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Cornea & External Disease

# Virulence Genes of *Staphylococcus aureus* Associated With Keratitis, Conjunctivitis, and Contact Lens–Associated Inflammation

**Madeeha Afzal[1], Ajay Kumar Vijay[1], Fiona Stapleton[1], and Mark Willcox[1]**

[1] School of Optometry and Vision Sciences, University of New South Wales, Sydney, NSW, Australia

**Correspondence:** Madeeha Afzal, School of Optometry and Vision science, UNSW, Gate14, barker street, Rupert Myers Building (North wing), level 3, Sydney, NSW 2033, Australia. e-mail: m.afzal@unsw.edu.au

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**Purpose:** *Staphylococcus aureus*, cause a range of ocular diseases in humans, including noninfectious corneal infiltrative events (niCIE), infectious conjunctivitis and sight threatening microbial keratitis (MK). This study aimed to determine the possession of known virulence genes of *S. aureus* associated with MK and conjunctivitis, in strains isolated from these conditions and niCIE.

**Methods:** Sixty-three *S. aureus* strains—23 from MK, 26 from conjunctivitis, and 14 from niCIE—were evaluated for possession of genes. Polymerase chain reaction was used for the detection of *mecA* and 10 known virulence genes involved in MK (*clfA*, *fnbpA*, *eap*, *coa, scpA, sspB*, *sspA*, *hla*, *hld*, and *hlg*), 2 associated with conjunctivitis (*pvl* and *seb*).

**Results:** *mecA* was present in 35% of infections and 7% of niCIE strains ($P = 0.05$). It was not seen in infection strains from Australia. Adhesion genes were found in all strains except *clfA*, which was found in 75% of infection and 93% of niCIE strains. Invasion genes were found in higher frequency in infections strains—*hlg* (100% vs. 85%; $P = 0.04$) and *hld* (94% vs. 50%; $P = 0.005$)—compared with niCIE strains. Evasion genes were common in infection strains except *scpA,* which was found at a significantly higher frequency in niCIE strains (86%) compared with infection strains (45%; $P = 0.001$).

**Conclusions:** The higher rates of *hlg* and *hld* in strains isolated from infections than niCIE may have a role in pathogenesis, whereas *scpA* may be an important virulence factor during niCIEs.

**Translational Relevance:** This study has identified virulence factors involved in the ocular pathogenesis of *S. aureus* infections and niCIE.

## Introduction

*Staphylococcus aureus* is an opportunistic human pathogen causing both community and hospital acquired infections. Approximately 30% of humans are asymptomatic carriers and these are at higher risk of infection as well as being a source of infection for others.[1,2] *S. aureus* can cause infection of various ocular sites such as keratitis,[3] blepharitis,[4] and conjunctivitis.[5] *S. aureus* can also cause infectious[6] and noninfectious or inflammatory contact lens–related keratitis,[7] the later are collectively called noninfectious corneal infiltrative events (niCIE).[8]

*S. aureus* secretes toxins and other virulence determinants that play an important role in the pathogenesis of infections.[9] *S. aureus* virulence factors are categorized based on their biological activities and include products involved in adhesion to host tissues or fomites (adhesins), evasion of host defense systems (evasins), and invasion of host tissue (invasins). Adhesins called microbial surface components recognizing adhesive matrix molecules recognize extracellular components.[10,11] For *S. aureus*, these include fibronectin-binding proteins, collagen-binding proteins, clumping factors, and coagulase.[12,13]

Evasion of the host immune system is facilitated by collagen-binding proteins, staphylokinase, enterotoxins, toxic shock protein toxin, lipase, protein A, v8 protease, and leucocidin.[14–16] The toxins alpha, beta, and gamma hemolysins; exfoliative toxins A and B; enterotoxin B; staphopain A and B; phospholipase;

Panton-Valentine leukocidin; and hyaluronidase are involved in the invasion of cells and tissues.[17–23] Some of these virulence factors can function within multiple biological activities, such as collagen-binding protein that is involved both in adhesion and invasion.[24] Alpha toxin and gamma toxin are involved in evasion and invasion.[25,26]

The virulence factors of *S. aureus* that have been associated with microbial keratitis (MK) include genes encoding adhesins *clfA*,[27] *fnbpA*,[18,28,29] *eap*,[18] and *coa*[24] and evasins *scpA*, *sspB*,[18,30], *hla*,[20,21] *sspA*,[31] and invasins *coa*[24] *hlg*.[25,26,32] The virulence factor of *S. aureus* that has been associated with conjunctivitis is *pvl*[23]; *seb* is reported to be involved in contact lens corneal infiltrative events.[19]

Most research has focused on investigating the virulence determinants associated with keratitis, with less information on those associated with conjunctivitis or niCIEs. Therefore, the aim of this study was to explore previously known virulence factors of *S. aureus* isolated from keratitis, conjunctivitis, and niCIE.

