Emerging ST121/*agr*4 community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) with strong adhesin and cytolytic activities: trigger for MRSA pneumonia and fatal aspiration pneumonia in an influenza-infected elderly

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Abstract

The pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) pneumonia in influenza-infected elderly individuals has not yet been elucidated in detail. In the present study, a 92-year-old man infected with influenza developed CA-MRSA pneumonia. His CA-MRSA was an emerging type, originated in ST121/*agr*4 *S. aureus*, with diversities of Panton–Valentine leucocidin (PVL)$^{-}$/*spa*t5110/SCC*mec*V^{+} versus PVL^{+}/*spa*t159$^{(etc.)}$/SCC*mec*$^{-}$, but with common virulence potentials of strong adhesin and cytolytic activities. Resistance to erythromycin/clindamycin (inducible-type) and gentamicin was detected. Pneumonia improved with the administration of levofloxacin, but with the subsequent development of fatal aspiration pneumonia. Hence, characteristic CA-MRSA with strong adhesin and cytolytic activities triggered influenza-related sequential complications.

Keywords: Community-associated methicillin-resistant *Staphylococcus aureus*, elderly community-acquired pneumonia, fatal aspiration pneumonia, influenza, ST121/*agr*4 lineage

Introduction

Staphylococcus aureus is a major human pathogen that causes skin and soft-tissue infections, life-threatening infections such as pneumonia and sepsis, and toxinoses including toxic shock syndrome. Methicillin-susceptible *S. aureus* (MSSA) has evolved as methicillin-resistant *S. aureus* (MRSA) through the acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*) [1–3].

Two types of MRSA have been identified [1]: traditional MRSA, which emerged in hospitals in 1961 and is now classified as healthcare-associated MRSA (HA-MRSA) with global examples of ST239/SCC*mec*III and ST5/SCC*mec*II [4,5], and community-associated MRSA (CA-MRSA), which emerged in the community in 1997–1999 with global examples of ST8/SCC*mec*IV (USA300), ST30/SCC*mec*IV, ST80/SCC*mec*IV and ST59/SCC*mec*V [3,5,6,7]. Hence, globally disseminated ST30/*agr*3 (but not ST121/*agr*4) MSSA actually evolved as global CA-MRSA [8].

CA-MRSA possesses distinct virulence factors from those of HA-MRSA, for example, it more strongly expresses cytolytic peptides, such as phenol-soluble modulins (PSMs) [6,9,10], and frequently produces Panton–Valentine leucocidin (PVL) [6,7,10,11]. Successful CA-MRSA also has unique virulence factors, as typically shown with USA300 [3,6,10].

The elderly are susceptible to *S. aureus* community-acquired pneumonia (CAP) that requires hospitalization [12], and this may be due to a functionally dysregulated host immune system [13]. Moreover, influenza-infected elderly individuals are at risk of bacterial pneumonia; influenza impairs host immunological mechanisms [14] and promotes co-infections or sequential infections with *S. aureus* or CA-MRSA [14–16]. Although PVL is not necessary [16], the pathogenesis of influenza-related CA-MRSA CAP remains unclear.

We herein isolated ST121/*agr*4 CA-MRSA from influenza-related MRSA CAP for the first time. We then attempted to elucidate the molecular features of CA-MRSA and gain novel

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insights into the pathogenesis of influenza-related, CA-MRSA-triggered sequential complications.

Case

A 92-year-old man was diagnosed with influenza A using an influenza rapid diagnostic (antigen detection) test on 25 January 2013, and was treated with oseltamivir phosphate (150 mg per day); however, no improvements were noted in his symptoms. He was admitted to a hospital on 27 January 2013 (day 1) with fever and progressive dyspnoea. He had a previous history of cerebral infarction, but did not need regular home visits by healthcare workers and did not have a urinary catheter. He did not have established risk factors for HA-MRSA infections such as a history of hospitalization, surgery, haemodialysis, the presence of a permanent indwelling catheter or percutaneous medical device, or residence in a long-term care facility in the past year [1]. He also had no previous MRSA infections or history of recent antibiotic use. On admission, his white blood cell count and C-reactive protein level were 20,200/μL and 33.3 mg/dL, respectively. Chest radiography revealed bilateral pulmonary infiltrates (Fig. 1): an infiltrative shadow in the right lung (arrow 1) and consolidation with air bronchograms on the lateral side of the left middle lung field (arrow 2). The pattern of the chest radiography findings, a peripherally distributed parenchymal abnormality (arrow 2), is more frequent with MRSA pneumonia than with MSSA pneumonia [17]. Respiratory failure (SpO_2, 76%) was noted. Chemotherapy was initiated with a drip infusion of piperacillin (2 g every 12 h). On day 2, sputum was examined, MRSA was detected in cultures (at 10^7 CFU/mL), and the presence of intracellular MRSA in polymorphs was confirmed microscopically; no other pathogens were detected. On day 5, oral levofloxacin (500 mg every 24 h) was initiated based on the results of drug-susceptibility testing for MRSA (see Supplementary material, Table S1). His respiratory status rapidly improved, and his white blood cell count and C-reactive protein level also improved (4,500/μL and 1.7 mg/dL, respectively) on day 14 (7 February 2013). Hospitalization was continued due to severe anorexia; however, he developed aspiration pneumonia on 11 April and died on 19 April. This case of MRSA, epidemiologically classified as CA-MRSA [1], was named KT1.

