Pathogenicity of Ocular Isolates of *Acinetobacter baumannii* in a Mouse Model of Bacterial Endophthalmitis

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Submitted: October 8, 2013

Accepted: March 4, 2014

Citation: Talreja D, Kaye KS, Yu F-S, Walia SK, Kumar A. Pathogenicity of ocular isolates of *Acinetobacter baumannii* in a mouse model of bacterial endophthalmitis. *Invest Ophthalmol Vis Sci*. 2014;55:2392–2402. DOI:10.1167/iovs.13-13401

PURPOSE. To determine the virulence properties of ocular isolates of *Acinetobacter baumannii* in causing endophthalmitis in a mouse model.

METHODS. Endophthalmitis was induced by intravitreal injections of the bacteria into C57BL/6 (B6) mouse eyes. The disease progression was monitored by ophthalmoscopic, electroretinography (ERG), histologic, cell death (TUNEL labeling), and microbiological parameters. The expression of cytokines/chemokines was checked by quantitative RT-PCR (qRT-PCR) and ELISA. Flow cytometry was used to determine cellular infiltration. The role of neutrophils was determined using neutropenic mice. The virulence traits (biofilm formation, adherence, and cytotoxicity) of the ocular isolates were tested using corneal epithelial cells.

RESULTS. Among the three clinical isolates and a standard ATCC 19606 strain tested, a biofilm producing multidrug resistant (MDR) strain of *A. baumannii* AB12 caused severe endophthalmitis (100% destruction of the eyes) leading to the loss of retinal function as assessed by ERG analysis. Elevated levels of inflammatory mediators (TNF- α , IL-1 β , CXCL2, and IL-6) were detected in AB12-infected eyes. Histologic and TUNEL staining revealed increased retinal cell death and the flow cytometry data showed the presence of inflammatory cells, primarily neutrophils (CD45⁺/Ly6G⁺). Neutropenic mice showed an increased bacterial burden, reduced inflammatory response, and severe tissue destruction.

CONCLUSIONS. These results indicate that *A. baumannii* causes severe intraocular inflammation and retinal damage. Furthermore, neutrophils play an important role in the pathogenesis of *A. baumannii* endophthalmitis.

Keywords: endophthalmitis, inflammation, innate response, cell death, bacteria

Acinetobacter baumannii is a slow-growing, drug-resistant Gram negative bacterium generally an inhabitant of soil, vegetation, and aquatic environments.¹ It is considered an opportunist pathogen and often dismissed by healthcare providers as a less virulent bacterium.² In the last decade, an increasing number of reports have shown that it causes a wide variety of infections ranging from asymptomatic colonization on the skin, intestinal, and respiratory tracts to severe, life-threatening infections like pneumonia, necrotizing fasciitis,^{3,4} meningitis,⁵ and septicemia.^{6–8} Moreover, the majority of the clinical isolates are multidrug resistant (MDR),^{9–11} making the management of their infections a significant burden on healthcare,¹² and conferring their status as a “red alert” human pathogen.¹³

Among the ocular infections, endophthalmitis is rare, but the most vision-threatening complication of ocular surgeries or trauma that requires prompt diagnosis and treatment to prevent vision loss. The overall incidence of endophthalmitis after ocular surgery or injection is 0.016% to 0.46%, and after trauma is 0.9% to 17%.¹⁴ Moreover, in recent years, increased use of multiple intravitreal (IVT) injections for the treatment of AMD and diabetic retinal diseases is also contributing toward increased incidence of endophthalmitis.¹⁵ Studies have shown that during the injection procedure, IVT injection needles can directly inoculate ocular surface flora into the vitreous cavity

resulting in post injection endophthalmitis.^{16,17} As most postoperative endophthalmitis cases are caused by the introduction of bacteria from the ocular surface to the posterior segment either during surgery or trauma, the composition of the ocular flora is critical in determining the outcome of such infections.^{14,18} Thus, mere colonization of the ocular surface with *A. baumannii* increases the chances to develop aggressive ocular infections because their transferable MDR nature (1) prevents eradication by preoperative antibiotic therapy, and (2) makes it difficult to treat, in case there is an infection.

