



Molecular Characterization of *Staphylococcus aureus* Isolates Implicated in Bovine Mastitis

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Abstract

Staphylococcus aureus is a well-known contagious and opportunistic pathogen responsible for bovine mastitis worldwide, and in Malaysia, the sequence types (STs) of these isolates associated with bovine mastitis had not been previously characterized. This study therefore aimed to characterize *S. aureus* isolates from bovine mastitis cases in Malaysian dairy herds, using isolates recovered following standard procedures and analyzed via multi-locus sequence typing with a boiling method. A total of ten isolates were analyzed, revealing six distinct sequence types: ST97 (CC97) was the most prevalent (n=4), followed by ST1 (CC1) (n=2), and then ST221 (CC5) (n=1), ST1496 (CC1) (n=1), ST4427 (CC97) (n=1), and ST2125 (CC1) (n=1). Notably, ST2125 and ST1496 were identified for the first time in association with bovine mastitis. The most widespread *S. aureus* ST linked to subclinical mastitis in Malaysia was ST97, and the molecular epidemiology of this ST, along with the other STs identified, was compared to *S. aureus* STs reported globally.

Keywords: Molecular Characterization, *Staphylococcus aureus*, Bovine, Mastitis, MLST

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Introduction

Staphylococcus aureus is recognized as a major cause of various infections in both humans and animals (Hata *et al.*, 2008). In humans, it is associated with a wide spectrum of diseases, ranging from mild skin infections to severe and life-threatening conditions such as endocarditis and hemolytic pneumonia (Lindsay & Holden, 2004). Other notable illnesses caused by *S. aureus* include scalded skin syndrome, toxic shock syndrome, and

staphylococcal food poisoning (von Eiff *et al.*, 2015). In animals, *S. aureus* serves as a primary etiological agent of intramammary infections, a condition that results in substantial economic losses for dairy farmers (Fitzgerald, 2012). Transmission among dairy cows commonly occurs through contact with contaminated surfaces or milk, leading to intramammary infections that are often difficult to detect in their early stages (Feng *et al.*, 2016a). Numerous epidemiological

studies have indicated that specific *S. aureus* strains are responsible for most mastitis cases worldwide (Kapur *et al.*, 1995; Zadoks *et al.*, 2002; Budd *et al.*, 2016), though farm-associated strains have also been identified (Rabello *et al.*, 2007; Pereira *et al.*, 2011). Multilocus sequence typing (MLST) has been widely utilized as a molecular tool for investigating the global transmission and genetic distribution of microbial populations. This approach involves sequencing several housekeeping genes, with the resulting data being stored in online databases that facilitate the comparison of isolates and their sequence types across different laboratories. Consequently, MLST provides a robust platform for worldwide epidemiological studies (Yang *et al.*, 2018). However, the sequence types (STs) of *S. aureus* isolates associated with bovine mastitis in Malaysia have not yet been characterized. Therefore, the objective of this study is to determine the STs of *S. aureus* isolates implicated in intramammary infections (IMIs) from Malaysian dairy farms.

Materials and Methods

Isolation and Identification of Bacterial Isolates

S. aureus isolates were obtained from cows with bovine mastitis cases. Isolation of bacterial pathogens were carried out as

previously described (Abebe *et al.*, 2016). Briefly, milk samples were aseptically taken and streaked on blood agar and MacConkey agar (Oxoid, England). They were then incubated at 35°C for 24 hours. All plates were examined for bacterial growth, and other colony appearance. Colonies of interest were picked for subculture on blood agar. Isolates were identified using analytical profile index (API) following manufacturer's manual.

Confirmation of *S. aureus* isolates by polymerase chain reaction (PCR)

Staphylococcus aureus isolates were confirmed using PCR by targeting 270bp of thermo-stable nuclease gene (*nuc*), which is specific for *S. aureus* in 25µL volume. The volume was composed of 14µL Toptaq master mix (Qiagen) containing DNA polymerase; PCR Buffer (3 mM MgCl₂); and 50µM of each dNTPs; 5µL DNA template, 2µL (0.2µM) of each primer (forward and reverse) (Integrated DNA Technologies, Singapore) (Table 1.5) and 4µL of RNase free water (Qiagen). The PCR reaction was performed in a thermocycler (BIO-RAD) under the following condition described by Merlino *et al.* (2002). The initial denaturation was performed for 5 min at 94°C, 30 cycles of denaturation at 94°C for 1min, annealing for 30s at 50°C and extension for 1min 30s at 72°C.

