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# Effects of Glycyrrhizin on a Drug Resistant Isolate of *Pseudomonas aeruginosa*

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## Abstract

**Purpose:** This study aimed to characterize a non-ocular drug resistant isolate (RS1) of *Pseudomonas aeruginosa* and test its susceptibility to glycyrrhizin (GLY).

**Methods:** Antibiotic sensitivity of RS1 was tested using a HARDYISK™ antimicrobial sensitivity test. Type III toxins were amplified using polymerase chain reaction (PCR). GLY minimum inhibitory concentration (MIC) and assays for its effects on: time kill and biofilm/adhesion were done. Bacterial proteomic profiles were analyzed using mass spectroscopy. *In vivo*, C57BL/6 (B6) mice were infected with RS1 and treated with GLY. Clinical score, photography with a slit lamp, myeloperoxidase (MPO) assay, bacterial plate count, real time (RT) PCR and enzyme-linked immunosorbent assay (ELISA) were used to assess treatment effects.

**Results:** Amplification of virulence genes (*exoS*, *T* and *U*) was seen in RS1. MIC of GLY was 40 mg/ml and was bacteriostatic for RS1. Proteomics revealed significant differences between RS1 and PAO1 (control), and included significant increase in expression of outer membrane, efflux pump and fimbrial proteins. GLY significantly reduced RS1 biofilm formation and adherence to cultured mouse corneal epithelial (CMCE) and human corneal epithelial-transformed (HCET) cells compared to control (no GLY). *In vivo*, GLY vs phosphate-buffered saline (PBS)-treated RS1 infected corneas exhibited less disease, lower clinical scores, reduced neutrophil infiltrate and bacterial load. Treatment also reduced mRNA and protein levels of interleukin 1 beta (IL-1β), chemokine (C-X-C Motif) ligand 2 (CXCL2) and other proinflammatory molecules.

**Conclusions:** Results reveal that GLY is bacteriostatic for RS1, reduces bacterial growth, biofilm formation and adherence to mouse and human corneal epithelial cells, and improves disease outcome by reducing proinflammatory molecules, neutrophil infiltrate and plate count.

***Keywords:*** *Bacterial Keratitis; Drug Resistance; Pseudomonas; Mice; Glycyrrhizin*

## Abbreviations

*P. aeruginosa: Pseudomonas aeruginosa*; RS1: Non-Ocular Drug Resistant Isolate; GLY: Glycyrrhizin; PCR: Polymerase Chain Reaction; MIC: Minimum Inhibitory Concentration; B6 mice: C57BL/6 Mice; MPO Assay: Myeloperoxidase Assay; RT-PCR: Real Time Polymerase Chain Reaction; ELISA: Enzyme-Linked Immunosorbent Assay; CMCE cells: Cultured Mouse Corneal Epithelial cells; HCET cells: Human Corneal Epithelial Cells; PBS: Phosphate Buffered Saline; IL-1β: Interleukin 1 Beta; CXCL2: Chemokine (C-X-C Motif) Ligand 2; TFP: Type IV Pili; ARVO: Association for Research in Vision and Ophthalmology; ATCC: American Type Culture Collection; PTSB: Peptone Tryptic Soy Broth; p.i.: Postinfection; i.p.: Intraperitoneally; DEPC: Diethylpyrocarbonate; LiDS: Lithium Dodecyl Sulfate; BCA: Bicinchoninic Acid; cfu: Colony Forming Units; CV: Crystal Violet; MOI: Multiplicity of Infection; DTT: Dithiothreitol; OD: Optical Density; KBM: Keratinocyte Basic Me-

dium; BSA: Bovine Serum Albumin; HTAB: Hexadecyltrimethylammonium Bromide; M-MLV: Moloney-Murine Leukemia Virus; HMGB1: High Mobility Group Box 1; TLR: Toll-Like Receptor; NLRP3: NLR Family Pyrin Domain Containing 3; NLRC4:NLR Family CARD Domain Containing 4

## Introduction

*P. aeruginosa* produces numerous virulence factors, including toxins and enzymes [1] that contribute to the outcome of disease (e.g. keratitis). Toxins are secreted through various protein secretion systems (e.g. type I, III and VI) [2]. *P. aeruginosa* has type III toxin genes *exoS, exoT* and *exoU* that encode cytotoxins which are released by type III secretion system. These toxins are associated with the ability to evade the host response [3,4], bacterial dispersal [3] and inhibition of host DNA synthesis [5]. Other physiological features of the bacterium including adhesion and biofilm formation are regulated by additional virulence factors (e.g. type IV pili (TFP) [6,7] and quorum sensing systems [8,9]) and together with cytotoxicity/invasiveness, contribute to disease pathogenesis [3,10]. In this regard, in order to establish an infection, bacteria must first colonize the host [11]. Colonization is preceded by adherence, an important first step for initiating disease and is the first step in biofilm formation. As the biofilm matures, it consists of a complex polymeric matrix [12,13]. These biofilms shield the bacteria from host recognition [14,15] and support resistance to antimicrobials [16]. All of these are rationale to develop better antimicrobials for treatment [17,18].

This problem of resistance is global [19,20]. Specifically, there is an increasing trend in Gram negative multi drug resistant (MDR) bacterial infections viewed with serious concern, as there is a lack of effective antibiotics against them and few antibiotics in the pipeline [21].

*P. aeruginosa* induces infection of the cornea that is difficult to treat, particularly if the isolate is drug resistant [22,23]. The disease progress is rapid, evoking a vigorous inflammatory response in the cornea, which itself may contribute to corneal destruction [24]. Pertinent to treatment of experimentally induced antibiotic susceptible *P. aeruginosa* keratitis, we recently showed [25-27] that glycyrrhizin (GLY), a glycoconjugated triterpene, extracted from *Glycyrrhiza glabra* is both anti-inflammatory and antibacterial, decreasing disease pathogenesis.

Nonetheless, despite these encouraging data, there is no information regarding the effects of GLY on antibiotic resistant *P. aeruginosa* keratitis. Therefore, the present study tested a non-ocular drug resistant clinical isolate (RS1), which when tested showed intermediate/susceptible sensitivity to gentamicin, and piperacilin/tazobactam, intermediate resistance to meropenem and resistance to trimethoprim/sulfamethazole. The type III secretion system gene amplification of RS1 was tested next and then we tested the effects of GLY on MIC, time kill, adhesion and biofilm formation. Proteomic analysis was used to comparatively characterize the proteomic profile of RS1 to PAO1, a common non-MDR strain. *In vivo* studies tested whether GLY was effective in the treatment of RS1 induced keratitis in B6 mice. Evidence shows that GLY treatment is bacteriostatic for RS1, decreases adhesion/biofilm and *in vivo*, reduces disease by downregulation of the neutrophil infiltrate, bacterial plate count and proinflammatory molecules. These results suggest that GLY is effective against RS1 and may provide an alternative to treat drug resistant strains when antibiotics are no longer effective.