## Methods

### S. aureus Isolates

Sixty-three *S. aureus* clinical isolates recovered from ocular diseases were evaluated (Supplementary Table S1). Strains from the Bascom Palmer Institute (Miami, FL) was kindly provided by Dr Darlene Miller; those from Prince of Wales Hospital (Australia) were kindly provided by Dr Monica Lahra. All strains were donated without identifiable patient data. All strains were stored at −80°C in the culture collection of the School of Optometry and Vision Science at the University of New South Wales (UNSW). The genera and species of each strain was confirmed using the automated identification system VITEK 2 for gram-positive bacteria (BioMérieux, Baulkham Hills, NSW, Australia) according to the manufacturer's instructions.

### Virulence Factors of *S. aureus* Strains

Genomic DNA from each *S. aureus* strain was extracted using QIAGEN DNeasy blood and tissue extraction kit (Hilden, Germany). The quantity of the extracted DNA was assessed using a spectrophotometer (Nanodrop ND-1000, ThermoFisher Scientific, Waltham, MA). The eluted DNA was stored at −20°C. Polymerase chain reaction (PCR) amplification and detection of the virulence genes was carried out using gene specific primers (Supplementary Table S2) as described previously.[19,24,26,27,32,34–46,73] PCR was performed in a 25-µL reaction mix, containing 10 to 15 ng of template DNA. PCR amplification reactions were carried out using the PCR Master mix (ThermoFisher Scientific, Vilnius, Lithuania). The thermocycler conditions for amplification were initial denaturation at 94°C for 5 minutes for each primer, with various annealing temperatures and cycles specific for each primer (Supplementary Table S2) and final extension at 72°C for 2 minutes. Synthesized DNA fragments were visualized on 1.0% to 1.5% agarose gel containing GelRed (Biotium, Fremont, CA).

Bands of PCR products in agarose gels after electrophoresis were randomly sampled and sent to the Ramaciotti Centre for Genomic (UNSW, Sydney) for Sanger sequencing using their forward primers to confirm the gene sequences (Supplementary Table S2). Amplified PCR products were cleaned using Exosap-IT kit (ThermoFisher Scientific, Victoria, Australia) with BigDye v3.1 (ThermoFisher Scientific, Victoria, Australia) using Applied Biosystems 3730 DNA analyzer for Sanger sequencing, at a standard annealing temperature (50°C). The sequencing reaction clean-up was carried out using BigDye Xterminator Purification (Life Technologies, Vitoria, Australia). Fast Qc version 0.117 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to assess the quality of sequenced nucleotides using raw reads. Sequences were used for basic local alignment search tools searches conducted against the National Center for Biotechnology Information database to examine the similarity of sequences with available genes sequences of *S. aureus*.

### Statistical Analysis

Differences in virulence gene between the disease groups; infection strains (MK+ conjunctivitis) and noninfectious (niCIE) were analyzed using the $\chi^2$ test (GraphPad Prism 2019, v8.0.2.263). Correlations between possession of *mecA* and oxacillin resistance or possession of virulence genes or being multidrug resistant and possession of virulence genes were analyzed using Spearman's rho. For all analyses, a $P$ value of less than 0.05 was considered statistically significant.

## Results

The genes randomly selected for Sanger sequencing were *fnbpA*, *pvl*, *sspB*, and *clfA* and were confirmed to belong to their respective genes in three US

