

Susceptibility of *Staphylococcus aureus* Isolates Causing Bloodstream Infection to the Antimicrobial Peptide LL-37

Sinead McNicholas^{1,2}, Hilary Humphreys^{1,2}, Deirdre Fitzgerald Hughes^{1*}

¹Department of Clinical Microbiology, RCSI Education and Research Centre, Beaumont Hospital, Dublin, Ireland

²Department of Microbiology, Beaumont Hospital, Dublin, Ireland

Email: *dfitzgeraldhughes@rcsi.ie

Received January 2, 2013; revised February 8, 2013; accepted February 25, 2013

ABSTRACT

Both bacterial and host factors contribute to complicated bloodstream infection (BSI) caused by *Staphylococcus aureus* including methicillin-resistant *S. aureus* (MRSA). One bacterial factor that may affect the persistence of *S. aureus* in complicated BSI is reduced susceptibility to the innate immune defence peptide LL-37. LL-37 susceptibility among *S. aureus* isolates causing uncomplicated and complicated BSI was investigated. Following incubation with 2.5 µg/ml LL-37 for 1 h, the mean percentage survival was 67.6% and 74.9% for isolates causing uncomplicated and complicated BSI, respectively. Reduced LL-37 susceptibility may contribute to the persistence of *S. aureus* in complicated BSI.

Keywords: *Staphylococcus aureus*; Bloodstream Infection; LL-37

1. Introduction

The clinical outcome of bloodstream infection (BSI) caused by *Staphylococcus aureus* is variable and both bacterial and host innate immune factors may contribute to the clinical course of infection. The innate immune system acts as the first line of defence against microorganisms such as *S. aureus* and cationic anti-microbial peptides (CAMPs) are an important component of the innate immune response [1]. LL-37 is a cathelicidin, found in various cell types including neutrophils, monocytes, epithelial cells, salivary glands and sweat glands, and this molecule has important functions which include; the protection of skin and epithelial surfaces from invading organisms, the direct killing of phagocytosed bacteria and immune-regulation [1,2]. Its concentration on unstimulated mucosa ranges from 2 - 5 µg/ml but may increase in response to infection to levels above 30 µg/ml [3]. *S. aureus* has evolved mechanisms that confer reduced susceptibility to CAMPS such as LL-37, allowing it to evade this important component of innate immunity. Although these mechanisms are poorly understood, a number of potential factors have been identified and include aureolysin, which inactivates LL-37 and both polysaccharide intracellular adhesion (PIA) and fibrinogen-binding protein (fnBP) which have been shown to reduce susceptibility to LL-37 [4-6]. In addition, increased surface charge, achieved by the incorporation of D-alanine into teichoic acid or of lysine into phosphati-

*Corresponding author.

dylglycerol may reduce susceptibility to LL-37. Twenty *S. aureus* isolates causing BSI were assessed to determine if isolates differed in their susceptibility to LL-37 at physiological concentrations and whether *in-vitro* susceptibility to this peptide correlated with persistence or potential for complications of *S. aureus* BSI.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Twenty *S. aureus* isolates that caused BSI in Beaumont Hospital Dublin between the years 2008-2011 were selected for this study. Ten isolates had caused uncomplicated BSI and 10 had caused a complicated infection, defined as a persistent (lasting >3 days despite appropriate antibiotics) or disseminated infection (e.g. osteomyelitis). Isolate details are summarized in **Table 1**. Three reference strains were also included, Methicillin-resistant *S. aureus* (MRSA) strain COL, and *S. aureus* SH 1000 (kind gifts from Dr. Timothy Foster, Trinity College Dublin and Dr. Simon Foster, University of Sheffield, UK) and *S. aureus* ATCC 44330.

2.2. DNA Microarray and *spa* Typing

A combination of DNA microarray and *spa* typing was used to determine the clonal complex (CC) to which each isolate belonged. DNA microarray was also used to determine the presence or absence of genes that may contribute to LL-37 susceptibility. Genomic DNA was

Table 1. Details of *S. aureus* isolates from uncomplicated and complicated BSI, used for LL-37 bactericidal assay.

