Association of capsular polysaccharide locus 2 with prognosis of Acinetobacter baumannii bacteremia

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ABSTRACT
Acinetobacter baumannii causes healthcare-associated infections worldwide. Capsular polysaccharide (CPS) is a major virulence factor of A. baumannii both in vitro and in vivo. Capsule locus 2 (KL2) for CPS is the most common KL type and is associated with carbapenem resistance. It is unclear whether KL2 is related to the clinical outcome of invasive A. baumannii infection. Here we followed patients with A. baumannii bacteremia prospectively between 2009 and 2014. One-third of the unduplicated blood isolates were randomly selected each year for microbiological and clinical studies. The KL2 gene cluster was identified using polymerase chain reaction. A total of 148 patients were enrolled randomly. Eighteen isolates (12.2%) carried KL2, and 130 isolates (87.8%) didn’t. Compared with non-KL2 isolates, KL2 isolates had significantly higher resistance to imipenem, sulbactam, and tigecycline. Compared with the non-KL group, in the KL2 group, the hospital stay before development of bacteremia was longer (P < 0.001), a higher percentage had pneumonia (P = 0.004), and the white blood cell count was lower (P = 0.03). Infection with KL2 A. baumannii predicted mortality (adjusted hazard ratio [aHR], 2.03; 95% confidence interval [CI], 1.09–3.78; P = 0.03), independently of the Pitt bacteremia score (aHR, 1.34; 95% CI, 1.23–1.46; P < 0.001) and leucopenia (aHR, 2.16; 95% CI, 1.30–3.57; P = 0.003). Thrombocytopenia contributed to the effect of KL2 on mortality in bacteremia (Sobel test P = 0.01). Large-scale studies are warranted to confirm these findings and the underlying mechanisms deserve further investigation.

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Introduction
Acinetobacter baumannii is a major pathogen implicated in hospital-acquired infections across the globe over past two decades [1]. Infection with A. baumannii is associated with a high mortality rate [2–4]. Appropriate empirical antimicrobial therapy is important for the treatment of A. baumannii infections [5], but the threat remains because of emerging resistance worldwide. The mortality rate of A. baumannii bacteremia remains high even after appropriate treatment [5]. Therefore, the mechanisms of A. baumannii virulence warrant investigation.

In A. baumannii bacteremia, different clones can cause different outcomes [6]. Sequence type (ST) 2 had been reported as the dominant multilocus sequence type of A. baumannii, and exhibits many virulence-related traits, such as high ability of biofilm formation and adherence to lung epithelial cells [7]. Genotype ST2 is associated with worse clinical outcomes in infections [8]. In A. baumannii, capsular polysaccharide (CPS) is a major virulence factor that is crucial for bacterial survival in vitro and in vivo [9]. Specific capsule types are proved to overwhelm mammalian defences in vivo, including K2 [10]. K2 capsular polysaccharide is associated with capsule locus (K locus) 2 gene cluster, which encodes enzymes for biosynthesis and export of CPS [11]. Capsule locus 2 (KL2) is the most common KL type in analysis of posted whole genome sequences [11,12], and most of KL2 strains belong to ST2 [11].

The primary aim of this study was to examine whether KL2 A. baumannii is more virulent and leads to higher mortality in invasive infection. We also aimed to delineate the KL2 distributions among the patients with A. baumannii bacteremia in Taiwan.

Materials and methods
Hospital setting, bacterial isolates, and patients
This study was conducted at the National Taiwan University Hospital, a 2200-bed medical centre, located in northern Taiwan. Between January 2009 and

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December 2014, patients who had bacteraemia caused by \textit{A. baumannii} complex were enrolled prospectively. For patients who experienced multiple episodes of \textit{A. baumannii} complex bacteraemia, only the first episodes were included. We randomly selected one third of the non-duplicated \textit{A. baumannii} complex in each year for microbiological studies. Bacterial isolates of genospecies-identified \textit{A. baumannii} were subjected for KL2 gene cluster identification, and these patients were included for clinical investigation. Only adults older than 18 years were included. This study was approved by the Research Ethics Committee of NTUH (NTUH 201008047R). The isolates were collected as part of clinical routine practice, and the informed consent process was waived by the ethics committee.

Microbiological studies

Blood cultures were processed by the clinical microbiology laboratory at NTUH and the isolates of \textit{A. baumannii} complex identified using the VITEK 2 system (bioMérieux Inc., La Balme les Grottes, France) were collected. Genospecies were identified according to 16S–23S ribosomal RNA gene intergenic spacer region, as described previously [4].

