TITLE

A multi-locus sequence typing method of *Staphylococcus aureus* DNAs in a sample from human skin

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ABSTRACT

The skin and mucous membranes are the primary sites of *Staphylococcus aureus* colonization, particularly those of healthcare personnel and patients in long-term care centers. We found that *S. aureus* colonized with a higher abundance ratio on skins which had recovered from pressure injury than on normal skins in our earlier research on the skin microbiota of bedridden patients. Multilocus sequence typing (MLST) is useful tool for typing *S. aureus* isolated from clinical specimens. However, the MLST approach cannot be used in microbiota DNA owing to the contamination from other bacteria species. In this study, we developed multiplex-nested PCR method in determining *S. aureus* MLST in samples collected from human skins. The seven pairs of forward and reverse primers were designed in the upstream and downstream regions, which were conserved specifically in *S. aureus*. The first amplifications of the seven were conducted in a multiplex assay. The samples were diluted and applied to conventional PCR for MLST. We confirmed that the method amplified the seven allele sequences of *S. aureus* specifically in the presence of untargeted DNAs from human and other skin commensal bacteria. Using this assay, we succeeded the typing STs of *S. aureus* in the DNA samples which were derived from the skins healed from pressure injury. Peaks obtained by Sanger sequencing showed that each sample contained one ST, which were mainly categorized

into clonal complex 1 (CC1) or CC5. We propose that this culture-free approach may be used in detecting *S. aureus* in clinical specimens without isolation.

KEYWORDS: *Staphylococcus aureus*, multilocus sequence typing, culture-free approach, clinical samples

List of Abbreviations:

CC, clonal complex; MLST, multilocus sequence typing; MRSA, methicillin-resistant *S. aureus*; NGS, New generation sequencing; PI, pressure injury; RPI, recurrent pressure injury; ST, sequence type.

INTRODUCTION

Staphylococcus aureus produces a wide range of extracellular proteins and polysaccharides that are linked to virulence (1, 2) This bacterium causes skin infections and foodborne disease in people, as well as sepsis in hospitals and nurseries (3) Colonization of *S. aureus* in humans is mostly seen in the nose cavity and is regulated by nasal microbiota (4). A high rate of *S. aureus* colonization has been reported in health care workers, persons who used needles regularly for drug injections, hospitalized

67 patients, and immunocompromised individuals (2, 5–7). Various reports have shown that
68 long-term care facilities for the elderly, such as long-term care hospitals and nursing
69 homes, pose as reservoirs for methicillin-resistant *S. aureus* (MRSA) (8–13). Senn et al.
70 reported an outbreak of ST228 HA-MRSA at a long-term hospital (14). Despite its low
71 colonization rate and low abundance in normal skin, it has been reported that *S. aureus* is
72 responsible for about 80% of all skin and soft-tissue infections, whereas healthy skin does
73 not suffer from the infection (2, 15–18).

74 *S. aureus* is classified by using a variety of gene-based techniques which target
75 *agr* (19), *coa* (20, 21), *spa* (22), PCR-based ORF typing (23, 24) and multilocus sequence
76 typing (MLST) (25). The seven housekeeping genes, *arcC*, *aroE, glpF*, *gmk*, *pta*, *tpi*, and
77 *yqiL* are used in the MLST of *S. aureus*. A recent study on *S. aureus* pan-genomes reported
78 that core gene distributions are different among ST5, ST8, and ST22 (26). Sequence types
79 (STs) which match the central genotype at four or more loci are known as clonal
80 complexes (CCs) of *S. aureus*, unless they more closely match another central genotype
81 (27).

82 Although MLST is a useful tool for genotyping *S. aureus*, isolation was required
83 in order to type *S. aureus* by the methods described above. Microbiota DNA samples,
84 including *S. aureus* DNAs, were not available for the conventional MLST. Due to the

85 unavailability, it is difficult to analyze prevalence of STs in the microbiota DNA samples.
86 In this study, we developed the multiplex-nested PCR assay which allows MLST from
87 mixed genomic DNA samples such as DNA extracted from human skins. After the proof-
88 of-concept, we applied this assay for analysis of the prevalence and typed *S. aureus*
89 colonized on skins healed from pressure injury (PI) by using the skin DNAs which had
90 been utilized for skin microbiome analysis (28, 29).
91
92 **MATERIALS AND METHODS**
93 1. Bacteria culture collection
94 *S. aureus* strain N315 (NCBI Reference Sequence: NC_002745.2) was provided
95 by Dr. Keiichi Hiramatsu (30). *S. caprae* strain JMUB145 was isolated in the blood of a
96 patient receiving chemotherapy for uterine cancer who had fever at Jichi Medical
97 University Hospital in Japan (31). *S. epidermidis* JCM 2414 (ATCC 14990) and
98 *Pseudomonas aeruginosa* JCM 6119 (ATCC 27853) were collected from the Japan
99 Collection of Microorganisms, RIKEN BRC, which is a partner in National BioResource
100 Project of the MEXT, Japan. *Corynebacterium tuberculostearicum* NBRC 113812 and
101 *Cutibacterium acnes* NBRC 107605 (ATCC 6919) isolates were provided by the National
102 Institute of Technology and Evaluation, Japan. *S. aureus* strain Cowan I (NCTC 8530)

was purchased from ATCC.

S. aureus strain N315, *S. caprae* strain JMUB145, and *S. epidermidis* JCM 2414 were cultured by using Todd-Hewitt broth (Becton and Dickinson, New Jersey, USA) containing 0.5% yeast extract (Becton and Dickinson); *Pseudomonas aeruginosa* JCM 6119 in BBLTM Select APSTM LB broth (Becton and Dickinson). Further, *Cutibacterium acnes* NBRC 107605 and *Corynebacterium tuberculostearicum* NBRC 113812 were cultured in modified Gifu Anaerobic Broth (GAM) broth (Nissui Pharmaceutical, Tokyo, Japan).

2. Cell line and culture conditions

The human keratinocyte cell line (HaCaT) was cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) which contains 8% fetal bovine serum (FBS; BIOSERA, Nuaille, France), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (FUJIFILM Wako Pure Chemical Corporation) at 37 °C under 5% CO_2 atmosphere.

