ExoU Expression in Clinical Isolates of *Pseudomonas aeruginosa*

Background

Pseudomonas aeruginosa (Pa) is a Gram-negative bacterium present in high-human contact environments.[1] Pa infectious are typically nosocomial, or hospital-acquired, and most hosts typically have co-morbidities such as immunodeficiencies or cystic fibrosis (CF).[2] Pa is also associated with ventilator-associated pneumonia patients, as well as infections from urinary catheters or of post-surgical wounds due to its near-ubiquitous prevalence in the environment and arsenal of resistance mechanisms.[2,3] Pa can establish chronic infections in the mucus secreted into the lower respiratory tract of CF patients, where the bacteria often adapt to this environment by reducing or entirely shutting off expression of prominent virulence factors, such as ExoU secretion.[2]

The Pa bacterium has genes expressing many virulence factors that enhance its infection, spread, and persistence in the typical environment of the respiratory system. The type-III secretion system (T3SS) is a needle-like apparatus responsible for transducing effector molecules across the host cell membrane.[3] These effector molecules are ExoU, a prominent cytotoxin that causes rapid and complete apoptosis, ExoS, ExoY, and ExoT, which produce a less cytotoxic phenotype.[2] Most strains of Pa contain portions or the entirety of genes required for T3SS, but only an estimated 44-77% of strains secrete effector proteins.[4]

Any secretion from the T3SS increases the risk of mortality and severe infection.[4,5] Pa strains typically express either ExoU or ExoS, with very few strains expressing both, and most expressing various combinations of ExoY and/or ExoT.[2] ExoS secretors typically make up the majority of strains in case study populations, accounting for approximately 52-75% of a case study's population.[4–6] Pa preferentially targets phagocytes within the lung, with its main target being macrophages and neutrophils.[7] The T3SS allows Pa to inject cytotoxins into these cells to prevent immune interference in Pa colonization.[7] ExoU is a cytotoxin with phospholipase activity that can cause immune cell lysis, protecting the bacterium from phagocytosis and reducing the cytokine and chemokine signals released by these cells.[2,7] ExoU secretion is associated with higher virulence, severe disease, and mortality in clinical settings.[3,6]

Goals and Significance

Pseudomonas aeruginosa strains are growing increasingly resistant to antibiotics, with some strains resistant to even drugs of last resort like carbapenem.[8] The ability of the bacterium to form biofilms within the respiratory tract and efflux pumps that can effectively counteract antibiotic treatment make it increasingly difficult to treat Pa infections.[2] Pa infections account for 20% of all ICU pneumonias and is a significant mortality factor for cystic fibrosis patients.[7] The mortality rate for Pa infection ranges from 33-61%, making it a pathogen of interest for optimized clinical treatment strategies.[9]

Genetic analysis of clinical strains can have an impact on treatment course, as ExoU expression can be predicted from the bacterial genome its expression is often an indicator for more severe clinical disease.[7] This project aimed to study 31 Pa strains isolated from clinical samples and test whether they expressed ExoU. The clinical strains were collected from patients hospitalized at Northwestern University from the period of 2018-2022 with Pa pneumonia. The strains were previously sequenced and analyzed for the presence of T3SS genes by Travis Kochan and Sophie Nozick, members of the Hauser Laboratory.

Methodology

An Excel file containing clinical outcomes and genetic information on 32 strains of Pa collected from Northwestern Hospital was obtained from Nozick. One strain, 1653, was excluded because it was not properly annotated. Another strain, 1466-IPA0-09, had clinical data but no genetic data, so it does not have a predicted ExoU site. A graphic depiction of the Western blot sample preparation is depicted in Figure 1. Samples were prepared by growing overnight plates and then subsequent overnight liquid cultures of each Pa isolate in LB media. The liquid cultures were sub-cultured for three hours at 1:50 in fresh LB, 1M $MgCl_2$ and 0.5M EGTA. The $MgCl_2$ addition prompted type III secretion, if possible, in each strain.[10] The bacteria were then pelleted, and the culture supernatant containing any T3SS effector proteins was isolated and purified using a 0.22-micron filter and overnight precipitation in TCA. The protein precipitate was spun at max speed for 45 minutes, then aliquoted to smaller tubes after three acetone washes. The protein product was resuspended in 1X Loading Buffer and BME, then heated and stored properly for Western Blotting.