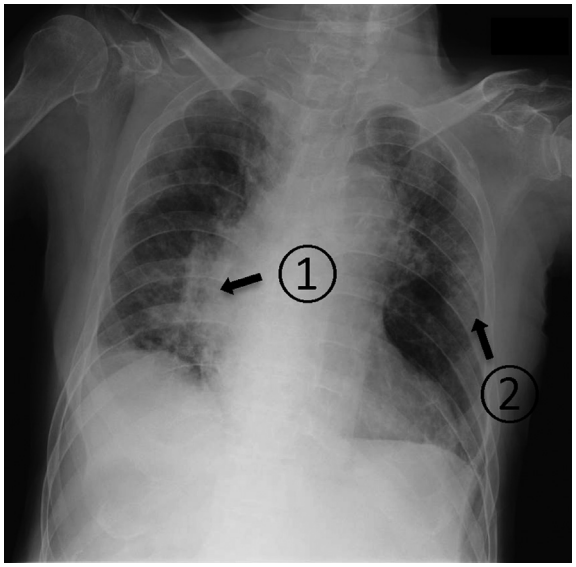

FIG. 1. Chest X-ray of the patient on admission day 1. A chest radiograph shows bilateral pulmonary infiltrates. Arrow 1 indicates an infiltrative shadow overlapping the second arch in the middle to lower field of the right lung, while arrow 2 indicates consolidation with air bronchograms on the lateral side of the left middle lung field.

Characterization of microbes

The molecular typing of MRSA, such as sequence type (ST), clonal complex (CC), *spa*, *agr*, SCC*mec* [2], and Coagulase (Coa), was performed as described previously [18]. Forty-nine virulence genes were analysed by PCR [18]: three leucocidin genes (*luk*$_{PV}$*SF*, *lukE-lukD* and *lukM*), five haemolysin genes (*hla*, *hlb*, *hlg*, *hlg-v* and *hld*), the peptide cytolysin, PSMα (*psmα*), 19 staphylococcal superantigen genes, named enterotoxin (SE) or enterotoxin-like (SEl) (*tst*, *sea-e*, *seg-j*, *selk-r* and *selu*), staphylococcal exotoxin (*set*) genes, a staphylococcal superantigen-like gene cluster (*ssl*), 3 exfoliative toxin (ET) genes (*eta*/*b* and *etd*), the epidermal cell differentiation inhibitor gene (*edin*), 14 adhesin genes (*icaA*/*D*, *eno*, *fib*, *fnbA*/*B*, *ebpS*, *clfA*/*B*, *sdrC-E*, *cna* and *bbp*), and the arginine catabolic mobile element-*arcA* gene. MRSA plasmids were analysed as described previously [18]. Bacterial susceptibility testing was performed using the agar dilution method with Mueller–Hinton agar [18]. The mRNA expression level of the PSMα gene (*psmα*) was examined using an RT-PCR assay [18]. Data were analysed statistically using the Student's *t*-test. The level of significance was defined as $p < 0.05$.

KT1 exhibited ST121/*agr*4 and was positive for the enterotoxin gene cluster (*egc*) with *seg*, *sei*, *selm*, *seln*, *selo* and *selu* [19] (Fig. 2a), similar to global ST121/*agr*4 MSSA [8]. However, KT1 was negative for PVL, and its *spa* type (*spa*1493(t5110)) was divergent from global ST121/*agr*4 MSSA [8,20].

