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Characterization of a Mycoplasma Pneumoniae CARDS Toxin Mutant

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Pathobiology Honors Thesis

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<u>Acknowledgements:</u>

<u>Abstract:</u>

Mycoplasma pneumoniae is a high-burden pathogen which causes mild to significant infections of the respiratory system. According to the CDC, an estimated two million cases occur yearly in the United States alone, demonstrating the widespread effect of the pathogen. In addition to being the cause of respiratory infections, *M. pneumoniae* has also been implicated in exacerbating pre-existing asthma conditions. These morbidities make finding a vaccine candidate a vital part of easing the healthcare burden caused by the pathogen. The current mechanism of infection is unknown, but recent evidence points to the Community Acquired Respiratory Distress Syndrome (CARDS) toxin as being involved in that mechanism. We believe that if a mutant was created that is a knockout for the CARDS toxin, then the virulence will be diminished. This will provide further evidence to the theory that the CARDS toxin is responsible for the pathogenesis of the bacteria. In this experiment, we isolated a *M. pneumoniae* mutant and placed it in a mouse model to test for virulence. After using transposon insertion to find a possible mutant, polymerase chain reaction (PCR) was run to confirm the insertion of the transposon. The mutant was then placed in a mouse model to compare its virulence with that of wild-type *M. pneumoniae*. When placed in the model, the mutant *M. pneumoniae*-infected mice had lower lesion scoring and lower bacterial counts in serial dilutions than those of the wild-type *M. pneumoniae*. This gave proof that the isolated mutant was in fact attenuated for virulence. Further steps must be taken to ascertain the identity of the mutant, such as DNA sequencing and in vitro studies. Given the results, this experiment was a success in isolating a virulence-attenuated *M. pneumoniae* mutant, and is a step closer to finding a possible vaccine for the disease.

Introduction:

      Mycoplasma pneumoniae is a small atypical bacteria known for its ability to cause significant respiratory tract infections in humans worldwide. They are well-documented for causing upper and lower respiratory infections. It is widespread in Africa, North America, and Europe, but data suggests the pathogen is endemic all over the world. Past research proved *M. pneumoniae* has the ability to exacerbate pre-existing asthma conditions in patients already suffering from airway irritation (Atkinson *et al.,* 2008). The current hypothesis states that the Community-Acquired Respiratory Distress Syndrome (CARDS) toxin may be a contributing factor to the virulence of the pathogen. If the genome of *M. pneumoniae* can be mutated to disrupt the CARDS toxin, then the virulence of the pathogen should be reduced. In this paper, a mutant form of *M. pneumoniae* was isolated, in the hopes of finding the desired attenuation.

      The infection caused by the bacteria *M. pneumoniae* is a significant burden on healthcare systems worldwide. Climate and weather are not thought to significantly affect the spread of the pathogen (Atkinson *et al.,* 2008). It has a long incubation period and low transmission rate, and can survive in respiratory tracts for long periods of time (Atkinson *et al.,* 2008). It can manifest in several pathologies, ranging from milder upper respiratory infections to much more severe cases of community-acquired pneumonia (Kashyap *et al.,* 2010). Typically, the final stage of the disease results in community-acquired pneumonia, but up to 20% of patients can be asymptomatic (Atkinson *et al.,* 2008). The global burden of the pathogen can be seen in *Fig. 1* (Parrott *et al.,* 2016).
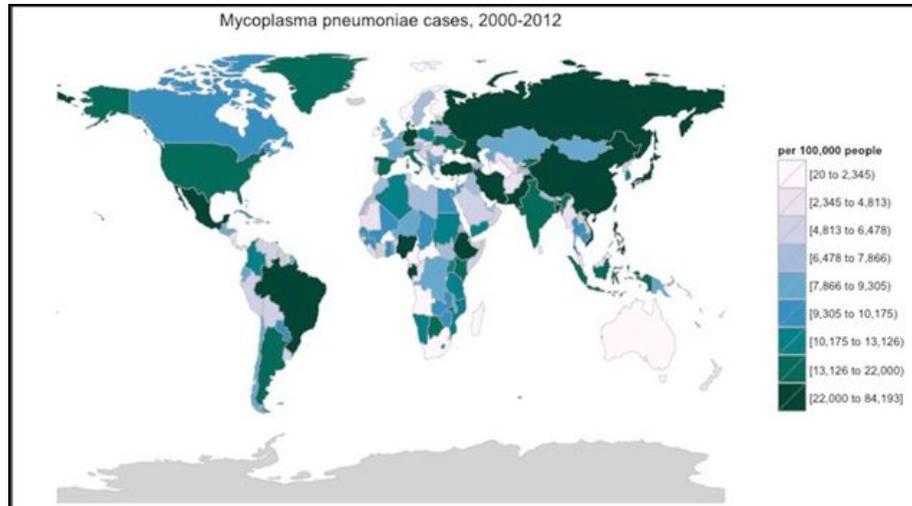
Figure 1 (adapted from Parrott et al., 2016): This image shows the number of *M. pneumoniae* cases worldwide from 2000-2012. Russia, China, and Brazil have high numbers of cases as evidenced by the key on the right of the image.