The Center for Disease Control classified MDR *A. baumannii* as a “serious threat” pathogen.¹⁹ Thus, it’s an emerging nosocomial pathogen and increasing numbers of studies are documenting its transmission and pathogenesis.^{20,21} However, studies related to pathogenicity of *A. baumannii* in ocular infections are limited to case reports implicating them as a causative agent of endophthalmitis.^{22–24} Considering the importance and understudied area of investigation, the objective of this study was to establish virulence properties of ocular *A. baumannii* isolates in causing endophthalmitis. The results from this study should create awareness among clinicians regarding MDR *A. baumannii* as an emerging, but rare ocular pathogen with a propensity to cause vision loss. We

used both in vitro (human corneal epithelial cells [HCECs]) and in vivo (a mouse model of bacterial endophthalmitis) approaches to prove/disapprove our hypothesis that *A. baumannii* can cause endophthalmitis in murine models. Our data, for the first time, demonstrate that intraocular infection MDR *A. baumannii* can cause severe vision morbidity.

MATERIALS AND METHODS

Bacterial Isolation and Antibiotic Susceptibility Testing

Twelve ocular isolates of *A. baumannii* were obtained from the clinical microbiology laboratory of the Detroit Medical Center (DMC). The identification and antibiotic susceptibility was determined using MicroScan Walk-Away (Siemens Healthcare, Malvern, PA, USA), as described previously.²⁵ The description of the characteristics of these isolates is summarized in Talreja et al.²⁶

In Vivo Induction of Endophthalmitis

Endophthalmitis was induced in the eyes of female C57BL/6 mice (8 weeks; Jackson Labs, Bar Harbor, ME, USA) by IVT injection of the bacteria as described previously with slight modifications.²⁷ Briefly, the left eyes were injected with 2 μ L of the ocular isolate suspension in PBS ($\sim 5 \times 10^4$ CFU/eye), whereas the right eyes of each mouse were left untreated/uninfected. In the control group, the left eyes were injected with sterile PBS. The eyes were examined by two ophthalmologists in a masked fashion using slit-lamp and fundus microscopy. The ocular disease was graded, and clinical scores from 0 to 4.0 were assigned using the previously described scale.^{27–29} Mice were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University.

Analysis of Retinal Function

Scotopic electroretinogram (ERG) was used to assess retinal function during the course of endophthalmitis as described previously.²⁷

Flow Cytometry Analysis

The retinas were eviscerated from the mice as described previously^{30,31} and were digested in Accumax (Millipore, Billerica, MA, USA) for 10 minutes at 37°C. Retinas from two eyes were pooled together to obtain sufficient number of cells. Following digestion, the retinal tissue was passed three to four times through a 23-G needle/syringe and filtered through a 40- μ m cell strainer (BD Falcon, San Jose, CA, USA). The cells were incubated with Fc block (BD Pharmingen, San Jose, CA, USA) for 30 minutes followed by a washing step with PBS containing 0.5% BSA. Cells were then incubated with conjugated monoclonal antibodies CD45-PECy5, Ly6G-FITC, and the respective isotypes (BD Pharmingen) for 30 minutes in the dark. After a washing step, the cells were acquired on a BD Accuri C6 (BD Immunocytometry Systems, San Jose, CA, USA), and the data was analyzed using Flow Jo (Tree Star, Inc., Ashland, OR, USA).

Histologic and TUNEL Assay

For histologic analysis, the eyes were kept overnight in fixative and sent to Excalibur Pathology, Inc. (Oklahoma City, OK, USA)

for paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining. As per manufacturer's instructions, TUNEL assay was performed on retinal cryosections using Millipore Apop Tag Fluorescein In Situ Apoptosis Detection Kit.

Neutrophil Depletion

Mice were made neutropenic by intraperitoneal injection of 200 μ g of an anti-mouse Gr-1Mab (IA8; R&D Systems, Minneapolis, MN, USA) in 200 μ L of PBS for 24 hours, followed by the bacterial challenge. The control mice received the same dose of isotype rat immunoglobulin G (IgG).