3. DNA extraction

Bacterial DNA was extracted by using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. *S. aureus*, *S. caprae*, and *S.*

121 *epidermidis* were suspended in TE buffer and treated with lysostaphin (FUJIFILM Wako
122 Pure Chemical Corporation) and lysozyme (FUJIFILM Wako Pure Chemical
123 Corporation) at 37°C for one hour before the extraction. DNA from HaCaT cells was
124 extracted as based on the manufacturer's instructions by using the QIAamp UCP
125 Pathogen Mini kit (Qiagen, Hilden, Germany).
126 4. Skin samples from healthy subjects
127 We swabbed an area of 5cm x 5cm, centered around 5cm from the elbow fossa
128 on the forearms of three healthy subjects. Following the stirring of the cotton swabs in
129 TE buffer and treatment with lysostaphin and lysozyme at 37°C for one hour, DNA (100
130 μL) was extracted from them using a QIAamp DNA mini kit. Skin bacteria were also
131 collected by swabbing, moved to Mannitol salt agar (Nissui Pharmaceutical) containing
132 3% egg yolk, and cultivated for 48 h to examine whether the skin samples contain *S.*
133 *aureus*.
134 5. Skin samples recovered from pressure injury
135 In our prior investigation, DNA of the skin which had recovered from pressure
136 injury was obtained by wrapping the affected areas in breathable medical tape (4.0 × 5.0
137 cm) with urethane adhesive (29, 32). The control samples of the healed skins were
138 collected from opposite the healed site when the healed site was on a trochanter or 5 cm

139 superior to the healed skin on the sacrum or coccyx (32). The tapes were cut, suspended
140 in TE, and treated with lysostaphin and lysozyme at 37°C for one hour. DNAs (200 μL)
141 were extracted from them using a QIAamp DNA mini kit.
142 6. Alignment of nucleotides
143 *S. aureus*-specific regions around the seven alleles were detected by using
144 genomic sequences of 20 *S. aureus* strains (two each of CC1, CC5, CC8, CC15, CC22,
145 CC30, CC45, CC93, CC97, CC121) and two each of *Mammaliicoccus lentus, M. sciuri,*
146 *S. borealis, S. capitis, S. caprae, S. cohnii, S. epidermidis, S. equorum, S. hominis, S.*
147 *lugdunensis, S. pasteuri, S. pettenkoferi, S. saprophyticus, S. warneri,* and *S. xylosus*. The
148 seven genes of *S. aureus* strain N315 (NC_002745.2) containing 300 bp upstream and
149 300 bp downstream regions were used as queries. Homologous regions were searched by
150 using BLASTn tool (33), extracted by SeqKit tool (34) and alignment was carried out
151 using GENETYX software (GENETYX Corporation, Tokyo, Japan). The genome data
152 were obtained from the pubMLST website (27)
153
154 7. MLST typing
155 A total of seven loci were used in *S. aureus* MLST (35, 36). The first PCR
156 (multiplex PCR) was conducted using a Multiplex PCR Assay Kit (Takara Bio Inc., Shiga,

157 Japan). The seven primer pairs (each 0.2 μM) and 1 μL of DNA were added to the sample
158 mixture (10 μL) (Table 1). The thermal cycles were as follows: one cycle at 94°C for 1
159 min; 30 cycles of denaturing at 94°C for 30 sec; primer annealing at 55°C for 1 min;
160 extension at 72°C for 1 min. After the final cycle, held at 72°C for 10 min and the PCR
161 products were kept in a thermocycler at 4°C. PCR samples were diluted 50-fold and
162 incubated with ExoSAP-IT (ThermoFisher SCIENTIFIC, MA, USA) at 37°C for 20 min
163 and 80°C for 1 min. According to the previous report (25) with a slight modification, the
164 second PCR (conventional MLST PCR) was performed with 1 μL of the ExoSAP-IT
165 treated sample as a template and primers for MLST (0.2 μM each) using Tks Gflex™
166 DNA Polymerase (Takara Bio Inc., Shiga, Japan). The thermal cycles were as follows:
167 one cycle at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 sec, primer annealing
168 at 55°C for 1 min, and extension at 72°C for 1 min. In case that the second PCR failed
169 (no band detected in electrophoresis), the first PCR was conducted by single plex PCR
170 using only one pair of primers. The PCR samples were treated with ExoSAP-IT at 37°C
171 for 20 min, and then Sanger DNA sequencing was performed using one of the primers of
172 the second PCR. The pubMLST database provided allele counts, STs and CCs (27).
173 8. Proof-of-concept
174 DNA concentrations of bacterial and cell line samples were calculated by using

a Qubit assay kit (ThermoFisher SCIENTIFIC). For proof-of-concept, we prepared a reaction mixture which contains bacterial DNA (10^3 copies/μL/sample) except *S. aureus*, bacterial DNA with *S. aureus*, bacterial DNA except *S. aureus* and the human DNA (10^4 copies/μL), or the bacterial DNAs with *S. aureus* and the human DNA. One microliter was applied to the first multiplex PCR.

9. Ethical statement

The Medical Ethics Committee of Kanazawa University gave approval for this investigation (for the bedridden patients, permission number 44, and for healthy volunteers, approval number 113825). The study was performed in compliance with the Helsinki Declaration and the Kanazawa University Microorganism Safety Management Regulations.

RESULTS

Primer design of first multiplex PCR

In order to detect the ST of *S. aureus* in a mixed sample of DNA sample (Figure S1), PCR must target specific genomic regions that are located around the allele sequences for typing (Figure S2). We found that the DNA sequences of six housekeeping genes except the *arcC* gene of *S. aureus* have homology with those of other staphylococci

193 by multiple alignment of 20 *S. aureus* and 30 closely related species, indicating that the
194 genes were not relevant to the target of the PCR. Instead, the *S. aureus*-specific regions
195 were found in 300 bp upstream and downstream regions of each gene. The specific
196 regions in their upstream and downstream were chosen as forward and reverse primer
197 sequences, respectively (Figure S2). After a multiplex PCR using the seven pairs of
198 primers, PCR products were diluted 50 times, treated with commercial exonuclease
199 I/Shrimp alkaline phosphatase reagent (Exo-SAP-IT), and utilized as a template for
200 nested PCR (conventional PCR for MLST) (27, 35).
201
202 **Specificity of amplification**
203 Using DNA from skin microbes such as *S. aureus*, *S. caprae*, *S. epidermidis*, *P.*
204 *aeruginosa*, *C. acnes*, and *C. tuberculostearicum*, we tested the primers' specificity
205 (Figure 1). In the absence of *S. aureus* DNA, none of the seven alleles were amplified by
206 our multiplex-nested PCR, all of the bands were detected during electrophoresis when *S.*
207 *aureus* DNA (10^3 copies in a reaction mixture) was present. We next investigated at how
208 contamination with human DNA (10^4 copies in a reaction mixture) affects specificity
209 since clinical specimens included human DNA. Although results showed that addition of
210 human genomic DNA from HaCaT cells caused non-specific amplifications of *glpF* and