## Materials and Methods

### Mice

C57BL/6 (B6) mice (8 weeks of age) were purchased (Jackson Laboratory Bar Harbor, ME, USA), and National Institutes of Health guidelines for husbandry were followed. All animal usage complied with ARVO guidelines.

### Microbial Culture and Corneal Infection

*P. aeruginosa* including: RS1 isolate (Detroit Medical Center, Detroit, MI, USA), a clinical isolate KEI 1025 (Kresge Eye Institute, Detroit, MI, USA); strains 19660, 19142 and PAO1 [American Type Culture Collection (ATCC) Manassas, VA, USA]; were grown in peptone tryptic soy broth (PTSB) medium at 37°C in a rotary shaker water bath at 150 rpm for 18 h to an OD (measured at 540 nm) between 1.3 - 1.8. Bacteria were pelleted by centrifugation at 5500xg for 10 minutes, washed once with sterile saline, recentrifuged, resuspended, and di-

luted in sterile saline (0.85%, pH=7.4). A HARDYISK™ antimicrobial sensitivity test (Hardy Diagnostics, Santa Maria, CA, USA) was used for semi quantitative *in vitro* susceptibility testing of RS1 by the agar diffusion test procedure (Kirby-Bauer) of rapidly growing pathogens for sensitivity to a panel of 12 antibiotics. To infect animals, they were anesthetized, cornea wounded and RS1 ($1 \times 10^8$ cfu/µl) was added (5µl) onto the scarified cornea. All were done as described before [25,26].

### Corneal Disease

The disease induced by RS1 was graded and representative corneas were photographed at 5 days after infection with a slit lamp camera, as described before [28].

### Treatment Using Licorice Root Derivative

B6 mice were injected subconjunctivally before infection (1 day) with PBS (5 µl) or GLY (5 µl; 2 mg/ml, Sigma-Aldrich Corp., St. Louis, MO, USA). At 1 and 3 days p.i. they received an i.p. injection (100 µl/day) of PBS or GLY (2 mg/ml). GLY concentration was determined based on previous reports from this and other laboratories [26,29].

### Detection of Virulence Genes Encoded by Type III Secretion System

20 µl of tissue buffer (0.25% SDS + 0.05 M NaOH) was mixed with a single colony of bacterial isolate/strain [RS1, KEI 1025 (positive control for *exoT* and *exoS*) or 19660 (positive control for *exoU*)], and the mixture was incubated at 95°C for 10 minutes. After incubation, the mixture was centrifuged for 1 minute at 13,000xg and 180 µl of diethylpyrocarbonate (DEPC) -treated water (ThermoFisher Scientific, Waltham, MA, USA) was added. Virulence genes *exoS, exoT* and *exoU* were amplified by PCR (specific primers shown in table 1), and the PCR products were analyzed by electrophoresis, all as described before [27,30,31].

| Cytotoxin gene | Nucleotide Sequence(5'-3') | Product size(bp) | Reference |
|---|---|---|---|
| *exoS* | F - ATC CTC AGG CGT ACA TCC | 328 | [4] |
| | R - ACG ACG GCT ATC TCT CCA C | | |
| *exoT* | F - AGA ACC CGT CTT TCG TGG CTG AGT T | 351 | [4] |
| | R - CAG CTC GCT CGC CTT GCC AAG T | | |
| *exoU* | F - CCT TAG CCA TCT CAA CGG TAG TC | 911 | [5] |
| | R - GAG GGC GAA GCT GGG GAG GTA | | |

***Table 1:*** *Primers used for the amplification of different cytotoxin genes among P. aeruginosa isolates.*

*F: Forward; R: Reverse.*

### MIC Assay

MIC of GLY was determined for the RS1 isolate similar to previous work [27], but with some changes. Serial dilutions of GLY (0 - 60 mg/ml) were prepared in PTSB in sterile tubes (5 ml/tube) and 10 µl of the bacterial culture (adjusted to 1.5 X $10^8$ cfu/ml using 0.5 McFarland standard) was added to each tube and incubated at 37°C for 18h. MIC of GLY for RS1 was assigned as described before [27].

### Time Kill Assay

The conventional colony counting based method was used in this study as described before [27,32] to assess the bacteriostatic/bactericidal activity of GLY on RS1. Briefly, at 0, 1, 3, 6 and 9h, aliquots were removed from the culture, diluted, plated (X3), incubated for 18h at 37°C and colonies were counted. Bacteriostatic/bactericidal activity was defined as described before [27,32].

### Proteomic analysis of RS1 vs PAO1

RS1 and PAO1 were grown in 25 ml of PTSB at 37°C for 18h, as described before, and pellets (1.5 ml of 4 separate cultures from each bacterial isolate/strain) were analyzed by the WSU Proteomics Core facility. In brief, each bacterial pellet was heated at 95°C for 5 minutes in 1% lithium dodecyl sulfate (LiDS) to extract the proteins. Each extract was passed through a coarse filter and total protein concentra-

tion was assayed with a bicinchoninic acid (BCA) assay. Equal protein amounts from each extract was reduced with DTT, alkylated with iodoacetamide and digested with trypsin overnight. Digestion conditions were < 0.1% LiDS, 10% acetonitrile, 80 mM Tris pH 7.5. The digested samples were run directly on a Thermo Fusion mass spectrometer. MS1 spectra were collected at 120K resolution followed by higher energy collisional dissociation (HCD) and ion trap analysis of MS2 spectra for the most abundant features. These spectra was then searched against the Uniprot *Pseudomonas aeruginosa* proteome (ID = UP000042235, 6,563 entries) using MaxQuant. Resulting intensities for each protein was normalized to obtain same median intensity for each sample. Fold change in normalized protein intensities (average intensity of RS1/average intensity of PAO1) accompanied with a p value calculated based on moderated t-test was used to test the difference in protein expression in RS1 vs PAO1.

