Antagonistic interactions between *Pseudomonas aeruginosa* isolated from different age groups of cystic fibrosis patients

Undergraduate Honors Thesis

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Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen affecting cystic fibrosis (CF) patients and immunocompromised individuals contributing significantly to morbidity and mortality. Once infected with *P. aeruginosa*, patients are chronically colonized and unable to clear the infection even with antimicrobial treatment. *P. aeruginosa* is inherently resistant to many antimicrobials which has encouraged the hunt for novel potential therapeutics. Recently, *P. aeruginosa* isolates obtained from the same CF patient were shown to interact antagonistically where secreted signals from one isolate decreased protease production by another isolate. This antagonistic interaction also decreased the virulence of the virulent *P. aeruginosa* isolate. The purpose of this study was to determine if *P. aeruginosa* isolates from a variety of patients in three different age groups (under 13, between 13 and 18, and above 18) would respond to the antagonistic signals. Two antagonistic signal producing *P. aeruginosa* isolates (14672 and 14651) were co-cultured with *P. aeruginosa* isolates obtained from various patients. After *P. aeruginosa* cultures are grown and mixed they are spotted onto skim milk plates to test for the levels of protease production. Protease production is measured by a zone of clearing around the bacterial culture. The mixed cultures are then compared to individual strains also spotted on the skim milk plates. This method was used to test *P. aeruginosa* isolates from all age groups and isolates from all groups did respond to the antagonistic signals. These results suggest that *P. aeruginosa* from a wide range of patients responds to the antagonistic signal, indicating this signal may have potential as a future therapeutic agent for CF patients.

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Introduction

Cystic Fibrosis

Approximately 30,000 children, adolescents, and adults are afflicted with cystic fibrosis (CF) in the United States. CF is the most common lethal autosomal recessive genetic disorder among Caucasians, appearing in about 1 in 2,500 births (Pier, 1985). In 1989 a molecular basis was provided for the disease. The gene responsible for CF has been located on chromosome 7 and encodes a protein known as cystic fibrosis transmembrane conductance regulator (CFTR) (Kerem et al., 1989). A vast range of mutations on this gene can be responsible for the disease (Tsui, 1992). Evidence shows that CFTR functions as a chloride channel. CF epithelia have abnormally low chloride conductance across the apical membrane and as a consequence the surfaces become dehydrated, the mucous becomes viscous and bacteria are not easily cleared, resulting in chronic infection (Guggino, 1999). CF is often diagnosed by the age of two and is caused by the production of a faulty protein in the pancreas that causes the body to produce abnormally thick and profuse mucus. This hyperproduction of mucus leads to a buildup of thick mucus in the patient's lung which provides an ample environment for the growth of pathogens entering through the respiratory tract. CF patients are predisposed to severe lung infections, which ultimately prove fatal for 80% to 95% of CF patients by the age of 50 (Pier et al., 1996).

Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative bacterium with a versatile metabolism. Although *P. aeruginosa* is ubiquitous environmentally, *P. aeruginosa* is an opportunistic human pathogen, as it normally does not affect healthy individuals but often infects immunocompromised individuals. The pathogen has been isolated from burn victims, surgical patients, CF patients, cancer or bone marrow transplant patients, as well as AIDS patients (Driscoll, Brody, & Kollef,

2007). The cornerstone of *P. aeruginosa*'s virulence is its inherent resistance to a wide variety of antibiotics and disinfectants (Hancock, 1998). These characteristics prove treatment of these infections to be exceedingly difficult. This enhanced resistance is multifactorial resulting from the presence of multidrug efflux pumps (Kohler et al., 1997), both beta-lactamases (Hancock & Woodruff, 1988), and decreased permeability of the outer membrane(Hancock, 1998). *P. aeruginosa*'s vast virulence characteristics aid to the range of infections it is able to cause. In addition to these cellular virulence characteristics, *P. aeruginosa* also produces extracellular virulence factors that enhance the organism's pathogenesis. These extracellular factors include exotoxin A, exoenzyme S, elastase, proteases, rhamnolipids, siderophores, gelatinase, lipase, and phospholipase C (Woods & Sokol, 1986). These virulence factors enable *P. aeruginosa* to manipulate a host environment, enhancing its ability to survive. Proteases produced by *P. aeruginosa* have the ability to destroy host proteins and tissues during the infection process and aid greatly to the pathogenesis of the microorganism (Galloway, 1991).