Real Time RT-PCR

Total RNA was isolated from mouse retinal tissue using TRIzol solution, (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 1 μ g of total RNA was reverse-transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). Quantitative assessment of gene expression was carried out by Taqman probe based real time PCR using predesigned assays (IDT, Coralville, IA) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Bacterial Load and Cytokine ELISAs

At various days post infection (DPI), eyes were enucleated, disrupted, and homogenized with metal beads in a tissue lyser (Qiagen, Valencia, CA, USA) in 250 μ L of PBS for 2 minutes at the maximum speed. Fifty microliters of tissue homogenates was serially diluted, and aliquots (10 μ L) were plated onto Tryptic soy agar (TSA) plates (BD Biosciences, San Jose, CA, USA), and incubated overnight at 37°C. The remaining 200 μ L of the lysate following centrifugation (20,000g for 15 minutes at 4°C) and protein estimation was used for quantitation of cytokines/chemokines with the mouse specific ELISA kits (R&D Systems), according to the manufacturer's protocol.

Bacterial Biofilm, Adherence, and Cytotoxicity Assays

The biofilm forming capabilities of *A. baumannii* isolates was measured using the standard crystal violet staining method of biofilms on microtiter plates.³² For the bacterial adherence assay, HCECs were infected with clinical isolates at a multiplicity of infection (MOI) of 100:1. At various time points, the HCECs were rinsed three times with sterile PBS and harvested with TrypLE (Invitrogen). The harvested samples were serially diluted and plated for enumeration of CFU following overnight incubation. The adherence was determined as the percentage of cells associated with HCECs in comparison to the original inoculum. The cytotoxicity induced by *A. baumannii* on HCECs was determined by using a Cytotoxicity Detection Kit LDH (Roche, Indianapolis, IN, USA) as per the manufacturer's recommendation.

Statistical Analysis

All statistical analysis and graphing were performed on Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). An unpaired, two-tailed Student's *t*-test was used to determine statistical significance for data from the cytokine ELISA, and bacterial count. A nonparametric Mann-Whitney *U* test was performed for clinical score.

TABLE. Characteristics of Ocular *A. baumannii* Isolates

Isolate ID	Antibiotic Resistance Pattern	Adherence Properties	Biofilm Formation	In Vitro Toxicity/Cell Death	In Vivo Toxicity/Cell Death
ATCC 19606	Sensitive, non-MDR reference strain	+	+	++	++
AB7	AMP, CFZ, AZT, NFT, CTX, CHL, TET	++++	+	++	++
AB8	AMP, CFZ, CTZ, NFT, CTX, CHL	+	++++	++	+++
AB12	AMP, ASL, CFZ, CTZ, CPM, AZT, IPM, MRP, TOB, CIP, MOX, TIG, NFT, TMS, CTX, CHL, TET, GEN	++++	+++	++++	++++

PTZ, piperacillin/tazobactam; CTR, ceftriazone; +, poor; ++, weak; +++, moderate; +++, strong.

FIGURE 1. Pathogenicity of the ocular *A. baumannii* AB12 in a mouse model of bacterial endophthalmitis. The eyes of C57BL/6 mice (4–6 per group) were inoculated intravitreally with 5×10^4 CFU/eye of an ocular isolate AB12 or PBS. Percentage of eyes destroyed were calculated based on clinical scores (range, 0–4) assigned to each eye at 1, 2, and 3 DPI (A). Eyes were enucleated at appropriate time points for assessment of bacterial load (B). Electroretinogram responses to a 6-dB flash were recorded at indicated time points and the percentage amplitude of a- and b-wave retained in infected eyes were compared to that of PBS-injected controls and presented as mean $\pm$ SD ERG amplitudes (C). Light microscopy examination (D), and histologic analysis of the anterior (E) and the posterior segment (F), were performed to assess retinal damage. * $P < 0.05$ (Student's *t*-test, PBS versus infected). Original magnification, $\times 20$. C, cornea; AC, anterior chamber; L, lens; VC, vitreous chamber; R, retina; OD, optic disk.