Table 1. Primers used for *nuc* gene (276bp) in confirmation of *S. aureus* Isolates (Merlino *et al.*, 2002)

Primers	Oligonucleotide sequences
<i>nuc</i> F	5'-GCGATTGATGGGATACGGTT-3'
<i>nuc</i> R	5'-AGCCAAGCCTTGACGAACATAAGC-3'

Genomic DNA extraction

The Genomic DNA used as template DNA in multilocus sequence typing of *S. aureus* isolates was extracted using boiling method described by Dashti *et al.* (2009) with little modification. Briefly, a loopful of freshly cultures *S. aureus* isolates was suspended in 1.5 mL micro-centrifuge tubes containing 1mL of sterile distilled water. After incubation at room temperature for 5 min, it was then heated on dry water bath at 96°C for 10 min. the content was then centrifuged for 10 min at 13rpm, the supernatant was then collected into

new 1.5 ml micro-centrifuge tubes which were used as DNA template.

Multi-locus sequence typing (MLST)

The internal fragments of seven *S. aureus* housekeeping genes, consisting of *arcC*, *aroE*, *glpE*, *gmk*, *pta*, *tpi*, and *yqiL* (Table.2.0) were amplified as described previously (Enright *et al.*, 2000). The amplification of the targeted gene fragments was illustrated using gel electrophoresis and then viewed by gel documentation machine (Alpha Imager). The PCR amplicons were submitted to First Base sequencing Company (Malaysia) for DNA

sequencing of both the forward and reverse strands. The sequences were checked for quality using BioEdit software Version 7.2. The sequences of each locus were compared to allele sequences in the MLST database (<http://saureus.mlst.net>). The combination of

alleles at the seven loci was used to define the allelic profile for each isolate and assigned to a sequence type (ST). Comparison of the STs was conducted by goeBURST at <http://www.phyloviz.net/goeburst/>.

Table 2. The sets of primers used for the amplification of seven housekeeping genes in *S. aureus*

Primers	Oligonucleotide Sequences Forward and Reverse (5'-3')	Size
Carbamate kinase (<i>arcC</i>)	F-TTGATTACACCAGCGCGTATTGTC R-AGGTATCTGCTTCAATCAGCG	456
Shikamate hydrogenase (<i>aroE</i>)	F-ATCGGAAATCCTATTTTCACATTC R-GGTGTTGTATTAATAACGATATC	456
Glycerol kinase (<i>glpF</i>)	F-CTAGGAACTGCAATCTTAATC R-TGGTAAAATTTCGGGACCATC	465
Guanylate kinase (<i>Gmk</i>)	F-ATCGTTTTATCGGGACCATC R-TCATTAACACTACAACGTAATGCTA	429
Phosphate acetyltransferase (<i>Pta</i>)	F-GTTAAAATCGTATTACCTGAAGG R-GACCCTTTTGTTGAAAAGCTTAA	474
Triosphosphate isomerase (<i>Tpi</i>)	F-TCGTTCAATTCTGAACGTCGTGAC R-TTGCACCTTCTAACAATTGTAC	402
Acetyle coenzyme A acetyle tranferase (<i>yqiL</i>)	F-CAGCATACAGGACACCTATTGGC R-CGTTGAGGAATCGATACTGGAAC	516

Results

Multilocus sequence typing (MLST)

The result of the electrophoresis for seven targeted gene fragments analysed using the gel documentation (Alpha Imager) is shown in Figure 1.0. A total of ten (n=10) *S. aureus* isolates were analysed by MLST. Six

different sequence types (STs) were identified (Table 3.0). These includes ST97 (CC97) (n=4) which is the most prevalent, followed by ST1 (CC1) (n=2), and then ST221 (CC5) (n=1), ST1496 (CC1) (n=1), ST4427 (CC97) (n=1) and ST2125 (CC1) (n=1). Among the STs ST2125 and 1496.