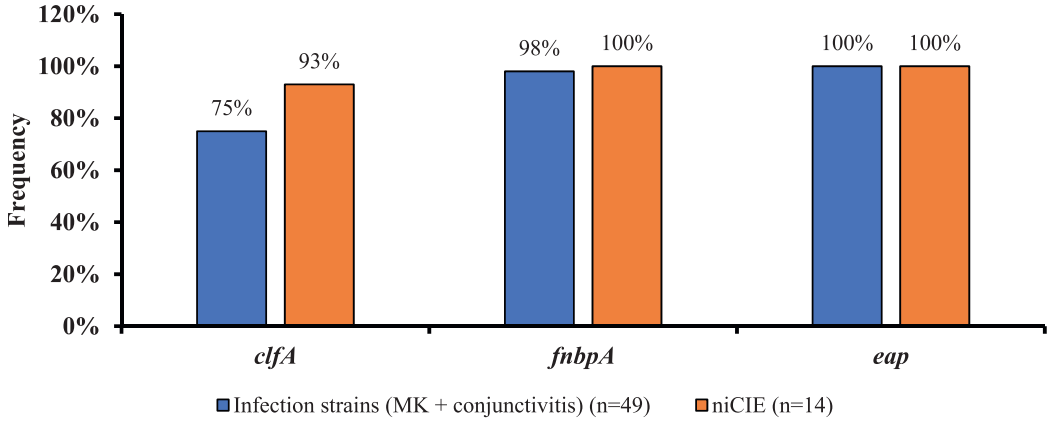


**Figure 1.** Frequency of three virulence genes involved in bacterial adhesion in ocular conditions (differences in frequency of possession of these genes were not significant between isolates from infections or niCIE).

conjunctivitis (100, 102, 103) and three Australian niCIE (12, 20, and 24) strains.

## Methicillin Resistance Gene (*mecA*)

In *S. aureus* isolates, possession of the methicillin resistance gene (*mecA*) was determined using PCR. A higher frequency of *mecA* gene was observed in infection strains 35% (MK, 22%; conjunctivitis, 46%) than niCIE strains (7%) ($P = 0.05$). However, *mecA* was not seen in any infection strains from Australia (0%) vs infection strains from the United States (35%; $P = 0.0001$) (Supplementary Table S3). Oxacillin susceptibility of these strains have been previously published,[33] and there was a significant correlation between possession of *mecA* and oxacillin resistance (correlation coefficient, rs = 0.48, $P = 0.005$). The only other correlation was a negative association between possession of *mecA* and *scpA* (rs = −0.40, $P = 0.001$).

As previously reported, 87% of the isolates were multidrug resistant (MDR); that is, resistant to three or more antibiotics from different antibiotic groups. The following positive correlations between MDR and possession of virulence genes were found: *coa* (rs = 0.34, $P = 0.007$) and *hlg* (rs = 0.29, two-tailed $P = 0.023$), but there was a negative correlation between MDR and possessing *seb* (rs = –0.28; $P = 0.025$). Not surprisingly, there was a strong correlation between possession of *mecA* and oxacillin resistance (s = 0.48; $P = 0.005$). The only other correlation with *mecA* was for possession of *scpA* which was negatively correlated (rs = −0.402; $P = 0.001$).

The correlation between possession of *mecA* and oxacillin resistance was expected. The negative association between possession of *mecA* and *scpA* reflected the sensitivity of the niCIE strains to oxacillin and their possession of *scpA* and might suggest some incompatibility between possession of these genes or their associated mobile genetic elements, and this should be examined in future studies.

## Adhesin Genes

Figure 1 shows the possession of adhesin genes. Most genes were present in all strains. However, there was a difference in the frequency of possession of *clfA*, which was present in 75% of infection strains (MK, 69%; conjunctivitis, 85%) and 93% of niCIE strains; however, this difference was not significant ($P = 0.26$) (Supplementary Table S3).

## Evasin Genes

Most of the evasin genes (Fig. 2) were more common in infection strains than niCIE strains, except *scpA*. For *seb*, there was a trend ($P = 0.06$) for possession of this gene to be at a higher frequency in infection compared with niCIE isolates. For strains from infections, 45% (MK, 48%; conjunctivitis, 42%) possessed *scpA*, whereas 86% of niCIE strains possessed this gene ($P = 0.001$); *scpA* was more frequently observed in infection strains from Australia 83% (MK, 78%; conjunctivitis, 100%) than infection strain from the United States 22% (MK, 0%; conjunctivitis, 68%; $P = 0.0001$) (Supplementary Table S3). Infection strains (MK, 35%; conjunctivitis, 65%) had a higher frequency of *seb* than niCIE strains (21%; $P = 0.0060$ (Fig. 2). Most strains (84%) possessed *sspB* and, although there was a slightly higher frequency of *sspB* in the US MK strains (100%) compared with the