Fig. 1 shows how prevalent the infections caused by *M. pneumoniae* are globally. Work on finding a vaccine candidate has been stymied by the need to elucidate the disease mechanism. Beyond respiratory illnesses, this pathogen has also been linked to exacerbating chronic asthma symptoms (Sutherland *et al.,* 2004). Much work has been done on finding the mechanism of virulence of *M. pneumoniae*. Finding the right mechanism can lead to finding a possible vaccine.

 M. pneumoniae are atypical bacteria with an extremely small genome. They are among the smallest bacterial organisms, with a one to two micrometer-long spindle shape (Atkinson *et al.,* 2008). Their genome is 600 to 1,350 kbp long (He *et al.,* 2016). The pathogen is not only found in humans, but is known to infect insects, animals, and plants. *M. pneumoniae* causes disease by interacting with the host's epithelial tissue. It survives by adhering to the respiratory epithelium, where it is protected from the mucus in the nose and throat via its adhesive properties

(Kashyap *et al.*, 2010). The damage it causes through adhesion is extensive. Several proteins such as P1, P40, and P90 come together to form an adhesion protein complex. This complex allows *M. pnuemoniae* to anchor itself onto cellular membranes (He *et al.,* 2016). Through this adherence process, *M. pneumoniae* causes the trademark symptoms often seen in patients.

Through the process of cytoadherence, *M. pneumoniae* produces hydrogen peroxide, superoxide radicals, and induces the formation of endogenous toxic oxygen molecules (Waites *et al.,* 2004). These compounds cause severe distress to the respiratory lining where the bacteria adheres to, and causes the characteristic cough experienced by patients. In the lower respiratory airway pathways, *M. pneumoniae* may be opsonized and taken up by macrophages. The activation of immune responses will result in the production of TNF-alpha, IFN-gamma, and various interleukins (Waites *et al.,* 2004). It has been speculated that the release of proinflammatory cytokines during a *M. pneumoniae* infection is related to the exacerbation of pre-existing chronic conditions such as bronchial asthma in the host (Waites *et al.,* 2004).

Several studies in the past have been done to ascertain the link between *M. pneumoniae* and chronic lung diseases such as asthma. A 1970 study found that 32% of asthma patients involved in the research showed evidence for infection by either *M. pnuemoniae* or another respiratory virus (Berkovich *et al.,* 1970). Another study conducted further analysis, and found that 19% of asthma patients had mycoplasma or viral infections after severe asthmatic episodes (Huhti *et al.,* 1974). Further research has drawn even more correlation; a 2004 study found 20% of patients with previously diagnosed asthma faced exacerbated asthmatic conditions when a *M. pneumoniae* infection occurred (Biscardi *et al.,* 2004). In 2009, Varshney *et al.,* in a study conducted among Indian children with asthma, found *M. pneumoniae* antibodies in 22% of those

who reported moderate to severe asthma symptoms (Varshney *et al.,* 2009). In a similar study, IgM antibodies against *M. pneumoniae* were found in 24% of children presenting with respiratory infections (Shenoy *et al.,* 2005). Asthma symptoms were found to be exacerbated in another study, after an infection by *M. pneumoniae* (Yeh *et al.,* 2016). PCRs were used to determine the presence of *M. pneumoniae* in pulmonary airways of adult chronic asthma patients. 45% of the patients tested had *M. pneumoniae* in their respiratory tracts (Kraft *et al.,* 1998). These results were corroborated by findings which showed the presence of *M. pneumoniae*-specific IgM antibodies in patients who presented with exacerbated asthma conditions (Seggev *et al.,* 1986). Clearly, there is an association between the respiratory viral infections and the presence of *M. pneumoniae* within the respiratory tract. However, the exact molecular mechanism of action has yet to be elucidated.