211 *gmk* fragments, Sanger sequencing for *S. aureus* MLST was successful for all seven allele
212 sequences, showing that these non-specific amplifications did not interfere MLST. The
213 amplification was successful using another S. aureus (ST30 type strain Cowan I) (Figure
214 S3).
215
216 **Skin samples of healthy volunteers**
217 Skin DNA samples from three healthy volunteers were tested before clinical
218 samples. The skin microbiome samples were collected by swabbing and used to extract
219 DNA. All the alleles except a *pta* of volunteer #2 were amplified. Amplification of the
220 *aroE* allele was weak when the #2 sample was used. These results indicated that the
221 quantity of *S. aureus* is low. However, we cultivated the skin bacteria on mannitol salt
222 agar plates and confirmed that all the three skin samples contained *S. aureus*. The
223 amplification of *pta* allele of the #2 sample succeeded when the gene was amplified by
224 single plex reaction using only the pair of *pta* primers (Figure S4), suggesting that the
225 failure of the second PCR could be overcome via retrial of the first PCR using only one
226 pair of primers.
227
228 **Typing of *S. aureus* in skins healed from pressure injury**

229 Frequency of recurrent pressure injuries (RPIs) is much higher (25.9%) (30) to
230 26.7% (29) compared with that of primary PI incidence (1.9%) (37). We assumed that the
231 skin microbiome affects the physiology of PI-healed skins and has a role in recurrence.
232 In our prior publication, eight of the 30 patients (26.7%) had RPI within six weeks (29).
233 We extracted DNA from PI-healed skin samples. After excluding one RPI sample
234 obtained immediately after bathing (RPI, N = 7; non-RPI, N=22), the 29 skin samples
235 were analyzed for relative abundance ratios of *Staphylococcus* species at the collection
236 time points using 16S rRNA metataxonomics and *Staphylococcu*s-specific typing (28,
237 38). Previously we found that relative abundance ratios of *S. aureus* were higher in the
238 healed skins than normal skins at the collection time, and that two of the seven healed
239 skins that later suffered from RPI were predominated by *S. aureus* at high ratio of more
240 than 70% (28). In this study, we typed the seven healed and control skins. In comparison,
241 seven non-RPI skins with a high *S. aureus* ratio were also typed. As a result, the typing
242 for the 14 healed skins was successful (Table 2). CC1 or CC5 were found in the RPI group,
243 whereas the non-RPI group comprised CC1, CC5, CC97, and new types. The non-RPI #3
244 contained different CC between the healed and normal skins. Five of the 14 normal
245 (control) skins were not typed due to a failure in *tpi* and/or *yqi* allele amplification.

Using multiplex/nested PCR, we found that *S. aureus* predominated in the two RPI samples (RPI #5 and #7) belonged to ST764 (CC5) and ST7910 (new) (CC1), respectively. The other five RPI healed skins were also dominated by CC1 or CC5. The typing also revealed that *S. aureus* on eight non-RPI healed skins belonged mostly to CC1 or CC5, while CC97 predominated in one sample (non-RPI #3). Typing failed in the *pta* alleles of the one non-RPI control (normal) skins (#7), most likely due to a low abundance of *S. aureus* genome.

DISCUSSION

Nested PCR is utilized to increase the accuracy and sensitivity for pathogen detection in clinical samples. Hsu et al., tested several PCR-based methods and concluded that combination of nested-PCR and probe-based real time PCR showed best accuracy for Acanthamoeba genotyping (39). Sun et al., developed rapid and sensitive detection method of *Mycobacterium tuberculosis* based on hybridization of nested PCR products and their specific probes (40). The combination of multiplex and nested PCR has also found widespread use in pathogen detection. It has been reported that MLST with nested PCR of *Trichomonas vaginalis* was a sensitive typing method that could be used without

264 patient sample culture (41). FilmArray, an automated nested multiplex PCR System, has
265 been used in several clinical facilities (42). In this study, we developed a multiplex-nested
266 PCR method to determine ST of *S. aureus* in a DNA mixture (Figure S1). In the presence
267 of additional skin commensal bacteria and human DNA, our PCR technique demonstrated
268 specificity to all the seven allele sequences (Figures 1 and S3). We validated our
269 procedure works for clinical samples by testing skin samples from healthy individuals
270 (Figures 2 and S4).

271 Next generation sequencing (NGS) is now mainstream for microbiome analysis.
272 While shot-gun sequencing may reveal virulence factors in numerous bacteria, the costs
273 are expensive. NGS-based 16S rRNA analysis, which targets amplified V3-V4
274 hypervariable regions, may identify bacteria at genus level, such as *Staphylococcus*, but
275 not at the species level. A recently developed novel amplicon NGS to categorize a genus
276 into species, however this approach only targets one genus in each sequencing (38).
277 Assembly of the two amplicon NGS data can detect relative abundance ratio of *S. aureus*
278 cost-effectively. *S. aureus* may also be typed for MLST utilizing our multiplex-nested
279 PCR.

280 In this study, we examined STs of *S. aureus* colonized on the skins healed from
281 PI (Table 2). As results, the STs were mainly categorized into CC1 or CC5. There is a

282 paper on MRSA epidemiology that covers CC1 and ST5 (43). CC1 has been mainly
283 detected in livestock in many countries. Community-acquired MRSA (CA-MRSA) clone
284 MW2/USA400 (ST1-SCC*mec*IV lineage) from the United States have emerged in
285 hospitals worldwide. Recently, regional outbreaks of ST2425 (CC1)/t1784 MRSA strains
286 were detected in Japan (44, 45) On the other hand, ST5 MRSAs, have been identified as
287 both healthcare-associated MRSA (HA-MRSA) and livestock-associated MRSA (LA-
288 MRSA). ST5-SCC*mec*Ⅱ, which was previously referred as the New Yolk/Japan clone,
289 is one of the most widely distributed lineages (35, 46). Previous epidemiological studies
290 indicate spread of SCC*mec*Ⅱ ST764, a variant of the ST5 lineage, during nosocomial
291 MRSA infection in Japan and Thailand (47-49). In this study, ST764 predominated on the
292 healed skin of Patient #5 of RPI group with high abundance (=0.77). CA-MRSA and HA-
293 MRSA lineages were genetically distinct (50). Further research is required to determine
294 the pathogenicity of the CC1 and CC5 *S. aureus* colonies found on the healed skins.
295 There are some limitations on our study. Firstly, it remains unclear why different
296 STs were detected among the healed and control skins from the skins of non-RPI #3, #4,
297 and #5 (Table 2). Our Sanger sequencing results revealed that one kind of ST was found
298 in each sample used in this study. However, it is possible that *S. aureus* of different STs
299 may be mixed in the bacterial flora. In such case, NGS analysis would be required. We

300 can determine the proportion of STs of *S. aureus* present in the bacterial flora by adding
301 adapter sequences to the primer sequences used for this study, creating a library, and
302 running it through the NGS sequencer. Secondly, skin samples contain other bacterial
303 DNA, human DNA, and contaminants, the amounts of which vary among individuals.
304 Therefore, no uniform threshold can be established as to whether a skin sample is
305 applicable to MLST of *S. aureus*. Thirdly, the current primer set failed to determine the
306 *S. aureus* STs at a frequency of 21% (6 out of 28 skin samples) due to failure for four
307 alleles such as *gmk*, *pta, tpi*, and *yqiL* (Table 2). Optimization of primer sequences for the
308 first PCR may improve sensitivity of the approach.

