The Origins of *Staphylococcus aureus* Isolated from Blepharitis Based on Panton-Valentine Leukocidin and Antibiotic Susceptibility Testing

Angela R. Elam[*,1], Tyler A. Kowalski[2], Eric G. Romanowski[2,3] and Regis P. Kowalski[2,3]

[1]*Department of Ophthalmology and Visual Sciences, University of Michigan Kellogg Eye Center, Ann Arbor, MI 48105 USA*

[2]*The Charles T. Campbell Ophthalmic Microbiology Laboratory at the University of Pittsburgh Medical Center, Pittsburgh, PA 15213 USA*

[3]*The UPMC Eye and Ear Institute, Department of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213 USA*

Abstract: *Introduction*: Blepharitis is a common condition, sometimes associated with *Staphylococcus aureus*. Panton-Valentine Leukocidin (PVL) toxin is a *Staphylococcus aureus* virulence factor that can be associated with skin and soft tissue infections. Hospital-acquired *Staphylococcus aureus* infections are generally multi-resistant to antibiotics and PVL-negative. Community-acquired *Staphylococcus aureus* infections tend to be broadly susceptible to antibiotics and PVL-positive. Though blepharitis is a common clinical diagnosis, the source of infection is not fully understood. Focus on the origin of disease could aid in better treatment and reduction of anti-infective resistance. The purpose of this study is to determine if *Staphylococcus aureus* blepharitis is predominantly a community-acquired or hospital-acquired infection based on antibiotic susceptibility and PVL testing. *Materials and Methods*: Fifty-nine de-identified *Staphylococcus aureus* isolates collected from patients with blepharitis were tested for antibiotic susceptibility by disk diffusion using multiple antibiotics from several different classes. The isolates were also tested for the presence of *Staphylococcus aureus* nuclear DNA and PVL toxin gene using PCR. Multi-resistance was defined as resistance to 3 or more classes of antibiotics. *Results*: Of the 59 isolates with PCR-identified *Staphylococcus aureus* DNA, 13 (22%) were multi-resistant; 12 (20%) were methicillin-resistant; and, 3(5%) were PVL-positive. Ten (17%) were multi-resistant and PVL-negative, consistent with hospital-acquired infection. None of the isolates were broadly susceptible to antibiotics and PVL-positive, which would be consistent with community-acquired infection. Forty-nine isolates (83%) (p=0.0001) could not be designated to either group. *Conclusion*: Based on PVL and antibiotic susceptibility testing, our results reject the hypothesis that *Staphylococcus aureus* blepharitis is a community-acquired infection. *Staphylococcus aureus* blepharitis appears not to be predominantly community- or hospital-acquired based on these parameters.

Keywords: Blepharitis, *Staphylococcus aureus*, Panton-Valentine Leukocidin.

INTRODUCTION

Blepharitis is a common condition that involves inflammation or infection of the eyelids. When caused by *Staphylococcus aureus*, treatment is important to avoid other ocular infections, including corneal ulcer, orbital cellulitis and panophthalmitis [1-5]. Panton-Valentine Leukocidin (PVL) is a virulence factor produced by *Staphylococcus aureus* that has been associated with severe skin and soft tissue infections [6]. It is a pore-forming leukotoxin with the ability to lyse leukocytes [6]. There is basis in the literature linking PVL to community-acquired *Staphylococcus aureus* infections [7]. Its presence, or lack thereof, has been linked to classification of community-acquired and hospital acquired staphylococcal infections [6]. *Staphylococcus aureus* isolates that are PVL-positive and susceptible to most antibiotics (including methicillin) are categorized as community-acquired, while PVL-negative isolates that are multi-resistant (including methicillin) are identified as hospital-acquired. While it may be suspected that *Staphylococcus aureus* blepharitis is a community-acquired condition, this has not been determined using PVL testing and antibiotic susceptibilities. Given the nature of the condition, one may reason that the *Staphylococcus aureus* isolates involved in blepharitis would be PVL-positive and pan-susceptible to antibiotics. No studies have been done to classify how *Staphylococcus aureus* behaves in blepharitis versus other sites of the body.