Much work has been done to find out what the exact mechanism that underlies *M. pneumoniae*-induced infection. One possible mechanism is the Community Acquired Respiratory Distress Syndrome toxin, or the CARDS toxin. The CARDS toxin was recently characterized as a possible mechanism used by *M. pneumoniae* in its cytoadherence function. The toxin is encoded by the mpn372 gene in the pathogen genome (Kannan *et al.,* 2010). CARDS is an exotoxin that binds to surfactant protein A, allowing it to become internalized by cells. (Kannan *et al.,* 2010). This virulence factor is known for its ADP-ribosylating and vacuolating abilities (Becker *et al.,* 2015). This toxin is made up of three domains which form its tertiary structure, which can be seen in *Fig. 2*. Domain 1 is associated with ADP-ribosylating activity. Domains 2 and 3 are more linked in function, and are associated with the vacuolating ability of the toxin (Becker *et al.,* 2015).
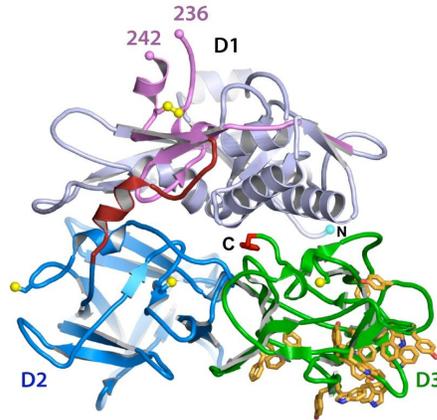
Figure 2 (adapted from Becker et al., 2015): This image shows the tertiary structure of

the CARDS toxin. The three domains are highlighted in different colors.

The exact arrangement of the domains can be seen more clearly in *Fig. 3*. This image shows how

the domains are arranged in the gene, and what purposes each one serves.

Figure 3: This image shows the three domains of the CARDS toxin, as well as the linker.

When it was purified, the toxin was able to cause significant dose-dependent cytopathology in

mammalian cell and organ cultures. These researchers showed that an exposure to the purified

recombinant CARDS toxin presented pathological signs that were similar to an infection by *M.*

pneumoniae. This provided evidence that the toxin is related to the pathologic effects seen during

an infection (Kannan *et al.,* 2010).

Further research was conducted on the role of the CARDS toxin in *M. pneumoniae*

infection. One such study was conducted using recombinant-CARDS toxin in naive mice

(Medina *et al.,* 2012). When the mice were exposed to the rCARDS toxin, mucus expression was

heightened, and the major mucin gene Muc5AC was expressed at greater levels (Medina *et al.,* 2012). The increased production of mucus is an indicator of both *M. pneumoniae* infection and allergic lung inflammation (Chu *et al.,* 2005). An image (*Fig. 4)* taken from this paper is shown below to highlight the increased mucus production on lung slides (Medina *et al.,* 2012).

Day 7 post CF (PAS)   Day 7 post CARDS (PAS)
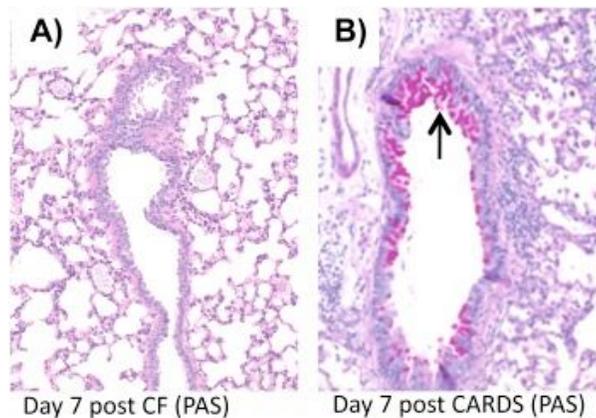
Figure 4 (adapted from Medina et al., 2012): Slide A shows pathology of a lung section from a mouse treated with carrier fluid and stained with PAS stain. Slide B shows lung pathology taken from a mouse treated with rCARDS toxin, and stained with PAS. The arrow indicates the buildup of mucus production in the rCARDS-treated animal.