309 In conclusion, we created a culture-free MLST technique for the rapid detection
310 of *S. aureus* using nested PCR. This approach provides sensitive and specific typing,
311 allowing direct patient materials to be typed.

312

313 **Data Availability Statement**

314 The data that support the findings of this study are available from the corresponding
315 author upon reasonable request.

316

317 **Conflict of interest**

We declare that we have no known competing financial interests or personal relationships that influence the work in this study. SW is an Editorial Board member of Microbiology and Immunology and co-author of this article. To minimise bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

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Author Contribution

HF conducted the experiments. KoO designed this study and wrote the manuscript. KoO and NT analyzed the data and designed the primers. SW conceived this study. NT, SW, and AY revised the manuscript for the intellectual content. KaO contributed to the ethical approval. JS contributed to the sample collection. SO supervised HF. All authors reviewed the manuscript.

FIGURE LEGENDS

Figure 1. Proof-of-concept using DNA samples of bacteria and human. Agarose gel electrophoresis of the second PCR products of multiplex/MLST PCR. The first multiplex PCR was performed under the four experimental conditions with different templates to be utilized. Lane 1: DNA mixture of five bacteria (each 10^3 copies of *Staphylococcus caprae*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa, Cutibacterium acnes* and *Corynebacterium tuberculostearicum*) in a reaction mixture. Lane 2: DNA mixture of the five bacteria (each 10^3 copies) and *S. aureus* strain N315 (each 10^3 copies). Lane 3: HaCaT cells (10^4 copies in a mixture) and the five bacteria (each 10^3 copies). Lane 4: DNA mixture of HaCaT cells (10^4 copies), the five bacteria (each 10^3 copies), and *S. aureus* strain N315 (each 10^3 copies). The second PCR was conducted using Tks Gflex DNA Polymerase as described in the Materials and Methods.

Figure 2. Preliminary test using the skin samples of three healthy volunteers. Agarose gel electrophoresis of the second PCR products of multiplex/MLST PCR. The skin samples were obtained from the three healthy volunteers by swabbing their forearms,

followed by DNA extraction (Materials and Methods). The first multiplex PCR was performed using one microliter of the extracted DNA solution.

Figure S1. Graphical abstract of our developed MLST.

Figure S2. Alignment of the seven genes.

Figure S3. Proof-of-concept using DNA samples of bacteria, human, *S. aureus* strain Cowan I (ST30). Agarose gel electrophoresis of the second PCR products of multiplex/MLST PCR. The first multiplex PCR was performed using DNA mixture of HaCaT cells (10^4 copies), the five bacteria (each 10^3 copies), and *S. aureus* strain Cowan I (ST30) (each 10^3 copies). After ExoSAP-IT treatment and 50-fold dilution, the second PCR was conducted using Tks Gflex DNA Polymerase as described in the Materials and Methods.

Figure S4. Amplification of the *pta* allele using the healthy volunteer #2. Agarose gel electrophoresis of the second PCR products of the *pta* allele. The first single-plex PCR was performed using the pair of *tpi* primers with Tks Gflex DNA Polymerase or Multiplex PCR Assay Kit. After ExoSAP-IT treatment and 50-fold dilution, the second PCR was conducted using Tks Gflex DNA Polymerase as described in the Materials and Methods.

REFERENCES

1. Foster T. (1996) Staphylococcus, 4th editio. University of Texas Medical Branch at Galveston, Galveston (TX)
2. Taylor T.A., Unakal C.G. (2022) Staphylococcus Aureus. StatPearls. https://doi.org/10.1128/9781555819972.ch21
3. Grace D., Fetsch A. (2018) Staphylococcus aureus —A Foodborne Pathogen. In: Staphylococcus aureus. Elsevier, pp 3–10
4. Krismer B., Weidenmaier C., Zipperer A., Peschel A. (2017) The commensal

lifestyle of Staphylococcus aureus and its interactions with the nasal microbiota. Nat Rev Microbiol 15:675–687

5. Lowy F.D. (1998) Staphylococcus aureus infections. N Engl J Med 339:520–532
6. Rasigade J.P., Vandenesch F. (2014) Staphylococcus aureus: a pathogen with still unresolved issues. Infect Genet Evol 21:510–514
7. Chambers H.F. (2005) Community-associated MRSA--resistance and virulence converge. N Engl J Med 352:1485–1487
8. Baldwin N.S., Gilpin D.F., Hughes C.M., Kearney M.P., Gardiner D.A., Cardwell C., Tunney M.M. (2009) Prevalence of methicillin-resistant Staphylococcus aureus colonization in residents and staff in nursing homes in Northern Ireland. J Am Geriatr Soc 57:620–626
9. Cox R.A., Bowie P.E.S. (1999) Methicillin-resistant Staphylococcus aureus colonization in nursing home residents: a prevalence study in Northamptonshire. J Hosp Infect 43:115–122
10. Stark L., Olofsson M., Löfgren S., Mölstad S., Lindgren P.E., Matussek A. (2014) Prevalence and molecular epidemiology of Staphylococcus aureus in Swedish nursing homes - as revealed in the SHADES study. Epidemiol Infect 142:1310–1316
11. Lee B.Y., Bartsch S.M., Wong K.F., Singh A., Avery T.R., Kim D.S., et al (2013) The importance of nursing homes in the spread of methicillin-resistant Staphylococcus aureus (MRSA) among hospitals. Med Care 51:205–215
12. Zhang J., Gu F.F., Zhao S.Y., Xiao S.Z., Wang Y.C., Guo X.K., Ni Y.X., Han L.Z. (2015) Prevalence and molecular epidemiology of staphylococcus aureus among residents of seven nursing homes in Shanghai. PLoS One. https://doi.org/10.1371/journal.pone.0137593
13. Hudson L.O., Reynolds C., Spratt B.G., Enright M.C., Quan V., Kim D., et al (2013) Diversity of methicillin-resistant Staphylococcus aureus strains isolated from residents of 26 nursing homes in Orange County, California. J Clin Microbiol 51:3788–3795
14. Senn L., Clerc O., Zanetti G., Basset P., Prod'hom G., Gordon N.C., et al (2016) The Stealthy Superbug: the Role of Asymptomatic Enteric Carriage in Maintaining a Long-Term Hospital Outbreak of ST228 Methicillin-Resistant Staphylococcus aureus. mBio. https://doi.org/10.1128/MBIO.02039-15
15. Oh J., Byrd A.L., Park M., Kong H.H., Segre J.A. (2016) Temporal Stability of the Human Skin Microbiome. Cell 165:854–866
16. Parlet C.P., Brown M.M., Horswill A.R. (2019) Commensal Staphylococci