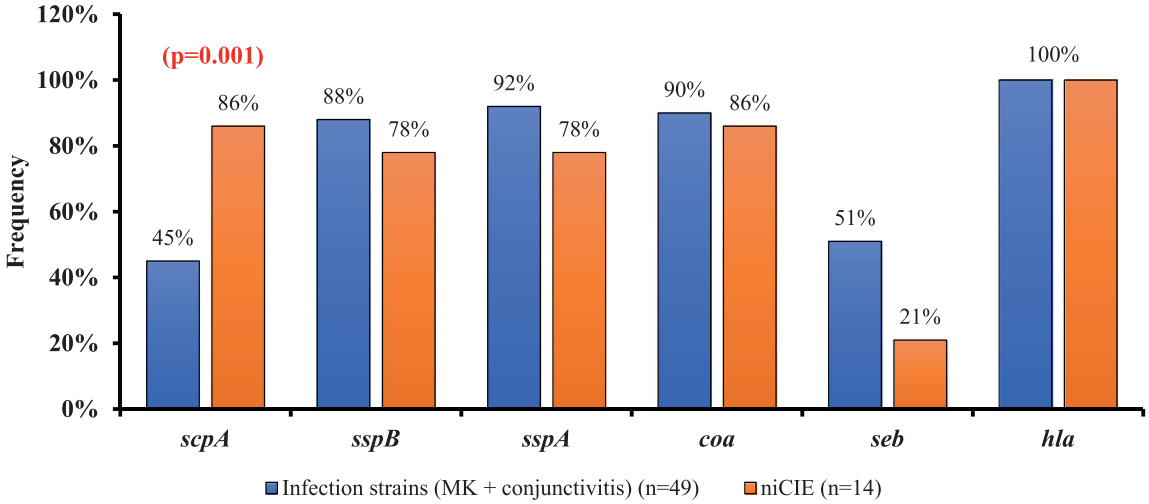


**Figure 2.** Frequency of virulence genes involved in bacterial evasion in ocular conditions, gene showing *P* values, indicates a significant difference between strains isolated from infections and niCIE.

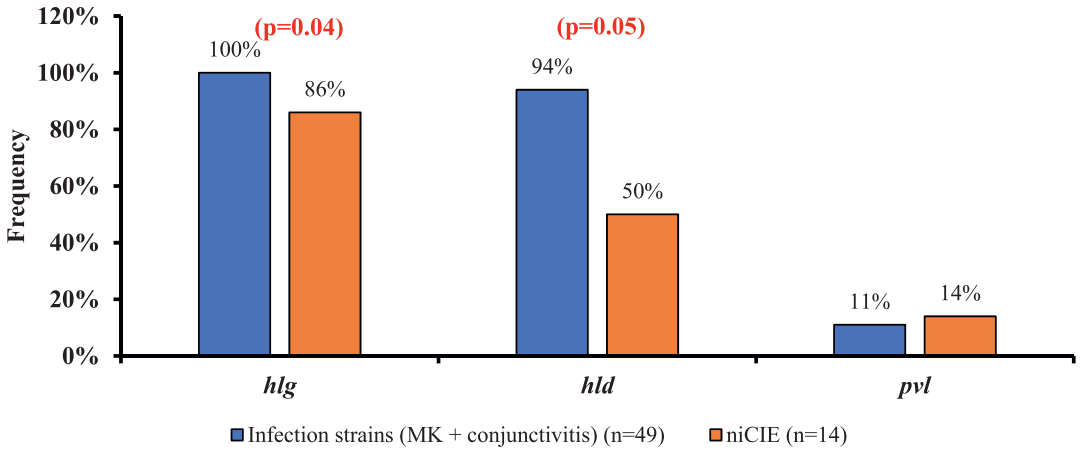


**Figure 3.** Frequency of *S. aureus* virulence genes involved in bacterial invasion in ocular conditions, genes showing *P* values indicates a significant difference between strains isolated from infections or niCIE.

Australian MK strains (78%), this difference was not significant ($P = 0.22$) (Supplementary Table S3). The gene *sspA* that encodes the v8 protease was present in infection strains 94% (MK, 100%; conjunctivitis, 82%), which was slightly more common than in niCIE strains (78%; $P = 0.17$) (Supplementary Table S3). Most infection strains (90%) (MK, 100%; conjunctivitis, 77%) possessed *coa*, as did most niCIE strains (86%; $P = 0.6$).