### Biofilm: Effect of GLY

Biofilm formation was tested (19142, PAO1, RS1) with a colorimetric assay as described before [27,33,34]. In a similar experiment 90 µl of PTSB (alone=control) and GLY (0, 5 and 10 mg/ml) plus 10 µl of RS1 was added to wells of a microtiter plate (X3). All plates were incubated for 24h in aerobic conditions (no shaking) at 37°C, air dried, fixed with 99% methanol, and stained with 2% crystal violet (CV; Sigma-Aldrich). CV bound to biofilm was released with 100 µl acetic acid (33%) and the OD at 570 nm was measured as described before [27] to determine final OD. Strains 19142 (mucoid) and PAO1 (non-mucoid) were controls for biofilm production.

### Adhesion of Bacteria to Corneal cells

Isolates PAO1 and RS1 were processed (described above) and their growth evaluated as done previously [27]. HCET cells (10.014 pRSV-T) were cultured in KBM (Lonza, Walkersville, MD) with growth factors. Cultured B6 mouse corneal epithelial (CMCE) cells were grown as described before [35]. Both groups of cells (2 X $10^5$ cells) were seeded onto chambered slides followed by overnight incubation (37°C). Chambers were washed (X2, PBS) and fresh cell culture media (1 ml) added (no antibiotics). Bacteria were added in a volume equal to 10 MOI/cell to each chambered slide, incubated (3h, 37°C, aerobic conditions) and washed (X3, PBS). Air dried slides were stained (Wright-Giemsa, Sigma-Aldrich) for half a min, and PBS (0.5 ml, 1 minute) added. Then, chambers were gently decanted and washed with PBS. After air drying, chambered slides were mounted with permount, observed (brightfield microscope) and photographed as before [27]. Bacteria adhered to cells were quantified (n = 100 cells/group), averaged and reported as number of adherent bacteria per cell [27]. In a similar adherence assay, immediately before the addition of RS1 (volume equal to 10 MOI) to the chambered slides, bacteria were reconstituted in sterile saline containing GLY (0, 5 or 10 mg/ml). Then the bacteria were added to the chambered slides containing HCET or CMCE cells and incubated for 3h similar to the adherence assay described above.

### Neutrophil Assay (MPO)

Individual corneas were removed at 3 and 5 days p.i. after RS1 infection, and GLY or PBS-treated corneas were processed and tested as described before [25,26]. MPO (1 unit) is equal to ~2 × $10^5$ neutrophils [36].

### Quantification of Viable Bacteria

Mice (n = 5/group/time) were sacrificed at 3 and 5 days p.i. and RS1 infected, GLY or PBS-treated corneas were harvested. Each cornea was processed, dilutions plated and after overnight incubation at 37°C, colonies counted as described before [25,26]. Results are expressed as $\log_{10}$ cfu/cornea.

### RT-PCR

Normal (uninfected) and infected (RS1) corneas were removed at 5 days p.i. from B6 mice after GLY- or PBS-treatment and process for PCR as described before [26]. The primers used are shown in table 2.

| Gene | Nucleotide Sequence | Primer | GenBank |
|---|---|---|---|
| *b-actin* | 5'- GAT TAC TGC TCT GGC TCC TAG C -3' | F | NM_007393.3 |
| | 5'- GAC TCA TCG TAC TCC TGC TTG C -3' | R | |
| *HMGB1* | 5'- TGG CAA AGG CTG ACA AGG CTC -3' | F | NM_010439.3 |
| | 5'- GGA TGC TCG CCT TTG ATT TTG G -3' | R | |
| *IL-1b* | 5'- CGC AGC AGC ACA TCA ACA AGA GC -3' | F | NM_008361.3 |
| | 5'- TGT CCT CAT CCT GGA AGG TCC ACG -3' | R | |
| *TLR2* | 5'- CTC CTG AAG CTG TTG CGT TAC -3' | F | NM_011905.3 |
| | 5'- TAC TTT ACC CAG CTC GCT CAC TAC -3' | R | |
| *TLR4* | 5'- CCT GAC ACC AGG AAG CTT GAA -3' | F | NM_021297.2 |
| | 5'- TCT GAT CCA TGC ATT GGT AGG T -3' | R | |
| *CXCL2* | 5'- TGT CAA TGC CTG AAG ACC CTG CC -3' | F | NM_009140.2 |
| | 5'- AAC TTT TTG ACC GCC CTT GAG AGT GG -3' | R | |
| *TNF-α* | 5'- ACC CTC ACA CTC AGA TCA TCT T -3' | F | NM_013693.2 |
| | 5'- GGT TGT CTT TGA GAT CCA TGC -3' | R | |
| *NLRP3* | 5'- TGC CTG TTC TTC CAG ACT GGT GA -3' | F | NM_145827.3 |
| | 5'- CAC AGC ACC CTC ATG CCC GG -3' | R | |
| *NLRC4* | 5'- CTG GAA AAG GAT GGG AAT GA -3' | F | NM_01033367.3 |
| | 5'- CCA AGG CAG CAT CAA TGT AG -3' | R | |

***Table 2:*** *Nucleotide sequence of the specific primers used for PCR amplification.*

*F: Forward; R: Reverse.*

### Enzyme-Linked Immunosorbent Assay

After GLY or PBS treatment, normal (uninfected) and infected corneas were harvested at 5 days p.i. and prepared as described before [25,26,37]. IL-1β and CXCL2 protein levels were assayed using ELISA kits (R&D Systems (Minneapolis, MN, USA) as described before and with similar sensitivities [25,26].

### Determination of Significance

The Mann-Whitney U test was used to determine the significance of clinical scoring. 1-way ANOVA followed by the Bonferroni's multiple comparison test (GraphPad Prism) was used for comparison of three or more groups [e.g. Figure 4A, 4B and Figure 4I, 4M)]. For all other experiments involving comparison of two groups an unpaired, two-tailed Student's t-test was utilized. $p < 0.05$ +/- SEM determined significance. To guarantee reproducibility, experiments were repeated once.

## Results and Discussion

### Results

#### Drug resistance patterns of RS1 isolate

Non-ocular RS1 clinical isolate was collected from urine, by the Microbiology Laboratory of the Detroit Medical Center Hospital (DMC, Detroit, MI, USA) and tested by disk diffusion for drug resistance. Figure 1D shows the outcome of evaluation of the RS1 for resistance to twelve antibiotics. RS1 shows a pattern of resistance that is intermediate/susceptible to antibiotics gentamicin and piperacilin/tazobactam, intermediate for meropenem and resistant to trimethoprim/sulfamethazole.