FIGURE 2. Infiltration of PMNs in *A. baumannii*-infected mouse retina/vitreous cavity. Eyes were given intravitreal injections of PBS or 5×10^4 CFU of AB12. At the indicated time points, eyes were enucleated and retina/vitreous from two eyes were pooled to make single-cell suspensions and stained with anti-CD45 and anti-Ly6G mAbs. Post acquisition, the cells were size gated to differentiate them from debris. The percentage of dually positive PMNs was determined using a CD45 versus Ly6G dot plot (upper-right right [Q2] quadrant). The data are representative of duplicate experiments. * $P < 0.001$ (Student's *t*-test).

RESULTS

Pathogenicity of Ocular *A. baumannii* (AB12) in Causing Endophthalmitis

The ocular isolates of *A. baumannii* were characterized for MDR, biofilm formation, cytotoxicity, and adherence properties. The results are presented in the Table. Multidrug resistance was a common characteristic of all the three isolates, AB7 was resistant to seven antibiotics (Ampicillin [AMP], Cefazolin [CFZ], Aztreonam [AZT], Nitrofurantoin [NFT], Cefotaxime [CTX], Chloramphenicol [CHL], and Tetracycline [TET]), AB8 was resistant to six antibiotics (AMP, CFZ, Cefazidime [CTZ], NFT, CTX, CHL), and AB12 was resistant to 18 antibiotics (AMP, Ampicillin-sulbactam [ASL], CFZ, CTZ, CPM, AZT, Imipenem [IPM], Meropenem [MRP], Tobramycin [TOB], Ciprofloxacin [CIP], Moxifloxacin [MOX], Tiglicine [TIG], NFT, Trimethoprim/sulfomethoxazole [TMS], CTX, CHL, TET, Gentamicin [GEN]). Strain AB12 showed coresistance to β -lactam, aminoglycosides, TET, and quinolones. Because of the broad and extended resistance pattern of AB12, we initiated our studies to determine the virulence properties of this isolate in causing endophthalmitis using a mouse model of bacterial endophthalmitis.^{27,33,34} As shown in Figure 1A, *A. baumannii* AB12 infection resulted in 50% destruction of the eyes at 1 DPI, 75% at 2 DPI, and 100% at 3 DPI. After clinical scoring, the eyes were enucleated to determine the intraocular growth of the bacteria. Higher viable bacteria were recovered at 1 DPI (6.5×10^7 CFU/eye) and 2 DPI (8.6×10^7 CFU/eye). However, at 3 DPI, the bacterial load (4.1×10^7 CFU/eye) is reduced, but is still significantly higher than the original inoculum (i.e., 5×10^4 CFU/eye; Fig. 1B). To determine whether *A. baumannii* infection influences retinal function, we measured scotopic ERG responses. The amplitudes of a- and b-waves in control eyes were considered 100%. At 12 hours post

infection, there was a significant reduction in both a- (43%) and b-wave (64%) amplitudes in infected eyes, and this trend continued in a time-dependent manner with only 10% to 15% amplitude retained at 3 DPI (Fig. 1C).

To determine whether declined ERG response is due to retinal destruction, histologic examination was performed on infected eyes. As shown by light microscopy, the control (PBS-injected) eye exhibited clear cornea and anterior chamber with no visible signs of inflammation, whereas increased corneal opacity, hemorrhage, and inflammation is evident at 1, 2, and 3 DPI (Fig. 1D). The histologic analysis revealed the presence of inflammatory cells into the anterior (Fig. 1E) and posterior (Fig. 1F) segment of the infected eyes. Flow cytometry analysis used for identification and quantification of infiltrated cells revealed that the major cell types are CD45⁺ and Ly6G⁺ cells, suggesting the presence of neutrophils (Fig. 2). The time-course studies revealed a time-dependent increase in polymorphonuclear neutrophils (PMNs) infiltration in infected eyes with approximately 75% PMNs at 3 DPI. This also coincided with increased retinal damage as quantitated by retinal folds with an average 8 ± 2 -fold/retina at 1 DPI, 13 ± 1 -fold at 2 DPI, and 18 ± 1 -fold at 3 DPI. To further assess retinal damage, TUNEL staining was performed to determine retinal cell death. The quantification data showed the presence of 9 ± 1 TUNEL+ve cells/retina (red arrows) at 12 hours post infection, and their number increased to an average of 429 ± 15 TUNEL+ve cells/retina at 24 hours, 1274 ± 60 at 48 hours, and 2086 ± 100 at 72 hours (Fig. 3A). The majority of TUNEL-positive cells were localized in areas adjacent to optic disk at 2 and 3 DPI (Fig. 3B). In contrast, no retinal cell death was observed in PBS-injected control eyes.