KT1 carried SCC*mec*V, a characteristic SCC*mec* of CA-MRSA [2,3,5], and oxacillin and imipenem resistance levels were low, which is consistent with CA-MRSA [5,18]. KT1 carried the characteristic combination of the adhesin genes, *cna* (for collagen binding) and *bbp* (for bone sialoprotein binding). Moreover, the

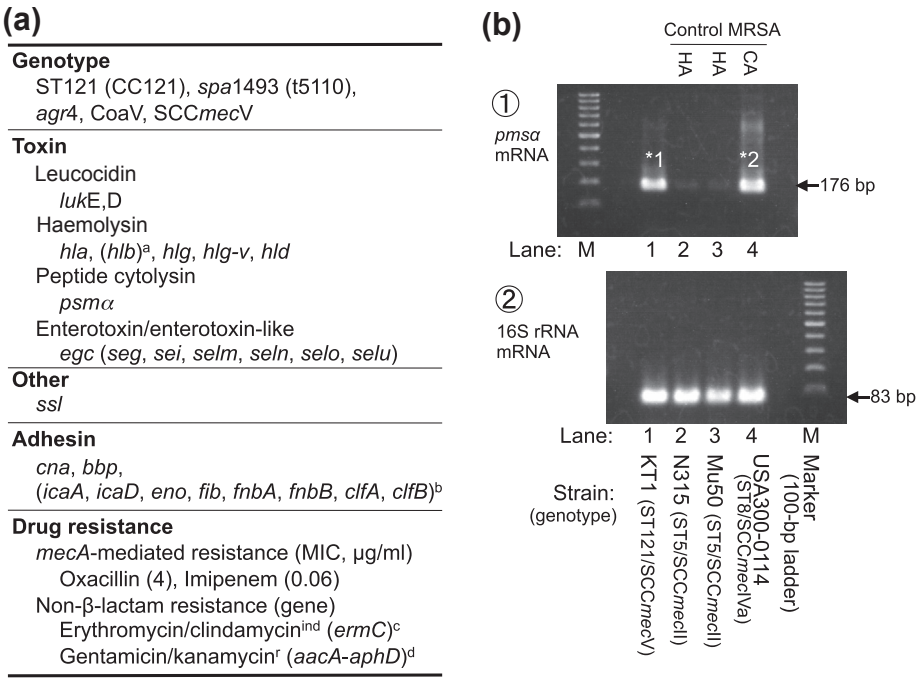

FIG. 2. Molecular characteristics (a) and mRNA expression levels of the cytolytic peptide gene (*psm*α) (b) of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strain KT1. In (a), superscript letters indicate the following: [a]the split *hlb* gene due to the insertion of phage Sa3; [b]eight adhesin genes are common among *S. aureus*; [c]the 2,473-bp plasmid pWKT1 (GenBank Accession number, LC086373) carried *ermC* with the leader peptide sequence, responsible for inducible (ind) resistance to clindamycin; pWKT1 was 100% homologous to the pKH19 of MRSA *spa*t1081 (GenBank Accession number, EU350089) and 99% homologous to the pOC160-1 of ST8 CA-MRSA from Russia (GenBank Accession number, AB982226); and [d]*aacA-aphD* encoded for resistance (r) to gentamicin. (b) Strains KT1 and USA300-0114 are CA-MRSA, and strains N315 and Mu50 are healthcare-associated (HA-) MRSA. The mRNA expression levels of the phenol-soluble modulin α (PSMα) gene (*psm*α), normalized to 16S rRNA expression levels, were 0.64 ± 0.13, 0.05 ± 0.02, 0.07 ± 0.02 and 0.63 ± 0.13 for strains KT1, N315, Mu50 and USA300-0114, respectively; the *psm*α expression level of KT1 (no. 1) was similar to that of USA300-0114 (no. 2), and was significantly higher than that of HA-MRSA ($p < 0.01$).

PSMα gene expression level of KT1 was significantly higher than that of HA-MRSA ($p < 0.01$), similar to CA-MRSA USA300 (Fig. 2b). Possible ancestral ST121/*agr*4 MSSA strains also exhibited the same virulence characteristics (see Supplementary material, Fig. S1); those ST121/agr4 MSSA strains (n=14) were PVL$^+$, Coa V, *cna*$^+$, *bbp*$^+$, and *egc*$^+$, and harbored stronger PSMα activities than other MSSA strains [21].

KT1 carried an *ermC* plasmid (pWKT1), specifying for inducible clindamycin resistance, and the *aacA-aphD* gene encoding for gentamicin resistance (Fig. 2a; see Supplementary material, Table S1). KT1 was susceptible to generally recommended anti-MRSA agents (see Supplementary material, Table S1).