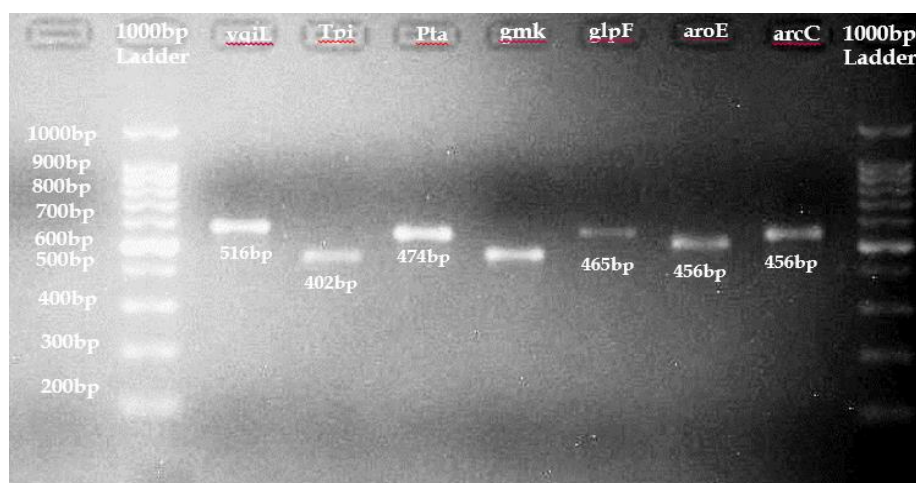


Figure 1. Illustration of the bands for the seven housekeeping genes obtained from gel electrophoresis

of *S. aureus* isolates associated with subclinical mastitis in selected dairy herds from Malaysia.

In the present study 10 *S. aureus* isolates were subjected to MLST. Six different STs (ST1, ST97, ST221, ST1496, ST2125, and ST4427) were identified in this study. To the best of our knowledge, this is the first time some *S. aureus* STs were identified in Malaysia. These are the ST2125, ST221, ST4427 and ST1496. Generally, the most widely spread ST identified was the ST97 (40%). The ST97, belong to the clonal complex 97 (CC97). A Clonal complex (CC) is defined as a group of isolates that share at least six of the seven alleles with one another (Feil *et al.*, 2004). The ST97 (CC97) has been reported to be commonly associated with infections in ruminants (Fitzgerald, 2012). Additionally, the ST97 has been reported widely by previous studies as the most common *S. aureus* ST associated with bovine mastitis cases from countries like but not limited to Brazil, Italy, Japan, Norway, Spain, Netherlands and United States (Smith *et al.*, 2005; Rabello *et al.*, 2007; Hata *et al.*, 2008). The widely distribution of this ubiquitous lineage strongly indicates that the strain is specifically adopted to thrive on cow hosts (Smith *et al.*, 2005; Budd *et al.*, 2016). A closely related ST to ST97 that was also identified in this study is ST4427. This ST is a single locus variant (SLV) of ST97 differing at single locus on gene encoding for shikimate hydrogenase (*aroE*). The second most prevalent ST identified was ST1 (CC1). This ST has been isolated in association with both mastitis and human infections (Rabello *et al.*, 2007; Fitzgerald, 2012). In Malaysia, it was reported along with other STs as the predominant methicillin resistant clones associated with nosocomial infections (Ghaznavi-Rad *et al.*, 2010). It was also reported to cause mastitis in sheep and goats (Caruso *et al.*, 2016). This is an evidence of inter- species transmission of *S. aureus* strains as suggested by different studies (Lowder *et al.*, 2009; Resch *et al.*, 2013; Senghore *et al.*, 2016). Another ST related to ST1 (CC1) identified was ST2125 (CC1). It is single locus variant (SLV), differing at one locus encoding

for Acetyl coenzyme Acetyl transferase (*yqiL*). This ST was previously reported in association with livestock handlers in China (Ye *et al.*, 2016).

This study also identified ST221 belonging to the CC5. It has been reported in association with bovine mastitis in China (Chen *et al.*, 2013). This ST was also reported as most widely spread ST in association with swine in Japan (Baba *et al.*, 2010). According to MLST database

(<http://saureus.mlst.net/sql/burstspadadvanced.asp>), elsewhere in France and Scotland, ST221 was also reported to be isolated in hospitals in 2002 and 2003 respectively. This is an evidence to the claim that there is continuous global dissemination of *S. aureus* strains especially those belonging to CC5 from human to other domestic animals (Lowder *et al.*, 2009).

Conclusion

In this study, the most widely spread *S. aureus* ST associated with subclinical mastitis in Malaysia identified was the ST97. The molecular epidemiology of this ST along with other STs identified in this study in relation to different *S. aureus* STs around the world was defined. This knowledge would increase our understanding of the epidemiology and population structure of this important bacterium, which has been regarded as the most common cause of bovine mastitis that results in huge economic losses.

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