**Gel Electrophoresis of PCR Products for RS1 Isolate**

The type III secretion-toxin encoding gene patterns of RS1 isolate is shown in figure 1 (A-D). For RS1, amplification of all three virulence genes, *exoT* (Figure 1A), *exoS* (Figure 1B) and *exoU* (Figure 1C) was seen. Keratitis isolate KEI 1025 (*exo*T and *exo*S, Figure 1A, 1B) and ATCC strain 19660 (*exo*U, Figure 1C) were used as positive controls for testing gene amplification.

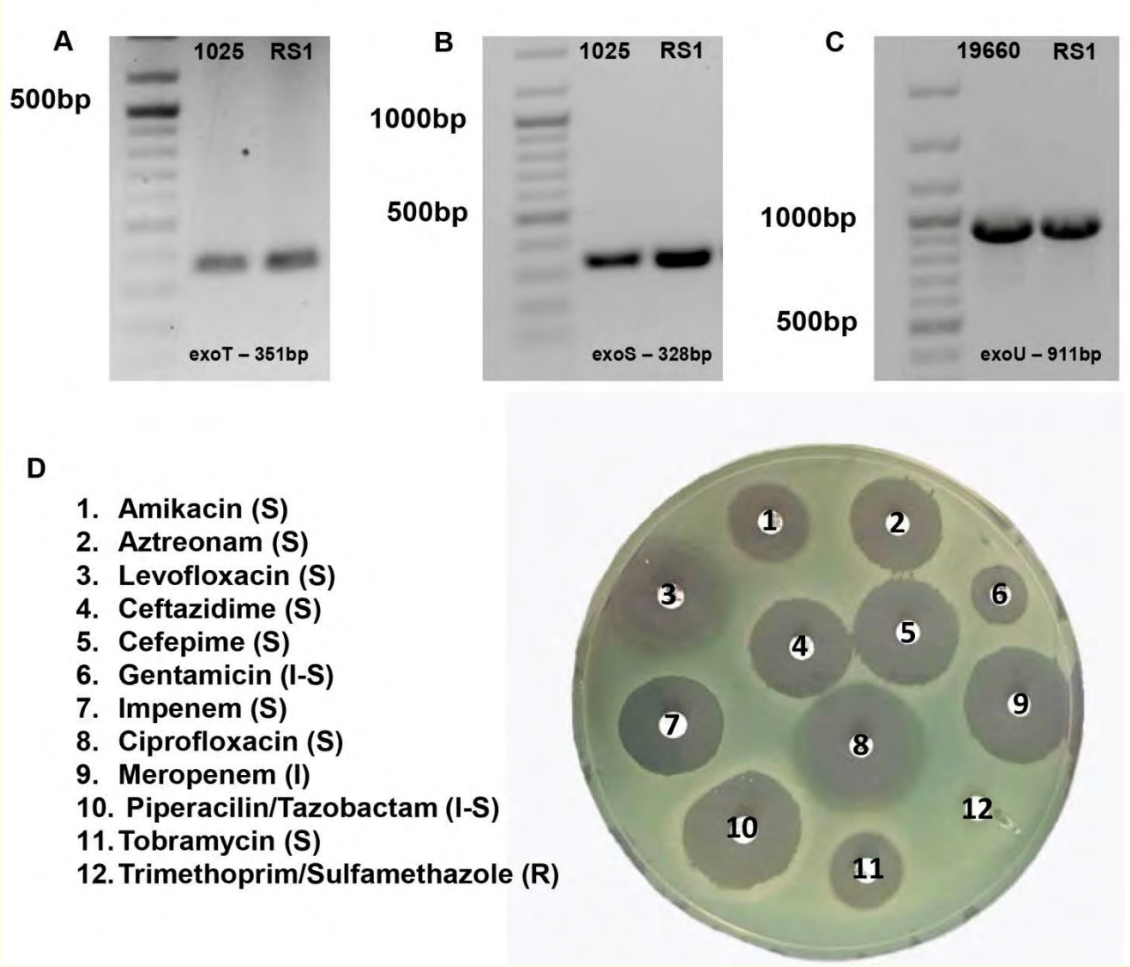



***Figure 1:*** *Gel electrophoresis of PCR products of the type III secretion genes of RS1 and its antibiotic sensitivity. Amplification of exoT (A), exoS (B) and exoU (C) genes was seen. Clinical isolate KEI 1025 was the positive control for amplification of exoT (A) and exoS (B). ATCC strain 19660 was the positive control for amplification of the exoU gene (C). RS1 shows a pattern of resistance that is intermediate/susceptible to antibiotics gentamicin and piperacilin/tazobactam, intermediate for meropenem and resistant to trimethoprim/sulfamethazole (D).*

**Effect of GLY on RS1 Growth and Time Kill**

The MIC of GLY was determined for RS1. Absorbance values were reduced at 5, 10 and 20 mg/ml GLY, with no visible growth detected at 40 or 60 mg/ml (Figure 2A). For time kill determination, bacteria were treated with 0.25, 0.5, 1.0, and 1.5 × MIC of GLY (MIC = 40 mg/ml). Figure 2B illustrates time kill curves for isolate RS1. At all concentrations × MIC of GLY, a decrease in colony counts (less than 3 $\log_{10}$

cfu/ml) was seen after 1 and/or 3h, and indicates GLY is bacteriostatic. At 6h, exposure to MIC and 1.5 × MIC showed similar effects, with no effect at the other concentrations. No bacteriostatic activity was detected for any × MIC tested at 9h.

