The Interplay of Antibiotic Resistance and Virulence Attenuation in *Acinetobacter baumannii*: Profiling Alterations in Pathogenicity in Response to Antibiotic Pressure Over 14 Days

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ABSTRACT

Purpose: In immunocompromised patients with severe bloodstream infections due to *Acinetobacter baumannii* (AB), there is a paucity of data regarding the interplay between the emergence of antibiotic resistance and virulence capacity. The objective was to analyze the relationship between resistance and virulence of AB when under selective antimicrobial pressure of the last-line-of-defense therapy Polymyxin B (PB), using the non-mammalian *Galleria mellonella* waxworm model.

Methods: AB isolates were taken from a strain with a demonstrated MIC baseline of 1.0 and resistant to all carbapenems, at pre-Polymyxin B therapy (0h) and during therapy (24hr, 48hr, 72hr, 96hr, 336hr). 20 waxworms were injected with 10^5CFU/ml of each isolate into the right pro-leg. Controls: 20 waxworms were injected with phosphate-buffered-saline; 20 waxworms were not inoculated. Waxworms were incubated at 37˚C and mortality was accounted for daily over 6 days. Kaplan Meier survival analysis was conducted to examine statistical differences, with $p<0.05$ defined as significant.

Results: The greatest mortality of G. *mellonella* occurred in the 0hr, 24hr, and 48hr isolates, which had mortality rates of 80%(16/20), 95%(19/20), and 100%(20/20) respectively. After 6 days, the 72hr, 96hr, and 336hr inoculates had mortality rates of 70%(14/20), 45%(9/20), and 20%(4/20). In the 24hr group, 14 waxworms deceased after day 1 while the first waxworm in the 336hr group died on day 3. The PB MICs revealed sequential increases in drug resistance after prolonged drug exposure. The 0hr isolate had a susceptible MIC of 1.0 whereas the 336hr isolate had a resistant MIC greater than 64.

Conclusion: The increasing MIC values and diminished mortality rates revealed that PB antibiotic exposure counter selected for resistance in AB over 336hrs, attenuating virulence capacity. Our data may have significant implications for severely ill patients treated with prolonged courses of antibiotics and highlight the significance of the interplay between antibiotic resistance and virulence.

BACKGROUND

- *A. baumannii* is an opportunistic infection which can cause hospital acquired pneumonia, sepsis, urinary tract infections, hospital acquired meningitis and other soft tissue and bone infections.
- *A. baumannii* has a developed survival mechanisms which contribute to the development of drug resistance, consequently potentiating hospital outbreaks . This has posed therapeutic challenges as resistance increases in last-line-of-defense therapy.
- The *A. baumannii strain 03-149-01* demonstrated resistance to all carbapenems, but was susceptible to Polymixin B and Colisitn, at pre-Polymixin B therapy (0hr) with a MIC of 1.0.
- The non-mammalian *G. mellonella* (waxworms) is a commonly accepted model to showcase virulence factors. *G. mellonella* can be used an in vitro model because they posses both humoral and cell mediated immune response pathways, the inoculum can be administered precisely via injection, and they can withstand incubation at 37˚C.
- The collateral impact of antibiotic resistance on virulence is unclear. This is particularly important to consider in multidrug resistant strains such as *A. baumannii* 03-149-01