In addition to the increased mucus production, the researchers also witnessed an inflammation of eosinophilic-lymphocytic composition. Bronchoalveolar lavage fluid (BALF) is a prime indicator of immune responses in the lungs. Fluid was pumped through the trachea into the lungs, and then withdrawn with a syringe to obtain BALF (Hardy *et al.,* 2009). This fluid contained cells found in the lungs. These cells are washed and counted to enumerate the amount of immune cells found in the lungs. The rCARDS toxin-treated mice had higher counts of neutrophils, eosinophils, lymphocytes, and macrophages by day 7 post infection (Medina *et al.,* 2012). In addition to the higher immune responses, rCARDS toxin-treated mice also had higher

lesion scoring in lung sections. These inflammatory and immunologic effects gave evidence that the CARDS toxin is important to the disease-causing ability of *M. pneumoniae*. Based on this data, a hypothesis was formed for this project.

In this thesis, a mutant form of *Mycoplasma pneumoniae* was created and tested in a mouse model to assess its effects on disease virulence. The goal was to create a mutant which was a CARDS toxin-knockout, and demonstrated a lower virulence when compared to the wild-type pathogen. This would provide further evidence that the CARDS toxin is an important component in the disease-causing ability of *M. pneumoniae*.

Methods:

Creation of a CARDS-attenuated mutant *Mycoplasma pneumoniae.* Previous work in our lab

resulted in the creation of a transposon-mutant library for *M. pneumoniae* (Szczepanek *et al.,*

2010)*.* This library contains 1658 mutants, and given that *M. pneumoniae* has roughly 690

protein-encoding genes, the library provides roughly ~2.5x coverage per gene.

To create the CARDS-toxin knockout mutant, a transposon was inserted into a binding

region in domain 1 of the gene (mpn372). Domain 1 is an ADP-ribosylating region, and the

arrangement of the full CARDS toxin gene can be seen in *Fig.3.* The desired insertion point of

the transposon can be seen in *Fig. 5.* Using the pMT85 plasmid, the transposon will be inserted

into this region (Janis *et al.,* 2008). The amplicon size was estimated to be 1821 base pairs long.

Gentamicin and kanamycin were inserted along with the plasmid to ensure proper plasmid

insertion.
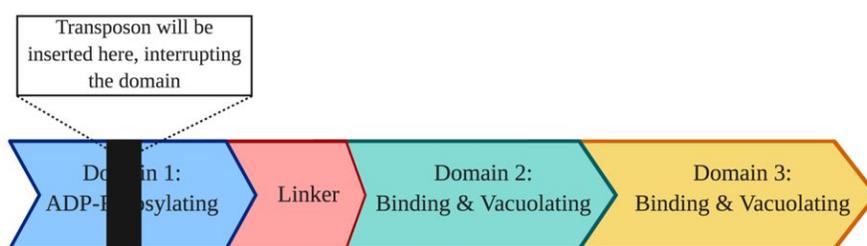
Figure 5: The domain arrangement of mpn372, and the desired insertion point of the transposon.

Given that the mutant library was generated via "haystack mutagenesis," the transposon

insertions are random, requiring PCR screening to identify transposon insertions and putative

mutants. This technique was used to screen for transposons which disrupted the promoter region.

Insertions in this region are more likely to result in absolute gene knockouts, but insertions in the

full open reading frame were also considered. We designed CARDS-specific primers with the

forward primer sitting upstream of the transcriptional start site. The primers consisted of 2 groups which run in opposite directions. Group 1 consisted of primers running in the reverse direction of the CARDS toxin (5' → 3') and primers running in the reverse direction of the transposon (3' → 5'). Group 2 was the same but vice versa; it consisted of primers running in the reverse direction of the CARDS toxin (3' → 5') and the forward direction of the transposon (5' → 3'). These combinations of primers showed whether the transposon is properly inserted into domain 1 at the binding region. Several different primers were run with both the wild-type and mutant *M. pneumoniae* DNA to confirm which primers work best without spurious amplification, and to find the best screening primer pairs.