## Invasin Genes

The genes *hla* and *eap,* which are involved in invasion of host tissues, were present in all strains, whereas there were differences in possession of *hlg* and *hld* (Fig. 3). Most infection strains (94%) (MK, 100%; conjunctivitis, 86%) possessed *hld*, whereas only 50% of niCIEs strains possessed this gene ($P = 0.005$) (Fig. 3). Similarly, 100% of infection strains (MK, 100%; conjunctivitis, 100%) possessed *hlg*, whereas only 86% of niCIEs strains possessed this gene ($P = 0.04$). Possession of *pvl* varied with condition and origin of the isolates, but those differences did not reach statistical significance (16% of US infections vs. 0% of Australian infection strains; $P = 0.14$ [Supplementary Table S3]; 14% of niCIE strains from Australia vs. 0% of infection strains from Australia; $P = 0.18$).

# Discussion

This study has demonstrated distinct differences in *S. aureus* virulence determinants in strains isolated

from ocular infections and niCIE. The hypothesis of the current study was that there would be more potential virulence determinants produced by infection strains than noninfectious strains. The hypothesis was confirmed, with PCR of infection strains frequently possessing the virulence genes *scpA*, *seb*, *hld*, and *hlg* compared with the niCIE strains.

Previous studies have reported the virulence factors of *S. aureus* that have been associated with corneal infections such as *clfA* as an important virulence factor in skin and soft tissue infections, *fnbpA*[18,28] and *coa*[24] described as mediating adhesion and initiating keratitis. Similarly, *eap* was shown as an important virulence determinant in a clinical *S. aureus* keratitis isolate.[18] Alpha-toxin (*hla)* was shown as major virulence factor in both rabbit and murine models of keratitis.[21] *SspA*/*v8* caused severe pathology in rabbit corneas.[31] Another study showed involvement of *sspA* and *sspB* in adhesion and invasion of human corneal epithelial cells.[35] Similarly *hlg* was reported to contribute in corneal virulence,[25,32] whereas *pvl*[23] and *seb*[19] were reported to contribute in conjunctivitis and contact lens infiltrative events virulence, respectively.

All ocular strains in the current study possessed the adhesins *eap* and *fnbpA* and most possessed *clfA*. The product of extracellular adhesion protein (*eap)* is an important virulence determinant of *S. aureus*, promoting adhesion and internalization of the organism into epithelial[47] and other mammalian cells,[48] and binding to plasma proteins including fibrinogen and fibronectin.[49,58] Most (97.9%) of the *S. aureus* possess *eap,* and it is important in the colonization of cornea in *S. aureus* keratitis.[50] So, the possession by all strains in the current study reinforces its role in adhesion and in ocular infections and inflammatory conditions. Most strains in the current study (98%) also possessed *fnbpA*. The product of *fnbpA,* fibronectin binding protein A, promotes adhesion to mammalian cells and initiation of biofilm formation.[51] *FnbpA* and *eap* play contributary roles in the internalization of *S. aureus.*[52] A study comparing a clinical *S. aureus* isolate with a less virulent laboratory strain identified *fnbpA* and *eap* as important virulence factors. The clinical *S. aureus* isolate produced more potentially important virulence factors than the less virulent laboratory strain, accounting for the ability of the clinical *S. aureus* isolate to cause more severe keratitis.[18] Ocular surface cell injury, which may occur during contact lens wear[53,54] or ocular surface disease,[17] increases the presence of fibronectin on the ocular surface.[34] This property may enhance the ability of *S. aureus* to cause ocular infection or inflammation. Similarly, the high frequency of possession of *clfA*, the product of which is a fibrinogen binding protein of *S. aureus*, suggests an important role for this gene/protein in ocular surface disease. Clumping factor is an important virulence factor of *S. aureus* in other skin and soft tissue infections[55,56] and blood,[57] often by increasing bacterial survival in the bloodstream.