Western blots were run using SDS-PAGE gels with 20 µL of sample per well. I experienced some initial errors in loading the gels, as seen in Figure 4, where the earlier wells leaked or overflowed, creating inconsistent results on the first few lanes of these gels. Blots were run at 100 and 150 V for approximately 10 and 45 minutes, respectively. The gels were transferred onto a membrane using transfer buffer at 100 V for 50 minutes, then blotted with a primary anti-ExoU antibody purified by Nozick. A secondary goat antibody was used, along with TBS-T washes, to complete gel processing. The gels were imaged for 2 minutes at 700 nm absorbance, and results can be seen in Figures 1-5.

Results and Figures

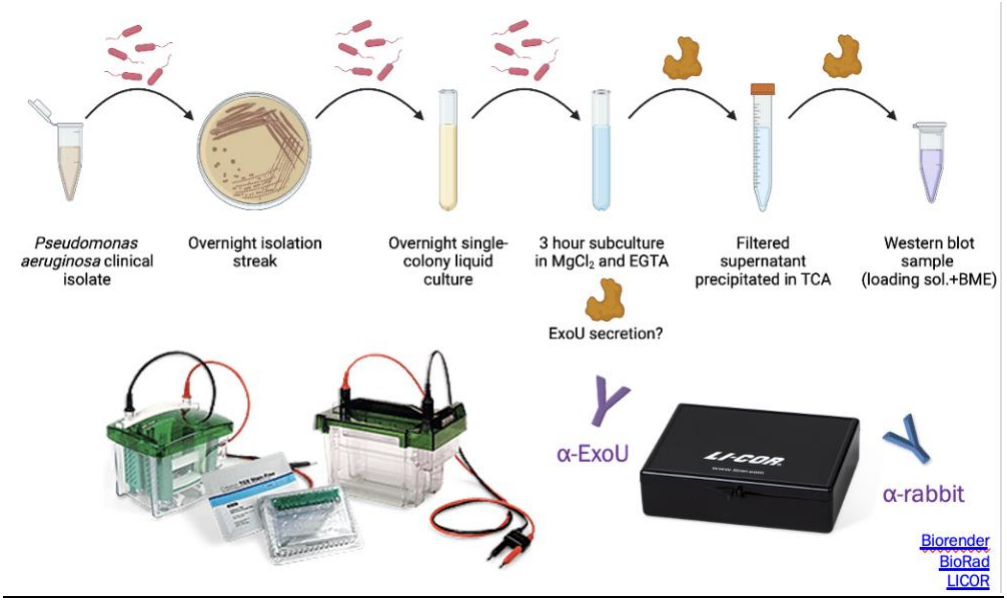

Figure 1 Western blot sample preparation and visualization protocol. Made in Biorender.

Clinical isolates were streaked for single colony isolation, then challenged with $MgCl_2$ to induce type-III secretion of ExoU. The bacterial supernatant was prepared and run on an SDS-PAGE gel, then transferred to a membrane for visualization. ExoU secretion was detected using anti-ExoU primary antibody and anti-rabbit goat secondary antibody.