After finding primers that resulted in the least spurious amplification, they were run through a temperature gradient to optimize the annealing temperature. Other PCR conditions were fine-tuned to reduce spurious amplification in the selected pair. These conditions included determining cycle time and number.

Because the mutant library is extensive, pooling was used to screen large numbers of mutants for an insertion in the CARDS gene. 30 mutants were pooled together, and a PCR was run on each pool. Individual screening was conducted on the pool with the best insertion, and the mutant with the best transposon insertion in that pool was grown in cell culture. This mutant was harvested and run in a PCR to assess where the transposon was inserted and whether it was attenuated.

Assessment of mutant virulence compared to wild-type virulence in a mouse model. After the mutant was grown and checked for genomic attenuation, it was tested in an animal model to assess virulence attenuation. BALB/c mice procured from Jackson Laboratory were used as the animal study. 2 groups of mice, each containing 10 8-week old female mice, were set up for the experimental protocol. Mice were housed at 5 mice/cage, with 2 cages per experimental group. Group 1 received 5x10^7 CFU of wild-type *Mycoplasma pneumoniae* strain PI128 on D0 intranasally. Group 2 received 5x10^7 CFU of the mutant strain intranasally on D0 as well. 4 days post infection, the mice were sacrificed. Carbon monoxide cannot be used to sacrifice these animals as the compound may interfere with bacterial recovery later. Instead, the mice were anesthetized with isoflurane and cervical dislocation was used to euthanize the animal. A paw pinch was used as a confirmation of death. *Fig. 6* shows the timeline of events during animal trials.
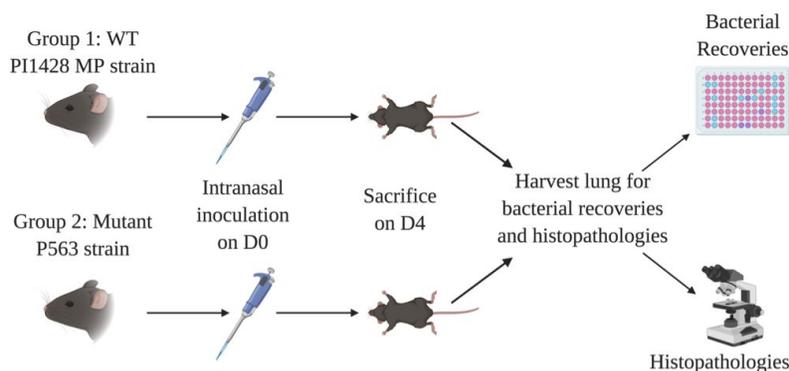

Figure 6: A brief timeline showing the steps taken during animal trials.

Once death has been confirmed, the mice were dissected following previously characterized protocols (Morton *et al.,* 2017). Portions of the lower right lobe of the lung were harvested and stored in complete Fortified Commercial Medium in order to perform bacterial

recovery, and the rest of the lung was fixed in 10% neutral buffered formalin. The resected tissue was used for both histopathology and bacterial recoveries. Bacterial recoveries were performed by placing the resected lower right lung in 3 mL of FC, followed by two minutes of steady vortexing. The tissue sample was then incubated for 3 hours, filtered, and used to perform serial 10-fold dilutions. This gave CCU counts for the mutant and WT pathogens. The histopathology obtained from the samples was used for lesion scoring. Lesions were graded on a scale of 0 to 4, with 0 having no visible lesions and 4 having severe lesions, in increments of 1. These scores were assigned in blinded fashion by an experienced individual trained by a board certified veterinary pathologist (Szczepanek *et al.,* 2012).
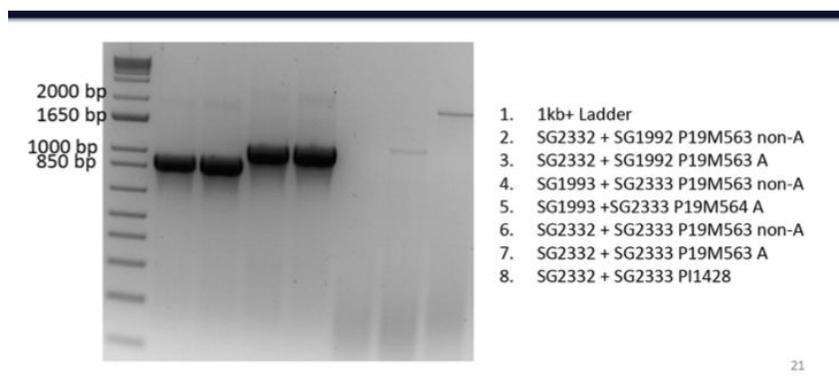
Results:

