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

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) is a common multidrug-resistant (MDR) pathogen. We herein discussed MRSA and its infections in Krasnoyarsk, Siberian Russia between 2007 and 2011. The incidence of MRSA in 3,662 subjects was 22.0% and 2.9% for healthcare- and community-associated MRSA (HA- and CA-MRSA), respectively. The 15-day mortality rates for MRSA hospital- and community-acquired pneumonia (HAP and CAP) were 6.5% and 50%, respectively. MRSA CAP cases included pediatric deaths; of the MRSA pneumonia episodes available, 27.3% were associated with bacteremia. Most cases of HA-MRSA examined exhibited ST239/spa3(t037)/SCCmecIII.1.1.2 (designated as ST239Kras), while all CA-MRSA cases examined were ST8/spa1(008)/SCCmecIV.3.1.1 (IVc) (designated as ST8Kras). ST239Kras and ST8Kras strongly expressed cytolytic
peptide (phenol-soluble modulin α, PSMα; and δ-hemolysin, Hld) genes, similar to CA-MRSA. ST239Kras pneumonia may have been attributed to a unique set of multiple virulence factors (MVFs): toxic shock syndrome toxin-1 (TSST-1), elevated PSMα/Hld expression, α-hemolysin, the staphylococcal enterotoxin SEK/SEQ, the immune evasion factor SCIN/SAK, and collagen adhesin. Regarding ST8Kras, SEA was included in MVFs, some of which were common to ST239Kras. The ST239Kras (strain OC3) genome contained: a completely unique phage, φSa7-like (W), with no att repetition; S. aureus pathogenicity island SaPI2R, the first TSST-1 gene-positive (tst+) SaPI in the ST239 lineage; and a super copy of IS256 (>22 copies/genome). ST239Kras carried the Brazilian SCCmecIII.1.1.2 and United Kingdom-type tst. ST239Kras and ST8Kras were MDR, with the same levofloxacin resistance mutations; small, but transmissible chloramphenicol resistance plasmids spread widely enough to not be ignored. These results suggest that novel MDR and MVF+ HA- and CA-MRSA (ST239Kras and ST8Kras) emerged in Siberian Russia (Krasnoyarsk) associated with fatal pneumonia, and also with ST239Kras, a new (Siberian Russian) clade of the ST239 lineage, which was created through stepwise evolution during its potential transmission route of Brazil-Europe-Russia/Krasnoyarsk, thereby selective advantages from unique MVFs and the MDR.

**Introduction**

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a major multidrug-resistant (MDR) pathogen since the early 1960s [1], with recent threats, such as intensive care unit (ICU)-associated bacteremia in London [2], serious invasive infections in the United States (US) [3], and global antimicrobial resistance in common infections, being alerted by the World Health Organization (WHO) [4].

Traditional MRSA is now classified as healthcare-associated MRSA (HA-MRSA) [3], and another class of MRSA, which emerged in community settings between 1997 and 1999, as community-associated MRSA (CA-MRSA) [3,5,6]. HA- and CA-MRSA both carry staphylococcal cassette chromosome *mec* (SCCmec) [7,8], and each have several genetic backgrounds [9–14], which are generally identified based on multilocus sequence types (ST types), protein A gene (*spa*) types, and SCCmec types [6,8,12,14].

The most disseminated HA-MRSA worldwide includes the ST239 lineage, such as ST239/SCCmecIII [15] and ST239/SCCmecIII [2,16–20], as well as the ST5 lineage, such as ST5/SCCmecI carrying the toxic shock syndrome toxin-1 (TSST-1) gene (*tst*) [21,22]. The most characterized CA-MRSA includes the ST8 lineage, such as ST8/SCCmecIVa (USA300) [12,23], and also the lineages of ST30/SCCmecIV [6,24–26], ST59/SCCmecV [27–29], and ST80/SCCmecV [6,24,30]. Although CA-MRSA, unlike HA-MRSA, is generally less MDR [31], CA-MRSA also has the capacity to become MDR (representative, USA300) [32,33]. Some CA-MRSA are MDR from their emergence (representative, the Taiwan clone) [29].

HA-MRSA infections most frequently occur among inpatients [34,35], while CA-MRSA infections occur in healthy individuals. CA-MRSA mainly causes skin and soft tissue infections (STTIs), but also occasionally invasive infections [6,13,34,36]. The median ages of HA-MRSA and CA-MRSA patients are 68 and 23 years, respectively [34]. CA-MRSA often produces Panton-Valentine leukocidin (PVL) [6,12,13,37–39], and exhibits elevated expression of cytolytic peptides (phenol-soluble modulins, PSMs; or δ-hemolysin, Hld) [12,40].
MRSA evolution includes horizontal gene transfer, mediated by mobile genetic elements, plasmids, and phages, and also through mutations [8,37,41–44]. The mosaicism of the genome has also been reported; ST239 MRSA is a bacterial hybrid between clonal complex (CC) 30 (founder, ST30) and CC8 (founder, ST8) [19,45]. MRSA occasionally spreads by intercontinental transmission [9,20,46], and replacement often occurs [18,47,48]. The evolution of MRSA is still dynamic, and, therefore, may attack humans posing a new threat.

In Russia, dominant MRSA are ST239/SCCmecIII and PVL-negative (PVL−) ST8/SCCmecIV [49]. Although we previously reported PVL-positive (PVL+) ST30 CA-MRSA [50], tst ST239 HA-MRSA [51], and a whole genome structure [52], information on MRSA in Russia is still limited at the molecular level, especially in Siberian Russia, which is located between the European and Far Eastern regions. In the present study, we focused on episodes (and mortality rates) of MRSA hospital-acquired pneumonia (HAP) and community-acquired pneumonia (CAP) with pediatric deaths in Krasnoyarsk, Siberian Russia, as has been reported with initial fatal pediatric MRSA CAP episodes in the US North areas [5]. We discussed possible MRSA multiple virulence factors (MVFs), implicated in fatal cases of MRSA HAP and CAP. We then demonstrated their unique genomic structures and evolution of representative fatal-pneumonia-associated MRSA.

**Materials and Methods**

**Ethics statement**

The Ethics Review Boards of Krasnoyarsk State Medical University (Ethics Review Board No28/2010), Krasnoyarsk, Russia; Far Eastern Federal University School of Biomedicine, Vladivostok, Russia; National Taiwan University College of Medicine, Taipei, Taiwan; Niigata University School of Medicine, Niigata, Japan; and International Medical Education and Research Center, Niigata, Japan, specifically approved this study. Written informed consent was obtained from patients, if necessary.

**Patients and bacterial strains**

A total of 3,662 subjects were examined in Krasnoyarsk between 2007 and 2011. *S. aureus* specimens including MRSA were isolated in four hospitals in Krasnoyarsk, and all bacterial strains were isolated from different individuals. The data obtained are summarized in Table 1. The follow-up period used to determine the mortality for pneumonia was 15 days in this study; and 15-day mortality rates were compared between MRSA HAP and MRSA CAP cases. HA-MRSA was defined as MRSA isolated from inpatients 48 h after hospitalization while CA-MRSA was defined as MRSA isolated from outpatients who had no history of hospitalization within at least the past year and presented with no other established risk factors for MRSA infections [3]. Russian MRSA strains also included ten strains from inpatients (age, 1–41 years) with burn wound infections and respiratory tract infections in Vladivostok in 2012 and 2013; and nine strains from patients with burn and wound infections, osteomyelitis, respiratory tract infections, and blood stream infections in Moscow and Saint-Petersburg (European Russia) and in Kurgan (Ural Federal Region, Russia) in 2011 and 2012.

The following were used as reference or control strains. Strain RS08 (PVL+ST30/spa19-t019/SCCmecIVc) was isolated from a female badminton player in her twenties with furunculosis in Vladivostok in 2006 [50]. Of the ST239/spa351(t030)/SCCmecIII.1.1.4 strains, 16K was isolated from a 20-year-old male outpatient with urethritis in Vladivostok between 2006 and 2008 [52]. Another 11 strains were collected from Vladivostok [52]. Of the ST239/spa3(t037)/SCCmecIII.1.1.1 MRSA (tst+) strains, nine (including strain 6K) were from Vladivostok [52] and four (PM3, PM14, PM27, and PM38) were from Taiwan [28]. Of the PVL−
ST8/SCC mec IVa CA-MRSA (USA300) strains, USA300-0114 was a type strain kindly provided by L. K. McDougal and L. L. McDonald and NN36 [53] and NN47 [54] were Japanese isolates. Of the tst+ ST5/SCC mec II HA-MRSA (NY/J clone) strains, N315 and Mu50 were reference strains that were kindly provided by K. Hiramatsu, I6 was a Japanese isolate [55], and PM29 was a Taiwanese isolate [28]. The reference strains HU25 (ST239/SCC mec III.1.1.2-IIIA, Brazilian clone) and ANS46 (ST239/SCC mec III.1.1.1) were kindly provided by H. de Lencastre.