In this study, our goal was to determine whether *Staphylococcus aureus* blepharitis has similar characteristic to the origins of many skin infections. We hypothesize that *Staphylococcus aureus* isolated from blepharitis would possess the *pvl* gene and be pan-susceptible to most antibiotics. This would classify these isolates as community-acquired infections.

*Address correspondence to this author at the Department of Ophthalmology and Visual Sciences, University of Michigan Kellogg Eye Center, 1000 Wall Street, Ann Arbor, MI 48105, USA; Tel: 734-763-5874; Fax: 734-047-3555; E-mail: aelam@med.umich.edu

E-ISSN: 2309-6136/14

MATERIALS AND METHODS

Antibiotic Susceptibility

The antibiotic susceptibility profiles of *Staphylococcus aureus* isolates from blepharitis (1999-2010) were reviewed from the de-identified clinical bank of bacteria collected for yearly susceptibility monitoring as required for laboratory certification. We preliminarily determined that a minimal sample size of 38 isolates was required for a power of 0.9 (testing proportions, Minitab, State College, PA) based on an estimate of 0.5 testing PVL-positive versus 0.75 actually testing PVL-positive. The Clinical and Laboratory Standards Institute methods were used to interpret susceptibility based on serum concentrations because there are no guidelines for interpreting topical antibiotic therapy [8]. The *Staphylococcus aureus* isolates were tested using disk diffusion susceptibility to the following classes of antibiotics: polypeptides (bacitracin and polymyxin B), macrolides (erythromycin), aminoglycosides (gentamicin and tobramycin), fluoroquinolones (ciprofloxacin, ofloxacin, gatifloxacin, and moxifloxacin), miscellaneous (trimethoprim and sulfamethoxazole), oxacillin and cefoxitin. We determined methicillin resistance using cefoxitin. Multi-resistance was defined as resistance to three or more classes of antibiotics. Only robust or heavy isolation of *Staphylococcus aureus* from the eyelids of patients with clinical signs of blepharitis were included in the battery of isolates.

PCR Testing of *Staphylococcus aureus* for *nuc* and *pvl* DNA

The de-identified *Staphylococcus aureus* isolates from blepharitis were retrieved from stocks frozen at -80^{o}C that were saved for new drug validation. The isolates were sub-cultured at 37^{o}C on 5% trypticase soy agar supplemented with 5% sheep blood red cells (BBL™, Sparks, Maryland).

Nucleic acids were obtained from the *Staphylococcus aureus* isolates cells using QuickExtract DNA Extraction Solution (Epicentre). Briefly, 30 microliters were placed in a thin walled PCR tube (0.2 ml) into which a single colony of *Staphylococcus aureus* cells from a plate was placed. The tubes were vortexed briefly to suspend the cells, placed at 65°C for 6 minutes, again vortexed briefly, shifted to 98°C for 2 minutes, and cooled on ice. The resulting lysates were used directly for RT-PCR analysis.

All *Staphylococcus aureus* isolates were PCR tested for the *nuc* gene as a positive control for PCR analysisand the *pvl*gene [9]. The SmartCycler® II system (Cepheid, Sunnyvale,CA) was used to detect both genes, separately. This is a “closed” PCR system where amplification and detection is accomplished concurrently with TaqMan® technology using fluorescent probes to detect amplification after each replicating cycle [8]. All PCR reactions contained 15 µl of master mix and 10 µl of extracted *Staphylococcus aureus* DNA in 25 µl SmartCycler® II tubes (Cepheid, Sunnyvale, CA). The master mix for two reactions comprised of a forward primer, reverse primer, probe, DNAase-free water, and an OmniMix® HS bead [3 units TaKaRa Hot Start Taq™ polymerase, 200µM dNTP, and 4 mM MgCl2 in 25mM HEPES buffer, pH 8.0] [9]. The concentrations of forward primer, reverse primer, and probe in the final reaction tube for the *nuc* and *pvl*genes were 0.05 µM, 0.05 µM, 0.05 µM, and 0.3 µM, 0.3 µM, 0.1 µM, respectively. The primers and probe for the *nuc* gene: [forward] 5’-CAA AGC ATC AAA AAG GTG TAG AGA-3’; [reverse] 5’-TTC AAT TTT CTT TGC ATT TTC TAC CA3’; [probe] 5’-56-FAM –TTT TCG TAA ATG CAC TTG CTT CAG GAC CA-36-TAM-3’(Integrated DNA Technologies, Coralville, Iowa). The primers and probe for the *pvl* gene : [forward] 5’-ACA CAC TAT GGC AAT AGT TAT TT-3’; [reverse] 5’-AAA GCA ATG CAA TTG ATG TA-3’; [probe] 5’-56-FAM- ATT TGT AAA CAG AAA TTA CAC AGT TAA ATA TGA-36-TAM-3’ 3’ (Integrated DNA Technologies, Coralville, Iowa). ATTC *Staphylococcus aureus* isolates (BAA-1680, USA300, *pvl* positive, ATCC, Manassas, Virginia) and (BAA-1681, USA100, *pvl* negative, ATCC, Manassas, Virginia) were used for positive and negative controls for *pvl* PCR testing and both were positive controls for *nuc* PCR testing.