Isolate No.	Organism	<i>mprf</i> ^a	<i>aur</i> ^a	<i>ica</i> ^a	<i>fnBPA</i> ^a	Capsule type ^a	Clonal complex (CC) ^b
Uncomplicated							
11	MRSA	+	+	+	+	5	CC22
13	MSSA	+	+	+	+	8	CC15
16	MSSA	+	+	+	+	8	CC15
22	MSSA	+	+	+	+	5	CC25
23	MSSA	+	+	+	+	5	CC5
24	MSSA	+	+	+	+	5	CC22
30	MSSA	-	+	+	+	8	CC45
32	MSSA	+	+	+	+	8	CC15
50	MSSA	+	+	+	+	5	CC5
58	MSSA	+	+	+	+	8	CC30
Complicated							
3	MSSA	+	+	+	+	8	CC12
7	MRSA	+	+	+	+	5	CC20
15	MSSA	+	+	+	+	8	CC30
17	MSSA	+	+	+	+	5	CC8
31	MRSA	+	+	+	+	5	CC22
33	MSSA	-	+	+	+	8	CC45
37	MSSA	+	+	+	+	8	CC30
48	MSSA	+	+	+	+	8	CC15
49	MRSA	+	+	+	+	5	CC22
53	MSSA	+	+	+	+	5	CC5

^aPresence or absence of gene determined by DNA microarray; ^bClonal lineage determined by a combination of DNA microarray and *spa* typing as described in the methods. *Mprf*, encodes multiple peptide resistance factor, *aur* encodes aureolysin, *ica* encodes intracellular adhesion, *fnBPA* encodes fibrinogen binding protein.

extracted using a DNeasy[®] blood and tissue kit (Qiagen, Crawley, UK). *Spa* typing, which involves PCR amplification and sequencing of the polymorphic 24 base pair variable number tandem repeat region within the 3' end of the protein A gene *spa*, was carried out according to the SeqNet website (<http://www.seqnet.org>). Sequencing was performed by Beckman Coulter Genomics (Takeley, UK) and Source BioScience (Dublin, Ireland). Genetic characterization of isolates was undertaken using the StaphyType Kit (Alere Technologies Germany) as previously described [7].

2.3. LL-37 Bactericidal Assay

A modification of the method described by Ouhara *et al.* (2008) [8] was used. Briefly, colony survival was determined from viable cell counts following incubation of 5×10^5 CFU/ml *S. aureus* with 2.5 µg/ml LL-37 in 10 mM

sodium phosphate buffer, pH 7.4 for 1 h and dilution (1/10) with 0.95% w/v NaCl. Percentage survival was calculated from viable counts (CFU/ml) from assays containing LL-37 compared to control assays containing no LL-37. The assay was performed in duplicate on three occasions for each isolate and the reference strains. Preliminary investigation of laboratory strains of *S. aureus* demonstrated concentration-dependent susceptibility to LL-37. The lowest concentration tested (2.5 µg/ml) allowed the observation of susceptibility differentials between strains (24%, 54%, 92% survival for COL, SH 1000 and 8325-4 respectively) and this concentration was therefore chosen for investigation of clinical isolates of *S. aureus*.

2.4. Statistical Analysis

Mean survival following incubation with LL-37 was

compared between isolates from uncomplicated *Vs* complicated infection using the Student's t-test for statistical significance.