Genotyping and virulence gene analysis

ST typing, CC assignment, spa typing, agr typing, and SCC mec typing were performed as described previously [8,56]. Coagulase (Coa) typing was conducted using a staphylococcal coagulase antiserum kit (Denka Seiken, Tokyo, Japan). Virulence genes were analyzed by PCR [56]; the target genes in PCR included 49 genes: 3 leukocidin genes (lukPVSF, lukE-lukD, and lukM), 5 hemolysin genes (hla, hlb, hlg, hlg-v, and hld), a peptide cytolyisin (psma), 19 staphylococcal enterotoxin (SE) genes (tst, sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser, and set), 1 putative SE gene (sea), 3 exfoliative toxin genes (eta, etb, and etd), a staphylococcal superantigen-like gene cluster (sll), the epidermal cell differentiation inhibitor gene (edin), 14 adhesin genes (icaA, icaD, eno, fib, fnbA, fnbB, ebpS, clfA, clfB, sdrC, sdrD, sdrE, cna, and bbp), and the arginine catabolic mobile element (ACME)-arcA gene.

Table 1. Clinical and bacteriological information on MRSA isolated in Krasnoyarsk between 2007 and 2011.

| Study group | Diseases | Subjects | Bacterial isolation | Fatal cases |
|-------------|----------|----------|---------------------|-------------|
|             |          | Total number | Age (Y) | Isolation year | S. aureus (MRSA) | MRSA/total subjects | MRSA/S. aureus | /subjects | /S. aureus | /MRSA |
| Inpatients  | Pneumonia| 710 0–81 | 2007–2011<sup>b</sup> | 221 (62) | 8.7% | 28.1% | 0.6% | 0% | 6.5%<sup>c</sup> |
|             | SSTIs<sup>a</sup> | 874 20–84 | 2010–2011 | 210 (31) | 3.5% | 14.8% | 0.1% | 0% | 3.2% |
|             | Osteomyelitis | 208 21–80 | 2010–2011 | 77 (19) | 9.1% | 24.7% | 0% | 0% | 0% |
| Outpatients | Pneumonia | 310 0–67 | 2007–2008 | 93 (8) | 2.6% | 8.6% | 1.3% | 0% | 50%<sup>c</sup> |
|             | SSTIs<sup>a</sup> | 126 27–84 | 2010–2011 | 41 (2) | 1.6% | 4.9% | 0% | 0% | 0% |
|             | Colitis | 401 0–9 | 2011 | 357 (4) | 1.0% | 1.1% | 0% | 0% | 0% |
| Carriers    | Students in the community | 287 18–20 | 2010–2011 | 77 (1) | 0.3% | 1.3% | 0% | 0% | 0% |
|             | Athletes in the community | 108 11–28 | 2011 | 42 (0) | 0% | 0% | 0% | 0% | 0% |
|             | Hospital workers | 638 22–52 | 2008–2011 | 183 (2) | 0.3% | 1.1% | 0% | 0% | 0% |

<sup>a</sup>SSTIs, skin and soft tissue infections; in this study, SSTIs include dactylitis, paronychia, hidradenitis, wound infection, skin abscess, furuncle, carbuncle, bursitis, cellulitis, erysipelas-like necrotic cellulitis, and necrotizing fasciitis.

<sup>b</sup>Number of MRSA isolates in 2007–2009 was 42, and that in 2010–2011 was 20 (total 62).

<sup>c</sup>P<0.01

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TSST-1 and SEA assays

The amounts of TSST-1 and SEA in the supernatants of bacterial cultures at 2.0 X 10^9 CFU/ml were examined using a TSST-RPLA kit (Denka Seiken) and SET-RPLA kit (Denka Seiken), respectively, according to the instructions of the manufacturer.

Pulsed-field gel electrophoresis (PFGE) analysis

Bacterial DNA for PFGE was digested with SmaI and electrophoresed in 1.2% agarose with marker DNA (Lambda ladder; Bio-Rad Laboratories, Inc., Hercules, CA, USA), as described previously [52].

Plasmid analysis

The plasmid DNA of MRSA was prepared using a Plasmid Midi Kit (QIAGEN Sciences, Tokyo) or according to the method by Kado and Liu [57] with a modification to the lysostaphin treatment. Plasmid DNA was analyzed by agarose (0.6–1.0%) gel electrophoresis. The Tn554 circular intermediate was detected by PCR (PCR product size, 772 bp), as previously described [52].

Conjugative transfer

Donor strains were mated with S. aureus RN2677, a recipient strain, which is resistant to rifampicin (Rif^r) and novobiocin and carries no plasmids, on tryptic soy agar (Difco, Sparks, MD, USA), with or without membrane filters [52].

Susceptibility testing

Susceptibility testing of bacterial strains was performed using the agar dilution method with Mueller-Hinton agar [58]. Inducible clindamycin resistance (Clir^r) was tested, as above, by using agar plates containing erythromycin (Em) at 0.1 to 1 μg/ml.

Drug resistance gene analysis

The genes for drug resistance, antiseptic resistance, and heavy metal resistance were analyzed by PCR [28,29]. The genes (resistance phenotypes) analyzed were: meca (resistance to methicillin, oxacillin, and cephalosporins), blaZ (resistance to ampicillin), ermA and ermC (Em^r/Cir^r), cat (resistance to chloramphenicol, Cp^r), aacA-aphD (resistance to gentamicin, Gm^r, and kanamycin), aadD (resistance to neomycin), tet (resistance to tetracycline), spc (spectinomycin, Sp^r), ble (resistance to bleomycin), qacA (resistance to acriflavin/quatetnary ammonium, such as benzalkonium chloride and benzethonium chloride/chlorhexidine gluconate/ethidium bromide), cad (resistance to cadmium), and mer (resistance to mercury).

Genome analysis

The ST239 MRSA OC3 genome was analyzed by pyrosequencing using a genome sequencer FLX system with the assembler software GS De Novo Assembler version 2.6 (Roche Diagnostics, Branford, CT, USA). The GenBank accession numbers for the OC3 genome (144 contigs with ≥20 bp in size) are BBKC01000001-144. The OC3 contigs were mapped on the 3,043,210-bp complete genome (GenBank accession number FN433596) of TW20 (the most characterized ST239) using MUMmer software (http://mummer.sourceforge.net/). The gene or open reading frame (orf) was searched for using the software in silico MolecularCloning (version 4.2) (In Silico Biology, Yokohama, Japan).
Entire sequencing of mobile genetic elements, phages, and plasmids

The gaps between contigs were filled by PCR and sequencing. We also assembled contigs using an LA PCR in vitro cloning kit (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. In brief, after digestion with suitable restriction enzymes and ligation with the corresponding cassette adapters, amplification was performed with cassette primers and target-specific primers.

Phylogenetic and homology analyses

Multiple alignments were performed up to 1,000 times using default settings with ClustalW software (version 2.1) and a phylogenetic tree analysis was performed using TreeViewX software (version 0.5.0) (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). A homology analysis was performed using the software BLAST (http://blast.ddbj.nig.ac.jp/top-e.html) and FASTA (http://fasta.ddbj.nig.ac.jp/top-j.html).

mRNA expression assay

The mRNA expression levels of the cytolytic peptide (PSMα and Hld) genes (psma and hld) and 16S rRNA genes were examined by an RT-PCR assay [56,59]. psma and hld expression levels were normalized to 16S rRNA expression levels. The mRNA expression levels of the transcriptional regulator genes (sarA, sarR, mgrA, saeR, saeS, sarX, rot, and srrAB) were also examined.

Statistical analysis

Data were evaluated by Fisher’s exact test for MRSA incidence and by an analysis of variance with repeated measurements for the mRNA expression assay. The level of significance was defined as a P value of <0.05. Regarding 15-day mortality rate estimates, 95% confidence intervals (95% CIs) were included.