The PCR settings were set in two stages: Stage 1) 95^{o}C for 10 minutes to activate the “Hot Start” Taq-polymerase, and Stage 2) 45 cycles of 95^{o}C for 3 seconds (denaturing); 55^{o}C for 10 seconds (annealing); and, 65^{o}C for 60 seconds (extension) with a temperature increase of 0.5^{o}C per second.

Statistical Analysis

Comparisons between antibiotics and PVL groups were analyzed at a p-value of 0.05 significance using chi-square testing (Minitab, State College, PA).

RESULTS

Figure **1** details the *in vitro* susceptibility to commonly used antibiotics of the 59 isolates included in the study. All of the antibiotics demonstrated *in vitro* efficacy (greater than 80% susceptible) against the

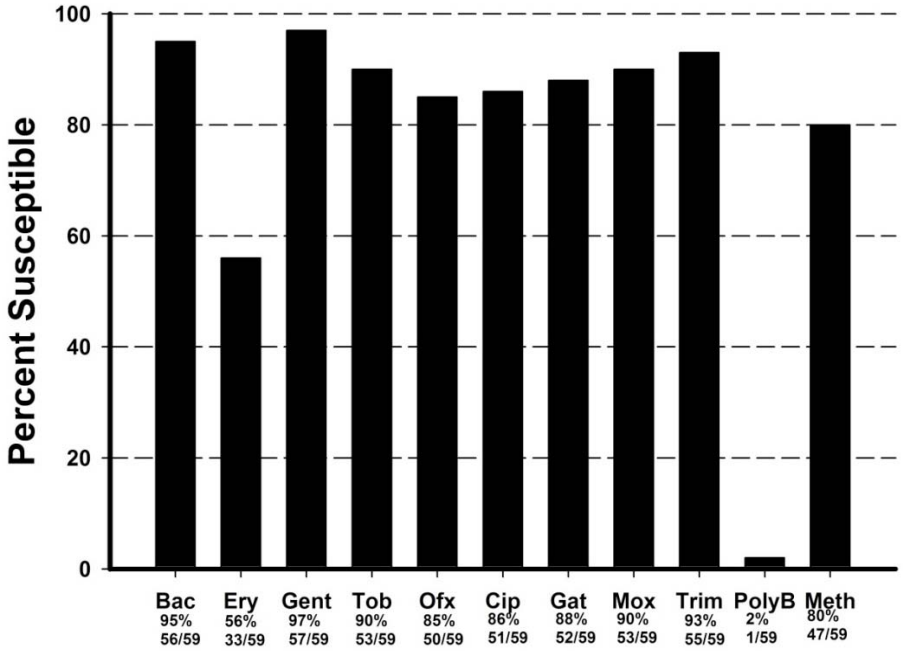

Figure 1: Summary of Bacterial Antibiotic Susceptibilities of 59 Staphylococcus aureus Isolated from Blepharitis as Determined by Disk Diffusion Testing.

Staphylococcus aureus isolates, with the exception of erythromycin and polymyxin B, which demonstrated 56% and 2% susceptibility, respectively. Thirteen (22%) of the isolates were resistant to 3 or more classes of antibiotics tested (multi-resistant). Twelve (20%) isolates were found to be methicillin-resistant.