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of colistin, tigecycline and imipenem for \textit{A. baumannii} isolates were determined using broth microdilution. The MICs of levofloxacin, ampicillin-sulbactam, cefepime, amikacin and minocycline were determined using Sensititre GNX3F (Trek Diagnostics, West Sussex, England), and were interpreted according to the Clinical and Laboratory Standards Institute [13]. Isolates for which tigecycline MIC of \(\leq 2\) \(\mu g/mL\) were considered as susceptible [14].

Multilocus sequence typing

Primers for the seven house-keeping genes (\textit{cpn60}, \textit{fusA}, \textit{gltA}, \textit{pyrG}, \textit{recA}, \textit{rplB}, and \textit{rpoB}) were designed, and sequencing were performed according to the methods of Diancourt et al. [15]. Sequence chromatograms were edited and stored using BioNumerics software (version 6.0). Allele sequences and allelic profiles were compared with those available on the MLST website of the Institute Pasteur (www.pasteur.fr/mlst), and sequence type were assigned.

Capsule locus 2 identification

Two sets of primers were used to detect KL2 gene cluster by polymerase chain reaction according to the method of Kenyon et al. [11]. Firstly, primers psa\textsubscript{F}\_f (5′-TGGTGAAGCAATTCAAGCTG-3′) and psa\textsubscript{F}\_r (5′-AATAAGGCGATGCACCCAAAG-3′) were designed to detect any K locus with a \textit{psa} module responsible for synthesis of pseudaminic acid [16]. Secondly, among \textit{psa} module-carrying K loci, primers wzy\textsubscript{grt3}\_f (5′-CTCITTATCGGTTCAAAAATC-3′) and wzy\textsubscript{grt3}\_r (5′-GCCCATTTACTATCAACCG-3′) were used to detect the region between \textit{wzy} and \textit{grt3} that was specific for the KL2 [11].

Clinical data collection and definitions

We collected clinical data prospectively for enrolled patients. The data included demographic data, underlying diseases, infectious focus, regimens of antimicrobial therapy, and data of laboratory examination results at the time of bacteraemia onset. The site of infection was classified according to US Centres for Disease Control and Prevention [17]. Patients were classified as having primary bacteraemia when no obvious port of entry identified. The Charlson comorbidity index was used to adjust patient’s underlying diseases [18], and the Pitt bacteraemia score was used to measure the severity of bacteraemia [19]. All-cause in-hospital mortality was recorded.

The onset day of \textit{A. baumannii} bacteraemia was defined as the day when at least one set of blood culture that was positive for \textit{A. baumannii} was drawn. Appropriate empirical antimicrobial therapy was defined as using intravenous antimicrobial agent to which \textit{A. baumannii} isolate was susceptible within 2 days of bacteraemia onset. Tigecycline was considered an appropriate regimen only while treating infections caused by isolates for which tigecycline MIC of <1 \(\mu g/mL\) [20]. Healthcare-associated bloodstream infection was defined as bacteremia that occurred more than 48 h after hospitalization [21]. Use of immunosuppressive agents was defined as usage of antineoplastic, cytotoxic chemotherapy, or corticosteroids at a dosage of prednisolone \(\geq 20\) mg/day for \(\geq 2\) weeks or prednisolone 30 mg/day for \(\geq 1\) week within 6 weeks before the onset of bacteraemia [4]. The primary outcome was 28-day all-cause mortality following the onset of \textit{A. baumannii} bacteraemia.

Statistical analysis

The median and inter-quartile range (IQR) were calculated for continuous variables. The percentages were calculated for categorical variables. The Mann–Whitney \textit{U} test and Fisher’s exact test were used to compare continuous and categorical variables, respectively, between two groups. Multivariate Cox proportional-hazards models were used to analyse the outcome. Variables with a \(P\) value of \(\leq 0.2\) in univariate regression were added in a stepwise manner
and selected for the final model in multivariate analysis by minimizing Akaike’s information criterion [22]. Intermediate variables were identified using the Sobel test [23,24]. Because of the potential for overadjustment bias, intermediate variables were not included in outcome analysis in the final model [25]. The data were analysed using Stata software (version 14, Stata-Corp, College Station, TX, USA). Two-sided P values ≤0.05 were considered to be significant.