Results

MRSA infection in Krasnoyarsk, Siberian Russia

A total of 3,662 subjects were examined for S. aureus and MRSA infections, and the data obtained are summarized in Table 1. Regarding nosocomial infections (inpatients), MRSA infections accounted for 8.7% (62/710) of cases of pneumonia, 3.5% (31/874) of SSTIs, and 9.1% (19/208) of osteomyelitis; the overall incidence of MRSA (among S. aureus isolates) was 22.0% (112/508). Among the cases of MRSA HAP, 6.5% (4/62) were fatal (Tables 1 and 2); the ages of these four patients were 39, 46, 48, and 71 years old (average, 51 years old), and these deaths occurred in 2007 (three cases) and 2011 (one case). One fatal case of nosocomial SSTIs (3.2%, 1/31) was noted and may have been due to sepsis.

Among the cases of community-acquired infections (outpatients), MRSA infections accounted for 2.6% (8/310) of cases of pneumonia, 1.6% (2/126) of SSTIs, and 1.0% (4/401) of colitis; the overall incidence of MRSA (among S. aureus isolates) was 2.9% (14/491). The fatal case of MRSA CAP was 50% (4/8) (Tables 1 and 2); the ages of these four patients were 4 months old and 1, 40, and 41 years old (average, 20.5 years old), and these deaths occurred in 2007 (one case) and 2008 (three cases).

The incidence of MRSA (among S. aureus isolates) was significantly higher in hospitals (22%) than in the community (2.9%) (P < 0.01). However, the 15-day mortality rates for MRSA CAP and MRSA HAP were 50% (95% CI, 17.5%-82.6%) and 6.5% (95% CI, 2.1%-16.5%), respectively (P < 0.01).
Regarding healthy carriers (Table 1), MRSA+ cases were observed in 0.3% (2/638) of hospital workers in routine surveillance in hospitals, and in 0.3% (1/395) of students and athletes in occasional examinations in the community; the incidence of MRSA (among S. aureus isolates) was 1.1% (2/183) and 0.8% (1/119), respectively (P > 0.05).

Among MRSA in Table 1, thirty-one isolates were subjected to molecular characterization (Table 2). When pneumonia was targeted between 2007 and 2009, eight MRSA were characterized, all of which were from fatal or severe cases only; MRSA was not available from other non-fatal pneumonia cases. When pneumonia, osteomyelitis, SSTIs, and colitis were mainly targeted in 2010 and 2011, MRSA from fatal cases were initially selected and characterized:

Table 2. List of MRSA strains characterized at molecular levels in the present study.

| Isolation year | Name of MRSA | ST type (epidemiological classification of MRSA) | Patients |
|---------------|--------------|---------------------------------------------|----------|
| 2007          | OC3          | ST239 (HA)                                  | Pneumonia, sepsis (bacteremia) | Death | 46Y | M |
|               | OC76         | ST239 (HA)                                  | Pneumonia                                    | Death | 71Y | F |
|               | OC8          | ST8 (CA)                                    | Pneumonia                                    | Death | 1Y  | M |
|               | OC11         | ST8 (HA)                                    | Pneumonia, sepsis (bacteremia)               | Death | 39Y | M |
| 2008          | OC22         | ST8 (CA)                                    | Pneumonia                                    | Death | 41Y | M |
|               | OC23         | ST8 (CA)                                    | Pneumonia                                    | Death | 40Y | M |
|               | OC59         | ST8 (CA)                                    | Pneumonia                                    | Death | 4M  | F |
|               | OC52         | ST8 (HA/CA)                                 |                                          | b    | b   | 34Y | F |
| 2009          | OC180        | ST239 (HA)                                  | Pneumonia                                    | Recovery | 34Y | M |
| 2010          | OC22B        | ST239 (HA)                                  | Osteomyelitis                                | Recovery | 22Y | M |
|               | OC70         | ST239 (HA)                                  | Osteomyelitis                                | Recovery | 40Y | M |
|               | OC66         | ST239 (HA)                                  | Erysipelas-like necrotic cellulitis          | Recovery | 59Y | F |
|               | OC159        | ST239 (HA)                                  | Erysipelas-like necrotic cellulitis          | Recovery | 57Y | F |
|               | OC114        | ST39 (HA)                                   | Wound infection, cellulitis                  | Recovery | 58Y | M |
|               | OC145        | ST39 (HA)                                   | Wound infection                              | Recovery | 50Y | M |
|               | OC1          | ST8 (CA)                                    | Skin abscesses                               | Recovery | 50Y | F |
|               | OC217        | ST8 (CA)                                    |                                          | c    | c   | 19Y | F |
|               | OC50         | ST12 (HA)                                   | Surgical site infection, (sepsis)            | Death | 84Y | M |
| 2011          | OC8C         | ST239 (HA)                                  | Pneumonia                                    | Death | 48Y | M |
|               | OC14         | ST239 (HA)                                  | Pneumonia                                    | d    | d   | F |
|               | OC35         | ST239 (HA)                                  | Pneumonia, sepsis (bacteremia)              | Recovery | 27Y | M |
|               | OC98         | ST239 (HA)                                  | Osteomyelitis                                | d    | 31Y | M |
|               | OC99         | ST239 (HA)                                  | Osteomyelitis                                | Recovery | 30Y | M |
|               | OC111        | ST239 (HA)                                  | Osteomyelitis                                | Recovery | 76Y | F |
|               | OC1A         | ST39 (HA)                                   | Burn infection                               | Recovery | 37Y | M |
|               | OC2          | ST39 (HA)                                   | Burn infection                               | Recovery | 53Y | M |
|               | OC5          | ST39 (HA)                                   | Wound infection                              | Recovery | 31Y | M |
|               | OC44         | ST39 (HA)                                   | Peritonitis                                  | Recovery | 30Y | M |
|               | OC14C        | ST39 (HA/CA)                                |                                          | b    | b   | 43Y | F |
|               | OC160        | ST8 (CA)                                    | Wound infection, cellulitis                  | Recovery | 53Y | M |
|               | OC1C         | ST8 (CA)                                    | Colitis                                      | Recovery | 3Y  | M |

aY, years; M, male; F, female; HA, healthcare-associated MRSA; CA, community-associated MRSA.
bHealthy carrier (hospital worker)
cHealthy carrier (student)
dInformation not available.

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two from MRSA HAP and nosocomial SSTI/sepsis. Eighteen isolates were randomly selected from non-fatal cases: two out of 19 pneumonia isolates, five out of 19 osteomyelitis isolates, nine out of 32 MRSA SSTI isolates, one out of four colitis isolates, and one isolate from peritonitis. All three MRSA isolates from healthy carriers were included in the molecular analysis.

In Table 2, at least three out of the 11 MRSA pneumonia episodes (pneumonia/sepsis) were associated with bacteremia with the incidence being ≥27.3%; at least two out of seven HAP episodes (>2/7) and one out of four CAP episodes (>1/4), or two out of six ST239 MRSA cases (>2/6) and one out of five ST8 MRSA cases (>1/5) were associated with bacteremia. Although two ST239 and ST8 HAP-related bacteremia cases in 2007 were fatal, one ST239 HAP-related bacteremia case in 2011 was not.

**Molecular characteristics of MRSA from Krasnoyarsk**

The molecular data of the 31 MRSA isolates are summarized in Table 3 and Fig 1. MRSA strains were classified into three groups, A to C (Table 3).

- **Group A (n = 20)** was the ST239 lineage with two types, ST239/ spa3(t037)/SCCmecIII.1.1.2 (group A1; 90%, 18/20) and ST239/ spa3(t037)/SCCmecIII.1.1.1 (group A2; 10%, 2/20). All strains in group A1 were *tst*+; TSST-1 production levels were similar to those (50–800 ng/ml; average, 313 ng/ml) of the ST5/SCCmecII NY/J clone. They were also highly MDR, including levofloxacin (Lvx) and Rif, and many (77.8%, 14/18) carried a Cp plasmid (pCp). In the PFGE analysis (Fig 1), group A1 comprised several divergent subclusters with a major cluster (type 239α; 44.4%, 8/18). Group A1 MRSA was designated as ST239 Kras. Three ST239 Kras strains (OC3, OC8C, and OC76) caused fatal HAP, and one strain (OC14C) was from a carrier (hospital worker).

- **The strains in group A2 were sea+** (Table 3). Their PFGE patterns were divergent from group A1 (Fig 1). Group A2 was a common ST239 HA-MRSA in Russia, whereas group A2 showed a slightly narrower MDR spectrum (Table 3). Group A (A1 and A2) exhibited a high level of resistance to imipenem and oxacillin (Table 3), which is consistent with the characteristics of HA-MRSA [31].