P. aeruginosa has a social repertoire consisting of interactions that include biofilm formation (O'Toole, Kaplan, & Kolter, 2000) and quorum sensing (Manefield & Turner, 2002). Quorum sensing regulates virulence factor production in many strains of *P. aeruginosa.* *P. aeruginosa* is known to upregulate its virulence within the biofilm. Organisms forming biofilms can undergo profound changes during their transition from free-living organisms to cells that are part of a complex, surface-attached community (O'Toole et al., 2000). These changes are reflected in new phenotypic characteristics developed by *P. aeruginosa* in response to a variety of environmental signals within the CF lung (O'Toole et al., 2000). Small signaling molecules are produced by individual bacteria within the community and are recognized by other community members

(Fuqua, Winans, & Greenberg, 1994). The concentration of the signals within the environment can regulate gene expression and the production of many virulence factors (Fuqua et al., 1994).

Cystic fibrosis and Pseudomonas aeruginosa

Not only is *P. aeruginosa* a difficult pathogen to treat, it is known to undergo pathoadaptive mutations to modify gene expression within the niche of the CF lung. Infections by *P. aeruginosa* are unique in that they adapt to the CF lung, it acquires loss-of-function mutations that decrease virulence factor production (Jain et al., 2004). *Pseudomonas* isolates from early infections are similar to environmental isolates in their phenotype; however as the infection prolongs, isolates become more resistant to antibiotics and are frequently mucoid (Govan & Harris, 1986). Once a mucoid infection has initiated, it becomes more difficult to treat due to the biofilm produced during growth of the pathogen (Henry, Mellis, & Petrovic, 1992). Treatments involving long term use of antibiotics often render *P. aeruginosa* to become multi drug resistant (Lyczak, Cannon, & Pier, 2000). Resistant characteristics are often not observed in the initial colonization of a patient; however, increasingly appear through the course of infection.

The CF lung is known to be a polymicrobial community, especially as the patient matures (Bittar et al., 2008). Considering the nature of the CF microbial community and social interactions among *P. aeruginosa* within, studying the isolates in a pure form provides a poor representation of the environment within the patient's lung. CF *P. aeruginosa* isolates from sub-populations display a variability in virulence as well as diversity in virulence factor production between isolates within the same sub-population as well as between sub-populations (Lutter, Faria, Rabin, & Storey, 2008). Isolates from the same patient show a wide range of diversity suggesting the harsh environment of the CF lung may not in fact be selecting for a single phenotype, but for multiple phenotypes that work in conjunction (Lutter et al., 2008). It has been previously shown

that certain combinations of CF isolates result in decreased lethality of a *P. aeruginosa* infection in a *D. melanogaster* infection, which correlates to decreased production of protease by the virulent *P. aeruginosa* isolate (Lutter et al., 2008).

This project focuses on the interactions among *P. aeruginosa* living within the CF patient's respiratory tract. Novel antagonistic signals produced by *P. aeruginosa* variants (14651 and 14672) found within the CF lung have been identified and serve as the basis for this project. The supernatant of *P. aeruginosa* CF isolates 14651and 14672 separately decrease the virulence of other responsive *P. aeruginosa* strains (Lutter et al., 2008). These antagonistic signals cause reduced production of protease, a well-known virulence factor of *P. aeruginosa* (Driscoll et al., 2007) as seen in co-culture experiments (Figure 1; (Lutter et al., 2008). Mixed infections between 14651 or 14672 with other *P. aeruginosa* CF isolates in the *D. melanogaster* infection model resulted in increased fly survival compared to individual infections with highly virulent strains (Lutter et al., 2008). This reduction of a virulent stain's lethality suggests that these novel antagonistic signals may have the potential to be developed in the future as an alternate therapeutic target to decrease the severity of infections. It is interesting to note that the strains producing the antagonistic