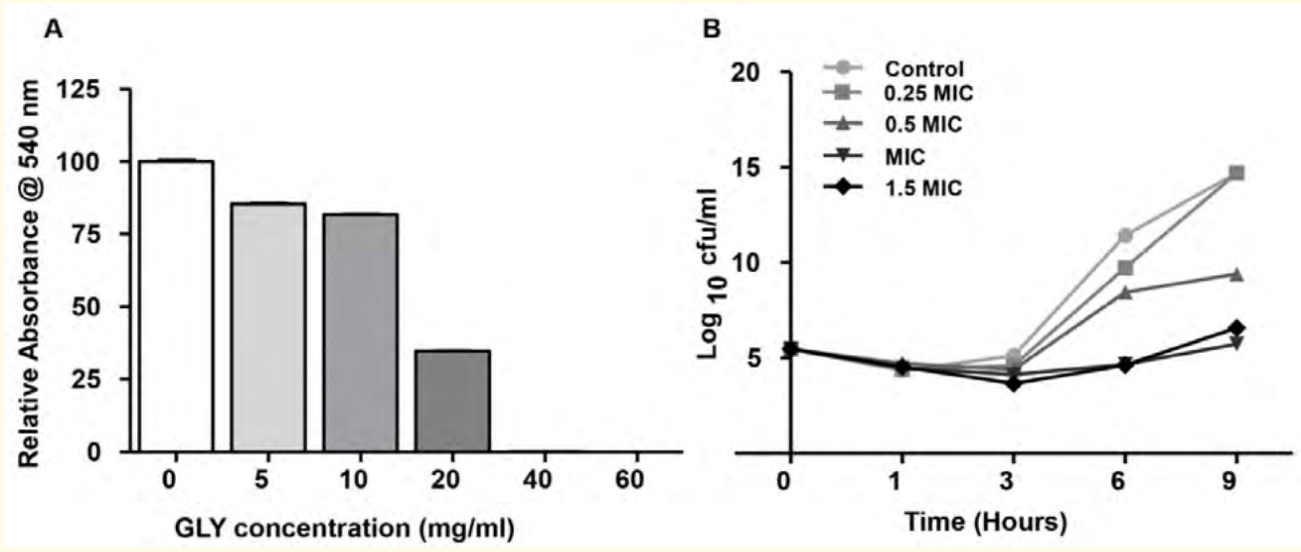
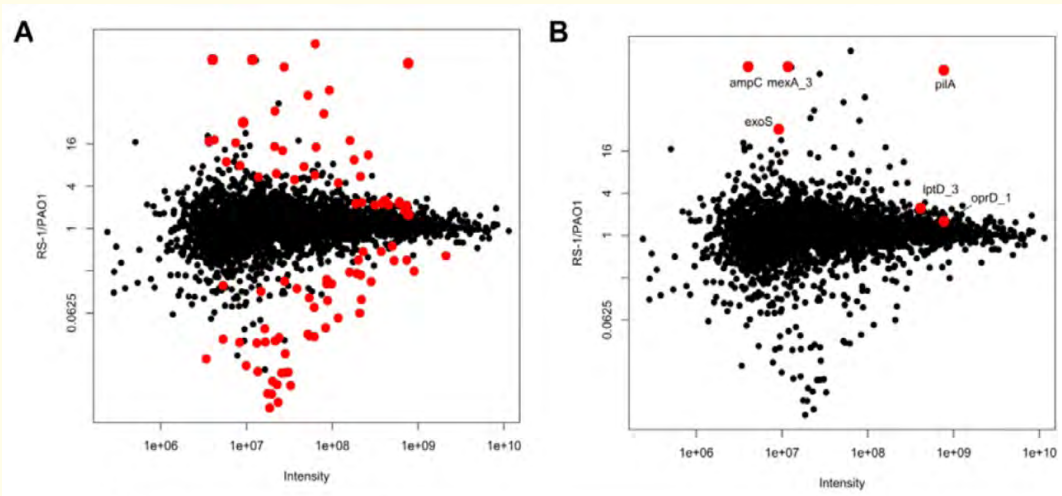



***Figure 2:*** *GLY MIC and time kill. For RS1 (A) the MIC for GLY was 40 mg/ml. GLY was bacteriostatic (> 3log10 cfu/ml) for RS1 at 1 and 3h (all concentrations X MIC of GLY) and at 6 h (1 and 1.5 X MIC of GLY) when compared with the starting inoculum at 0 h. At 9 h bacteriostatic activity was not seen. n = 3/group/isolate.*

**Proteomic analysis of RS1 vs PAO1**

After proteomic analysis (Figure 3A-C), 2990 proteins were identified and 2963 had quantitative data. The quantitative data were normalized so each sample had the same median intensity. To find proteins that were different between RS1 and a prototypic non-MDR PA strain, PAO1, a moderated t-test was used. 87 proteins (red dots in figure 3A) were significantly different between groups [moderated t-test, 10% false discovery rate (FDR)], confirming differences between RS1 and PAO1. Protein abundancies were normalized so each sample had similar median intensity [38]. Among the 87 proteins, table (Figure 3C) selected proteins with fold differences (= average intensity of RS1/average intensity of PAO1) and corresponding p values are listed. All are expressed significantly higher in RS1 vs PAO1 (LPS-assembly protein LptD, outer membrane porin D, exoenzyme S, fimbrial protein). Other proteins were only expressed in RS1 (multi-drug resistance protein mexA, beta-lactamase). The proteins listed in the table (Figure 3C) are also shown and labeled on a Volcano plot (Figure 3B).




C

| Accession # | Gene | Protein name | Fold difference | p value |
|---|---|---|---|---|
| A0A0F6RPI6 | lptD_3 | LPS-assembly protein LptD | 2.43 | 0.00003 |
| A0A069QD10 | oprD_1 | Outer membrane porin D | 5.34 | 0.00128 |
| Q93SQ0 | exoS | Exoenzyme S | 26.6 | 0.00009 |
| A0A0F6UGL2 | mexA | Multidrug resistance protein MexA | *Inf | |
| Q4H482 | ampC | Beta-lactamase | *Inf | |
| A0A0F6UHB3 | pilA | Fimbrial protein | 171 | 0.00000 |

Fold difference = Average intensity of RS1/Average intensity of PAO1

*Inf indicates proteins that were detected in RS1 samples but not in PAO1

***Figure 3:*** *Proteomic analysis of RS1 vs PAO1. Among the quantified proteins, 87 ("Hit" proteins; red dots) were significantly different [moderated t-test, 10% false discovery rate (FDR)] between groups (A). Selected proteins were labeled in the volcano plot (B) and are listed in a table (C).*

**Biofilm Formation and Bacterial Adherence Assay**

For this assay, a mucoid (ATCC 19142) and a non-mucoid (ATCC PAO1) biofilm producer, were used as positive controls for testing biofilm formation of RS1 (Figure 4A). All bacterial strains and the RS1 isolate produced a biofilm (Figure 4A). RS1 did not differ in the amount produced when compared to strain PAO1 (Figure 4A). In contrast, biofilm production of both PAO1 and RS1 was significantly less when compared to the mucoid producing strain 19142 (both = $p < 0.001$). Treatment of RS1 with GLY decreased biofilm formation at both 5 ($p < 0.001$) and 10 ($p < 0.001$) mg/ml compared with control (no GLY); however, there was no difference between the two treatment groups. Next, we compared adhesion of PAO1 and RS1 to CMEC and HCET cells and the data are shown in figure 4C-H. Stained adherent bacteria could be easily identified on the surface of CMCE (Figure 4D, 4E) and HCET (Figure 4G, 4H) cells. Upon incubation of RS1 for 3 h with either group of cells, the average number of adherent bacteria/cell was significantly higher for the RS1 isolate when compared to PAO1 [$p < 0.001$ for both epithelial cell lines (Figure 4C, 4F)]. Next, the effects of GLY (5 and 10 mg/ml) on RS1 adherence to both CMCE and HCET cells was tested (Figure 4I-4P). GLY significantly reduced the average number of adherent bacteria to both CMCE (Figure 4I-L, $p < 0.001$ for each concentration) and HCET cells (Figure 4M-P, $p < 0.001$, for each concentration) when compared to control. There was no GLY concentration dependent effect detected for either group, but a small shift downward was detected at the higher concentration of GLY.