- **Group B (n = 10)** was the ST8 lineage with the type *spa*1(t008)/SCCmecIV.3.1.1(IVc) (Table 3). Most strains (80%, 8/10) were sea+, and produced SEA at high levels (Table 3). All strains shared very similar PFGE patterns, with patterns of 8α (major type; n = 7) and 8β (n = 2) and with no more than a three-band difference, indicating the same clone (designated as ST8 Kras) (Fig 1). ST8 Kras (group B) exhibited a low level of resistance to imipenem and oxacillin (Table 3), in agreement with the characteristics of CA-MRSA [31]; however, all strains were Lvxr and most strains (90%, 9/10) carried a Cp plasmid.

- **Four ST8 Kras strains** (OC8, OC22, OC23, and OC59) caused fatal CAP. One ST8 Kras strain (OC11) caused fatal HAP, suggesting that ST8 Kras even spread in hospitals. The ST8 Kras strains associated with fatal pneumonia were all sea+. Two ST8 Kras strains were isolated from carriers; OC217 was from a student while OC52 was from a hospital worker.

- **Group C (n = 1, strain OC50)** exhibited ST12/ spaNew(t156)/untypable SCCmec, showed low imipenem and oxacillin resistance levels, and carried a Cp plasmid.

Regarding drug resistance (Table 3), all Lvx+ ST8 and ST239 carried *gyrA* (Ser84Leu) and grlA (Ser80Phe) mutations, and manifested minimum inhibitory concentrations (MICs) of 4–16 μg/ml. Rif+ ST239 Kras strains carried *rpoB* (His481Asn, Ile527Met) mutations, with MICs of ≥256 μg/ml. All Cp+ strains carried the *cat* gene, with MICs of 64 μg/ml. All strains were susceptible to trimethoprim, fusidic acid, vancomycin, teicoplanin, linezolid, and mupirocin.
Table 3. Molecular characterization of MRSA strains isolated in Krasnoyarsk.

| Types, gene, or resistance | Group A A1 (n = 18) | Group B (n = 10) | Group C (n = 1) |
|----------------------------|---------------------|------------------|----------------|
| Types                      |                     |                  |                |
| CC                         | 8                   | 8                | 12             |
| CC11                       | 239                 | 239              | 8              |
| spa                        | 3 (t037)            | 3 (t037)         | 1 (t008)       |
| agr                        | 1                   | 1                | 1              |
| SCCmec                     | III.1.1.2           | III.1.1.1        | IV.3.1.1 (IVc) |
| Coagulase                  | IV                  | IV               | III            |
| Toxins                     |                     |                  |                |
| Leukocidins                |                     |                  |                |
| lukP, SF                   | -                   | -                | -              |
| lukE-lukD                  | +                   | +                | +              |
| lukM                       | -                   | -                | -              |
| Hemolysins                 |                     |                  |                |
| hla, hlg, hlg-v            | +                   | +                | +              |
| hlb (split)                | (+)                 | (+)              | (+)            |
| Peptide cytolysins         |                     |                  |                |
| psmα, hld                  | +                   | +                | +              |
| Staphylococcus enterotoxins|                     |                  |                |
| sea                        | -                   | +                | + (8/10)       |
| tst                        | +                   | -                | -              |
| sec, sep                   | -                   | -                | +              |
| sek, seq                   | +                   | +                | -              |
| Exfoliative toxins         |                     |                  |                |
| eta, etb, etd              | -                   | -                | -              |
| Others                     |                     |                  |                |
| ssl                        | +                   | +                | +              |
| edin                       | -                   | -                | -              |
| Adhesins                   |                     |                  |                |
| c12agα                     | +                   | +                | +              |
| cna                        | +                   | +                | -              |
| bbp                        | -                   | -                | -              |
| ACME (arcA)                | -                   | -                | -              |
| Resistance                 |                     |                  |                |
| β-lactam                   |                     |                  |                |
| Imipenem (MIC, μg/ml)      | 16–64               | 32               | 0.125–0.5      |
| Oxacillin (MIC, μg/ml)     | 128–256             | 128              | 32–64          |
| Ampicillin (MIC, μg/ml)    | 32–64 (18/18)       | 32 (2/2)         | 4–8 (7/10)     |
| Non β-lactam               |                     |                  |                |
| Aminoglycosides            | Gm (17/18)          | Gm (3/10)        |
| Tetracyclines              | Tc (18/18)          | Tc (2/2)         |
| Macrolides                 | Em (18/18)          | Em (2/2)         | Em (2/10)      |
| Lincosamides               | Cli (18/18)         | Cli (2/2)        | Cli (2/10)     |

(Continued)
Distribution and transfer of drug resistance plasmids

The plasmid data of Krasnoyarsk MRSA are summarized in Table 3, Fig 1, S1 and S2 Figs. Many MRSA strains carried only a small pCpr, and the same 2.9-kb pCpr was present in 14 (77.8%) out of 18 ST239Kras strains (group A1) and in seven (70%) out of 10 ST8Kras (group B) strains (Fig 1). This 2.9-kb pCpr was 99.9% homologous to the 2.9-kb pCpr of emerging ST239 MRSA (spa351[t030]/SCCmecIII.1.1.4) from Vladivostok (S1 and S2A-a Figs). One ST8Kras strain carried a 3.9-kb pCpr (Fig 1, S1 and S2A-b Figs). Furthermore, three ST8Kras strains carried the new mosaic 4.5-kb pCpr (Fig 1, S1 and S2A-c Figs); two of these strains carried two distinct species (2.9- and 4.5-kb) of pCpr (Fig 1 and S1 Fig).

Three ST8Kras strains carried large (>25-kb) antibiotic and antiseptic resistance plasmids (alternatively defined as a penicillinase plasmid, pPCase), in addition to pCpr (Fig 1, S1 and S2A-b Figs). Furthermore, three ST8Kras strains carried the new mosaic 4.5-kb pCpr (Fig 1, S1 and S2A-c Figs); two of these strains carried two distinct species (2.9- and 4.5-kb) of pCpr (Fig 1 and S1 Fig).

All plasmids were transferred to S. aureus RN2677 (recipient) in the bacterial mixed culture at frequencies ranging from 10^{-5} to 10^{-7} (S1D Fig). Tn554, carrying the ermA, spc genes, of ST239Kras strains was also transferred to RN2677 (S1D Fig), most likely through a Tn554 circular intermediate (S1A and S1C Fig). Of these, the 2.9-kb pCpr exhibited superior transfer frequencies over transmissible (Tra+) pPCase (S1D Fig).

Comparative genomics of ST239Kras (strain OC3)

The OC3 genome was estimated to be at least 2.93-Mb in size, with a 2,908-bp pCpr (pOC3) A total of 2.91-Mb (approximately 99.3% of the determined genome sequences) was mapped on the TW20 genome (Fig 2).

The OC3 genome most likely consisted of two (ST30-like and ST8-like) sections, similar to TW20[19,60]. On the ST30-like section, OC3 carried the collagen-adhesin (Cna) gene (cna), IEC6013 (with the Tn552 insertion), and the spa gene (type3-t037) similar to TW20; however,
OC3 lacked phage φSa1, and SCCmec was divergent (SCCmecIII.1.1.2 for OC3 vs. SCCmecIII.1.1.1 for TW20).

On the ST8-like section, there were five characteristic insertions in the OC3 genome: i) a tst+ S. aureus pathogenicity island (SaPI2R), ii) a completely unique phage (designated as φSa7-like W), iii) a phage, φSa5, iv) a transposon, Tn4001, and v) a SaPI carrying the ferri-chrome ABC transporter homologue gene (fhuD) (designated as SaPIfhuD). There were also two characteristic deletions in the OC3 genome: i) a phage, φSPβ-like (carrying the sasX gene and Tn4001) and ii) the dfrG gene in Tn5801-like, resulting in a trimethoprim-susceptible phenotype.

Fig 1. Pulsed-field gel electrophoresis (PFGE) analysis (right and left) and plasmid carriage patterns (center) of MRSA strains isolated in Krasnoyarsk. The MRSA strains shown are those described in Table 2. Group A (A1 and A2) and group B are described in Table 3. The color of the strain name indicates fatal pneumonia (red), possible sepsis (brown), and carrier cases (green). Of the cases of fatal pneumonia, OC3, OC8C, OC11, OC76 were from hospital-acquired pneumonia (HAP), while OC8, OC22, OC23, and OC59 were from community-acquired pneumonia (CAP). Of the carrier cases, OC14C and OC52 were from hospital workers and OC217 was from a student.