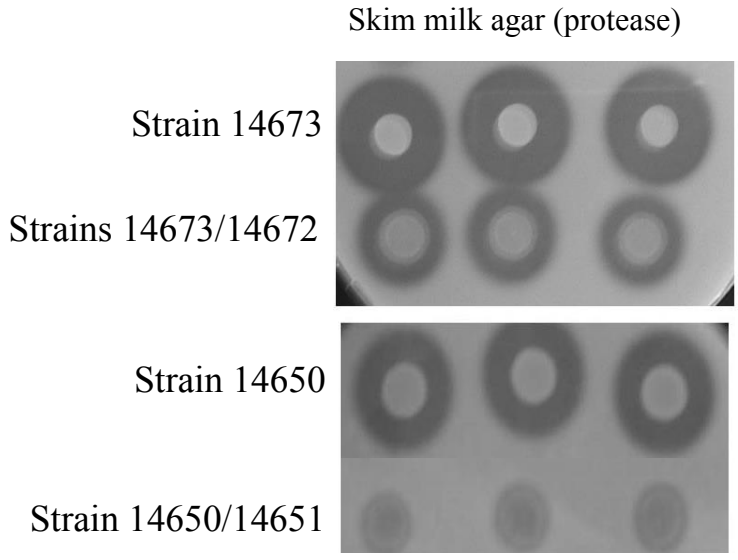

Figure 1: Illustrated action of 14651 and 14672 antagonistic signals. The top figure shows isolates from the same patient (14672 and 14673). When the two cultures are mixed, there is a significant decrease in protease production by strain 14673.The bottom figure illustrates the same action, within isolates from a different patient. Strain 14650 produces a large amount of proteases in a pure culture; however, this production is shut down when mixed with signal 14651.

signals themselves do not respond to the signal. The antagonistic signals only seems to affect other isolates. To determine if the signals produced by these strains (14651 and 14672) do indeed have therapeutic potential, it must first be shown that it can target *P. aeruginosa* CF isolates from multiple patients and in different age groups. Demonstrated in this project is the response of *P. aeruginosa* isolates from all age groups to the antagonistic signal of isolate 14651 and 14672.

Methods

P. aeruginosa strain 14651 and 14672 along with CF sputa isolates from teen, child, and adult patients obtained from the Cystic Fibrosis Clinic in Oklahoma City were cultured separately overnight in 96 well-blocks in brain heart infusion broth. From cryostock, 96 well blocks of CF patient *P. aeruginosa* isolates are stamped using a 48 pin replicator into 1.5 mL of BHI broth and placed in 37°C shaker/incubator overnight. *P. aeruginosa* strain 14651was cultured in 50 mL of BHI broth and also placed in 37°C shaker/incubator overnight. To observe modified protease production in a mixed infection, the antagonistic strain (14651 or 14672) was plated in parallel with a patient culture and the mixing of the two. In each experiment, 14672 or 14651 acted as an internal control since we know these isolates do not respond to their own signal. These separate cultures were mixed at equal OD_{600} and spotted onto a skim milk protease plate to measure protease production of each culture (Sokol, Ohman, & Iglewski, 1979). Protease plates are made with a dialyzed solution from of 9.25 grams of BHI broth powder in 25 mL of Nano Water. The solution is allowed to dialyze overnight in a 500mL graduated cylinder with 500 mL of Nano Water. The dialysate is added to 9.25 grams of agar and 250 mL of water which is then autoclaved. Fifteen grams of skim milk powder is separately mixed with 250 mL of Nano Water, then autoclaved on the same cycle. The two solutions are mixed after autoclaving. Autoclaving the two media in separate containers helps to prevent burning of the skim milk. The media is poured into petri dishes of 15mL aliquots into. Fifty µL of the patient culture was mixed with 50 µL of *P. aeruginosa* strain 14651 or 14672 in a 96 well plate. Strain 14651 or 14672 is diluted 1:10 with BHI broth to set an equal OD_{600} to the

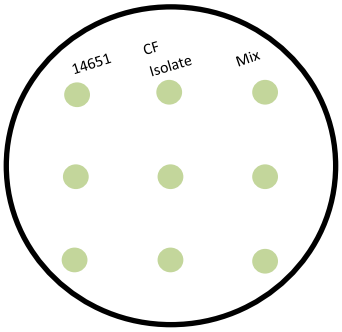

Figure 1: Schematic of cultures spotted on protease plates. Each isolate and co-culture were spotted 3x per plate

patient culture. The strains were spotted onto skim milk plates as demonstrated in Figure 2. Each culture is spotted in triplicate in 3 µL aliquots in triplicate.

Plates are incubated for 24 hours before recording results by measuring the zone of clearing from the edge of the bacterial culture to the edge of the zone of clearing using a hand ruler. Each individual isolate as well as the control strains (14651 or 14672) were independently measured. The data collected represents the actual zone of clearing for each isolate or strain tested. Proteases produced by the bacterial culture degrade casein in the skim milk plates which results in the zone of clearing. Decrease in protease production was analyzed and plotted using Prism 3 GraphPad. These methods were repeated on multiple patient isolates from all age groups (child, teen, and adult). Data of protease production was statistically analyzed using Prism3 software with a One-Way ANOVA test and a Newman-Keuls Posttest to compare all pairs of columns. Statistically significant differences were determined by a P value <0.005. Statistically significant decreases are notated with an asterisk on each graph.