OBJECTIVES

1. To define the relationship between the emergence of polymyxin B resistance and the pathogenicity of *A. baumannii*.

METHODS

1. *A. baumannii* strain 03-149-1 was front loaded with 3.61ug/ml of Polymyxin B at 0hr. Simulated pharmacokinetic profiles of antibiotics were assessed using the in vitro Hollow Fiber Infection Model for a duration of 14 days (336 hours).
2. Isolates were taken during incubation at 0hr, 24hr, 48hr, 72hr, 96hr, 144hr, 192hr, 240hr, 288hr, and 336hr. The 0hr isolate was plated on Mueller Hinton Agar while the remaining isolates were brought out on Polymyxin B 10mg/ml plates. MICs were evaluated for each isolate.
3. To purify the isolate, each was centrifuged (2,000g for 5 minutes) and washed with normal saline twice.
4. The virulence of the strains were tested using a *G. mellonella* (waxworm) assay. Ten microliters of each *A. baumannii* time isolate, at 10^7CFU/ml, was injected into the right pro-leg of each waxworm. Each waxworm was ultimately injected with a ~10^5 CFU/ml inoculum.
5. Twenty waxworms were used for every experimental group and were incubated at 37˚C after injection; waxworm mortality was accounted for once a day for 6 consecutive days. Waxworms were considered deceased if there was no response to touch
6. Two control groups were used as reference in conjunction with each experiment (no injection and normal saline injection. The results were discarded if more than 3 waxworms died in either control group. The 0hr, 24hr, 48hr, 72hr, 96hr, and 336hr isolates were completed in triplicate and the 144hr, 192hr, 240hr, and 288hr isolates were completed in duplicate.

Figure 1. Petri dish containing 10 *G. mellonella* waxworms during virulence assay.

RESULTS

Figure 2. Kaplan-Meier curves for waxworm survival comparing virulence of *A. baumannii* (AB) extracted sequentially from the Hollow Fiber Infection Model . **A.** The 0hr isolate represents the virulence of unexposed (polymyxin B susceptible) AB. **B, C, D, and E** represent 24hr, 48hr, 72hr, and 96hr exposure to polymyxin B respectively. The virulence among these groups was statistically significant as seen through Kaplan-Meier analysis ($p<0.001$). **F., G., H., I., and J.,** represent virulence in AB at 144hrs, 192hrs, 240hrs, 288hrs, and 336hrs respectively. Survival time following injection was significantly longer than with the 0hr-96hr isolates ($p<0.001$).