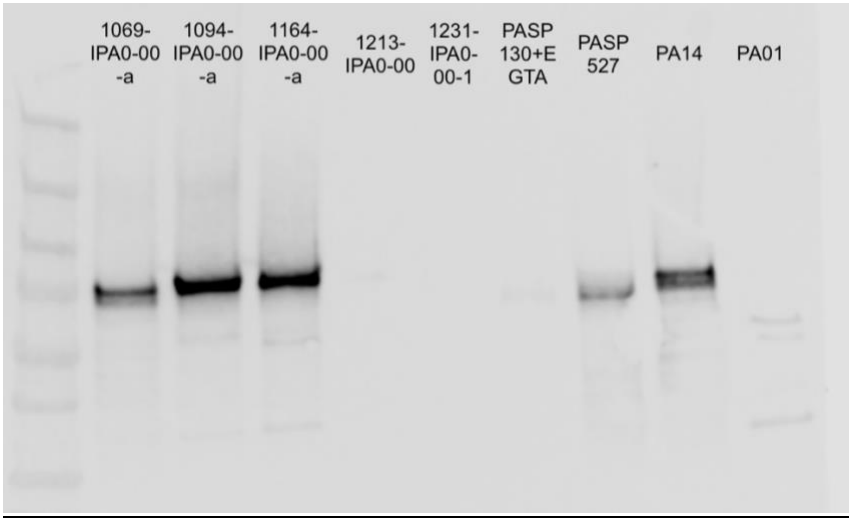

Figure 2: Western Blot of five clinical samples with additional samples from Aliki Valdes (PASP 130 + EGTA and PASP 527), along with positive (PA14) and negative (PA01) controls.

The clinical isolates 1069-IPA0-00-a, 1094-IPA0-00-a and 1164-IPA0-00-a are predicted ExoU secretors, which is supported by the appearance of bands in this Western blot.

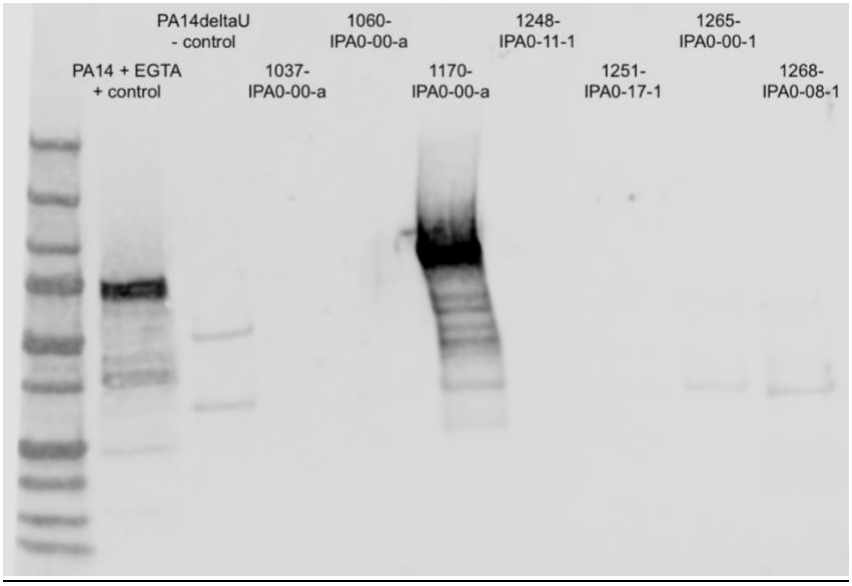

Figure 3: Western Blot of seven clinical isolates with positive (PA14+EGTA) and negative (PA14ΔU) controls.

The clinical isolate 1170-IPA0-00-a is a predicted ExoU secretor, which is supported by the appearance of a band in this Western blot.