Figure **2** characterizes the *Staphylococcus aureus* isolates from blepharitis in regards to *in vitro* antibiotic susceptibility and PVL testing. Three (5%) isolates were found to be PVL-positive. Of the 13 multi-resistant isolates, three (23%) were PVL-positive. Three of the 12 (25%) methicillin-resistant isolates were PVL-positive. None of the isolates were broadly susceptible to antibiotics and PVL-positive, which would have been consistent with community-acquired infection. Ten isolates were multi-resistant *in vitro* and PVL-negative, consistent with hospital-acquired infection. Forty-nine isolates (83%) (p=0.0001, Chi square) could not be designated to either group (i.e. were broadly-susceptible to antibiotics and PVL-negative).

DISCUSSION

Is *Staphylococcus aureus* blepharitis a typical skin infection that can be classified based on origin of

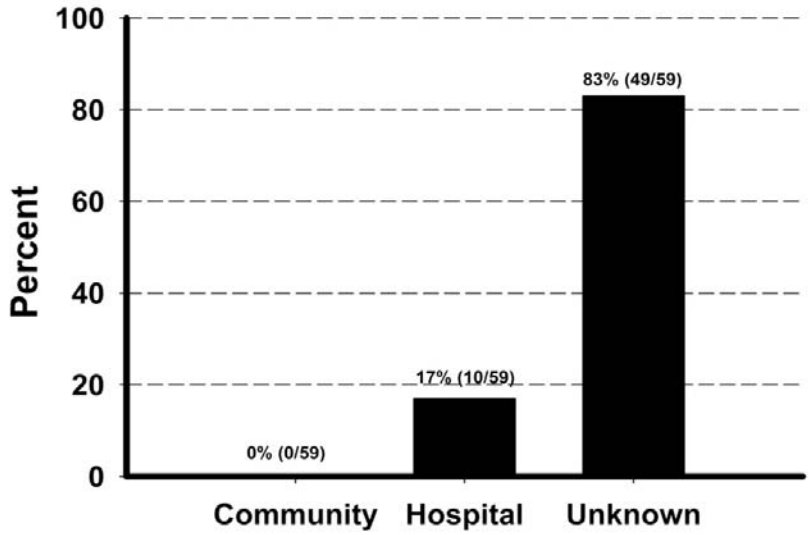

Figure 2: Characterization of Staphylococcus aureus from Blepharitis Based on Antibiotic Susceptibility and PVL Testing.

transmission by the presence of the Panton-Valentine Leukocidin toxin and *in vitro* antibiotic susceptibility testing? Many would presume that *Staphylococcus* aureus blepharitis is a community-acquired infection. However, based on the results of the current study, *Staphylococcus aureus* blepharitis is not easily classified as a community-acquired or hospital-acquired infection. None of the isolates were classified as community-acquired. Ten isolates (17%) were classified as hospital-acquired. The remaining 49 isolates (83%) were broadly-susceptible to antibiotics and PVL-negative. *Staphylococcus aureus* colonizes the skin and mucosa of approximately a third of all immunocompetent adults [9]. These isolates are usually PVL-negative. The fact that the majority of our isolates are PVL-negative, but fall neither into the community-acquired or hospital-acquired category, may shed light on an interesting hypothesis: that *Staphylococcus aureus* blepharitis may be a "self-acquired" condition, or of unknown origin, stemming from the normal flora of human eyelids.

Interestingly, while *Staphylococcus aureus* blepharitis is a skin/mucosal infection, based on PVL testing, it does not appear to behave like many skin/mucosal infections elsewhere in the body. We found it mainly to be PVL-negative, but as previously mentioned, many skin infections caused by *Staphylococcus aureus* are PVL-positive. PVL is strongly associated with skin and soft tissue infections. Colonization and more invasive disease, such as bacteremia or pneumonia, caused by *Staphylococcus aureus* tend to be PVL-negative [11]. This disputes the theory that PVL is associated with invasive disease and poor prognosis [12]. Again, our findings may support *Staphylococcus aureus* blepharitis being a "self-acquired" disease rising from colonization of the eyelids.