This method of testing protease production of CF patient isolates is repeated with a survey of patients in all age groups. Patient sputa samples testing include 3 adults: Patient 31314a (age 28), patient 32014b (age 41), patient 61214c (age 22); 1 adolescent: patient 32014a (age 16); and 1 child: patient 22714c (age 12). A sampling of isolates from each patient was selected and tested using the above methods.

Results and Discussion

Protease production of isolates from adult patients is shown in Figures 3 and 4.

Figure 3 demonstrates the effects on protease production by CF isolates from adult patients when co-cultured with antagonistic isolate 14672. Many isolates from patient 31314a (depicted in Figure3A) respond to the 14672 signal in co-culture. From this patient, all protease producing isolates tested show response to the signal. In contrast patient 32014b (depicted in figure 3B) shows fewer responders to the 14672 signal. We do observe two isolates from this patient that abolish their protease production when co-cultured; however the majority of isolates do not respond to the signal produced by 14672. Isolates from patient 61214c moderately respond to the 14672 signal.

Figure 4 depicts the response of the isolates from the same 3 adult patients, but now to the 14651 antagonistic signal. Patient 31314a isolates, although extensively responsive to 14672, are less responsive to 14651; however, we do see multiple responding isolates from patient 31314a to 14651. A variety of isolates from patient 32014b respond to the 14651 signal. Isolates from adult patient 61214c also shows wide response to the 14651 signal. When comparing the responding isolates of the same patient exposed to the two unique signals, we observe a difference in the isolates that respond with decreased protease production. Specifically, isolates that respond to the signal from 14651 do not necessarily respond to the signal from 14672 and vice versa. This provides us with preliminary evidence that these two antagonistic strains are different in the method of action.

We continue to observe this pattern of responding isolates to the 14651 and 14672 signals from adolescents and child patients, as well as the diversity in the response to 14651 or 14672.

Figures 5 and 6 illustrate the response of isolates from adolescent and child isolate to both 14651 and 14672 signals.

Response to the signal produced by 14672 is shown in Figure 5. Isolates of child patient 22714c shows few responding isolates; however, there are two statistically significant responding isolates within this child. Isolates from adolescent patient 32014a respond widely to the 14672 signal, similar to the response we saw in adult patients. When we look at the 14651 response in isolates from the same child and adolescent patients, we see that both age groups have a fair amount of responding isolates. Isolates from child patient 22714c show response to the 14651 signal when co-cultured, as well as isolates of adolescent patient 32014a.

Overall, we observed *P. aeruginosa* isolates that respond to the antagonistic signal in each age group; however the response of the isolates varied. Our goal was to see that there were at least a few responding isolates within each patient to the antagonistic signal. The hope is to utilize this antagonistic signal as a potential therapeutic in the future for CF patients. A decrease in the amount proteases produced by *P. aeruginosa* within the patient's lungs has the potential to decrease the severity of an infection and pulmonary decline in CF patients. In order for this future therapeutic to be of value to all patients in the CF community it was necessary to verify that *P. aeruginosa* isolated from patients in all age groups have the potential to respond with decreased protease production to antagonistic signals. As the *P. aeruginosa* are exposed to the signal, their protease production is reduced; however, it does not appear to kill the bacteria as *P. aeruginosa* growth is still observed on the skim milk plates. The antagonistic signals are inhibiting growth of the *P. aeruginosa* strains, only decreasing virulence. If used in treatment, this signal would be used in combination with existing antimicrobial therapies to decrease the severity of infection. Reducing the severity of infections and exacerbations would decrease the

rate of pulmonary decline, thus increasing the quality of life for CF patients and may enable patients to live longer.