Influence Staphylococcus aureus Skin Colonization and Disease. Trends Microbiol 27:497–507

17. Moran G.J., Krishnadasan A., Gorwitz R.J., Fosheim G.E., McDougal L.K., Carey R.B., Talan D.A. (2006) Methicillin-Resistant S. aureus Infections among Patients in the Emergency Department. New England Journal of Medicine 355:666–674

18. Sollid J.U.E., Furberg A.S., Hanssen A.M., Johannessen M. (2014) Staphylococcus aureus: Determinants of human carriage. Infection, Genetics and Evolution 21:531–541

19. Shopsin B., Mathema B., Alcabes P., Said-Salim B., Lina G., Matsuka A., Martinez J., Kreiswirth B.N. (2003) Prevalence of agr specificity groups among Staphylococcus aureus strains colonizing children and their guardians. J Clin Microbiol 41:456–459

20. Goh S.H., Byrne S.K., Zhang J.L., Chow A.W. (1992) Molecular typing of Staphylococcus aureus on the basis of coagulase gene polymorphisms. J Clin Microbiol 30:1642–1645

21. Sakai F., Takemoto A., Watanabe S., Aoyama K., Ohkubo T., Yanahira S., Igarashi H., Kozaki S., Hiramatsu K., Ito T. (2008) Multiplex PCRs for assignment of Staphylocoagulase types and subtypes of type VI Staphylocoagulase. J Microbiol Methods 75:312–317

22. Koreen L., Ramaswamy S. V., Graviss E.A., Naidich S., Musser J.M., Kreiswirth B.N. (2004) spa Typing Method for Discriminating among Staphylococcus aureus Isolates: Implications for Use of A Single Marker to Detect Genetic Micro- and Macrovariation. J Clin Microbiol 42:792–799

23. Takahashi H., Seki M., Yamamoto N., Hamaguchi S., Ojima M., Hirose T., et al (2015) Validation of a phage-open reading frame typing kit for rapid identification of methicillin-resistant Staphylococcus aureus (MRSA) transmission in a tertiary hospital. Infect Drug Resist 8:107–111

24. Suzuki M., Tawada Y., Kato M., Hori H., Mamiya N., Hayashi Y., et al (2006) Development of a rapid strain differentiation method for methicillin-resistant Staphylococcus aureus isolated in Japan by detecting phage-derived open-reading frames. J Appl Microbiol 101:938–947

25. Enright M.C., Day N.P., Davies C.E., Peacock S.J., Spratt B.G. (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. J Clin Microbiol 38:1008–15

26. Liu N., Liu D., Li K., Hu S., He Z. (2022) Pan-genome analysis of staphylococcus aureus reveals key factors influencing genomic plasticity. Microbiol Spectr.

https://doi.org/10.1128/SPECTRUM.03117-22
27. Jolley K.A., Bray J.E., Maiden M.C.J. (2018) Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. https://doi.org/10.12688/WELLCOMEOPENRES.14826.1
28. Ogura K., Furuya H., Takahashi N., Shibata K., Endo M, Watanabe S, et al (2022) Interspecies regulation between Staphylococcus caprae and Staphylococcus aureus colonized on healed skin after injury. front microbiol. https://doi.org/10.3389/fmicb.2022.818398
29. Shibata K., Ogai K., Ogura K., Urai T., Aoki M., Arisandi D., Takahashi N., Okamoto S., Sanada H., Sugama J. (2021) Skin physiology and its microbiome as factors associated with the recurrence of pressure injuries. Biol Res Nurs 23:75–81
30. Kuroda M., Ohta T., Uchiyama I., Baba T., Yuzawa H., Kobayashi I., et al (2001) Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet 357:1225–1240
31. Watanabe S., Aiba Y., Tan X.E., Li F.Y., Boonsiri T., Thitiananpakorn K., et al (2018) Complete genome sequencing of three human clinical isolates of Staphylococcus caprae reveals virulence factors similar to those of S. epidermidis and S. capitis. BMC Genomics 19:810
32. Arisandi D., Ogai K., Urai T., Aoki M., Minematsu T., Okamoto S., Sanada H., Nakatani T., Sugama J. (2020) Development of recurrent pressure ulcers, risk factors in older patients: A prospective observational study. J Wound Care 29:S14–S24
33. (2008) BLAST® Command Line Applications User Manual.
34. Shen W., Le S., Li Y., Hu F. (2016) SeqKit: A cross-platform and ultrafast toolkit for fasta/q file manipulation. PLoS One. https://doi.org/10.1371/JOURNAL.PONE.0163962
35. Enright M.C., Robinson D.A., Randle G., Feil E.J., Grundmann H., Spratt B.G. (2002) The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc Natl Acad Sci U S A 99:7687–7692
36. Jolley K.A., Feil E.J., Chan M.S., Maiden M.C. (2001) Sequence type analysis and recombinational tests (START). Bioinformatics 17:1230–1
37. Igarashi A., Yamamoto-Mitani N., Gushiken Y., Takai Y., Tanaka M., Okamoto Y. (2013) Prevalence and incidence of pressure ulcers in Japanese long-term-care hospitals. Arch Gerontol Geriatr 56:220–226
38. Ederveen T.H.A., Smits J.P.H., Hajo K., van Schalkwijk S., Kouwenhoven T.A.,