3. Results

3.1. Survival of *S. aureus* Following Incubation with LL-37

Among *S. aureus* clinical isolates causing complicated and uncomplicated BSI there was a wide variation in susceptibility to killing by LL-37 under the assay conditions used (uncomplicated BSI isolates, % survival; range = 24.2% - 100%, mean = 67.6%, complicated *S. aureus* BSI isolates, % survival; range = 52.8% - 100%, mean = 74.9%. However, overall, *S. aureus* isolates causing uncomplicated BSI were more susceptible to killing by LL-37 than isolates causing complicated BSI but this difference was not statistically significant ($p = 0.48$, Student's t-test) (Figure 1(a)). MRSA isolates were more resistant to LL-37 killing (percentage survival, range 57.94% - 100%, mean 80.5%) than MSSA isolates (percentage survival, range 24.2% - 100%, mean 68.9%) but this difference was not statistically significant ($p = 0.47$) (Figure 1(b)).

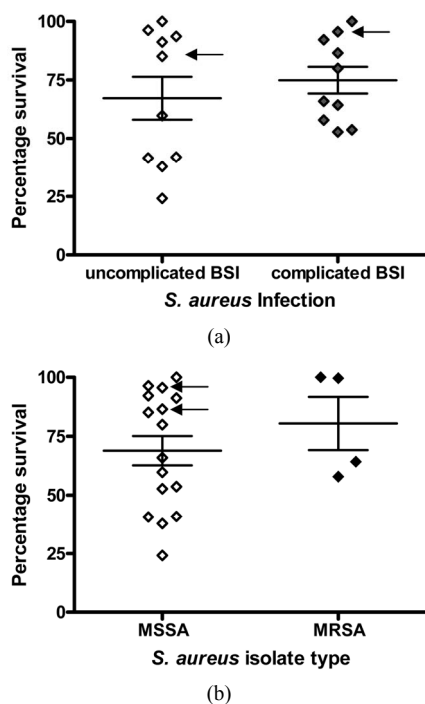


Figure 1. LL-37 Susceptibility of *S. aureus* isolates causing BSI. The percentage survival after exposure to 2.5 µg/ml LL-37 for 1 h is shown. Each symbol represents % survival calculated from assays carried out in duplicate on three separate occasions (a) *S. aureus* isolates causing uncomplicated BSI (n = 10) and complicated BSI (n = 10); (b) MSSA isolates causing BSI (n = 16) and MRSA isolates causing BSI (n = 4). Arrows indicate the *S. aureus* isolates that were *mprf*⁺.

3.2. LL-37 Susceptibility, Clonal Complex and Carriage of Selected Virulence Genes

S. aureus isolates causing both complicated and uncomplicated infections belonged to a variety of CCs (Table 1) but only CCs 5, 15, 22, 30, and 45 were common to isolates from complicated and uncomplicated infections. The LL-37 susceptibility of the most prevalent CCs (CC15(4), CC22(4), CC5(3) CC30(3)), were compared but there was no statistically significant difference in LL-37 bactericidal activity against isolates from different CCs (data not shown).

Genes that encode virulence factors that may influence LL-37 susceptibility among *S. aureus*, include *mprf*, *aur*, *ica* and *fnBPA*. Microarray analysis revealed the carriage of *mprf* in 18/20 *S. aureus* isolates (9/10 complicated and 9/10 uncomplicated) and the other three genes were found in all isolates investigated (Table 1). LL-37 susceptibility of the two *mprf*⁺ isolates was comparable to a number of *mprf*⁻ isolates. The two *mprf*⁺ isolates are highlighted in Figure 1. Genes encoding capsule types 3 and 8 were equally distributed among isolates from complicated and uncomplicated infections (Table 1). Furthermore, no statistically significant difference in LL-37 susceptibility between capsule type 5 and capsule type 8 *S. aureus* isolates was found (74.4% Vs 68.1% survival respectively, $p = 0.74$).

4. Discussion

There was marked variation in the *in-vitro* susceptibility of *S. aureus* isolates to LL-37 and high variation has been previously reported among other collections of *S. aureus* clinical isolates [9]. Cell survival following incubation with LL-37 was greater for *S. aureus* isolates causing complicated BSI compared to uncomplicated BSI. Although this is the first study that we are aware of, that assessed bloodstream isolates, the numbers studied may have precluded the demonstration of statistical significance. The relatively low rates of complicated cases of BSI caused by *S. aureus* (less than 15% of *S. aureus* BSI in our hospital during the study period) was a limiting factor in the present study. Therefore further studies are required on larger numbers of isolates from more than one centre.