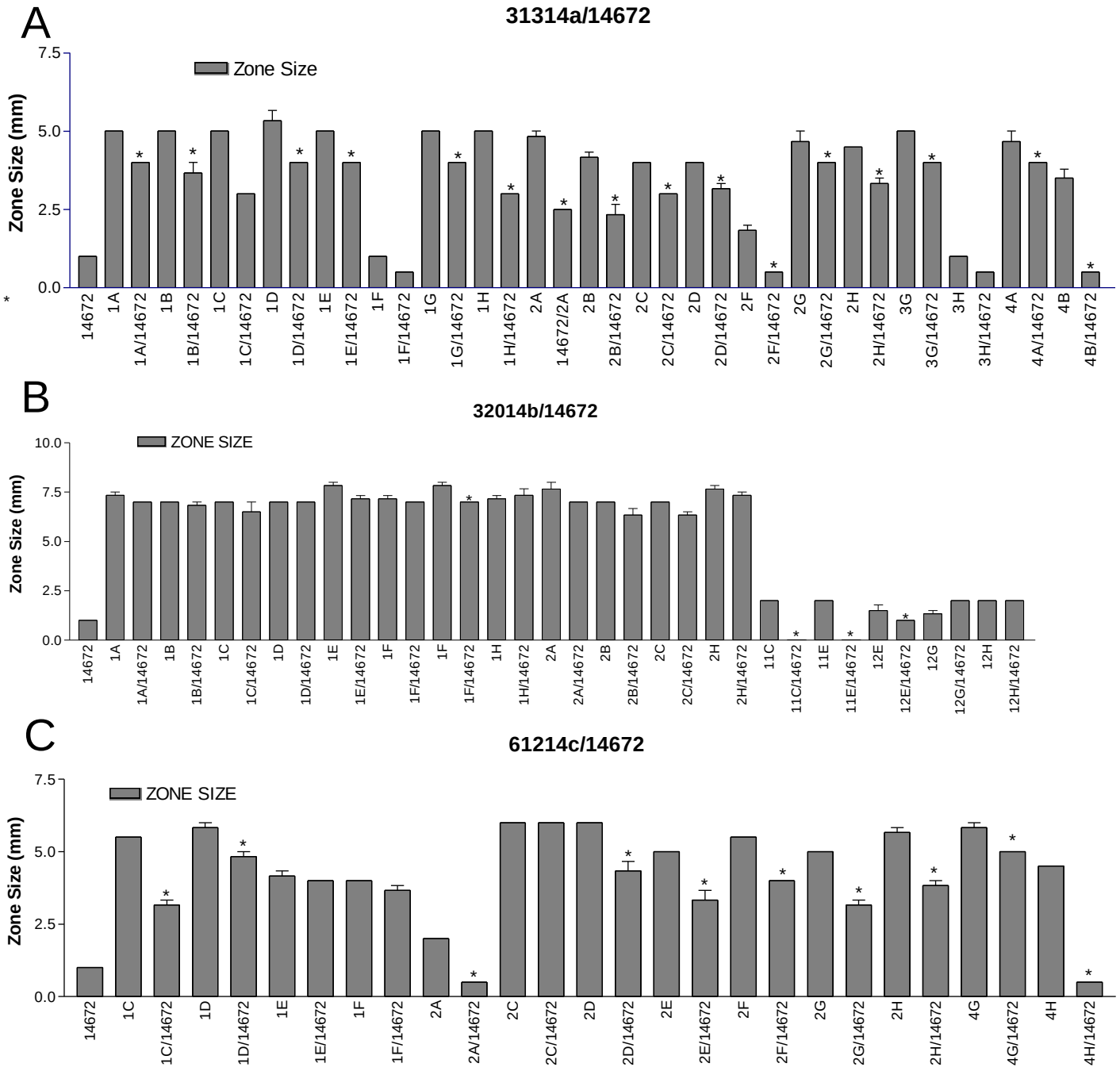

Figure 3: Response of *P. aeruginosa* isolates from adult patient to antagonistic *P. aeruginosa* strain 14672. Statistically significant decreases in protease production are indicated with an asterisk. Responding isolates are observed in within each patient.

(A): patient 31314a, 28 year old male, exhibits responding isolates to the 14651 signal when co-cultured with 1A,1B,1C,1D,1E,1G,1H, 2A,2B,2C,2D,2F,2G,2H,3G4A, and 4B

(B): patient 32014b, 41 year old male, exhibits responding isolates to the 14651 signal when co-cultured with 11C, 11E, and 11E.

(C): patient 61214c, 22 year old male, exhibits responding isolates to the 14651 signal when co-cultured with 1C, 1D, 2A, 2D, 2E, 2F, 2G, 2H, 4G, and 4H.