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Fig 2. Genome information for the ST239Kras strain OC3, in comparison with the ST239 MRSA strain TW20. The ST239Kras OC3 genome contigs (including filled contigs and complete structures; total 2.91-Mb) were mapped on the 3,043,210-bp TW20 genome (GenBank accession number FN433596); in the figure, the two genome structures were drawn as two circles on a common genome map, outside OC3 and inside TW20. Genome information included staphylococcal cassette chromosome mec (SCCmec), other drug resistance structures (such as a transposon, Tn, plasmid-related structure, and gene mutations), characteristic virulence genes, phages, S. aureus pathogenicity islands (SaPIs), genomic islands (νSa), and characteristic insertion sequences (ISs). SCCmec: SCCmecIIIa (in OC3), SCCmecII.1.2; SCCmecII (in TW20), SCCmecII.1.1 connected to SCCHg. Drug resistance (gene mutations): Lvx, levofloxacin resistance; Rif, rifampicin resistance; Su, sulfamethoxazole resistance. Virulence genes (region): tst, toxic shock syndrome toxin-1 gene; hld, δ-hemolysin gene; cna, collagen adhesin gene; spa, protein A gene; psmα, phenol-soluble modulin (PSM) gene; hla, α-hemolysin (α-toxin) gene; IEC, immune evasion cluster. The CC30 and CC8 genome sections are from Holden et al. [19], and the genetic element IEC6013 is from [60]. The plasmid pOC3 (2,908 bp; contig 75) of strain OC3 is not shown in the figure. The location of pSK41-related structure (with two IS431 repeats at both ends) currently remains uncertain.

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A large number of copies of the insertion sequence IS256 (>22 genome) were present in OC3, while TW20 only had eight copies. Regarding the MDR of OC3, the 15 genes identified were: i) nine drug resistance genes: mecA on SCCmecIII.1.1.2, ermA and spc on Tn554, blaZ on Tn552/ICE6013, aac(3)-aphD on Tn4001, tetM on Tn5801-like, cat on pOC3, and ble and aadD on a pSK41-related structure; ii) four drug resistance mutations: gyrA (S84L) and grlA (S80F) for Lvxr [61], rpoB (H481N, I527M) for Rifr [62], and folP (F17L, V30I, T31N, M37I, I58V, T59S, V60L, L64M, I101M, V117I, V126I; these replacements caused MIC of ≥512 μg/ml and corresponded to 11 out of 13 replacements in strain V21571 [63]) for sulfamethoxazole resistance; and iii) two heavy metal resistance genes: mer and cadA on SCCmecIII.1.1.2.

SCCmecIII.1.1.2

SCCmecIII.1.1.2 (OC3) was 61,780 bp in size, with 15-bp att direct-repeat sequences (attL, attR). Its structure was identical to that of reference strain HU25 of the Brazilian clone, with ancestral SCCmecIII, however, markedly distinct from TW20, which had the two-SCC cassette array SCCHg-SCCmecIII.1.1.1 (35,310 bp), as shown in Fig 3A.

SCCmecIII.1.1.1 (TW20) had four copies of IS431 (① to ④) in direct orientation at the boundary region of two SCCs, suggesting a recombination between two IS431 copies (② and ④) for SCCmecIII.1.1.2 conversion; the J3 region of SCCmecIII.1.1.2 (OC3) and SCCmecIII.1.1.1 (TW20) was, thus, divergent: 33,011 bp vs. 6,266 bp.

To confirm that all ST239Kras strains had the same IS431 recombination type, the PCR primer sets, Hgreg (F)/attM (R) (to detect attM) and Hgreg (F)/rec2/4 (R) (to identify recombination between IS431 copies ② and ④), were designed (Fig 3B). The results of the PCR assays clearly demonstrated that all ST239Kras strains had the SCCmecIII.1.1.2 (IIIA) structure of the Brazilian clone. In Russia (Krasnoyarsk and Vladivostok), SCCmecIII.1.1.4 and SCCmecIII.1.1.1 had no SCCHg linkage, as shown in Fig 3A.

ϕSa7-like (W)

ϕSa7-like (W) of OC3 was 42,359 bp in size and inserted into the huNaDC-1 gene. As shown in Fig 4, the integrase gene showed high similarity (mostly 100%) to the phage 7 (ϕSa7) integrase gene of the following strains: NM2 of the strain Newman (Fig 4), ST8 strains (GenBank accession numbers, AP009351.1 and CP007499.1), ST30 strain (GenBank accession number, LN626917.1), ST133 strain (GenBank accession number, CP001996.1), and ST239 strains (GenBank accession numbers, CP005288.1, CP006838.1, CP009681.1), indicating that ϕSa7-like (W) is a ϕSa7 family member. ϕSa7-like (W) had the ϕSa7 att-like 9-bp sequence on the left-side end.

However, the 9-bp right-side sequence of ϕSa7-like (W) was divergent; there was no ϕSa7 att on the right side (Fig 4). Moreover, the insertion site (huNaDC-1 gene) of ϕSa7-like (W) was divergent from that of ϕSa7, which was generally inserted into the intercistronic region between the isdB and rpmF genes (Fig 4, the figure on the lower right side). ϕSa7-like (W) only showed 66% overall homology to ϕSa7 (NM2).

SaPI2R carrying tst

SaPI2R (OC3) was 14,819 bp in size, flanked by directly repeated 20-bp att sequences (attL and attR), and inserted into the groEL gene (Fig 5A). SaPI2R exhibited high homology (91%) to the tst SaPI2 of strain ATCC25923, and the 6,810-bp left-side tst region of SaPI2R showed high homology (99%) to that of tst SaPI2 (strain RN3984) (Fig 5B). Although SaPI2R was closely related to tst SaPI2 (ATCC25923), SaPI2R markedly diverged from other tst SaPIs (Fig 5C).
In the \textit{tst} gene sequence comparison (Fig 5D-a), seven clusters were detected: i) cluster consisting of \textit{tst} from Russia (OC3), UK, USA, South Korea, and Argentine; ii) \textit{tst} cluster from USA (RN3984); iii) \textit{tst} cluster from USA (pRN6101); iv) cluster consisting of \textit{tst} from USA (including pRN6100), Japan (ST5/SCC\textit{mec}II HA-MRSA, NY/J clone; and ST8/SCC\textit{mec}IV).
CA-MRSA, ST8 CA-MRSA/J clone), and Kenya (camel strain); and v-vii) three \textit{tst} clusters from Ireland, Switzerland, and France (cow/bovine and ovine strains). The analysis at the deduced amino acid sequence levels produced very similar results (Fig 5D-b).

Russian (OC3) and UK TSST-1 precursors shared the same amino acid sequence with the same one amino acid replacement in the signal peptide region (S-11L; S\textsuperscript{!}L at position -11), when compared with purified TSST-1 protein (MN8) or the precursor protein, deduced from the first USA \textit{tst} gene (pRN6100) (Fig 5E). Regarding the \textit{tst} genes from clinical isolates, amino acid replacement (S-11L; S\textsuperscript{!}L at position -11), when compared with purified TSST-1 protein (MN8) or the precursor protein, deduced from the first USA \textit{tst} gene (pRN6100) (Fig 5E). Regarding the \textit{tst} genes from clinical isolates, amino
acid replacements in the mature toxin (TSST-1) region were very rare, in contrast to the \textit{tst} genes from animal isolates with distinct host specificity (Fig 5E). We were unable to determine why the \textit{tst} gene from camel (Kenya) had the clinical type sequence.

SaPI1 carrying \textit{sek} and \textit{seq}

SaPI1 (OC3) was 14,577 bp in size (with 17-bp \textit{att} at both ends), carried the superantigen (SE) genes (\textit{sek} and \textit{seq}), and was inserted into a non-coding region. SaPI1 (OC3) was highly homologous (99.9\%) to SaPI1 (ATCC25923). The SEK and SEQ amino acid sequences were the same between OC3 and TW20, with the unique amino acid replacement F119L and two unique amino acid replacements D194N and T201A (which corresponded to two out of seven replacements in USA300 SaPI5) (S3B-b Fig), respectively.