It has previously been shown that MRSA isolates are more susceptible to killing by LL-37 than MSSA isolates [5,8,10]. Although the number of isolates studied here were relatively low, this pattern of MRSA/MSSA comparative susceptibility was not found in *S. aureus* causing BSI. Removing the four MRSA isolates from the previous comparison of uncomplicated Vs complicated infections did not result in a significant change in the statistical comparison of LL-37 susceptibility (percentage survival 64.1% Vs 75.3%, $p = 0.39$).

LL-37 has been shown to play an important role in the innate immune response to *S. aureus* infection. However, *S. aureus* has evolved mechanisms that confer reduced susceptibility to LL-37 (e.g. increasing surface charge, inactivation of LL-37 by aureolysin) [4-6,10,11]. A number of other factors (PIA production, fnbP and the presence of a capsule) have also been shown to influence susceptibility of *S. aureus* isolates to killing by CAMPs, including LL-37 although the exact mechanisms are unknown [4,5]. The ability of certain *S. aureus* isolates to evade and inactivate this important antimicrobial peptide may contribute to their persistence in complicated BSI. However resistance to β -lactam antibiotics, capsule type or clonal type of the isolate did not significantly influence *in-vitro* susceptibility to LL-37.

Variations in LL-37 susceptibility among the isolates were also independent of the presence of genes encoding lysylphosphatidylglycerol synthetase (*mprf*), polysaccharide intracellular adhesin (*ica*), fibronectin binding protein (*fnbAB*) and aureolysin (*aur*) as all isolates investigated, contained them (except two which did not contain *mprf*). *S. aureus* causing BSI may produce other as yet unidentified virulence factors that may protect against innate immune defences. It is also possible that the expression of these genes varies among *S. aureus* isolates, which may contribute to the variations found. Expression of virulence genes is highly dependent on the dynamic environment encountered by the organism during infection and the killing assay used here does not facilitate investigation of the effects of these factors on LL-37 susceptibility.

In conclusion, LL-37 susceptibility may play a role in the persistence or potential for complications of *S. aureus* BSI, but the complex roles of LL-37 in the immune response against *S. aureus* and the significance of LL-37 resistance among *S. aureus* isolates, warrants further study.

5. Acknowledgements

We are grateful to the staff of the Microbiology Department, Beaumont Hospital, who provided *S. aureus* isolates. The authors acknowledge financial support from Pfizer Ireland through an Educational Award, No. WS 376235.