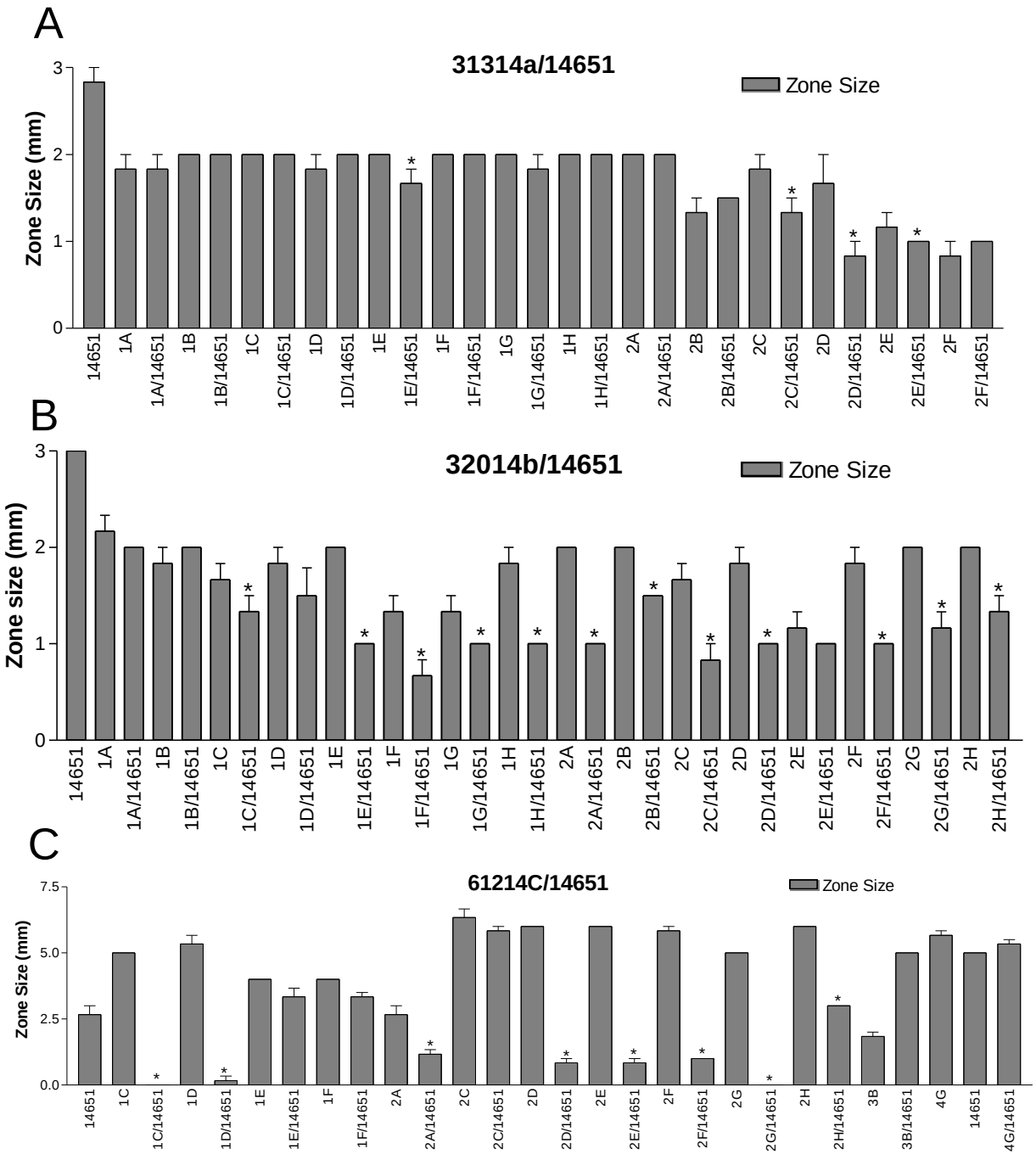

Figure 4: Response *P. aeruginosa* isolates from adult patient to antagonistic *P. aeruginosa* strain 14651. Statistically significant decreases in protease production are indicated with an asterisk. Responding isolates are observed in within each patient.

(A): patient 31314a, 28 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 1E, 2C, 2D, and 2E.
(B): patient 32014b, 41 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 1C, 1E,1F,1G,1H,2A,2B,2C,2D,2F,2G,and 2H.
(C): patient 61214c, 22 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 1C, 1D, 2A, 2D, 2E, 2F, 2G, and 2H.