Isolation of mutant P563. When the mutant library pools were run together, pool 19 showed a possible mutant with a good insertion of the transposon. This specific pool can be seen in *Fig. 7,* with pool 19 indicated.
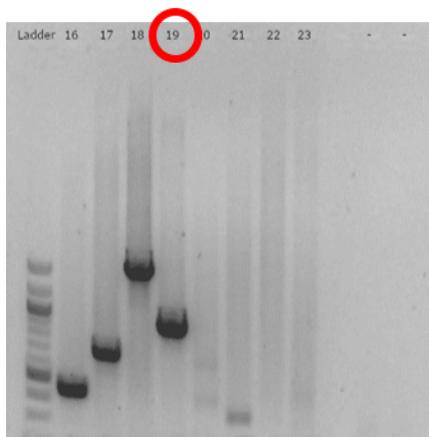
Figure 7: An example of some of the PCR pooling done to find a putative mutant. Pool 19, indicated by the red circle, showed a mutant with good insertion of the transposon.

When this pool was run individually, with each mutant in different lanes, mutant P563 showed good disruption of the binding region. A PCR was run on this mutant in the presence of the primers to see which primer combination gave the best disrupting insertion of the transposon. The results of that PCR can be seen in *Fig. 8*.

1. 1kb+ Ladder
2. SG2332 + SG1992 P19M563 non-A
3. SG2332 + SG1992 P19M563 A
4. SG1993 + SG2333 P19M563 non-A
5. SG1993 +SG2333 P19M564 A
6. SG2332 + SG2333 P19M563 non-A
7. SG2332 + SG2333 P19M563 A
8. SG2332 + SG2333 PI1428

Figure 8: PCR of mutant P563 in presence of several different primers. Non-A represents

non-adherent (floating) cells and A represents adherent cells.

Lane 1 contains a ladder. Lanes 2 and 3 contain a forward CARDS toxin primer and reverse

transposon primer. Lanes 3 and 4 contain a reverse CARDS toxin primer and forward transposon

primer. Adherent and non-adherent cells were run together, but no significant differences were

observed between the two. The adherent cells were used to grow the mutant and infect the mice.

Lanes 2-4 gave us fragment sizes that allowed us to map the insertion based on the sequence.

Fragment sizes are indicated on the left of the gel in Fig. 7. Lanes 2 and 3 had fragment sizes of

850 bp and 1650 bp. Lanes 3 and 4 had fragment sizes of 1000 bp and 2000 bp. By knowing the

fragment sizes from the PCR screening, sequencing can be used to map the insertion in the gene.

Attenuation of virulence in the mouse model. Lesion scoring of the animal lungs showed the mice dosed with mutant attenuated *M. pneumoniae* had lower scoring when compared to the WT PI428 strain *M. pnuemoniae.* The average scoring for the mutant strain was 1.0, while the average score for the WT strain was 1.5. This means the mutant strain had less lesion severity in the lungs, with less scarring being noted. The WT strain had more severe lesions, hence the higher scoring for the animal. *Fig. 9* shows the graph of the comparison between the two strains in lesion scoring.
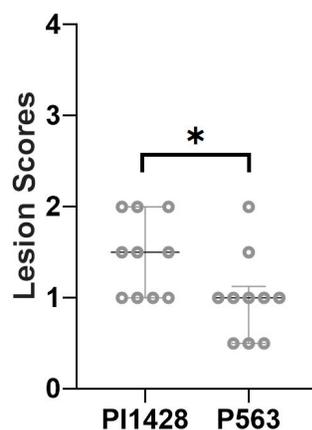
Figure 9: WT PI428 strain and P563 strain lesion scoring (0 to 4). Significance was noted here (p < 0.05).