REFERENCES

- [1] J. D. Heilborn, M. F. Nilsson, G. Kratz, G. Weber, O. Sorensen, N. Borregaard and M. Stahle-Backdahl, "The Cathelicidin Anti-Microbial Peptide LL-37 Is Involved in Re-Epithelialization of Human Skin Wounds and Is Lacking in Chronic Ulcer Epithelium," *Journal of Investigative Dermatology*, Vol. 120, No. 3, 2003, pp. 379-389. [doi:10.1046/j.1523-1747.2003.12069.x](https://doi.org/10.1046/j.1523-1747.2003.12069.x)
- [2] A. R. Kocuzilla and R. Bals, "Antimicrobial Peptides: Current Status and Therapeutic Potential," *Drugs*, Vol. 63, No. 4, 2003, pp. 389-406. [doi:10.2165/00003495-200363040-00005](https://doi.org/10.2165/00003495-200363040-00005)
- [3] J. Overhage, A. Campisano, M. Bains, E. C. Torfs, B. H. Rehm and R. E. Hancock, "Human Host Defense Peptide LL-37 Prevents Bacterial Biofilm Formation," *Infection and Immunity*, Vol. 76, No. 9, 2008, pp. 4176-4182. [doi:10.1128/IAI.00318-08](https://doi.org/10.1128/IAI.00318-08)
- [4] I. Fedtke, F. Gotz and A. Peschel, "Bacterial Evasion of Innate Host Defenses—The *Staphylococcus aureus* Lesson," *International Journal of Medical Microbiology*, Vol. 294, No. 2-3, 2004, pp. 189-194. [doi:10.1016/j.ijmm.2004.06.016](https://doi.org/10.1016/j.ijmm.2004.06.016)
- [5] H. Komatsuzawa, K. Ouhara, S. Yamada, T. Fujiwara, K. Sayama, K. Hashimoto and M. Sugai, "Innate Defences against Methicillin-Resistant *Staphylococcus aureus* (MRSA) Infection," *Journal of Pathology*, Vol. 208, No. 2, 2006, pp. 249-260. [doi:10.1002/path.1898](https://doi.org/10.1002/path.1898)
- [6] M. Sieprawska-Lupa, P. Mydel, K. Krawczyk, K. Wojcik, M. Puklo, B. Lupa, P. Suder, J. Silberring, M. Reed, J. Pohl, W. Shafer, F. McAleese, T. Foster, J. Travis and J. Potempa, "Degradation of Human Antimicrobial Peptide LL-37 by *Staphylococcus aureus*-Derived Proteinases," *Antimicrobial Agents and Chemotherapy*, Vol. 48, No. 12, 2004, pp. 4673-4679. [doi:10.1128/AAC.48.12.4673-4679.2004](https://doi.org/10.1128/AAC.48.12.4673-4679.2004)
- [7] S. McNicholas, A. C. Shore, D. C. Coleman, H. Humphreys and D. F. Hughes, "DNA Microarray Genotyping and Virulence and Antimicrobial Resistance Gene Profiling of Methicillin-Resistant *Staphylococcus aureus* Bloodstream Isolates from Renal Patients," *Journal of Clinical Microbiology*, Vol. 49, No. 12, 2011, pp. 4349-4351. [doi:10.1128/JCM.05017-11](https://doi.org/10.1128/JCM.05017-11)
- [8] K. Ouhara, H. Komatsuzawa, T. Kawai, H. Nishi, T. Fujiwara, Y. Fujiue, M. Kuwabara, K. Sayama, K. Hashimoto and M. Sugai, "Increased Resistance to Cationic Antimicrobial Peptide LL-37 in Methicillin-Resistant Strains of *Staphylococcus aureus*," *Journal of Antimicrobial Chemotherapy*, Vol. 61, No. 6, 2008, pp. 1266-1269. [doi:10.1093/jac/dkn106](https://doi.org/10.1093/jac/dkn106)
- [9] K. Midorikawa, K. Ouhara, H. Komatsuzawa, T. Kawai, S. Yamada, T. Fujiwara, K. Yamazaki, K. Sayama, M. A. Taubman, H. Kurihara, K. Hashimoto and M. Sugai, "*Staphylococcus aureus* Susceptibility to Innate Antimicrobial Peptides, Beta-Defensins and CAP18, Expressed by Human Keratinocytes," *Infection and Immunity*, Vol. 71, No. 7, 2003, pp. 3730-3739. [doi:10.1128/IAI.71.7.3730-3739.2003](https://doi.org/10.1128/IAI.71.7.3730-3739.2003)
- [10] T. J. Foster, "Immune Evasion by Staphylococci," *Nature Reviews Microbiology*, Vol. 3, No. 12, 2005, pp. 948-958. [doi:10.1038/nrmicro1289](https://doi.org/10.1038/nrmicro1289)
- [11] S. H. Rooijackers, K. P. van Kessel and J. A. van Strijp, "Staphylococcal Innate Immune Evasion," *Trends in Microbiology*, Vol. 13, No. 12, 2005, pp. 596-601. [doi:10.1016/j.tim.2005.10.002](https://doi.org/10.1016/j.tim.2005.10.002)