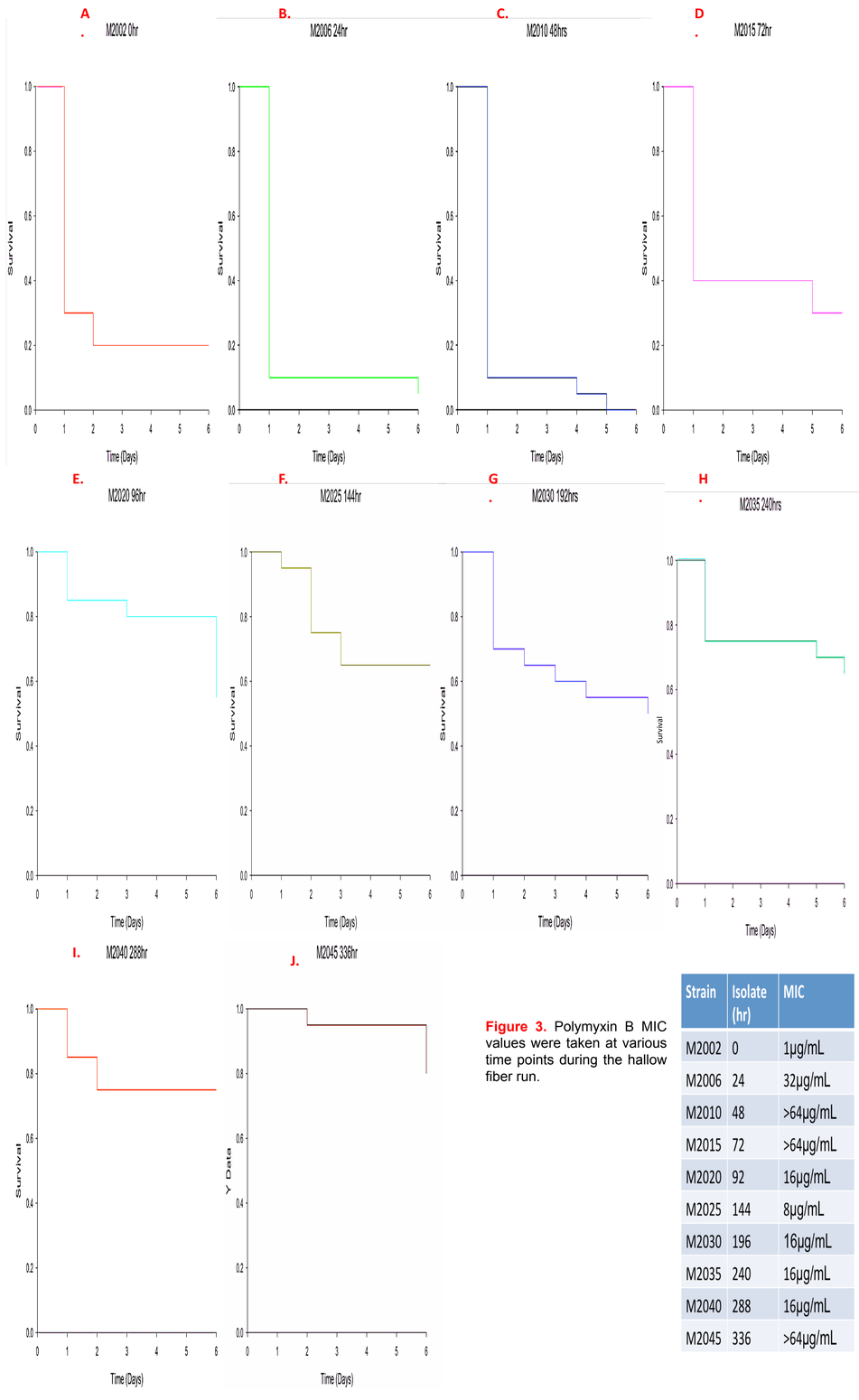

Figure 3. Polymyxin B MIC values were taken at various time points during the hallow fiber run.

Strain	Isolate (hr)	MIC
M2002	0	1µg/mL
M2006	24	32µg/mL
M2010	48	>64µg/mL
M2015	72	>64µg/mL
M2020	92	16µg/mL
M2025	144	8µg/mL
M2030	196	16µg/mL
M2035	240	16µg/mL
M2040	288	16µg/mL
M2045	336	>64µg/mL

1. Polymyxin B 10mg/ml exposure in the Hollow Fiber contributed to the development of resistance; the MIC at the 0hr time point was 1µg/mL, compared to the 336hr time point where the MIC was greater then 64µg/mL.
2. The lowest survival rate of 0% was seen in the 48hr isolate group, while the highest survival rate of 80% was seen in the 336hr isolate group, $p<0.001$.
3. Following intense polymyxin B selection pressure at 10mg/L, the isolates exposed in the Hollow Fiber Infection Model for ≥24hrs were resistant by the time of injection into the waxworms for the virulence assays.
4. Comparing the inocula used for the virulence assay, there were no statistically significant differences between any experimental or control groups.
5. In the virulence assay, the 0hr, 24hr, 48hr, 72hr, 96hr, 144hr, and 192hr isolates all had significantly lowered survival rates ($p<0.001$ for 0hr-92hr isolates, $p<0.05$ 144hr and 192 isolates).
6. Survival rates of waxworms injected with the 240hr, 288hr, and 336hr isolates did not show significant results when compared to the control group, $p>0.05$.

CONCLUSIONS

1. The development of resistant *A. baumannii* emerged as more time was spent in the Hollow Fiber Infection Model due to increasing polymyxin B exposure.
2. The emergence of polymyxin B resistance had a significant effect on the virulence of *A. baumannii*. The increasing polymyxin B MICs counter selected for resistance, which attenuated virulence capacity.
3. Overall, these findings could play a vital role in the pharmacotherapy of patients in the ICU with persistent infection. As prolonged antibiotic regimes may be necessary treatment, there still presents the risk of an attenuated strain due to the interplay between resistance and virulence.

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