Although, both the declined ERG response (Fig. 1C) and the localization of TUNEL-positive cells is suggestive of retinal cell death but not that of infiltrated cells (e.g., PMNs), to ascertain

FIGURE 3. Retinal cell death following *A. baumannii* challenge. Eyes were inoculated with clinical isolate AB12 as described in Figure 1. At the indicated time points, the eyes were enucleated and the cryosections were subjected to TUNEL staining; the blue 4',6-diamidino-2-phenylindole (DAPI) represents nuclei, and the green (FITC) represents TUNEL-positive cells. The total numbers of dead cells in the retinal sections were counted presented as mean $\pm$ SD of TUNEL-positive cells (**A**). Zones of dead cells in the indicated regions of the retina are shown (**B**). Dual labeling using NIMP-R14 (PMN marker) was performed to assess the death of infiltrated PMNs (**C**). In another set, retinal photoreceptor cell line (661W) was challenged with AB12 for indicated time points followed by TUNEL staining (**D**). * $P < 0.05$, ** $P < 0.001$ (Student's *t*-test, PBS versus infected); L, Left; R, Right.

these findings, we performed double staining to identify TUNEL positive and NIMP-R14 (PMN marker) cells. As shown in Figure 3C, only few cells showed dual positivity, a majority of them did not colocalize. To further confirm, we used a cultured mouse-derived photoreceptor cell line (661W)³⁵ and performed the TUNEL assay following *A. baumannii* challenge. To this end, our data showed that *A. baumannii* caused time-dependent photoreceptor cell death (Fig. 3D). Together, these results suggest that ocular isolates of *A. baumannii* have the potential to cause retinal damage leading to vision loss.

Inflammatory Responses in *A. baumannii* Endophthalmitis

As inflammatory mediators play an important role in orchestrating cellular infiltration to the site of infection, we examined the effect of *A. baumannii* endophthalmitis on retinal cytokine/chemokine responses. Total RNA was extracted from the retina of infected eyes over the course of infection and

assessed for mRNA expression of a selected panel of pro-inflammatory cytokines/chemokines by real-time RT-PCR (Fig. 4A). Among the tested cytokines and chemokines, there were substantial increases in IL-1 β , IL-6, TNF- α , and CXCL-2 mRNA expression in the retinas of the infected eyes over the course of the infection, but with different kinetics. The mRNA expression of IL-1 β peaks at 12 hours, IL-6 peaks at 24 hours, and the levels of TNF- α and CXCL-2 peaks at the 48 hour time point. In addition to retinal cytokine/chemokine mRNA levels, the levels of the corresponding cytokine and chemokine proteins were determined by ELISA (Fig. 4B). Similar to mRNA levels, the protein levels of cytokine/chemokine peaked between 12 and 48 hours and subsided by 72 hours, except for TNF- α .

Role of Neutrophils in *A. baumannii* Endophthalmitis

As neutrophils are the first innate immune cells recruited to the retina in endophthalmitis, to determine their role, we used

FIGURE 4. Inflammatory response in *A. baumannii*-infected eyes. Eyes were given intravitreal injection of sterile PBS or ocular AB12 isolate. At indicated time points, the first group of eyes ($n = 4$ per time point) were used for real-time RT-PCR analysis for expression of pro-inflammatory cytokines. The data are presented as fold increase by using a value of 1 for the ratio of the control value to that for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sample (A). The second group of eyes ($n = 4$ per time point) were enucleated, and 10- μ g protein lysate was used for detection of IL-1 β , IL-6, TNF- α , and CXCL2 by ELISA (B). * $P < 0.05$, ** $P < 0.001$ (Student's t -test, PBS versus infected).