Results

During the study period, a total of 148 A. baumannii blood isolates were collected for KL2 identification. Eighteen (12.2%) isolates had CPS associated with KL2. As shown in Table 1, KL2 isolates had significantly higher resistance to amikacin, cefepime, imipenem, levofloxacin, sulbactam, and tigecycline. There was no difference in colistin resistance between KL2 and non-KL2 isolates (P = 0.99).

The median (IQR) age of the 148 patients enrolled was 62.5 (22.2) years. The median PITT bacteraemia score was 4 (7) points, Charlson comorbidity index was 3 (4) points, and hospitalization duration before A. baumannii bacteraemia onset was 18 (28) days. Sixty-three (42.6%) patients had underlying malignancies, and 50 (33.8%) patients had received an immunosuppressive agent. The 28-day all-cause mortality rate was 45.9%.

The baseline characteristics differed between KL2 and non-KL2 groups (Table 2). The KL2 patient group had a longer hospital stay before bacteraemia onset (44.5 days vs. 15 days, P < 0.001), higher percentage of pneumonia (72.2% vs. 35.4%, P = 0.004), and lower white blood cell counts and platelet counts at the onset bacteraemia (P = 0.03, and 0.005 respectively). The underlying diseases and Charlson comorbidity index did not differ significantly between the KL2 and non-KL2 groups. Although the percentage of patients receiving appropriate empirical antimicrobial therapy (P = 0.80) and the PITT bacteraemia scores (P = 0.18) did not differ significantly between groups, the KL2 group had a higher risk of 28-day mortality than the non-KL2 group (P = 0.02).

Mortality analysis regarding capsule locus 2

Univariate Cox regression identified the patient characteristics associated with mortality as a longer duration of hospitalization before A. baumannii bacteraemia, underlying leukaemia, ventilator-associated pneumonia, higher PITT bacteraemia score, leucopenia, thrombocytopenia, and hyperbilirubinaemia. KL2 was significantly associated with a higher risk of mortality than non-KL2 (hazard ratio [HR], 2.09; 95% confidence interval [CI], 1.14–3.84; P = 0.02).

KL2 isolates were associated with high antimicrobial resistance to levofloxacin, imipenem, sulbactam and tigecycline. However, the percentage of patients receiving appropriate empirical antimicrobial therapy was not significantly lower in the KL2 than in the non-KL2 group (50% vs. 44.6%, P = 0.80). We used stratification analysis to identify interactions between KL2 and the appropriateness of empirical antimicrobial therapy. After stratification, the mortality rates did not differ significantly between patients who received appropriate empirical antimicrobial therapy and those who received inappropriate therapy within the KL2 patient group (66.7% vs. 77.8%; P = 0.99), and within the non-KL2 group (36.2% vs. 47.2%; P = 0.22). Even when receiving appropriate empirical antimicrobial therapy, the KL2 patient group still had a higher mortality rate than the non-KL2 group (HR, 2.51; 95% CI, 1.01–6.24; P = 0.048).

The presence of KL2 significantly predicted a lower platelet count (10,000/μL) (β-coefficient, −64.5; P = 0.01), and the Sobel test showed that the presence of KL2 had an indirect effect on mortality via platelet count (P = 0.02). In multivariate Cox regression model (Table 3), the presence of KL2 predicted mortality (adjusted HR [aHR], 2.03; 95% CI, 1.09–3.78; P = 0.03, Figure 1(A)), independently of the PITT bacteraemia score and leucopenia at the onset of bacteraemia.

Mortality analysis regarding sequence type 2 and capsule locus 2

Because ST2 was the most dominant sequence type, we further analysed the association between different capsule locus type and sequence type. Among 148 A. baumannii blood isolates, 69 (46.6%) were non-ST2/non-KL2, two (1.4%) were non-ST2/KL2, 61 (41.2%) were ST2/non-KL2, and 16 (10.8%) were ST2/KL2. Most of the KL2 isolates belonged to ST2 (16/18, 88.9%), only 16/77 (20.8%) of the ST2 isolates were KL2. Differences in mortality were analysed in patients with bacteraemia caused by ST2, non-ST2, KL2 or non-KL2 isolates. The mortality rate was least in patients with bacteraemia caused by non-ST2/non-KL2 isolates (30.4%), followed by those with bacteraemia caused by non-ST2/KL2 isolates (50%), and by ST2/non-KL2 isolates (55.7%). Patients with bacteraemia caused by ST2/KL2 isolates had the highest mortality rate (75%).