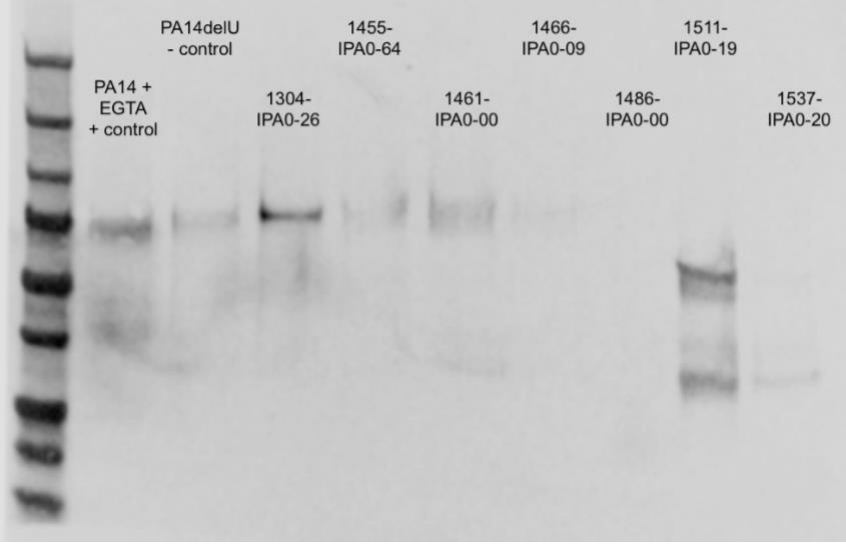

Figure 4: Western blot of clinical isolates with poor positive and negative controls.

The controls for this blot were likely incorrectly pipetted, creating overflow of the positive control sample into the first three wells. These samples were run again on a different gel.

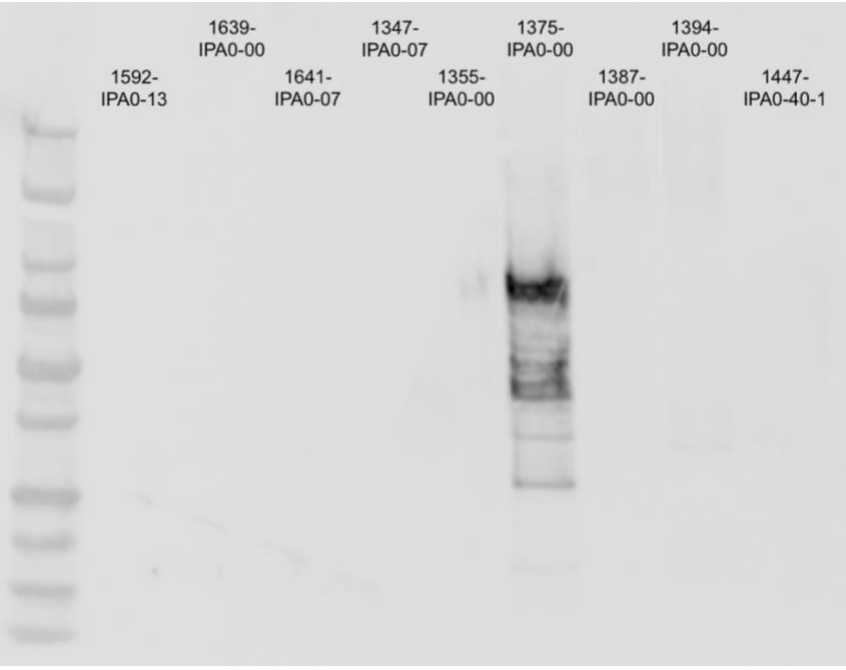

Figure 5: Western Blot of clinical strains featuring ambiguous result for strain 1375-IPA0-00.

This blot was run in succession with the blot shown in Figure 3, so only one set of positive and negative controls was used. While it initially seemed that 1375-IPA0-00 was expressing ExoU, a subsequent blot performed with a new sample preparation showed that these markings are likely artifacts.

