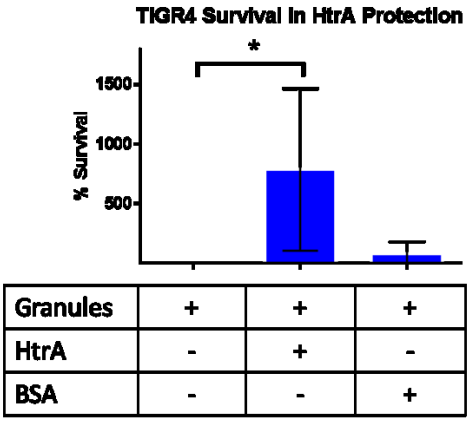

Figure B.34 Percent survival of TIGR4 exposed to granules and HtrA or BSA

Data shown are the means ± SD for the percent survival of TIGR4 to degranulate and TIGR4 exposed to degranulate and HtrA or BSA. Asterisk (*) indicates a significant difference in survival.

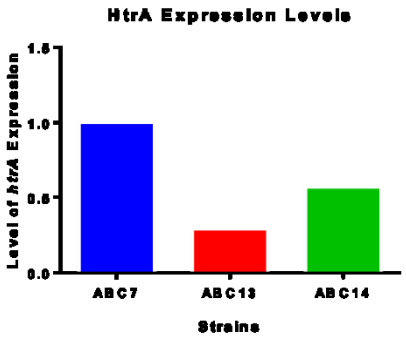

Figure B.35 Expression of ABC13 and ABC14 compared to ABC7

qPCR was conducted on ABC7, ABC13, and ABC14 targeting *htrA*. ΔΔct was calculated for the isolates 13 and 14 compared to ABC7 using mean cts of multiple runs. Shown are expression levels of ABC13 (red) and ABC14 (green) related to ABC7 (blue).