In multivariable Cox analysis, with ST2/KL2 as the reference, non-ST2/non-KL2 (aHR, 0.46; 95% CI, 0.22–0.95; P = 0.04) and ST2/non-KL2 (aHR, 0.46; 95% CI, 0.23–0.91; P = 0.03) were associated with lower mortality (Table 3). Within the ST2 group, KL2 independently predicted mortality (aHR, 2.26; 95% CI, 1.11–4.57; P = 0.02). However, the association with mortality did not differ significantly between the
non-ST2/KL2 group and non-ST2/non-KL2 group (HR, 2.85; 95% CI, 0.37–21.87; \( P = 0.31 \)). Survival differences between the groups with non-ST2/non-KL2, ST2/non-KL2 and ST2/KL2 are shown in the Kaplan–Meier curves in Figure 1(B).

**Table 1.** Comparison of the minimum inhibitory concentrations and resistance rates of capsule locus 2 (KL2) and non-capsule locus 2 (non-KL2) isolates of Acinetobacter baumannii.

| Variable | KL2 (n = 18) | Non-KL2 (n = 130) | \( P \) |
|----------|--------------|--------------------|------|
| MIC\(_{50}\), MIC\(_{90}\) (\( \mu \)g/mL) | Range (\( \mu \)g/mL) | Resistance rate (%) | MIC\(_{50}\), MIC\(_{90}\) (\( \mu \)g/mL) | Range (\( \mu \)g/mL) | Resistance rate (%) |
| Amikacin | >32, >32 | \( \leq \) to >32 | 94.4 | >32, >32 | \( \leq \) to >32 | 68.5 | 0.02 |
| Cefepime | >16, >16 | \( \leq \) to >16 | 94.4 | >16, >16 | \( \leq \) to >16 | 23.1 | 0.08 |
| Gentamicin | 1, 2 | 0.25 to 2 | 0 | 1, 2 | 0.25 to 4 | 0.8 | 0.99 |
| Imipenem | 32, 64 | 0.125 to >128 | 88.9 | 16, 64 | 0.125 to >128 | 56.3 | 0.009 |
| Levofloxacin | >8, >8 | \( \leq \) to >8 | 94.4 | 8, >8 | \( \leq \) to >8 | 68.5 | 0.02 |
| Minocycline | 4, 8 | \( \leq \) to 16 | 44.4 | 4, 8 | \( \leq \) to 16 | 23.1 | 0.08 |
| Sulfamethoxazole | >32, >32 | \( \leq \) to >16 | 94.4 | 16, >32 | \( \leq \) to >32 | 68.5 | 0.02 |
| Tigecycline | 4, 8 | 0.125 to 8 | 55.6 | 0.125 to 8 | 16.8 | 0.001 |

**Note:** MIC\(_{50}\) 50% minimum inhibitory concentration; MIC\(_{90}\) 90% minimum inhibitory concentration.

*The susceptibility to various antimicrobial agents was compared between A. baumannii capsule locus types using Fisher’s exact test.

**Discussion**

To our knowledge, this is one of only a few studies that describe the epidemiology of KL type of invasive A. baumannii infection. In this study, we found that the KL2 is associated with higher antimicrobial resistance. We found associations between the clinical outcome and the presence KL2 A. baumannii isolates in patients with bacteraemia. Independent to leucopenia and the Pitt bacteraemia score, KL2 is associated with higher mortality. We also found KL2 significantly predicted lower platelet count which contributed to the effect of KL2 on mortality in bacteraemia.

The K2 CPS is proved to overwhelm mammalian defences in vivo that administration of K2 capsule depolymerase protects mice from A. baumannii sepsis [10]. The short and branched tetrasaccharide unit of K2 CPS contains a complex uncommon negatively-charged aminosugar, pseudaminic acid, [11,26], and the glycosylated flagellin of Campylobacter jejuni [28]. The role of pseudaminic acid in K2 CPS of A. baumannii may be that pseudaminic acid contrib-
utes to bacterial immune evasion by molecular mimicry of human sialic acid [29], or modules host immune responses as it functions in C. jejuni [30].