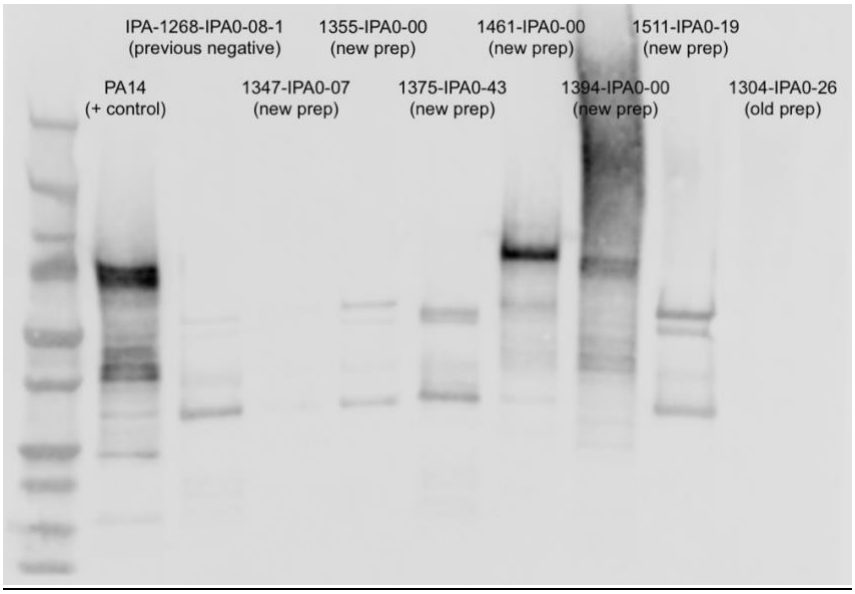

Figure 6 Western blot of seven clinical isolates with positive and negative controls.

The clinical isolates 1461-IPA0-00 and 1394-IPA0-00 are predicted ExoU secretors, which is supported by the appearance of bands in this Western blot.

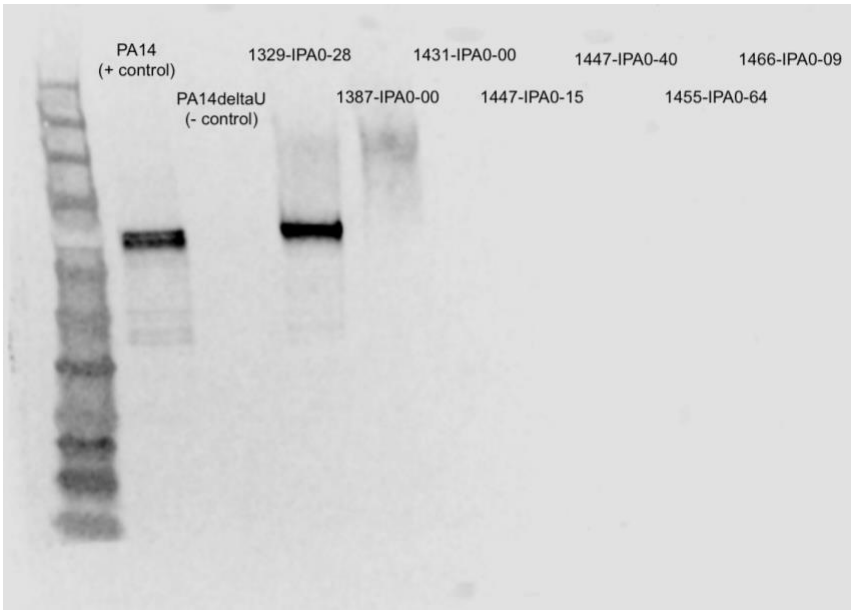

Figure 7 Western blot of seven clinical isolate samples with positive and negative controls.

The clinical isolate 1329-IPA0-28 is a predicted ExoU secretor, which is supported by the appearance of a band in this Western blot.