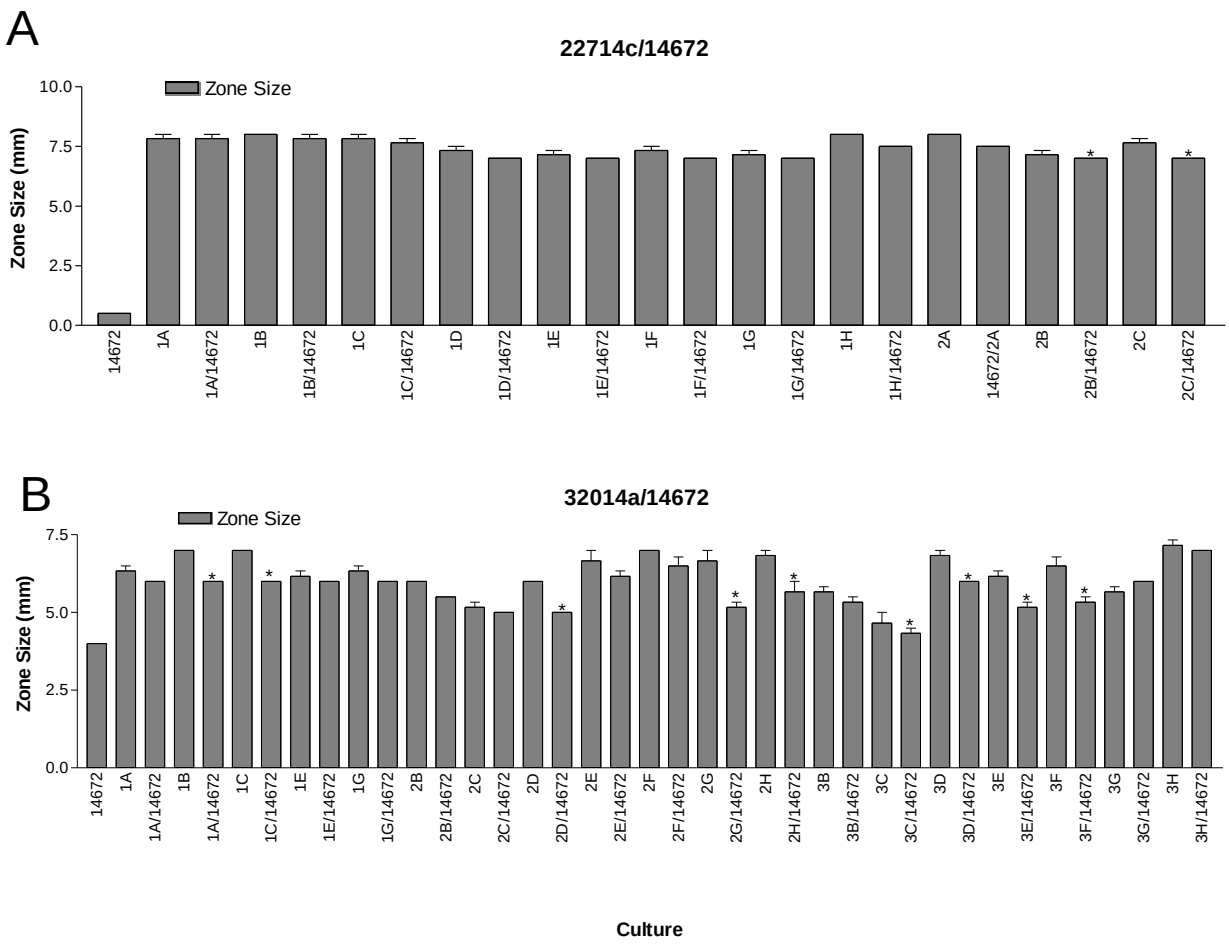

Figure 5: Response of *P. aeruginosa* isolates from child (A) and adolescent (B) patients to antagonistic *P. aeruginosa* strain 14672. Statistically significant decreases in protease production are indicated with an asterisk. Responding isolates are observed within each patient.

(A): patient 22714c, 12 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 2B and 2C.
(B): patient 32014a, 16 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 1B, 1C, 2D, 2G, 2H, 3C, 3D, 3E, and 3F.