Lesions were less severe in the P563-inoculated mice, as evidenced by the lower lesion count in those animals. This gives evidence that the P563 mutant was attenuated for virulence. In addition to the lesion scoring, bacterial recoveries were conducted to assess color-changing units (CCUs) via serial dilutions. The results of this experiment can be seen in *Fig. 10.*
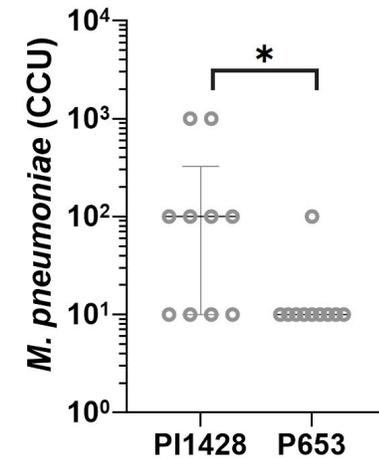
Figure 10: CCU counts from serial dilutions conducted on bacterial recoveries. Recoveries were taken from lower right lung tissue. Significance was noted here (p < 0.05).

The recoveries show that P563 had an average cell count of 10^1 CCU. In comparison, the WT strain PI1428 had an average count of 10^2 CCU. The highest value from the P563 recovery was 10^2 CCU, compared to 10^3 CCU from the same value for PI1428. The attenuated P563 was not able to grow and infect the host cells as much as the WT PI1428, giving further evidence to the virulence attenuation.

Discussion:

The burden *Mycoplasma pneumoniae* places on healthcare systems worldwide is significant. It causes high numbers of pneumonia and asthmatic cases in patients each year. Because of its effects, elucidating the mechanism of action of its infection is of great importance. As mentioned before, there is evidence linking the CARDS toxin and the primary virulence of *M. pneumoniae.* The goal of this project was to create an attenuated mutant with demonstrated reduced virulence. The lesion scoring from the *in vivo* testing showed significant reduction in virulence in the P563 mutant. The bacterial recoveries also showed less bacteria per milliliter when compared to the recoveries of the WT strain. This data, when taken together, shows that the mutant did have attenuated virulence.

There are several steps to take with the conclusions from this project. While the mutant was confirmed to have attenuated virulence, it still needs to be sequenced to determine if the transposon is properly inserted in the binding region in domain 1, disrupting the gene. The PCR results gave evidence that the transposon was able to insert properly. However, DNA sequencing is required to map the proper insertion, before this mutant can be used in further study.

In addition to DNA sequencing, *in vitro* studies should be conducted on the mutant. These studies will be used to phenotype the mutant. While we know the mutant is not as virulent as the WT pathogen, we still need to confirm this decrease in virulence is due to the disruption of the CARDS toxin. The CARDS toxin, as demonstrated by the domain layout, is capable of ADP-ribosylating, binding, and vacuolating functions. Assays can be designed to assess the ADP-ribosylating activity of the toxin. Cytotoxicity assays can also be used to determine the function of CARDS in the mutant. An important example of such an assay is determining

vacuolation in MRC-5 human lung cells (Kannan *et al.,* 2014). One of the CARDS toxin chief functions is its ability to form vacuoles in cells it infects. These vacuoles can be seen under microscopy. If the CARDS toxin is disrupted in our mutant, then its ability to form vacuoles will also be disrupted. These phenotypic characterizations will aid in determining if the mutant is actually a CARDS-toxin knockout.

As mentioned previously, isolating a CARDS-attenuated mutant of *Mycoplasma pneumoniae* is key in characterizing the pathogenesis of the disease. If a link between the CARDS toxin and virulence can be established, then a step is taken towards finding the mechanism of action of the pathogen. The mutant isolated in this study can play a central role in finding that link.

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