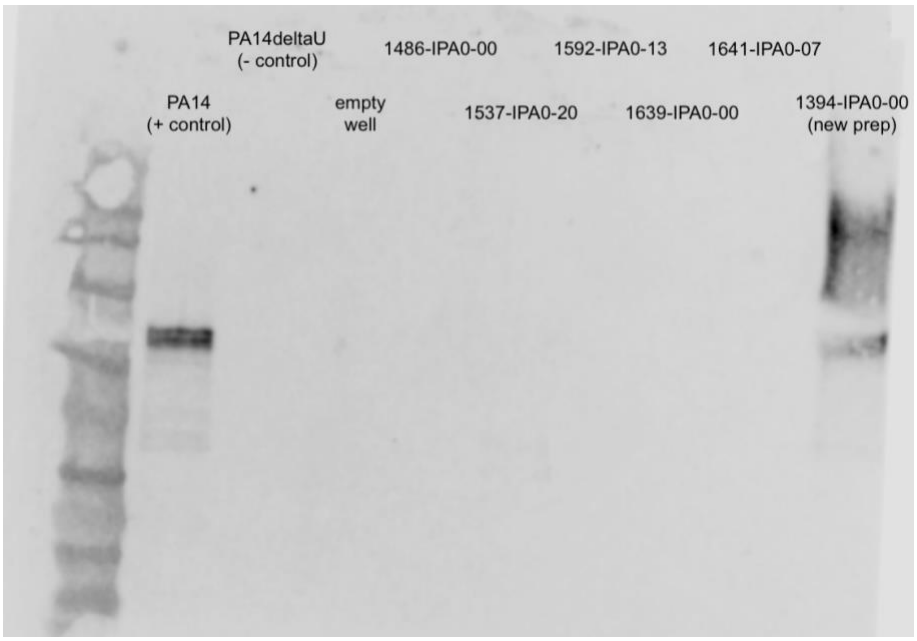

Table 1: Predicted and Measured ExoU Secretion for 31 Clinical Isolates of Pa

The 31 clinical isolate's IDs are listed below with their predicted effector secretion of either ExoU or ExoS. All of the predicted ExoU secretors, which made up 25.8% of the total samples, were positive for ExoU expression. A clinical outcome was recorded of either a cure, with a success score of 1, or an expiration, persistence, or indeterminate result resulting in a success score of 0. Indeterminate results did not meet either criteria for persistence or cure.

Strain ID	Predicted Effector (ExoS or ExoU)	ExoU WB Result (ExoU + or -)	Clinical Outcome	Success (1 or 0)
1037-IPA0-00-a	ExoS	-	Persistence	0
1060-IPA0-00-a	ExoS	-	Persistence	0
1069-IPA0-00-a	ExoU	+	Persistence	0
1094-IPA0-00-a	ExoU	+	Expired	0
1164-IPA0-00-a	ExoU	+	Cure	1
1170-IPA0-00-a	ExoU	+	Cure	1
1213-IPA0-00	ExoS	-	Cure	1
1231-IPA0-00-1	ExoS	-	Cure	1
1248-IPA0-11-1	ExoS	-	Cure	1
1251-IPA0-17-1	ExoS	-	Cure	1
1265-IPA0-00-1	ExoS	-	Cure	1
1268-IPA0-08-1	ExoS	-	Expired	0
1304-IPA0-26	ExoS	-	Cure	1
1329-IPA0-28	ExoU	+	Persistence	0

1347-IPA0-07	ExoS	-	Indeterminate	0
1355-IPA0-00	ExoS	-	Persistence	0
1375-IPA0-43	ExoS	-	Cure	1
1387-IPA0-00	ExoS	-	Expired	0
1394-IPA0-00	ExoU	+	Expired	0
1431-IPA0-00	ExoS	-	Indeterminate	0
1447-IPA0-15	ExoS	-	Indeterminate	0
1447-IPA0-40-1	ExoS	-	Cure	1
1455-IPA0-64	ExoS	+	Persistence	0
1461-IPA0-00	ExoU	+	Indeterminate	0
1466-IPA0-09	Not given	-	Cure	1
1486-IPA0-00	ExoS	-	Cure	1
1511-IPA0-19	ExoS	--	Persistence	0
1537-IPA0-20	ExoS	-	Persistence	0
1592-IPA0-13	ExoS	-	Cure	1
1639-IPA0-00	ExoS	-	Cure	1
1641-IPA0-07	ExoS	-	Indeterminate	0
	Total ExoU Secretors:	**8**	**Total Positive Outcomes:**	**14 (45.2%)**