FIGURE 5. Pathogenesis of *A. baumannii* endophthalmitis in neutropenic mice. Mice were injected intraperitoneally with 200 μ g of either anti-Ly6G (PMN^{-/-}) or control IgG2b (PMN^{+/+}) antibody 24 hours prior to AB12 challenge. At 72 hours post infection, eyes were enucleated and retinal single cell suspensions were prepared for flow cytometry analysis of PMN infiltration (A). The eyes were also subjected to bacterial load determination (B), cytokine ELISA (C), histologic (D), and cell death (E) analysis. * P < 0.05, ** P < 0.001 (Student's *t*-test, PMN depleted versus normal).

neutropenic mice. First, we assessed the recruitment of PMNs to infected retina, and as expected, drastically reduced PMNs were detected in neutropenic (~1%–2%) versus competent mice (~60%–65%) at 72 hours post *A. baumannii* infection (Fig. 5A). Depletion of PMNs resulted in increased survival of *A. baumannii* in the eyes (Fig. 5B). However, the levels of inflammatory mediators, noticeably TNF- α , were significantly reduced (Fig. 5C). Both histology (Fig. 5D) and cell death assays (Fig. 5E) revealed increased retinal damage in neutropenic mice compared with normal mice (Figs. 1, 3).

Comparison of Pathogenicity of AB12 With Other Ocular *A. baumannii* Isolates

Having shown the pathogenicity of ocular isolate AB12, we next sought to compare the virulence of other ocular isolates, which were susceptible to aminoglycosides, carbapenems, and quinolones. These classes of antibiotics are commonly used for the treatment of acute endophthalmitis.²² We found that all three isolates showed adherence to HCECs (Table). The ATCC 19606 strain showed poor adherence to HCECs, whereas the ocular isolates showed AB7 and AB12 showed strong adherence (Table, Fig. 6A). Both isolate AB8 and AB12 showed strong biofilm formation as compared with ATCC and AB7

(Table, Fig. 6B). Furthermore, the cytotoxicity (LDH release; Fig. 6C) and TUNEL assays (Fig. 6D) showed increased cell death by AB12 as compared with ATCC strain (Table). Overall, AB12 seems to cause more cell death followed by AB8 and AB7.

To test whether these in vitro virulence traits influence the in vivo pathology, eyes were inoculated with ATCC, AB7, AB8, and AB12. The eyes were collected at 72 hours post infection, a time point when there was increased retinal damage in AB12-infected eyes (Fig. 1). Intravitreal injections of all isolates led to cellular infiltration in both anterior and posterior chambers (Fig. 7A). Retinal damage as quantitated by counting retinal folds revealed that both AB8 caused significantly more retinal folding than AB7 and ATCC (Fig. 7B). Furthermore, TUNEL staining of infected retinal sections, also confirmed increased retinal damage in AB8 (Figs. 7C, 7D) and AB12-infected eyes (Fig. 3A).

DISCUSSION

Despite the importance of *A. baumannii* infections, relatively little is known about their pathogenesis and innate host defense mechanisms in the eye. In this study, we tested the

FIGURE 6. In vitro assessment of virulence factors of ocular *A. baumannii* isolates. Cultured HCECs were incubated with ATCC, and ocular isolates, AB7, AB8, and AB12. At indicated time points, cells were rinsed with sterile PBS and trypsinized. Serial bacterial dilutions were performed for enumeration of adhered CFU (A). Biofilm formation was determined by crystal violet staining, and the associated dye was measured at OD₅₅₀ and presented as mean absorbance (B). The conditioned media (100 μ L) of HCECs post infection was used to assess cytotoxicity using Roche LDH Assay Kit. Media without cells served as the low control for the experiment and cells lysed with Triton-X served as the high control. The percentage of cytotoxicity was calculated using the following formula: (Experimental Value – Low Control)/(High Control – Low Control) $\times$ 100 (C). To confirm cell death, TUNEL staining was performed on infected cells (D). Each experiment was performed in triplicate. * $P < 0.05$, ** $P < 0.005$.

pathogenicity of ocular isolates of *A. baumannii* in causing endophthalmitis using a mouse model. In contrast to general consideration as a “low virulence” pathogen,³⁶ our data show that *A. baumannii* causes significant retinal damage leading to declined retinal functions in B6 mouse eyes. We found predominant neutrophil influx and the inflammatory response in the infected eyes, which correlated well with the other bacterial endophthalmitis models.^{29,37} Moreover, the depletion of PMNs led to increased bacterial burden and rendered the eyes to develop severe *A. baumannii* endophthalmitis, suggesting the role of PMNs in retinal innate defense. Furthermore, the ocular isolates that make increased biofilms, tend to cause more retinal damage, implicating biofilm formation as a virulence factor in the development of *A. baumannii* endophthalmitis.