491 Lukovac S., et al (2019) A generic workflow for Single Locus Sequence Typing
492 (SLST) design and subspecies characterization of microbiota. Sci Rep 9:19834
493 39. Hsu T.K., Chen J.S., Tsai H.C., Tao C.W., Yang Y.Y., Tseng Y.C., Kuo Y.J., Ji D.,
494 Rathod J., Hsu B.M. (2021) Efficient nested-PCR-based method development for
495 detection and genotype identification of Acanthamoeba from a small volume of
496 aquatic environmental sample. Sci Rep. https://doi.org/10.1038/S41598-021-
497 00968-2
498 40. Sun Y., Chen J., Li J., Xu Y., Jin H., Xu N., Yin R., Hu G. (2017) Novel approach
499 based on one-tube nested PCR and a lateral flow strip for highly sensitive diagnosis
500 of tuberculous meningitis. PLoS One.
501 https://doi.org/10.1371/JOURNAL.PONE.0186985
502 41. van der Veer C., Himschoot M., Bruisten S.M. (2016) Multilocus sequence typing
503 of Trichomonas vaginalis clinical samples from Amsterdam, the Netherlands. BMJ
504 Open 6:e013997
505 42. Poritz M.A., Blaschke. AJ, Byington C.L., Meyers L., Nilsson K., Jones D.E., et
506 al (2011) FilmArray, an Automated nested multiplex pcr system for multi-pathogen
507 detection: development and application to respiratory tract infection. PLoS One
508 6:e26047
509 43. Hiramatsu K., Ito T., Tsubakishita S., Sasaki T., Takeuchi F., Morimoto Y., et al
510 (2013) Genomic basis for methicillin resistance in Staphylococcus aureus. Infect
511 Chemother 45:117–136
512 44. Mitsuboshi S., Yamaguchi T., Seino H., Fukuhara M., Hosokawa Y., Tsugita M.
513 (2021) Regional outbreak of methicillin-resistant Staphylococcus aureus ST2725-
514 t1784 in rural Japan. Infect Control Hosp Epidemiol 42:1294–1296
515 45. Ogura K., Kaji D., Sasaki M., Otsuka Y., Takemoto N., Miyoshi-Akiyama T.,
516 Kikuchi K. (2022) Predominance of ST8 and CC1/spa-t1784 methicillin-resistant
517 Staphylococcus aureus isolates in Japan and their genomic characteristics. J Glob
518 Antimicrob Resist. https://doi.org/10.1016/J.JGAR.2022.01.011
519 46. Coombs GW, Van Gessel H, Pearson JC, Godsell M-R, O'Brien FG, Christiansen
520 KJ (2007) Controlling a multicenter outbreak involving the New York/Japan
521 methicillin-resistant Staphylococcus aureus clone. Infect Control Hosp Epidemiol
522 28:845–52
523 47. Takano T, Hung W-C, Shibuya M, Higuchi W, Iwao Y, Nishiyama A, et al (2013)
524 A New Local Variant (ST764) of the Globally Disseminated ST5 Lineage of
525 Hospital-Associated Methicillin-Resistant Staphylococcus aureus (MRSA)
526 Carrying the Virulence Determinants of Community-Associated MRSA.

https://doi.org/10.1128/AAC.01147-12

48. Kondo S, Phokhaphan P, Tongsima S, Ngamphiw C, Phornsiricharoenphant W, Ruangchai W, et al (123AD) Molecular characterization of methicillin-resistant Staphylococcus aureus genotype ST764-SCCmec type II in Thailand. Scientific Reports | 12:2085

49. Aung MS, Kawaguchiya M, Urushibara N, Sumi A, Ito M, Kudo K, Morimoto S, Hosoya S, Kobayashi N (2017) Molecular Characterization of Methicillin-Resistant Staphylococcus aureus from Outpatients in Northern Japan: Increasing Tendency of ST5/ST764 MRSA-IIa with Arginine Catabolic Mobile Element. Microb Drug Resist 23:616–625

50. Lakhundi S., Zhang K. (2018) Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev. https://doi.org/10.1128/CMR.00020-18

Table 1. Primers utilized in this study.