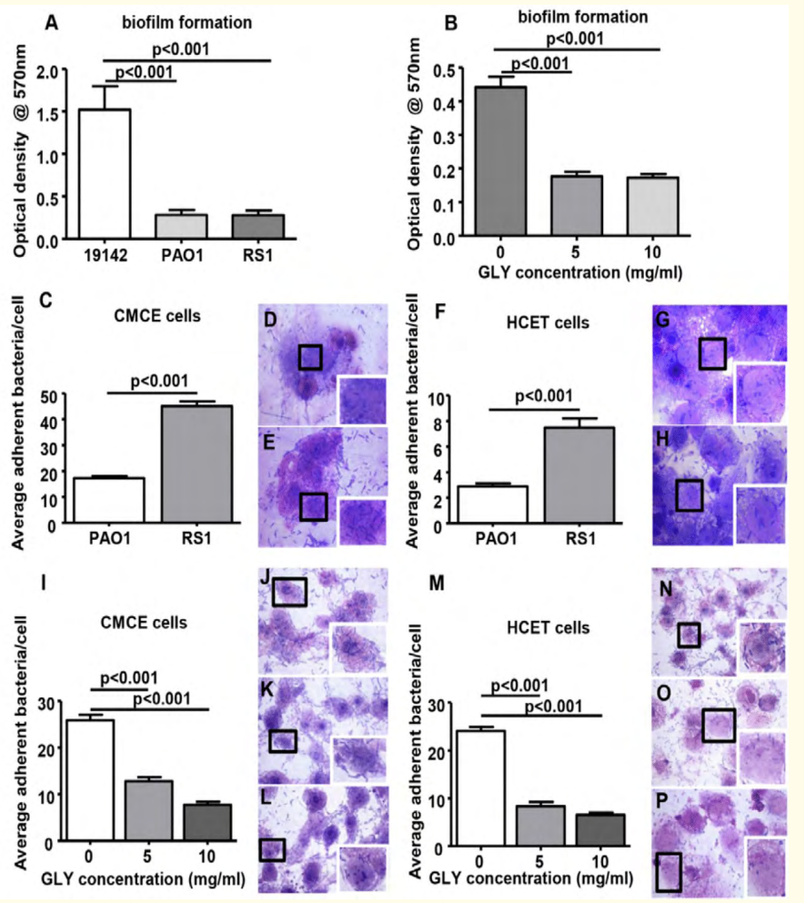



***Figure 4:*** *Biofilm formation and bacterial adherence to HCET and CMCE cells. The OD values of biofilm formation (A) produced by RS1 and PAO1 are significantly less than mucoid strain 19142, but there was no significance between RS1 and PAO1. Effect of GLY on biofilm formation of RS1 is shown (B). Biofilm formation activity of RS1 was reduced significantly with 5 and 10 mg/mL GLY when compared with the control (no GLY). The average number (C-H) of adherent RS1 bacteria per cell was significantly increased over PAO1 in mouse (C) and human (F) corneal epithelial cells. Adherent bacteria are shown in images (D, E, G, H). The average number of adherent RS1 for both cell types was decreased at 5 and 10 mg/ml of GLY (I, M). Adherent bacteria are shown in images taken using a bright field microscope without (J, N) and with GLY [5 mg/ml (K, O) and 10 mg/mL (L, P)] treatment. An inset is shown in all images (D, E, G, H, J-L, N-P), and represent an enlargement of the cells in each photomicrograph that are outlined by a black box. All data are mean ± SEM; biofilm assays were analyzed using the Bonferroni's multiple comparison test (n = 6/group/strain); bacterial adherence was analyzed using a two tailed student's t test (C, F) or Bonferroni's multiple comparison test (I, M). For adherence assays, n = 100 cells/group; magnification= X 20 µm; inset magnification= X40 µm.*

**Infectivity of Isolate RS1 and GLY Treatment**

Four concentrations of RS1 (1 X $10^6$ - 1 X $10^9$ cfu/µl) were tested *in vivo* in the mouse keratitis model to establish an optimum infecting dose (data not shown). At 5 days p.i. the lowest concentration produced only a mild infection, but 1 X $10^7$ did not provide consistent infection. 1 X $10^8$ produced a consistent infection, including corneal perforation and the highest concentration (1 X $10^9$) was similar to that of 1 X $10^8$. RS1 (1 X $10^8$ cfu/µl) was used for further studies in the *in vivo* B6 mouse model of keratitis. Clinical scores (Figure 5A) and photographs taken with a slit lamp (Figure 5B, C) revealed the severity of infection. When compared with PBS, GLY (Figure 5A) had reduced clinical scores that were significant at 3 ($p < 0.05$) and 5 ($p<0.01$) days p.i. Photographs taken with a slit lamp confirmed less opacity (disease) only in the GLY (Figure 5C) treated eyes. MPO assay (Figure 5D) revealed significant reduction in neutrophil infiltrate in the GLY -treated group both at 3 ($p < 0.001$) and 5 ($p < 0.001$) days p.i. Plate counts (Figure 5E) showed a significant decrease in bacterial load at 3 ($p < 0.01$) and 5 ($p < 0.05$) days p.i. after GLY treatment.