In the current study, we found that most of the *Staphylococcus aureus* isolates were susceptible to the most commonly-used antibiotics. Surprisingly though, the isolates were only susceptible to erythromycin about 50% of the time. Erythromycin is commonly used as a treatment for blepharitis, so this finding could potentially change our prescribing practices. Almost all isolates were susceptible to bacitracin, which could potentially provide our patients with an effective, inexpensive treatment.

Possible limitations to this study do exist. Because the *Staphylococcus aureus* isolates were de-identified, there was no clinical or patient information available. This was essentially a masked retrospective study. Perhaps repeating the study in a prospective manner with clinical information readily available would help to build upon the findings of this study. As with any *in vitro* study, the ability to translate the results *in vivo* is not completely certain.

REFERENCES

[1] Lindsley K, Matsumura S, Hatef E, Akpek EK.Interventions for chronic blepharitis.*Cochrane Database Syst Rev*. 2012 May 16; 5: CD005556.

[2] Rutar, T, Zwick O, Cockerham K, Horton J. Bilateral blindness from orbital cellulitis caused by community-acquired methicillin-resistant staphylococcus aureus. *Am J Ophthalmol* 2005; 140: 740-742. http://dx.doi.org/10.1016/j.ajo.2005.03.076

[3] Freidlin J, Acharya N, Lietman T, Cevallos V, Whitcher J, Margolis T. Spectrum of eye disease caused by methicillin-resistant staphylococcus aureus (MRSA) and methicillin-sensitive staphylococcus aureus (MSSA). *Am J Ophthalmol* 2007; 144: 313-315. http://dx.doi.org/10.1016/j.ajo.2007.03.032

[4] Zaidi T, Zaidi T, Yoong P, Pier GB. Staphylococcus aureus corneal infections: effect of the Panton-Valentine leukocidin (PVL) and antibody to PVL on virulence and pathology. Invest Ophthalmol Vis Sci 2013 Jul 1; 54(7):4430-8. http://dx.doi.org/10.1167/iovs.13-11701

[5] Sueke H, Shankar J, Neal T, Winstanley C, Tuft S, Coates R, Horsburgh MJ, Kaye S; Microbiology Ophthalmic Group. lukSF-PV in Staphylococcus aureus keratitis isolates and association with clinical outcome. Invest Ophthalmol Vis Sci. 2013 May 1; 54(5): 3410-6. http://dx.doi.org/10.1167/iovs.12-11276

[6] Boyle-Vavra S, Daum RS. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab Invest* 2007; 87: 3-9. http://dx.doi.org/10.1038/labinvest.3700501

[7] Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis.*Trends in Microbiology.* 2008;16:361-369. http://dx.doi.org/10.1016/j.tim.2008.05.002

[8] National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobials susceptibility tests for bacteria that grow aerobically, ed. 4. Approved standard. Villanova, Pennsylvania, *National Committee for Clinical Laboratory Standards*, 2000 document M7-A5, vol.20, No.2.

[9] McDonald RR, Antonishyn NA, Tansen T, Snook LA, Nagle E, Mulvey MR, Levett PN, Horsman GB. Development of a triplex real-time PCR assay for detection of panton-valentine leucocidin toxin genes in clinical isolates of methicillin-resistant *Staphylococcus aureus. J ClinMicrobiol*. 2005;43(12):6147-6149. http://dx.doi.org/10.1128/JCM.43.12.6147-6149.2005

[10] Kowalski RP, Thompson PP, Kinchington PR, Gordon YJ.The evaluation of the Smart Cycler II System for the real-time detection of viruses and chlamydia from ocular specimens.*Arch Ophthalmol.*2006;124:1135-1139. http://dx.doi.org/10.1001/archopht.124.8.1135

[11] Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, Fosheim GE, Jensen BJ, Killgore G, Tenover FC, Kuehnert MJ.Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004.*J Infect Dis.* 2008;197:1226-1234. http://dx.doi.org/10.1086/533494

[12] Shallcross LJ, Fragaszy E, Johnson AM, Hayward AC. The role of Panton-Valentine leucocidin toxin in staphylococcal disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2013; 13:43-54. http://dx.doi.org/10.1016/S1473-3099(12)70238-4

Received on 28-03-2014 Accepted on 01-04-2014 Published on 03-07-2014

DOI: http:///dx.doi.org/10.12974/2309-6136.2014.02.01.2