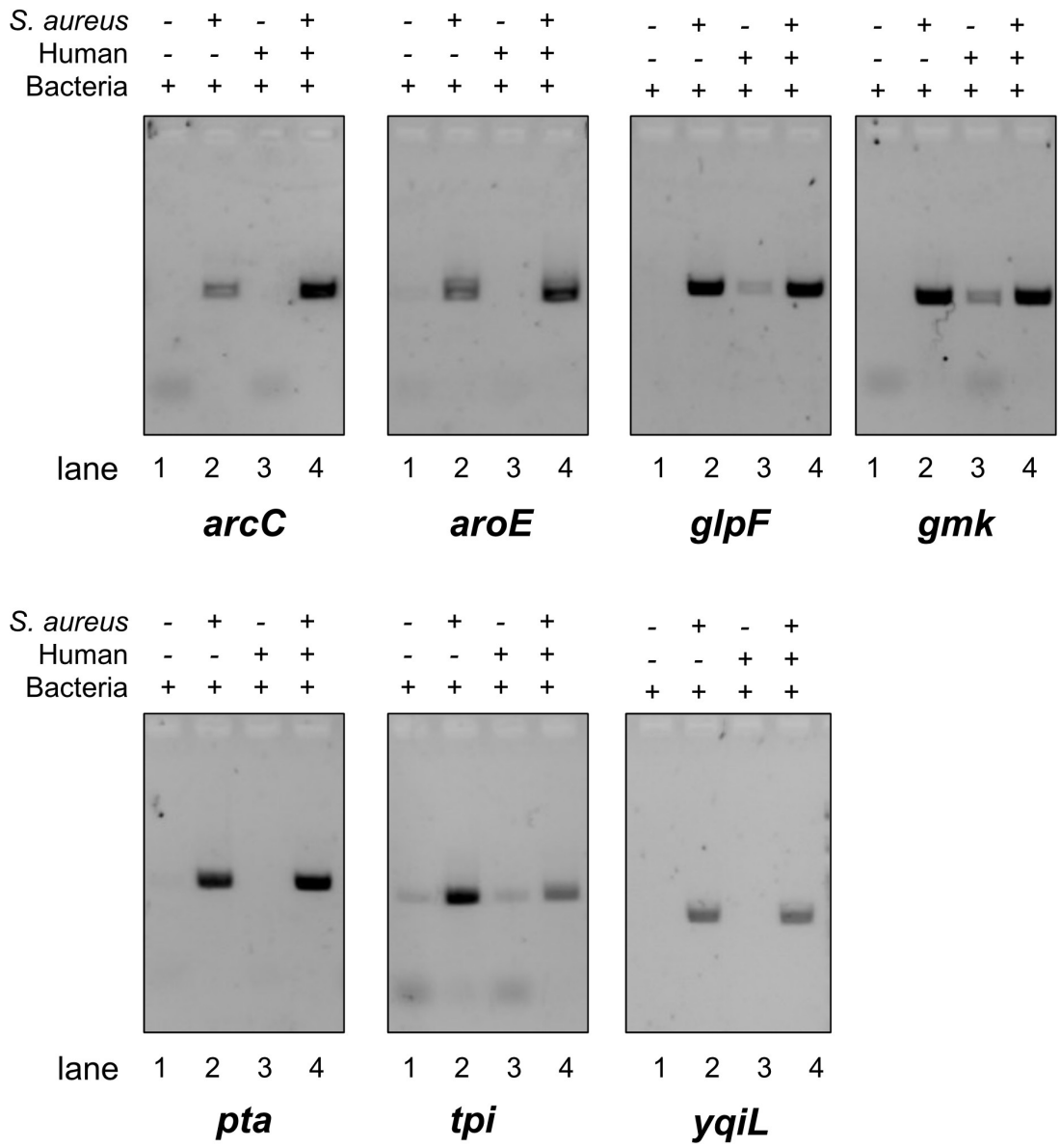
Genes		1st multiplex PCR (This study)	Sizes (bps)	2nd nested PCR (Previous studies) (25)	Sizes (bps)
arcC	Forward	5'-AACTTGGCCTTTCGTATAATGTCTGAC-3'	1405	5'-TTGATTCACCAGCGCGTATTGTC-3'	456
	Reverse	5'-GCTATATGGCTTATATATGCAGCAGG-3'		5'-AGGTATCTGCTTCAATCAGCG-3'	
aroE	Forward	5'-TTCGTTGATACCGCCTTTTCCA-3'	1128	5'-ATCGGAAATCCTATTTCACATTC-3'	456
	Reverse	5'-TATGGCGTAATCAACTTGGCGA-3'		5'-GGTGTTGTATTAATAACGATATC-3'	
glpF	Forward	5'-ACTGCTAGTGTAGGAATCTCTTTRT-3'	968	5'-CTAGGAACTGCAATCTTAATCC-3'	465
	Reverse	5'-ATCGCTAACCCATTGTACAAAATC-3'		5'-TGGTAAAATCGCATGTCCAATTC-3'	
gmk	Forward	5'-ATGGCATGCAATTTCAAATATGC-3'	1011	5'-ATCGTTTTATCGGGACCATC-3'	417
	Reverse	5'-CATAGGTGCGTTTGATTTGGAT-3'		5'-TCATTAACTACAACGTAATCGTA-3'	
pta	Forward	5'-CAGTGACAAGTGCATCGCGTTCGT-3'	1521	5'-GTTAAAATCGTATTACCTGAAGG-3'	474
	Reverse	5'-TGGCATTATAACCAATCTCATTC-3'		5'-GACCCTTTTGTTGAAAAGCTTAA-3'	
tpi	Forward	5'-TGATAGTTTAAAGTGATGTGGCATG-3'	1063	5'-TCGTTCATTCTGAACGTCGTGAA-3'	402
	Reverse	5'-GTTACCCATTTGTCCTTCAGGT-3'		5'-TTTGCACCTTCTAACAATTGTAC-3'	
yqiL	Forward	5'-GGATGTTAGTGGCGTATTGGTTC-3'	1554	5'-CAGCATACAGGACACCTATTGGC-3'	516
	Reverse	5'-CATTCGATACCGATGCATCTGTGG-3'		5'-CGTTGAGGAATCGATACTGGAAC-3'	

Table 2. MLST of skins healed from pressure injury and control (normal) skins

RPI group	Sample ID	Specimen	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqi*	MLST	**Clonal complex**	Abundance ratio*
RPI group	1	healed skin	1	4	1	4	12	1	10	ST5	**CC5**	0.0042
		control skin	1	4	1	N.D.	12	N.D.	N.D.	**Detection failure**	**Detection failure**	0.0023
	2	healed skin	1	4	1	1	1	1	1	ST7908	**CC1**	0.0048
		control skin	1	4	546	4	12	535	N.D.	**Detection failure**	**Detection failure**	0.0047
	3	healed skin	1	1	1	19	1	1	1	ST7909	**CC1**	0.4882
		control skin	1	4	1	14	12	N.D.	1	**Detection failure**	**Detection failure**	0.1011
	4	healed skin	1	4	1	15	12	1	10	ST7982	**CC5**	0.0072
		control skin	1	4	1	72	12	1	10	ST774	**CC5**	0.0117
	5	healed skin	1	136	1	4	12	1	10	ST764	**CC5**	0.7746
		control skin	1	4	1	4	12	N.D.	10	**Detection failure**	**Detection failure**	0.0084
	6	healed skin	1	1	1	4	1	1	656	ST4956	**CC1**	0.0105
		control skin	1	1	1	4	1	N.D.	1	**Detection failure**	**Detection failure**	0.0321
	7	healed skin	1	1	1	97	1	1	883	7910	**CC1**	0.7858
		control skin	1	4	1	1	1	724	10	402	**CC1**	0.0067
non-RPI group	1	healed skin	27	1	1	1	1	1	13	ST7911	**CC1**	0.8418
		control skin	1	1	1	1	1	324	1	ST7916	**CC1**	0.6011
	2	healed skin	1	1	1	1	1	1	1	ST1	**CC1**	0.2484
		control skin	1	1	1	97	1	324	1	ST7917	**CC1**	0.8195
	3	healed skin	3	1	1	1	1	862	3	ST7912	**CC97**	0.7503

	control skin	12	4	1	4	12	324	10	ST7918	**CC5**	0.0269
4	healed skin	623	1	1	2	10	26	897	ST7913	**-**	0.6546
	control skin	23	32	1	2	10	324	11	ST7920	-	0.3077
5	healed skin	3	35	19	89	20	26	462	ST7914	-	0.5856
	control skin	1	4	750	23	20	324	10	ST7921	-	0.0839
6	healed skin	1	4	1	4	12	1	1	ST83	**CC5**	0.1247
	control skin	1	4	1	455	12	324	10	ST7922	**CC5**	0.0232
7	healed skin	1	1087	1	169	1	1	10	ST7915	**CC1**	0.0522
	control skin	1	45	893	4	N.D.	324	10	**Detection failure**	**Detection failure**	0.0135

*The abundance ratios of *S. aureus* were calculated in our previous study (28).