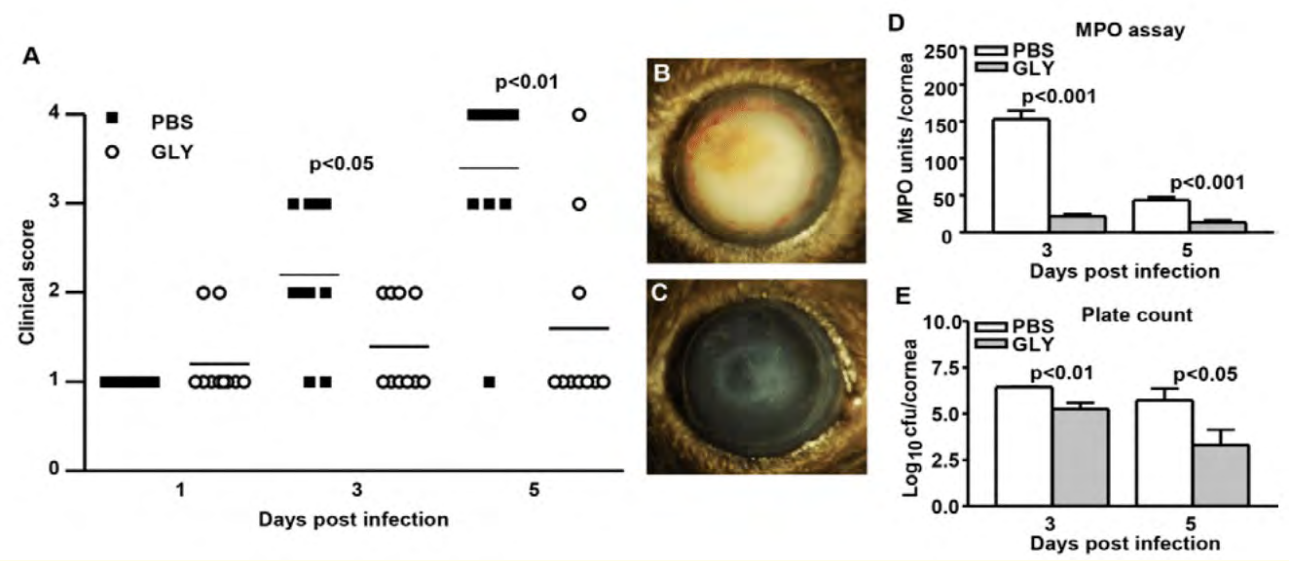



***Figure 5:*** *Treatment with GLY after infection with RS1. Clinical scores (A) were reduced significantly at 3 and 5 days p.i. in GLY vs PBS treated mice (n = 10/group/time). Photographs taken with a slit lamp at 5 days p.i. from PBS (B) and GLY (C) treated mice confirmed reduced opacity after GLY treatment. MPO (D) assay showed significantly reduced neutrophil infiltrate after GLY treatment both at 3 and 5 days p.i. Plate counts (E) showed a significant decrease in bacterial load at 3 and 5 days p.i. after GLY treatment. Horizontal lines indicate mean values. Data were analyzed using a nonparametric Mann-Whitney U-test. Photographs with a slit lamp are at a magnification of X 10. MPO assay and plate count data are mean ± SEM and were analyzed using a two tailed student's t test (n = 5/group/time).*

**RT-PCR and protein analysis of GLY effects**

Relative mRNA levels for proinflammatory mediators IL-1β and CXCL2 (Figure 6A and 6C) were decreased at 5 days p.i. ($p < 0.001$ for both) only after GLY treatment. ELISA analysis supported the mRNA data for IL-1β and CXCL2 (Figure 6B and 6D), which were downregulated significantly at 5 days p.i. ($p < 0.05$ and $p < 0.001$, respectively) in GLY vs PBS-treated mice. In the normal cornea, differences were not detected between groups for mRNA and protein levels.

**RT-PCR after GLY: Other molecules**

At 5 days p.i. GLY also significantly reduced mRNA levels of inflammatory molecules including HMGB1 ($p < 0.001$, Figure 6E), TNF-α ($p < 0.001$, Figure 6F), TLR2 ($p < 0.05$, Figure 6G), TLR4 ($p < 0.05$, Figure 6H), NLRP3 ($p < 0.001$, Figure 6I) and NLRC4 ($p < 0.05$, Figure 6J). For normal cornea, no difference was detected between groups.