Discussion

ST121/*agr*4 MRSA (KT1) met the CDC criteria for CA-MRSA [1]. Moreover, KT1 was bacteriologically consistent with CA-MRSA, based on the SCC*mec*V carriage [2,3,5], SCC*mec* (*mecA*) -mediated low level of resistance to oxacillin and imipenem [2,5,18], weaker multidrug resistance [5], and strong cytolytic activity [6,9,10]. Although elderly MRSA pneumonia in Japan is mostly from ST5/SCC*mec*II HA-MRSA and, hence, resistant to fluoroquinone [5], MRSA CAP in the present study was a rare fluoroquinone-susceptible case. KT1 exhibited gentamicin and inducible-clindamycin resistance, similar to CA-MRSA in Japan.

We herein described the first case of MRSA CAP from ST121/*agr*4 CA-MRSA (KT1), originated from PVL$^+$ ST121/*agr*4 MSSA (including *spa* type t159), which was globally disseminated in association with skin and soft-tissue infections and necrotizing pneumonia [8,20]. A unique feature of KT1 over other ST121/*agr*4 MSSA was PVL$^-$, *spa*t5110 and SCC*mec*V$^+$.

Influenza impairs host immunological mechanisms [14], promoting the development of CA-MRSA pneumonia [14,16]. The present case also had influenza-related CA-MRSA CAP, with chest radiography findings showing MRSA pneumonia [17], and was successfully treated with levofloxacin.

We strongly speculate that CA-MRSA associated with CAP harbours unique adhesin and toxic activities that act against the influenza-impaired respiratory tract mucosa. The present case was PVL-independent, which is consistent with previous findings [16]. KT1 (the ST121/*agr*4 lineage, irrespective of PVL^+ or PVL^- and MRSA or MSSA) harboured strong adhesin and toxic activities, as characterized by collagen and bone-sialoprotein adhesins, six *egc*-encoded superantigens, and the strong expression of PSMα. Collagen adhesin is considered to be associated with CAP, possibly through MRSA adherence to damaged tissues, for example, due to viral infection [22]. Globally disseminated ST30/*agr*3/SCC*mec*IV CA-MRSA, which has been associated with severe MRSA CAP, also harboured the same adhesin and toxic activities [23,24].

The present CA-MRSA CAP case, possibly in combination with his previous history of cerebral infarction, developed a decline in the activities of daily living. Pneumonia events have been associated with the loss of physical functioning [25]. In addition, in our patient, CA-MRSA CAP may also have damaged deglutition functions, possibly due to the strong adhesin and cytolytic activities of ST121/*agr*4 CA-MRSA, resulting in aspiration pneumonia.

ST121 CA-MRSA has been isolated from skin and soft-tissue infections, such as impetigo; however, its genetic characteristics remain unknown [26,27]; KT1 was negative for impetigo-associated ETs. The ST121/*agr*4 lineage may be evolving, in two ways, as PVL^+, ET^+ impetigo-associated CA-MRSA and as PVL^- (or PVL^+) CAP-associated CA-MRSA.

In conclusion, we isolated ST121/*agr*4 CA-MRSA (KT1) from influenza-related CA-MRSA CAP for the first time. KT1 originated from globally disseminated PVL^+ ST121/*agr*4 MSSA (with strong adhesin and cytolytic activities), with the unique KT1′ feature of PVL^-/*spa*t5110/SCC*mec*V^+. Pneumonia improved with levofloxacin administration, but with the subsequent development of fatal aspiration pneumonia. Hence, professional ST121/*agr*4 CA-MRSA, with high adhesin and cytolytic activities, triggered influenza-related sequential complications (CAP and aspiration pneumonia).

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.nmni.2016.05.011.

References

[1] Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 2007;298:1763–71.
[2] International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother 2009;53:4961–7.
[3] David MZ, Daum RS. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 2010;23:616–87.
[4] Harris SR, Feil EJ, Holden MT, Quail MA, Nickerson EK, Chantratita N, et al. Evolution of MRSA during hospital transmission and intercontinental spread. Science 2010;327:469–74.
[5] Yamamoto T, Hung WC, Takano T, Nishiyama A. Genetic nature and virulence of community-associated methicillin-resistant *Staphylococcus aureus*. BioMedicine 2013;3:2–18.
[6] Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol 2008;16:361–9.
[7] DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated methicillin-resistant *Staphylococcus aureus*. Lancet 2010;375:1557–68.
[8] Rasigade JP, Laurent F, Lina G, Meugnier H, Bes M, Vandenesch F, et al. Global distribution and evolution of Panton–Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus*, 1981–2007. J Infect Dis 2010;201:1589–97.
[9] Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 2007;13:1510–4.
[10] Thurlow LR, Joshi GS, Richardson AR. Virulence strategies of the dominant USA300 lineage of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA). FEMS Immunol Med Microbiol 2012;65:5–22.
[11] Zhang K, McClure JA, Elsayed S, Tan J, Conly JM. Coexistence of Panton–Valentine leukocidin-positive and -negative community-associated methicillin-resistant *Staphylococcus aureus* USA400 sibling strains in a large Canadian health-care region. J Infect Dis 2008;197:195–204.
[12] Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, et al. Community-acquired pneumonia requiring hospitalization among U.S. adults. N Engl J Med 2015;373:415–27.
[13] Fulop T, Le Page A, Fortin C, Witkowski JM, Dupuis G, Larbi A. Cellular signaling in the aging immune system. Curr Opin Immunol 2014;29:105–11.
[14] Rynda-Apple A, Robinson KM, Alcorn JF. Influenza and bacterial superinfection: illuminating the immunologic mechanisms of disease. Infect Immun 2015;83:3764–70.
[15] Hageman JC, Uyeki TM, Francis JS, Jernigan DB, Wheeler JG, Bridges CB, et al. Severe community-acquired pneumonia due to