**Fig 5.** Analysis of the \textit{tst}+ SaPI (SaPI2R) structure and \textit{tst} nucleotide and deduced amino acid sequences of the ST239Kras strain OC3. In A, the integration site (\textit{att}) and \textit{att} sequences of SaPI2R of the ST239Kras strain OC3 are shown. In B, the SaPI2R structure was compared with those of \textit{tst}+ SaPI (ATCC25923) and \textit{tst}− SaPI2 (RN3984). Homologous regions between SaPI structures are shaded with color. Genes: \textit{tst}, toxic shock syndrome toxin-1 gene; \textit{eta}, \textit{S. hyicus} exfoliatin A gene; \textit{ter}, terminase gene (which cleaves multimeric DNA); \textit{rep}, replication initiator gene; \textit{int}, the integrase gene. In C, the nucleotide sequences of \textit{tst}+ SaPIs and \textit{tst}− SaPI (ATCC25923) were analyzed for phylogenetic diversity. In D-a, the nucleotide sequences of the \textit{tst} genes were analyzed for phylogenetic diversity. In this figure, each GenBank record year is also shown. In D-b, the deduced amino acid sequences of the \textit{tst} gene products were analyzed for phylogenetic diversity. The origin (reported source) of each isolate is indicated by the color of the isolate name: red, Russia; yellow, United Kingdom (UK); blue, United States (USA); dark red, Korea; light blue, Argentina; purple, Japan; green, those for animal isolates. In C and D, the scale bars represent substitutions per single-nucleotide polymorphism site. In E, the representative \textit{tst} gene sequences were compared with the reference sequences (of pRN6100). Arrows indicate the positions of the nucleotide and amino acid changes for the representative \textit{tst} genes. At the bottom of the figure (green), different amino acids from the amino acid sequences of purified TSST-1 (MN8; GenBank accession number EFH95768) and the deduced amino acid sequence of the \textit{tst} gene (pRN6100) are indicated in red letters.
ϕSa3 carrying immune evasion genes

ϕSa3 (OC3) was 43,681 bp in size (with 13-bp att at both ends) and was inserted into the hlb gene. ϕSa3 (OC3) showed 76% homology to that of strain CN1, sharing the same att and same integration site (S4 Fig). ϕSa3 (OC3) had the immune evasion cluster (IEC) on the left-end side, with the immune evasion genes sak (for staphylokinase, SAK) and scn (for staphylococcal complement inhibitor, SCIN), but lacked chp (for chemotaxis inhibitory protein of S. aureus, CHIPS) present in CN1 (S4 Fig). The IEC region of TW20 and ST8Kras strain OC8 showed 99.3% homology and carried sea, in addition to sak, and scn (S4 Fig).

Other relevant genetic structures

Tn4001 (OC3), flanked by two IS256, was 6,483 bp in size and inserted into the noncoding region, located downstream of the ThiJ/PfpI family protein gene (Fig 2); in TW20, Tn4001 was present within ϕSPβ-like. The pSK41-related resistance structure, flanked by two IS431, was 4,039 bp in size and carried the two drug resistance genes ble and aadD (Fig 2); its location on the genome currently remains unknown.

SaPI (fhuD) was 15,756 bp in size and inserted into the noncoding region, located downstream of the SsrA-binding protein gene. It showed only 69% overall homology to fhuD’ SaPlm4 from the NY/J clone (Mu50). The 10.2-kb left-side half exhibited high homology (95%) to fhuD’ SaPlj50 from Japanese ST8/SCCmecIV CA-MRSA, suggesting that SaPI (fhuD) is a new mosaic SaPI (S5 Fig).

ϕSa5 (OC3), with 10-bp att at both ends, was 44,424 bp in size and inserted into the hypothetical gene (for protein AGY89988.1), located downstream of the Thi/Pfp family protein gene. It was the most similar to ϕSa5 (XN108), albeit with only 63% homology (S6 Fig); ϕSa5 (OC3) is a new mosaic phage.

Elevated mRNA expression of cytolytic peptide genes in ST239Kras

ST239Kras, including strain OC3, expressed the psmα and hld genes at high levels, similar to CA-MRSA USA300 and Russian CA-MRSA (RS08 and ST8Kras), but significantly higher than HA-MRSA, ST5/SCCmecII (the NY/J clone) and other ST239/SCCmecIII, including reference strains HU25 and ANS46 (P <0.05), as shown in Fig 6A.

Regarding the expression levels of transcriptional regulatory genes (Fig 6B), although no significant difference was observed among the ST239 strains for sarA, ST239Kras, including OC3, showed higher levels of expression for sarR, mgrA, saeR, saeS, sarX, rot, and srrAB (P <0.05), compared with the other ST239 strains, including reference strains HU25 and ANS46.

Comparison of ST239Kras and ST239 MRSA from other regions of Russia

The emerging HA-MRSA with the genotype ST239/spa351(t030)/SCCmecIII.1.1.4 was detected in the European region (Moscow and St. Petersburg), Ural region (Kurgan), and Far Eastern region (Vladivostok) (S1 Table), except for the Siberian region (Krasnoyarsk) (Table 3). Its spa variant, spaNew(t632), was distributed to the European region (St. Petersburg). ST239Kras was only distributed to the Siberian region (Krasnoyarsk).

Three major divergent clusters were detected in the PFGE analysis (S7 Fig): a large spa351/SCCmecIII.1.1.4 cluster, associated with the Far Eastern region; a large spa351-New(t632)/SCCmecIII.1.1.4 cluster, mainly associated with the European Russia/Ural mountain region (and also the Far Eastern region); and a large spa3/SCCmecIII.1.1.1-III.1.1.2 cluster, associated
Fig 6. mRNA expression levels of cytolytic peptide genes (psma and hld) in ST239_{Kras} and ST8_{Kras} strains (A) and of regulatory genes in ST239_{Kras} strains (B), in comparison with CA- and HA-MRSA reference strains and other ST239 MRSA strains. In A, in the right-side control MRSA box, CA-MRSA, which shows high expression levels, is marked in red; and HA-MRSA, which shows low expression levels, is marked in light blue. In the left-side MRSA box, CA-MRSA (strain RS08), which showed high expression levels as expected, is marked in red; CA-MRSA ST8_{Kras} (ST8/SCC{mec}IVc) strains, including OC8, also showed high expression levels (dark blue box on the right side). ST239 HA-MRSA strains, which showed low expression levels as expected, are marked in light blue. However, ST239_{Kras} (ST239/SCC{mec}III/spa3/tst+), including OC3, unexpectedly showed high expression levels, similar to CA-MRSA; this box is marked in dark blue on the left side. Regarding psma: *1, P < 0.05 vs. control ST239/SCC{mec}II and ST5/SCC{mec}II; *2, P < 0.05 vs. control ST239/SCC{mec}II and ST5/SCC{mec}II; *3, P < 0.05 vs. control ST239/SCC{mec}II and ST5/SCC{mec}II; *5, P < 0.05 vs. control ST239/SCC{mec}III and ST5/SCC{mec}II; *6, P < 0.05 vs. Russian ST239/SCC{mec}III and spa351. Regarding hld: *2, P < 0.05 vs. control ST239/SCC{mec}II and ST5/SCC{mec}II; *4, P < 0.05 vs. control ST239/SCC{mec}II and ST5/SCC{mec}II; *7, P < 0.05 vs. control ST239/SCC{mec}III and ST5/SCC{mec}II; *8, P < 0.05 vs. Russian ST239/SCC{mec}III/spa3 and spa351. In B, the psma expression levels of ST239 MRSA strains were examined. spa3/tst+; ST239_{Kras}. No significant difference was observed between ST239_{Kras} and other ST239 MRSA for sarA gene expression. *1, P < 0.05 vs. spa351; *2, P < 0.05 vs. spa3; *3, P < 0.05 vs. spa351; *4, P < 0.05 vs. spa3; *5, P < 0.05 vs. spa351; *6, P < 0.05 vs. spa3.

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with the Siberian region and Far Eastern region. The emerging ST239/spa351(t030)-SPA\new (t632)/SCCmecIII.1.1.4 type appeared to be slightly divergent between the European/Ural region and Far Eastern region. ST239Kras was divergent from those emerging types, and comprised spa3 subclusters with reference strains ASN46 and HU25.

Discussion

Regarding MRSA epidemiology in Russia, the incidence of MRSA (among S. aureus isolates) was 0–89.5% (average, 33.5%) in 2000 [64], 18% in 2004 [65], 54.4% in 2006–2008 [66], 32.1% in 2007 and 16.6% in 2012 [67]. The dominant MRSA types in Russia are ST239/spa3(t037)/SCCmecIII, followed by ST239/spa351(t030)/SCCmecIII and ST8/spa1 (t008)/SCCmecIV [49]. Russia is geographically classified into three major regions: European, Siberian, and Far Eastern. The present study described for the first time MRSA and its invasive infection in the Siberian region. In Krasnoyarsk (2007–2011), the incidence of HA-MRSA was at similar levels, while the incidence of CA-MRSA was a little bit lower.