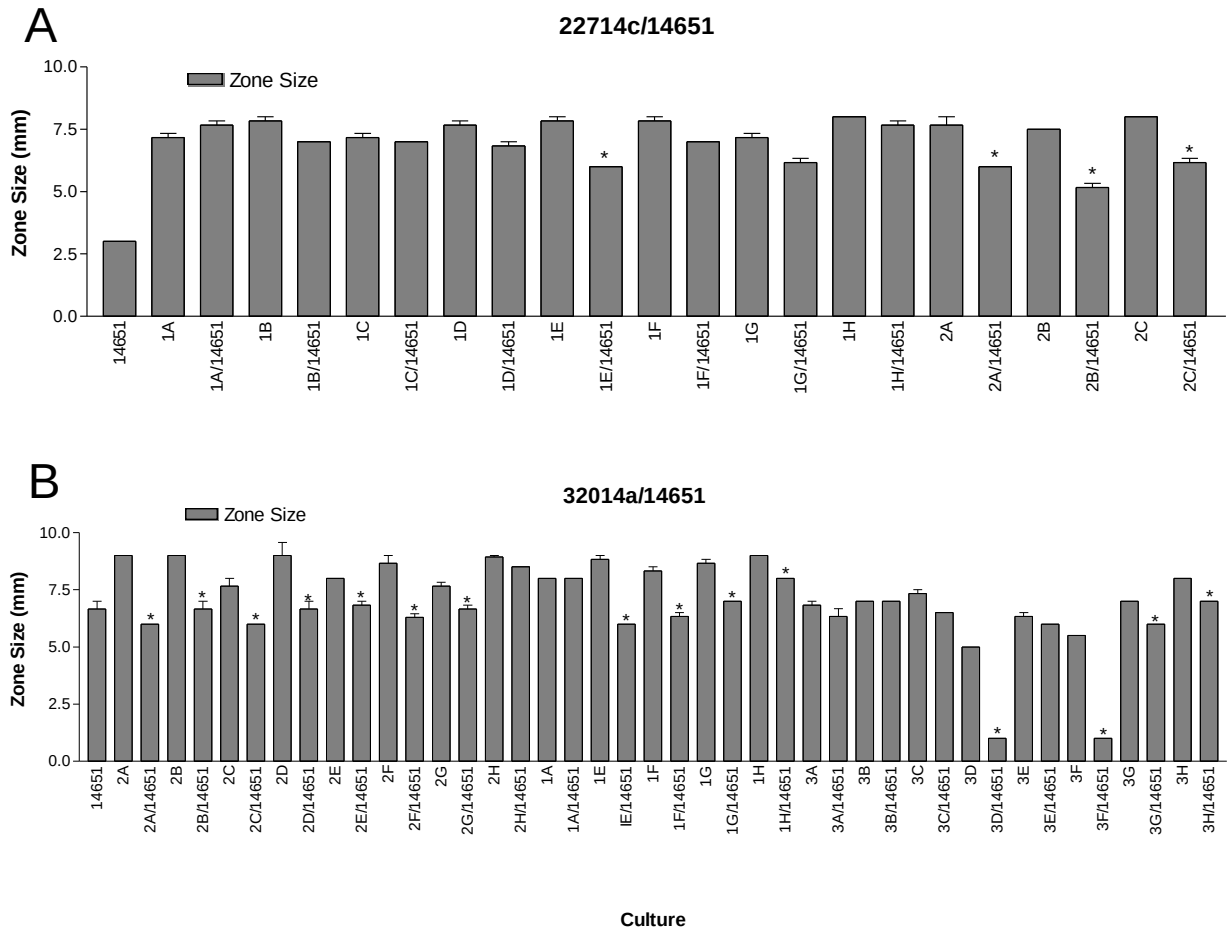

Figure 6: Response of *P. aeruginosa* isolates from child (A) and adolescent (B) patients to antagonistic *P. aeruginosa* strain 14651. Statistically significant decreases in protease production are indicated with an asterisk. Responding isolates are observed within each patient.

(A): patient 22714c, 12 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 1B, 1D, 1E, 1F, 1G, 2A, 2B, and 2C.
(B): patient 32014a, 16 year old male, exhibits responding isolates to the 14672 signal when co-cultured with 2A, 2B, 2C, 2D, 2E, 2F, 2G, 1E, 1F, 1G, 1H, 3D, 3F, 3G, and 3H.

Conclusions and Future Directions

In conclusion, CF isolate 14651 and 14672 both produce a signal that decreases the protease production by other *P. aeruginosa* CF isolates. We've found responding isolates from patients of every age group that respond to the 14651 and 14672 antagonistic signal. There is variability in the response of CF isolates to the signal within a patient and between patients. The reduction of protease production suggests that these novel antagonistic signals reduce the virulence of certain *P. aeruginosa* isolates and therefore has the potential to be developed in the future as an alternate therapeutic target to decrease the severity of infections. In future studies, the genomes of both 14651 and 14672 will be sequenced to determine what genes make these two variants unique from other *P. aeruginosa* isolates. Gene expression studies of *lasA* and *lasB* protease genes using qRT-PCR should be utilized to determine the alteration of virulent stains when exposed to 14651 and 14672 signaling.

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