Staphylococcus aureus, 2003–04 influenza season. Emerg Infect Dis 2006;12:894–9.

[16] Murray RJ, Robinson JO, White JN, Hughes F, Coombs GW, Pearson JC, et al. Community-acquired pneumonia due to pandemic A(H1N1)2009 influenza virus and methicillin resistant *Staphylococcus aureus* co-infection. PLoS One 2010;5:e8705.

[17] Morikawa K, Okada F, Ando Y, Ishii R, Matsushita S, Ono A, et al. Methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus* pneumonia: comparison of clinical and thin-section CT findings. Br J Radiol 2012;85:e168–75.

[18] Khokhlova OE, Hung WC, Wan TW, Iwao Y, Takano T, Higuchi W, et al. Healthcare- and community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) and fatal pneumonia with pediatric deaths in Krasnoyarsk, Siberian Russia: unique MRSA's multiple virulence factors, genome, and stepwise evolution. PLoS One 2015;10:e0128017.

[19] Collery MM, Smyth DS, Tumilty JJ, Twohig JM, Smyth CJ. Associations between enterotoxin gene cluster types *egc1, egc2* and *egc3, agr* types, enterotoxin and enterotoxin-like gene profiles, and molecular typing characteristics of human nasal carriage and animal isolates of *Staphylococcus aureus*. J Med Microbiol 2009;58:13–25.

[20] Rao Q, Shang W, Hu X, Rao X. *Staphylococcus aureus* ST121: a globally disseminated hypervirulent clone. J Med Microbiol 2015;64:1462–73.

[21] Sawanobori E, Hung WC, Takano T, Hachuda K, Horiuchi T, Higuchi W, et al. Emergence of Panton–Valentine leukocidin-positive ST59 methicillin-susceptible *Staphylococcus aureus* with high cytolytic peptide expression in association with community-acquired pediatric osteomyelitis complicated by pulmonary embolism. J Microbiol Immunol Infect 2015;48:565–73.

[22] de Bentzmann S, Tristan A, Etienne J, Brousse N, Vandenesch F, Lina G. *Staphylococcus aureus* isolates associated with necrotizing pneumonia bind to basement membrane type I and IV collagens and laminin. J Infect Dis 2004;190:1506–15.

[23] Ito T, Iijima M, Fukushima T, Nonoyama M, Ishii M, Baranovich T, et al. Pediatric pneumonia death caused by community-acquired methicillin-resistant *Staphylococcus aureus*. Japan. Emerg Infect Dis 2008;14:1312–4.

[24] Isobe H, Takano T, Nishiyama A, Hung WC, Kuniyuki S, Shibuya Y, et al. Evolution and virulence of Panton–Valentine leukocidin-positive ST30 methicillin-resistant *Staphylococcus aureus* in the past 30 years in Japan. Biomed Res 2012;33:97–109.

[25] Hoogendijk EO, Del Campo N, Rolland Y, Demougeot L, Gérard S, Vellas B. Adverse effects of pneumonia on physical functioning in nursing home residents: results from the INCUR study. Arch Gerontol Geriatr 2016;65:116–21.

[26] Chheng K, Tarquinio S, Wuthiekanun V, Sin L, Thaipadungpanit J, Amornchai P, et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* associated with pediatric infection in Cambodia. PLoS One 2009;4:e6630.

[27] Kikuta H, Shibata M, Nakata S, Yamanaka T, Sakata H, Akizawa K, et al. Predominant dissemination of PVL-negative CC89 MRSA with SCC*mec* type II in children with impetigo in Japan. Int J Pediatr 2011;2011:143872.