Most strains possessed all the genes associated with evasion of the host defense systems (*sspB*, *sspA*, *coa*, and *hla*). The product of *hla*, namely, alpha hemolysin, is a major virulence factor of *S. aureus* in rabbit models of keratitis[21,36] and is associated with corneal damage. Although an alpha toxin deficient strain of *S. aureus* can still produce the niCIE contact lens peripheral ulcers in a rabbit model,[37] the gene seems to have been retained by strains causing niCIE and so it may be needed for survival of these strains before them being isolated from niCIE. The product of *sspA*, a serine protease (also called *v8* protease), after maturation targets the Fc regions of immunoglobulins leading to partial loss of antigenic determinants, interfering in the interaction between cell surface antigens and immune effector cells mediated by immunoglobulins.[56] This serine protease is also important in biofilm remodelling.[59,60] This serine protease can cause severe pathology in rabbit corneas.[36] All strains had one or more of the protease genes *scpA* (encoding the cysteine protease staphopain A), *sspB* (encoding the cysteine protease staphopain B), or *sspA* (encoding the serine protease *v8*). There is a complex interplay between *scpA* and *sspB* in which the product of *sspA* is important in adhesion and internalization of the bacteria into human corneal epithelial cells by activation of *fnbp* and assists with evasion by delaying the host immune system, whereas *sspB* is involved in the modulation of bacterial invasion.[30] However for collagen binding adhesin (coa), a study used rabbit model, in which contact lenses soaked in *S. aureus* strains containing the collagen-binding adhesin (*cna*+) or its isogenic mutant lacking the adhesin (*cna*−), were placed on the injured cornea and the outcome showed that *cna* significantly contributed to bacterial adherence and corneal colonization and produced suppurative inflammation in a rabbit model of soft contact lens–associated bacterial keratitis more often than its collagen binding–negative isogenic mutant, which suggests that collagen-binding adhesin is involved in the pathogenesis of *S. aureus* keratitis.[24] The possession of this gene by most strains in the current study suggests that it may be important for virulence in ocular infection and inflammation.

In the current study, more strains isolated from infections than niCIEs possessed *seb* (Fig. 2). The product of *seb* had been previously shown to stimulate high level of cytokines in cultured corneal epithelial

cells to produce inflammation, especially interleukin 8, it did not stimulate the production of leukotriene B4 in an in vitro cell culture model.[19] Because leukotriene B4 has been associated with contact lens peripheral ulcers,[62] which are associated with the adhesion of *S. aureus* to contact lenses,[63] it is perhaps not surprising that possession of *seb* was low in strains from niCIEs in the current study. *S. aureus* isolates from various forms of allergic conjunctivitis with concurrent corneal ulceration possessed *seb* more frequently compared with patients with no ulceration,[64] which suggests a role in keratitis. The proinflammatory nature of the *S. aureus* super antigen *seb* has been shown to induce conjunctivitis with localized cutaneous swelling in 1 to 6 hours after accidental ocular exposure to *seb* in three US laboratory workers.[61]

There were also some differences in the frequency of genes often associated with the invasion of host tissues. All strains from infections possessed *hlg*, but this rate was significantly lower for strains from niCIEs. Strains deficient in *hlg* have decreased virulence in a rabbit model of MK[25]; the injection of gamma-toxin in cornea induced disease and caused corneal pathology,[32] but perhaps this toxin is not as important in survival of strains that go on to cause niCIE. Gamma toxin (*hlg*) and Panton Valentine leucocidin (*pvl*)[23] contribute to corneal virulence. In the current study, only 11% of the strains possessed *pvl*, and more conjunctivitis strains isolated from the United States (18%) possessed *pvl* than strains from Australia (0%). The possession of *pvl*, a bicomponent leukocidin that is responsible for leukocyte death, is often associated with community-acquired methicillin-resistant *S. aureus* (MRSA) strains.[51] In the current study, a higher frequency of the strains from the United States possessed *pvl* so they were probably more likely to be community acquired. According to literature data *pvl* gene is present in approximately 2% to 5% of *S. aureus* isolates.[65,66] The detection of *pvl* gene in strains, and their correlation with methicillin resistance, could be addressed in the future study. Eighty-four percent of all the strains in the current study possessed *hld* (MK, 100%; conjunctivitis, 88%), but this rate was significantly lower for strains from niCIEs (50%) (Fig. 3). However, strains producing *hld*, but not other hemolysins, produce minimal corneal virulence, suggesting that *hld* is not an important virulence factor in keratitis.[67] In the current study significantly higher rates of *hlg* and *hld* in infections (MK+ conjunctivitis) and lower rates for strains from niCIE (Supplementary Table S3) indicates that *hlg* and *hld* may have a role in the pathogenesis of keratitis.