Although, eyes are constantly exposed to microbial insults, they are remarkably resistant to microbial infections. These protective mechanisms are mediated by the strong innate defense both at the ocular surface³⁸ and within intraocular compartments.³⁹ However, ocular surgeries or trauma predisposes the eyes to develop intraocular infections such as bacterial endophthalmitis. Although the studies describing endophthalmitis caused by *A. baumannii* are limited, the clinical reports consensus poor visual and anatomical outcome, including evisceration.^{22,24} One of the contributing factors for the increased prevalence of *A. baumannii* in the hospital environment is their resistance to antimicrobials.⁴⁰

The antibiotic susceptibility testing of the ocular isolates used in this study exhibited diverse MDR phenotypes; in particular, one isolate AB12 was resistant to many classes of antibiotics including β -lactam, aminoglycosides, TET, CHL, and quinolones. One of the potential mechanisms of MDR phenotype of these isolates could be the presence of plasmids encoding MDR genes. Indeed these isolates were found to harbor large size (~90–120 Kb) plasmids.²⁶ The ocular infections caused by MDR bacteria are difficult to treat and require an aggressive antimicrobial therapy.^{41–45} To investigate the pathophysiology of *A. baumannii* endophthalmitis, we tested the virulence of AB12 in a mouse model of bacterial endophthalmitis.²⁷ Our data showed that intravitreally-injected AB12 grows significantly in the vitreous cavity at early (1 and 2 DPI) stages; however, at 3 DPI, the bacterial load is reduced 2-fold. Similar to other organs, the bacterial clearance in the eye is dependent on the early recruitment and infiltration of neutrophils. As they are the first effectors of the innate immune response and contribute to bacterial clearance through their direct antimicrobial capacity,⁴⁶ our data showed that the decrease in bacterial load coincided with increased PMN infiltration in the retina. These findings were supported by inducing endophthalmitis in neutropenic mice, which showed increased intraocular survival of *A. baumannii*, confirming an important role of neutrophils in limiting intraocular bacterial growth.

The infiltrated neutrophils exhibits its antimicrobial properties by phagocytosis, making neutrophil extracellular traps,⁴⁷

FIGURE 7. In vivo assessment of pathogenicity of *A. baumannii* ocular isolates. Eyes were given intravitreal injection of ATCC 19606 and ocular AB7, AB8 isolates. At 72 hours post infection eyes were subjected to light microscopy examination and histologic analysis of the anterior and the posterior segment (A). Retinal damage was assessed by counting the retinal folds (B). Another set of eyes were used for TUNEL staining to confirm retinal damage (C). The figure represents zones of dead cells in the left side, optic disk, and right side of the retina. The total numbers of dead cells in the retinal sections were counted and presented as mean $\pm$ SD of TUNEL-positive cells (D).

generation of reactive oxygen intermediates, and release of enzymes, such as myeloperoxidase, lactoferrin, and elastase.⁴⁸ It is postulated that the release of these inflammatory mediators may result in tissue damage.⁴⁹ Thus, neutrophils play important roles in clearance of infectious agents but, paradoxically, they are also involved in the pathology of endophthalmitis.^{14,15} We observed time-dependent increase in PMN infiltration with highest being at 48 and 72 hours post infection. Similarly, our histologic and TUNEL-staining data revealed significant retinal damage in infected eyes at same time points. This led us to postulate that the increased retinal damage could be due to persistent infiltration of the PMNs. However, PMNs depletion caused even more retinal damage in *A. baumannii*-infected eyes (Fig. 5). This could be due to the increased proliferation of the bacteria in the vitreous cavity in the absence of PMNs. Taken together, these results suggest that *A. baumannii* is pathogenic in the eye and possess the direct ability to cause retinal cell death. To further confirm these findings, we used a retinal photoreceptor cell line and showed that *A. baumannii* causes their cell death (Fig. 3D). These results also support our in vivo findings, which revealed a time-dependent decline of ERG response (Fig. 3C), suggestive of dysfunctional photoreceptors.