542

543 **Figure 1**

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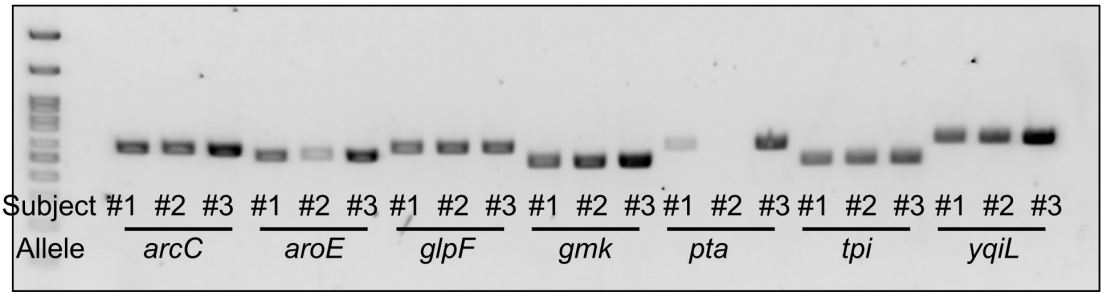

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546 **Figure 2**

547

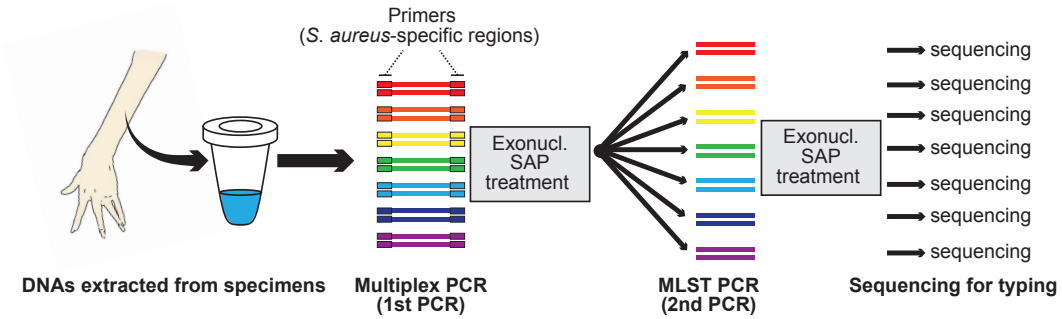

Figure S1. Graphical abstract of our developed MLST.

Figure S2. Alignment of the seven genes: Please see the other supplementary material.

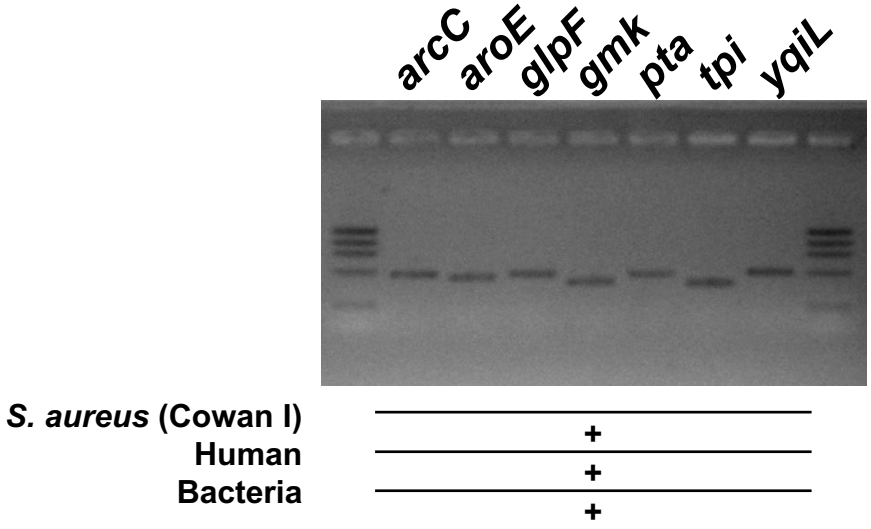

Figure S3. Proof-of-concept using DNA samples of bacteria, human, S. aureus strain Cowan I (ST30). Agarose gel electrophoresis of the second PCR products of multiplex/MLST PCR. The first multiplex PCR was performed using DNA mixture of HaCaT cells (10^4 copies), the five bacteria (each 10^3 copies), and *S. aureus* strain Cowan I (ST30) (each 10^3 copies). After ExoSAP-IT treatment and 50-fold dilution, the second PCR was conducted using Tks Gflex DNA Polymerase as described in the Materials and Methods.

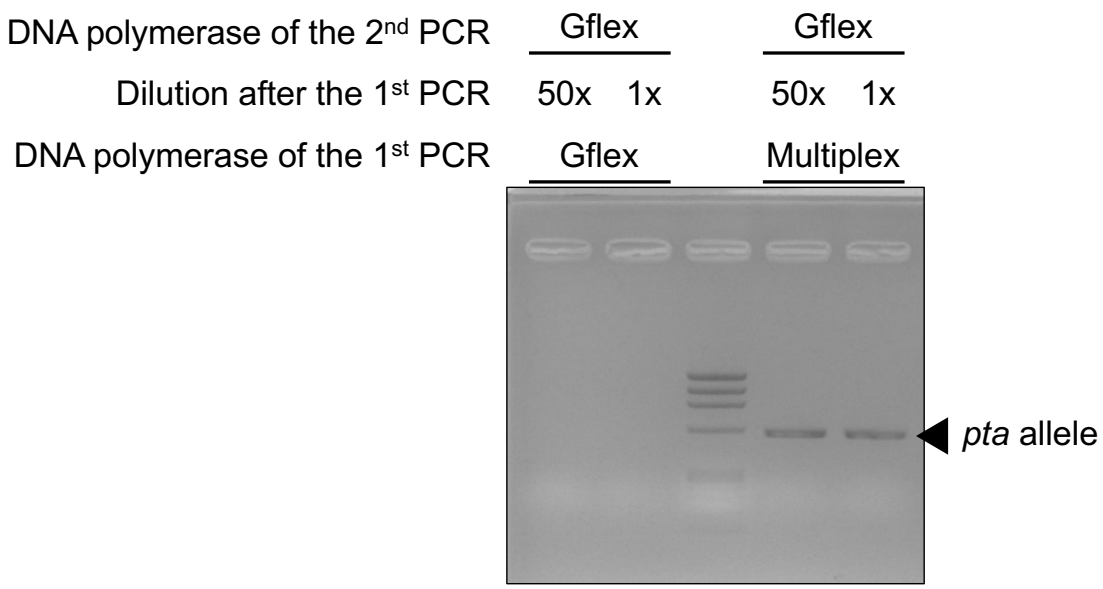

Figure S4. Amplification of the *pta* allele using the healthy volunteer #2. Agarose gel electrophoresis of the second PCR products of the *pta* allele. The first single-plex PCR was performed using the pair of *tpi* primers with Tks Gflex DNA Polymerase or Multiplex PCR Assay Kit. After ExoSAP-IT treatment and 50-fold dilution, the second PCR was conducted using Tks Gflex DNA Polymerase as described in the Materials and Methods.