However, regarding MRSA types, the most prevalent HA-MRSA was ST239Kras, a novel regional variant of the ST239 lineage (S2 Table, S8 Fig), in contrast to the European (Moscow and St. Petersburg), Ural (Kurgan), and Far Eastern (Vladivostok) regions, where the emerging ST239/spa351(t030)/SCCmecIII.1.1.4 type has recently become common [52]. ST239Kras is highly-virulent HA-MRSA with fatal HAP cases (with bacteremia) being reported. The ages of patients with fatal HAP were consistent with previous HA-MRSA data [34]. The genetic divergence in PFGE patterns strongly suggested that ST239Kras infections persisted and spread among patients and carriers (hospital workers) at least since 2007.

Regarding the ST8 lineage, a single unique MDR MRSA clone (ST8Kras) with similar PFGE patterns had persisted and spread. The spa type (spa1-t008) was the same as previous Russian ST8 [49], but divergent from that (spa826; t, unknown) of Vladivostok ST8 [52]. ST8Kras was LvxR with the same mutations as HA-MRSA ST239Kras. ST8Kras was a successful CA-MRSA, with not only fatal CAP, but also fatal HAP cases (with bacteremia). The fatal CAP cases included one infant and one young child death, consistent with previous CA-MRSA infections [34]. ST8Kras carriers were also identified, suggesting its potential to be become widespread.

PVL+ CA-MRSA, such as ST8 USA300 [12,23,68], has become a major public concern [3,5,12, 23,24,34,68,69]; however, MRSA invasive infections occurred regardless of PVL+ or PVL− [70–72]. In Russia, MRSA has mostly been PVL [49,65,67], with only two PVL+ cases [50,73]. In Krasnoyarsk, all MRSA were PVL-, albeit with PVL+ methicillin-susceptible S. aureus cases (at around 10%) associated with pyogenic skin infections (such as furuncles).

In the present study, we also focused onto the MVFs of MRSA. The hyper virulence of CA-MRSA USA300 has been attributed to MVFs, such as PVL, ACME-related factors, α-hemolysin (Hla), the elevated production of PSMs, and SEK (sek2) and SEQ (seq2) [37,68].

Regarding ST239Kras, a unique set of MVFs included TSST-1, the elevated expression of PSMα/Hld, Hla, SEK/SEQ, SCIN/SAK, and Cna. Of those factors, TSST-1 has been associated with toxic shock syndrome (TSS) [74–76] and neonatal TSS-like exanthematous disease [77] through a cytokine storm [78–82], is associated with invasive endocarditis [83], and is an immune evasion factor [84]. In Japan, major HA-MRSA and CA-MRSA are both tst+, and associated with invasive infections, including pneumonia and bacteremia [21,71,85].

ST239Kras strongly expressed PSMα (and Hld), a common characteristic of CA-MRSA [12,40], which is cytoplast against human cells [40] and possibly associated with bacteremia and abscesses [12] as well as the establishment of an MRSA niche [86]. Community infection (including necrotizing pneumonia) or colonization from HA-MRSA that strongly expresses PSMα/Hld includes ST5/SCCmecII (NY/l) [72] and ST764/SCCmecII cases [56].
In ST239Kras, some transcriptional regulatory genes, except for sarA [87,88], were also up-regulated. They included transcriptional-positive regulators such as mgrA [89], saeRS [89], and sarX [90]; and transcriptional-negative regulators such as sarR [87,88], rot [43], and srrAB [91]. Super IS256 copies in ST239Kras may be responsible for these transcriptional regulations, as has been reported with S. epidermidis [92] or rot [43]. IS256 also contributes to Tn4001 [93].

The acquisition of sek and seq with synonymous substitutions (sek2, seq2) may partly explain the hyper virulence of USA300 [37]. ST239Kras (OC3) shared the same (unique) SEK and SEQ sequences with TW20, which were distinct from those of USA300.

Immune evasion factor genes are generally clustered in the IEC locus in φSa7 [44,94], but are often detected in φSa7 [94] or vSaβ [29]. ST239Kras carried two those genes, sak and scn, in IEC, while USA300 carried three genes sak, ctp, and scn (GenBank accession no. CP000255). TW20 and ST8Kras (OC8) carried a distinct set of three genes, sak, scn, and sea.

Cna is a cell wall-associated adhesin [95,96], and associated with pneumonia [97] and bullous impetigo [98]. Cna is also a potential immune evasion factor [99]. Taken together, ST239Kras’ MVFs include professional factors for adherence, immune evasion, and specific lesions and symptoms.

ST8Kras possessed a distinct set of MVFs, which included SEA, the strong expression of PSMα/β/Hld, Sak/SCIN, and Hla. Of these factors, SEA is associated with the severity of infections (sepsis and shock) [100] and promotes bacterial survival in vivo [101]. ST8Kras has attracted attention because of its high mortality rate for MRSA CAP, including pediatric deaths. The whole genome of ST8Kras is now being investigated to further characterize ST8Kras’ MVFs.

Discussion on the evolution of MRSA is also the important points of the present research. Regarding the ST239/SCCmecIII lineage, this global HA-MRSA [10,20,49,52,102–113,114]) consists of more than five MRSA clades [20]. Historically, the Brazilian clone carried “SCCmecIIIa” [9,15,20], while the Hungarian clone carried “SCCmecIII” [17]. “SCCmecIIIa” is now one large fused SCC (SCCmecIII.1.1.2), derived from two SCC-linked “SCCmecIII” through IS431-recombination [8,9,115–117]. ST239Kras had the same IS431-recombination type as that of the Brazilian clone. In Russia, no SCCHg/SCCmecIII link was present [52].

Moreover, ST239Kras carried the tst gene on SaPI [42,118], for the first time in the ST239 lineage. The same tst gene was present in the United Kingdom before the isolation of ST239Kras, suggesting the potential salvage of tst in Europe. ST239Kras also carried a completely unique, domestic phage, φSa7-like (W). Phages [19,20,44,119–121] are a possible tool for S. aureus diversification, and classified according to the integrase gene types [120]. φSa7-like (W) was classified as integrase type 7 (Sa7int); however, φSa7-like (W) had no repeats of the terminal att sequence, similar to Tn554 [52,122–124], and had a unique insertion site distinct from φSa7. In addition, ST239Kras exhibited the characteristics of CA-MRSA, i.e., the strong expression of the cytolytic peptide gene (as described above).

Regarding ST239 MRSA transmission, the Brazilian clone spread to Portugal [9,46], Central Europe (Germany, Poland, and Czech Republic), Northern Europe (Finland) [17], and Eastern Europe/West Asia (Georgia) [125]. Krasnoyarsk has had a historically close relationship to the European region (St. Petersburg and Moscow). The (Southeast) Asian clade, including London strain TW20, which was likely transmitted from Southeast Asia [2,20], carried characteristic φSPβ-like (S2 Table) [19,20,126–128], while ST239Kras lacked φSPβ-like. Based on these findings and our results, we herein proposed a new Russian clade (representative strain, OC3) in the ST239/SCCmecIII lineage, and also speculated that ST239Kras originated in the Brazilian clone, with the possible transmission route of Brazil-Europe (West-Central-North/East)-Russia (European-Siberian) (S9 Fig). Further genome-level analysis is needed for the understanding of evolution.
The plasmid distribution in Krasnoyarsk was unique. Many MRSA only carried pCpr\(^r\) and often carried two pCpr\(^s\) species, in contrast to some other country’s cases with no pCpr\(^r\) [19,23,28,29,71,129,130], or Vladivostok’s cases with multiple plasmids [52]. In Russia, inexpensive Cp is commonly administered to patients without a doctor’s prescription as an ointment for skin injuries or burns, as a tablet for gastroenteritis, and as an eye lotion, providing MRSA with strong pressure to carry a pCpr\(^r\). A small (2.9-kb) pCpr\(^s\) must be transferred, even in nature, possibly through the rolling circle (RC) manner of replication [41,131,132], similar to the replication that occurs during the conjugation of large Tra\(^+\) plasmids [1,41,132–134]. pEM\(^r\) [135] and Tn554, with a circular intermediate [52,136], may follow pCpr\(^s\)-like transfer.