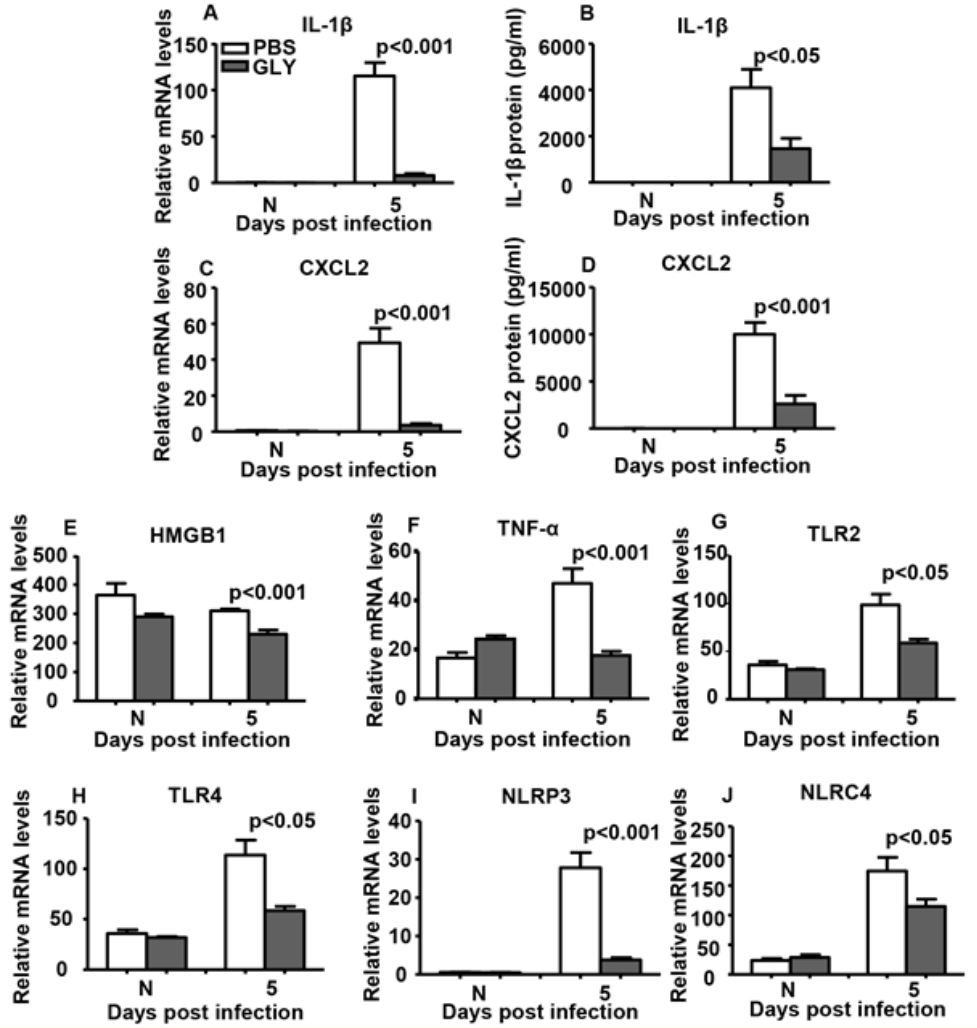



***Figure 6:*** *RT-PCR and ELISA of proinflammatory molecules after infection with isolate RS1. At 5 days p.i. corneal mRNA and protein levels of IL-1β (A, B) and CXCL2 (C, D) were reduced significantly after GLY treatment. Corneal mRNA levels of: HMGB1 (E), TNF-α (F), TLR2 (G), TLR4 (H), NLRP3 (I) and NLRC4 (J) also were reduced significantly at 5 days p.i. Data are mean ± SEM analyzed using a two tailed student's t test. n = 5/group/time.*

## Discussion

*P. aeruginosa* is an important pathogen and a global cause of microbial keratitis [39]. Clinically, presentation of this infection tends to be more severe compared with other bacterial ulcers and the infection progresses rapidly [40]. Increasing drug resistance in *P. aeruginosa* isolates has been reported over the past two decades, including those causing keratitis [22,41]. This increasing trend in antibiotic resistant bacterial infections is of serious concern, as there is a lack of effective antibiotics available and few in development [21]. GLY, derived from *Glycyrrhiza glabra*, has both antimicrobial and anti-inflammatory functions [42]. It possesses numerous pharmacologic effects [43] and has been tested with success in animal models of keratitis [26], colitis [44], sepsis [45], brain [46] and lung [47] injury. In addition, GLY has been used to treat blepharitis [48] and hepatitis [49,50] without signs of adverse events or drug toxicity. GLY has a host cell target, HMGB1, which is able to amplify inflammation in pseudomonas keratitis [37] and other diseases [51,52]. GLY does this by directly binding to HMGB1 and inhibiting many of its deleterious functions [53]. However whether GLY could function similarly against a drug resistant isolate was not tested.

The non-ocular RS1 isolate showed intermediate/susceptible resistance to gentamicin, and piperacilin/tazobactam and intermediate resistance to meropenem. RS1 was also resistant to trimethoprim/sulfamethazole. Based on this antibiotic sensitivity pattern of RS1, the isolate cannot be characterized as a MDR [54]. However, it shows intermediate and/or resistance to more than three antibiotic categories and was tested in this study.

RS1 amplified the type III secretion system genes *exoS, exoT and exoU which* encode for effector proteins ExoS, ExoT and ExoU in *P. aeruginosa* [1]. ExoS and ExoT have both ADP-ribosyltransferase and GTPase activating activity [55], while ExoU has phospholipase activity that targets eukaryotic membranes after cytoplasmic injection [56].

GLY exhibited antibacterial activity against the RS1 isolate, agreeing with previous work from this laboratory [26,27] and others who tested its effects against bacteria that were either Gram positive or negative [57]. All studies showed that the effects of GLY on bacterial growth were concentration dependent. And further, time kill assays showed that GLY at various concentrations × MIC was bacteriostatic after 3 or 6h. No bacteriostatic activity in any group was observed a 9h. These *in vitro* data are not in agreement with past *in vivo* work from this laboratory in which we have shown that GLY is bactericidal [26]. We reported that GLY reduced bacterial load in the cornea by about 4.5 log10 cfu/ml after infection with the clinical isolate KEI 1025 [26].

In this study, a mass spectrometry based proteomic analysis was used to comparatively analyze the proteomic profiles of RS1 vs PAO1. Among the proteins which were identified as significantly increased in RS1 vs PAO1, fimbrial protein, a major structural protein component of *P. aeruginosa* type IV pili (TFP). This protein, encoded by the gene *pilA* [58] was increased 171 fold in RS1. In *P. aeruginosa* these TFP play major roles essential to the bacterium's virulence and pathogenicity. TFP mediate motility, adhesion and biofilm formation [58-60] which promote chronic bacterial infections, reducing benefits of antimicrobial usage [55,61,62]. Adherence assays showed that RS1 had greater adherence capability than PAO1 to both mouse and human corneal epithelial cells. Although untested in our system, TFP have a critical role in *P. aeruginosa* adherence to tracheal epithelia [6]. In fact, *in vitro* TFP account for roughly 90% of *P. aeruginosa* adherence to human A549 (lung) cells [7,63,64]. Although we did not identify the adhesin involved, GLY also reduced biofilm formation and adherence for antibiotic sensitive *P. aeruginosa* (G81007), isolated from a diseased patient cornea [27].

GLY inhibits the activity of HMGB1 as well as other inflammatory molecules [52,65]. For RS1, treatment with GLY significantly reduced clinical scores, the host inflammatory (neutrophil) infiltrate and these data are supported by our previous work [25,26] and work done by others in different disease models [66,67]. In addition, after infection with RS1, GLY also reduced corneal bacterial load at 3 and 5 days p.i. when compared with PBS control, but only by about 2 logs. In contrast, GLY reduced plate counts in a drug susceptible keratitis isolate (KEI 1025) by 4.5 logs [26]. Whether or not this was the consequences of drug susceptibility is not known. IL-1β and CXCL2 are potent chemoattractants for neutrophils [68,69] and were also significantly reduced (mRNA and protein level) after GLY treatment. GLY also reduced mRNA levels of other proinflammatory molecules (HMGB1, IL-1β, CXCL2, TNF-α, TLR2 and TLR4) or inflammasome expression (NLRP3 and NLRC4) and agreed with data reported before [25,26,27].

## Conclusion

In summary, these studies provide evidence that GLY has antimicrobial and anti-inflammatory effects on a resistant non-ocular *P. aeruginosa* isolate which causes severe keratitis *in vivo*. Several mechanisms appear to be involved and include: reduction/inhibition of bacterial growth, viability and early events of biofilm formation.

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## Conflict of Interest

The authors declare no conflict of interest.

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**Volume 9 Issue 5 May 2018**