Previously reported antibiotic susceptibility data of these isolates[33] demonstrated that although most of the strains were MDR, noninfectious (niCIE) strains were more susceptible to antibiotics than conjunctivitis strains, and conjunctivitis strains were more susceptible than MK strains. MK strains from Australia were more susceptible compared with MK strains from the United States. One MDR strain from niCIE group (*S. aureus*, 27) had a higher minimum inhibitory concentration to all tested multipurpose disinfectant solutions.[33] The correlation between possession of *mecA* and oxacillin resistance was expected. The negative association between the possession of *mecA* and *scpA* might suggest some incompatibility between the possession of these genes or their associated mobile genetic elements, and this finding should be examined in future studies.

The presence of a gene does not necessarily mean the expression of that gene; the expression of *S. aureus* virulence factors is regulated by a complex regulatory system that enables the bacteria to adapt to different host environments. Several global regulators are influenced by environmental stimuli, such as nutrients and oxygen availability, cell density, pH, and osmolarity.[68–71] Moreover, studies showed that host niche–specific factors also have an impact on *S. aureus*.[72] Further gene expression study will help to better understand the *S. aureus* pathogenicity and the factors influencing the gene expression during ocular infections.

Because *S. aureus* MRSA strains are more likely to be MDR and difficult to treat, the association between ocular MRSA strains and *S. aureus* cytolysin (*hla, hlg* and *pvl*) has been studied widely.[75,77] One study found ocular MRSA population was dominated by two major clonal complexes, CC8 (40%) and CC5 (47%), which are also common causes of MRSA infections in other body sites. Alpha-toxin (*hla*) secreted by *S. aureus* is shown to interfere with the corneal epithelial wound healing, it also promotes invasion of pathogen within the inner layers of cornea. The virulence factors *hla*, *hlg*, and *pvl* contribute to ocular tissue damage and inflammation worldwide.[23,74] Because most community-acquired MRSA strains possess *pvl* and *pvl* has cytotoxic activity against many different types of immune cells,[23] ocular *S. aureus pvl*–positive strains showed worst clinical outcomes (greater treatment time, healing, and ulcer size) and more surgical interventions compared with *pvl*–negative ocular *S. aureus* strains.[76] Additionally, the prevalence of *S. aureus* virulence factors such as *pvl*, enterotoxin E (*sea*) or leucocidin E (*LukE*), among ocular isolates, have demonstrated that *pvl* and *lukE* were found in majority of ocular strains, whereas *sea* was less common.[78,79] However, a recent study showed that the enrichment of enterotoxin superantigens in *S. aureus* ocular strains when compared with nonocular *S. aureus* strains.[80]

The present study has some limitations. This study used a convenience sample of strains within the culture collection of School of Optometry and Vision Science, UNSW Sydney, Australia. All strains from the United States were isolated in 2004. The Australian isolates were isolated over a longer period, from 1995 to 2018, with the majority from infection being isolated between 2006 and 2018 (17/18). Although the number of virulence genes of the Australian isolates were on average $10 \pm 1$ between 1995 and 2006 and in 2018 and the types of genes did not change, the determination of virulence genes with additional new *S. aureus* isolates from the United States might be useful to provide further insights into the involvement of virulence genes in the pathogenesis of keratitis and conjunctivitis and to see if genes change over time. Another limitation that isolates with polymorphism or closely related isolates may show false-negative or false-positive PCR results. This limitation could be addressed in the future study by analyzing single nucleotide polymorphisms in the genomes of the strains. Because these strains have not been genotyped previously, exploring whether these strains possess these known virulence gene would help future studies to explore the genetic relationship of isolates (sequence types or clonal complexes) along with *agr* profiles, which could also shed light on the pathogenic potential of the isolates from the epidemiologic point of view.

Overall, the findings of this study illustrate that the genes involved in adhesion, except *clfA*, were observed in most of the strains. Conversely, infection strains had a higher frequency of the genes involved in evasion and invasion compared with niCIEs. Whole-genome sequencing can provide a powerful tool to understand the virulence determinants associated with pathogenesis of infections and noninfectious ocular conditions. Additionally, the presence of a gene does not indicate whether the gene is expressed during ocular infections, and further animal model studies using gene knockout for *hlg*, *hld*, *pvl*, *coa*, *seb*, *scpA*, and *sspB* and their effect on keratitis could help to understand their involvement in its pathogenesis and potential therapy.

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