In present study, thrombocytopenia contributed the effect of KL2 A. baumannii on mortality. Decrease in platelet counts is expected to weaken the important functions of platelet in sepsis, for example, helping to enourage bacteria in septic blood [31], or inhibiting inflammatory cytokines [32]. In addition, in sepsis induced by gram-negative bacteria, it is well known that LPSs can cause thrombocytopenia [33]. In A. baumannii, LPS triggers sepsis by interaction with host toll-like receptor 4 (TLR4), which lead to host lethality shown in murine sepsis model [34]. Besides, evasion of host innate immune defence is a key driver of A. baumannii virulence [35]. Therefore, the association of KL2 with mortality suggested that K2 CPS
may contribute to escape from clearance by innate immune system, which would further enable a higher bacterial density that triggers more severe sepsis mediated by LPS and TLR4 [35] and causes higher mortality.

In one analysis of *A. baumannii* whole genome sequences, KL2 was the most common KL type (24.2%) and accounted for a substantial percentage (32.2%) of total 2016 ST2 *A. baumannii* genomes [12]. In another analysis, most of the KL2-carrying strains (92.5%) belonged to ST2 [11]. Similar to the findings reported by Kenyon et al [11], most of the KL2 isolates (88.9%, 16/18) in our study belonged to ST2. A small but substantial percentage (20.8%, 16/77) of the ST2 isolates belonged to KL2. In the study by Kenyon et al., the clinical significance of KL2 in invasive *A. baumannii* infection was not confirmed because only three blood isolates were included [11]. In our study, KL2 isolates accounted for 12.2% of all *A. baumannii* blood isolates during the study period (2009–2014). A recent study reported that KL2 was the most common KL type (12.2%) of the *A. baumannii* blood isolates in two regions of Taiwan (2015–2017) [36]. The prevalence rate of KL2 was 19.3% among the carbapenem-resistant *A. baumannii* (CRAB) isolates in our study. This rate was comparable to those of other studies focusing on CRAB, ranging between 19.0% and 20.4% [36,37].

Therefore, KL2 may have clinical impact in invasive *A. baumannii* infection in Taiwan. In addition, we found that non-ST2/KL2 seemed to cause higher mortality than ST2/non-KL2 isolates did. However,
because there were only two isolates belonging to non-ST2/KL2, we didn’t demonstrate significant association with mortality compared with that of non-ST2/non-KL2 isolates (HR, 2.85; 95% CI, 0.37–21.87; \( P = 0.31 \)).

We showed that KL2 \textit{A. baumannii} was associated higher antimicrobial resistance and greater mortality in bacteremia. KL identification might be important to understand the virulence of \textit{A. baumannii} and serve as an outcome predictor of \textit{A. baumannii} bacteremia. The K2 capsular polysaccharide might be a potential target in treating \textit{A. baumannii} bacteremia via passive immunization, as the role of K1 CPS [38]. Pseudaminic acid-based antibacterial vaccine was proved to confer effective protection against \textit{A. baumannii} infection in an animal model [39]. In addition, other novel therapeutics targeting the bacteria surface glycoconjugates are under development, e.g. small-molecule inhibitors of the pseudaminic acid biosynthetic pathway enzymes [28,40].

There were several limitations in our study. Firstly, the sample size was small, especially the group of patients infected with KL2 isolates, which was smaller than expected. Although the presence of KL2 predicted mortality independent of the severity of bacteremia, we failed to show statistically significant effect of KL2 in non-ST2 patient group. Secondly, other CPS-typing methods should be used to examine the epidemiology and the clinical impact of different KL types. Thirdly, we found a tendency for lower mortality in patients who had received appropriate empirical antimicrobial therapy than those who did not.
However, because of the small sample size, the effect of appropriate antimicrobial therapy did not achieve statistical significance. Clinicians should pay attention to appropriate use of antimicrobial therapy as well as bacterial factors. Lastly, this study was conducted a few years ago. A recent study in Taiwan showed similar results that the KL2 prevalence rate was around 12.2% in A. baumannii blood isolates and might be associated with the high mortality of CRAB [36]. Nevertheless, whether the KL2 prevalence rate and clinical impact remain similarly in these years warrants further surveillance.

In conclusion, we found that most of the KL2 A. baumannii isolates identified in our study belonged to genotype ST2. The presence of KL2 was associated with high antimicrobial resistance. Infection with KL2 A. baumannii predicted mortality in patients with bacteremia independent of the bacteremia severity measured by the Pitt bacteremia score. Large-scale studies are warranted to confirm these findings and the underlying mechanisms deserve further investigation.

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No potential conflict of interest was reported by the author(s).

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