In conclusion, we identified novel regional variants of the ST239 and ST8 lineages (ST239\(_{\text{Kras}}\) and ST8\(_{\text{Kras}}\)), in Siberian Russia (Krasnoyarsk), in which international research had never previously focused on MRSA and its invasive infections. ST239\(_{\text{Kras}}\) and ST8\(_{\text{Kras}}\) were MDR and had clonally (albeit with divergence) and widely spread, with fatal cases of HAP and CAP with bacteremia. The 15-day mortality rate for MRSA CAP was significantly higher than that for MRSA HAP, and fatal cases of ST8\(_{\text{Kras}}\) CAP included infant and young child deaths. According to the recent accumulation of information showing that successful MRSA, associated with large epidemics, has a unique set of MVFs, we speculated that fatal cases of ST239\(_{\text{Kras}}\) HAP were caused by the unique combination of TSST-1, the strong expression of PSM\(_{\alpha}\)/Hld, Hla, SEK/SEQ, SAK/SCIN, and Cna, while fatal cases of ST8\(_{\text{Kras}}\) CAP were attributed to the combination of SEA, the strong expression of PSM\(_{\alpha}\)/Hld, Hla, and SAK/SCIN. ST239\(_{\text{Kras}}\) carried a completely unique phage and mobile DNA, and exhibited unique virulence phenotypes; therefore, ST239\(_{\text{Kras}}\) represented a new (Siberian Russian) clade of the ST239 lineage, which was created through regional stepwise evolution during its possible Brazil-Europe-Russia transmission. Small resistance plasmids spread widely enough to not be ignored and in a unique manner among MRSA.

**Supporting Information**

S1 Fig. Plasmid analysis (A to C) and plasmid transfer in a mixed bacterial culture (D) of MRSA from Krasnoyarsk, in comparison with the ST239 MRSA strain 16K from Vladivostok. In A; RN, RN2677 (recipient). Covalently closed circular (CCC) plasmid DNA, isolated from MRSA and transconjugants (RN2677 carrying plasmids), was electrophoresed in 1% agarose. Plasmid sizes were determined using reference plasmids with known molecular sizes. Plasmids (color): Cpr\(^s\) (yellow), chloramphenicol resistance plasmid; Em\(^s\) (blue), erythromycin resistance plasmid; Gm\(^s\) (red), gentamicin resistance plasmid. Regarding plasmids marked with \(\ast\), the entire plasmid sequence was determined. In B-a, CCC plasmid DNA was electrophoresed in 0.6% agarose. RN, RN2677. In B-b, CCC plasmid DNA was digested with EcoRI, and the digests were electrophoresed in 0.5% agarose. Marker 1, 2.5 kb DNA Ladder; marker 2, \(\lambda\)-HindIII digest. In C; RN, RN2677. The Tn554 circular intermediate was detected by PCR; the ST239\(_{\text{Kras}}\) strain OC3 (lane 2) and Em\(^s\) transconjugant (Em\(^s\) RN2677, lane 3) produced positive results (carried the Tn554 circular intermediate), while RN2677 (lane 4) had no such structure. In D, bacterial mating between MRSA (plasmid-donor) and RN2677 (recipient) was performed by filter mating and non-filter mating methods. Nov, novobiocin; Cp, chloramphenicol; Em, erythromycin; Gm, gentamicin; Cli, clindamycin; Spc, spectinomycin; Amp, ampicillin; Cd, cadmium; EtBr, ethidium bromide; Acr, acriflavin. Transfer frequency, plasmid-positive (drug-resistant) transconjugants/donor.

(TIFF)

S2 Fig. Structure analysis of small plasmids specifying for chloramphenicol resistance (A-a to c) and erythromycin/clindamycin resistance (B). Plasmid sequence data were from the
GenBank accession numbers described. Homologous regions are shaded in each comparison. Genes: cap, chloramphenicol resistance; rep, replication initiator protein; pre, pre protein; rlx, RLX protein; repL, replication initiator protein L. Em/Cli', constitutive resistance to erythromycin and clindamycin; Em/Cli'repL, inducible resistance to erythromycin and clindamycin (due to the presence of the leader peptide sequence in the promoter region upstream of ermC).

S3 Fig. Analysis of the sek+ seq+ SaPI structure of the ST239Kras strain OC3. In A, SaPI1 (OC3) showed the highest homology to SaPI1 (TW20). SaPI1 (OC3) was also compared with SaPI5 (USA300). Homologous regions between the SaPI structures are shaded with color. ear, penicillin-binding protein fragment. In B, the deduced amino acid sequences of the sek and seq genes (of OC3, TW20, and USA300) were compared with those of COL. Arrows indicate the positions of the amino acid changes. Different amino acids from the amino acid sequences of COL are indicated in red letters.

S4 Fig. Structure of φSa3 of the ST239Kras strain OC3. φSa3 (OC3) exhibited the highest homology to φSa3 (CN1). The left-side immune evasion cluster (IEC) region was also compared with those of φSa3 (TW20) and ST8Kras strain OC8. Homologous regions are shaded in each comparison. Genes in IEC: scn, staphylococcal complement inhibitor (SCIN) gene; chp, chemotaxis inhibitory protein of S. aureus (CHIPS) gene; sak, staphylokinase (SAK) gene; sea, staphylococcal enterotoxin A (ETA) gene. The IEC region, carrying scn and sak, of OC3 (a region from attL to sak) was 3,541 bp in size, and showed 99% homology to the corresponding region of TW20. The IEC region, carrying scn, sak, and sea, of OC8 (a region from attL to sea) was 6,022 bp in size, and showed 99.3% homology to the corresponding region of TW20.

S5 Fig. fhuD+ SaPI structure of the ST239Kras strain OC3. fhuD+ SaPI (OC3) showed only limited homology to any previous SaPI, suggesting a novel mosaic SaPI (fhuD). Homologous regions are shaded in each comparison. Genes: int, integrase gene; xis, excisionase; rep, replication initiator gene; ter, terminase gene (which cleaves multimeric DNA); fhuD, ferrichrome ABC transporter homologue.

S6 Fig. Structure of φSa5 of the ST239Kras strain OC3. φSa5 (OC3) exhibited the highest (but limited) homology to φSa5 (XN108), suggesting a new mosaic phage. Homologous regions are shaded in each comparison.

S7 Fig. Pulsed-field gel electrophoresis (PFGE) analysis of ST239 MRSA strains isolated from the European region (Moscow, St. Petersburg), Ural region (Kurgan), and Far Eastern region (Vladivostok), in comparison with ST239Kras. In the dendrogram (left side), a large spa3/SCCmecIII.1.1.1-III.1.2 cluster, associated with the Siberian region and Far Eastern region, is shadowed. In the middle of the figure, each Russian region is distinguished by color: red, Siberian region (Krasnoyarsk); green, Far Eastern region (Vladivostok); brown, European region (Moscow, St. Petersburg); purple, Ural region (Kurgan). Reference strains (HU25 and ANS46) are not marked. Regarding PFGE patterns (right side), the PFGE types of ST239 MRSA from Krasnoyarsk are those shown in Fig 1.

S8 Fig. Comparison of SCCmecIII structures of the whole genome-analyzed ST239 MRSA strains. Eight whole genome-analyzed ST239 strains, shown in this figure, are those described
in S2 Table. SCCmecIII structures were analyzed as shown in Fig 3. Homologous regions are shaded. SCCmecIII.1.1.new-a and SCCmecIII.1.1.new-b, SCCmecIII.1.1 with new J3 regions.

S9 Fig. A proposed model for the regional stepwise evolution of ST239_Kras, which emerged in Siberian Russia (Krasnoyarsk). ST239_Kras is characterized by SCCmecIII.1.1.2, tst, and φSa7-like (W). This figure indicates a possible Brazil-Europe-Russia transmission route for ST239_Kras, in addition to the territories of some other prevalent ST239 MRSA.

S1 Table. Molecular characterization of ST239 MRSA strains isolated from Moscow, St. Petersburg, Kurgan, and Vladivostok^a.

S2 Table. Comparison of the characteristic genetic structures on the ST239 MRSA whole genomes: virulence, drug resistance, and evolution.

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Author Contributions
Conceived and designed the experiments: TY ABS LJT GVR OEK WCH TWW YI OVP. Performed the experiments: OEK WCH TWW YI TY TT WH SVY OVT VVK YVK AN IVR SVS. Analyzed the data: WCH TWW LJT YI TY OEK WH AN. Contributed reagents/materials/analysis tools: WCH TWW TY YI OEK. Wrote the paper: TY WCH TWW YI OEK.

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