Table 2: Clinical Outcome for 31 Strains of *Pseudomonas aeruginosa* with Genetic Data

	Cure (1)	**Persistence (0)**	**Expired (0)**	**Indeterminate (0)**	**Total Samples**	**Poor Outcomes (Total 0)**
ExoU	2	3	2	1	**8** (25.8%)	**6** (75%)
ExoS	12	5	2	4	**23** (74.2%)	**11** (47.8%)
Total	**14** (45.2%)	**8** (25.8%)	**4** (12.9%)	**5** (16.1%)	**31**	**17** (54.8%)

Conclusions and Future Work

Western blotting analysis confirmed the genetic prediction of ExoU expression for this set of clinical isolates. In this dataset, the ExoU secretors had a greater percentage of poor clinical outcomes (75%) when compared to the predicted ExoS secretors (47.8%). Future work

will include purifying an antibody for the detection of ExoS secretion in these clinical isolates, which will also enable quantification of active or inactive T3SS.

My future thesis work will focus on characterizing bacteria extracted from previously healthy children with a rare presentation of Pa infection characterized as Shanghai fever. These clinical isolates can provide a good control population for comparison against the clinical samples isolated from Shanghai fever patients.

References

1. Crone S, Vives-Flórez M, Kvich L, et al. The environmental occurrence of Pseudomonas aeruginosa. *APMIS*. 2020;128(3):220-231. doi:10.1111/apm.13010

2. Gellatly SL, Hancock REW. *Pseudomonas aeruginosa* : new insights into pathogenesis and host defenses. *Pathog Dis*. 2013;67(3):159-173. doi:10.1111/2049-632X.12033

3. Hauser AR. The type III secretion system of Pseudomonas aeruginosa: infection by injection. *Nat Rev Microbiol*. 2009;7(9):654-665. doi:10.1038/nrmicro2199

4. Hauser AR, Cobb E, Bodi M, et al. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa. *Crit Care Med*. 2002;30(3):521-528. doi:10.1097/00003246-200203000-00005

5. Roy-Burman A, Savel RH, Racine S, et al. Type III protein secretion is associated with death in lower respiratory and systemic Pseudomonas aeruginosa infections. *J Infect Dis*. 2001;183(12):1767-1774. doi:10.1086/320737

6. Peña C, Cabot G, Gómez-Zorrilla S, et al. Influence of virulence genotype and resistance profile in the mortality of Pseudomonas aeruginosa bloodstream infections. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2015;60(4):539-548. doi:10.1093/cid/ciu866

7. Diaz MH, Hauser AR. *Pseudomonas aeruginosa* Cytotoxin ExoU Is Injected into Phagocytic Cells during Acute Pneumonia. *Infect Immun*. 2010;78(4):1447-1456. doi:10.1128/IAI.01134-09

8. Buehrle DJ, Shields RK, Clarke LG, Potoski BA, Clancy CJ, Nguyen MH. Carbapenem-Resistant Pseudomonas aeruginosa Bacteremia: Risk Factors for Mortality and Microbiologic Treatment Failure. *Antimicrob Agents Chemother*. 2016;61(1):e01243-16. doi:10.1128/AAC.01243-16

9. El-Solh AA, Hattemer A, Hauser AR, Alhajhusain A, Vora H. CLINICAL OUTCOMES OF TYPE III PSEUDOMONAS AERUGINOSA BACTEREMIA. *Crit Care Med*. 2012;40(4):1157-1163. doi:10.1097/CCM.0b013e3182377906

10. Zhu M, Zhao J, Kang H, et al. Modulation of Type III Secretion System in Pseudomonas aeruginosa: Involvement of the PA4857 Gene Product. *Front Microbiol*. 2016;7:7. doi:10.3389/fmicb.2016.00007