Compared with another tissue and organs, the influx of PMNs and other immune cells to the retina is tightly controlled by blood-retinal barriers (BRB). In the case of endophthalmitis this may happen through production of the pro-inflammatory cytokines such as TNF- α .³³ Our data showed increased levels of TNF- α at 3 DPI, a stage where there was increased PMN infiltration and severe retinal damage. Thus, TNF- α secreted by macrophages and neutrophils in response to *A. baumannii* infection may play an important role in the breakdown of the BRB. Indeed, a strong correlation has been reported between

the levels of expression of inflammatory mediators like TNF- α and severity of bacterial endophthalmitis.⁵⁰ Moreover, TNF- α further induces the expression of chemokines with strong chemotactic properties like CXCL2.⁵¹ Such a strong chemotactic drift causes rapid extravasation of neutrophils through the reduced BRB into the vitreous and the subretinal space, which through the secretion of inflammatory mediators further amplifies the extent of inflammation. Our data show increased levels of CXCL2 at later stages of infection and implicates its role in the persistent infiltration of PMNs to the *A. baumannii* infected retina. We observed that the depletion of neutrophils decreased the levels of inflammatory mediators in *A. baumannii*-infected eyes with a marked decline in the levels of TNF- α (Fig. 5C), implying that neutrophils play a critical role in the generation of the pro-inflammatory cytokine responses in the *A. baumannii* endophthalmitis.

Since the majority of postoperative bacterial endophthalmitis are caused by ocular surface flora,^{16,52} we tested the virulence traits of ocular *A. baumannii* isolates using corneal epithelial cells. We reasoned that as HCECs constitute the first line of innate defense against microbes,^{38,53,54} their interaction with ocular pathogens could be critical in determining the disease's outcome.⁵⁵ Biofilm formation and adherence are known virulence factors in the pathogenesis of Gram-negative bacterial infections.^{21,56} In this context, our data showed that ocular isolates of *A. baumannii* can adhere to and cause the cytotoxicity/cell death of corneal epithelial cells. We observed that an ocular isolate AB7 showed increased adherence but reduced biofilm formation, and reduced cytotoxicity. In contrast, the isolate AB8 showed increased biofilm formation and cytotoxicity but reduced adherence. The observed disparity in the adherence and biofilm formation traits of the ocular

isolates could be due to biotic versus abiotic surfaces employed for these assays as reported in previous studies.^{55,57,58} However, our in vivo data shows a correlation of increased biofilm formation and retinal damage. As bacterial biofilm formation has been implicated in the pathogenesis of bacterial endophthalmitis,^{59,60} we propose that biofilm forming ability of *A. baumannii* could be an important virulence factor in causing ocular infection. Further studies using mutant bacterial strains (lacking in biofilm formation) are in progress to elucidate the role of biofilms in *A. baumannii* endophthalmitis.

To conclude, our data support the notion that *A. baumannii* is an emerging but rare ocular pathogen capable of colonizing the ocular surface and causing severe endophthalmitis leading to blindness. Thus, the increasing threat of *A. baumannii* infections in hospitals, combined with the decreasing capacity to effectively treat MDR strains, should prompt more investigations to study the molecular pathogenesis of *A. baumannii* ocular infections.

Acknowledgments

The authors thank Pawan K. Singh (Kresge Eye Institute [KEI]) for his help in performing in vivo experiments and Shruti Agarwal for critically reading the manuscript. The authors also thank retina fellows at KEI for their help in clinical examination of mice, and the lab members for the inspiration and insightful discussion during the study.

Supported in part by a grant from the National Institutes of Health (EY19888); Fight for Sight; Midwest Eye-Banks; an unrestricted grant from Research to Prevent Blindness to the Department of Ophthalmology, Wayne State University School of Medicine; and a PhD fellowship from Oakland University (DT).

Disclosure: **D. Talreja**, None; **K.S. Kaye**, None; **F.-S. Yu**, None; **S.K. Walia**, None; **A. Kumar**, None

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