Two variants of staphylococcal cassette chromosome *mec* type IVA in community-associated meticillin-resistant *Staphylococcus aureus* strains in South Korea

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Meticillin-resistant *Staphylococcus aureus* (MRSA) strains harbouring staphylococcal cassette chromosome *mec* (SCC*mec*) type IVA are known to be more prevalent in South Korea than in other countries. Variations in the SCC*mec* IVA structure have been identified, including in sequence type (ST) 1 and ST72 strains. This study compared and investigated the genetic characteristics of two subtypes common in South Korea. Type IVA SCC*mec* of ST1 strains was characterized by type IV features with the linearized pUB110 at the junkyard (J) 3 region. However, that of ST72 strains carried a variant class B *mec* complex, *ccrA2*, with an identity of ~96% and the linearized pUB110 at the J3 region. In SCC*mec* of ST72 strains, the organization of the class B variant and the J3 region may be more similar to that of type IA than to other types, but the *ccr* type and other J regions seemed to be derived from type IV. These genetic characteristics showed that type IVA appears to result from the dynamic genetic exchange and recombination of SCC DNA.

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INTRODUCTION

Staphylococcus aureus is a versatile opportunistic pathogen, with a variety of factors that are associated with virulence (Dinges *et al.*, 2000; Lina *et al.*, 1997; Novick, 2000), causing benign skin and soft tissue infections, as well as fatal invasive disease.

The key to the appearance of meticillin-resistant *S. aureus* (MRSA) is the acquisition of *mecA* encoding PBP2A, which has been shown to be part of a mobile element referred to as staphylococcal cassette chromosome *mec* (SCC*mec*) (Hartman & Tomasz, 1984; Ito *et al.*, 1999, 2003). The main types of SCC*mec* are defined by the combination of the *mec* complex with the *ccr* locus (Ito *et al.*, 2001, 2004; Okuma *et al.*, 2002; Oliveira *et al.*, 2006a; Takano *et al.*,

2008). New types and subtypes have been reported, with diverse recombination of the *mec* complex, the *ccr* types and the junkyard (J) regions (Chongtrakool *et al.*, 2006; Heusser *et al.*, 2007; Hisata *et al.*, 2005; Ito *et al.*, 2003; Shore *et al.*, 2005).

Community-associated (CA)-MRSA strains have emerged as a major concern with regard to MRSA infections (Etienne, 2005; Gillet *et al.*, 2002; Ho *et al.*, 2004; Tristan *et al.*, 2007; Vandenesch *et al.*, 2003). However, in South Korea, there is limited information on the emergence of CA-MRSA strains. A recently reported study on MRSA in South Korea during 2004–2005 showed that there were two prevalent CA-MRSA clones [clonal complex (CC) 1 and sequence type (ST) 72] that carried type IVA SCC*mec* (Park *et al.*, 2007). The multiplex type IVA was first reported by Oliveira & de Lencastre (2002). In the multiplex pattern (downstream common sequence, pUB110 locus and *mecA* are amplified), type IVA appears to be related to IA, II and IV. Shore *et al.* (2005) reported multiplex type IVA carrying class A *mec* complex variants. However, type IVA found in South Korea was reported to harbour class B *mec* complex or variants (Park *et al.*, 2007). Chongtrakool *et al.* (2006) proposed a new nomenclature for SCC*mec* type in

Abbreviations: CA, community-associated; CC, clonal complex; HVR, hypervariable region; J, junkyard; MRSA, meticillin-resistant *Staphylococcus aureus*; MSSA, meticillin-sensitive *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the complete SCC*mec* sequence of the ST72 clone and the *mec* element sequence of the ST1 clone are EU437549 and EU437550, respectively.

which type IVA was described as 2B.N.2; however, the genetic variations in the class B *mec* element, the *ccr* type and the J regions were not fully examined, and the authors stated that the data for the *mec* class and *ccr* type had been given tentatively.

Type IVA SCCmec has not been classified clearly into types and subtypes to date. Here, we have described the genetic characteristics of type IVA found in South Korea and investigated its genetic relationship with other types. We have also reported two variants of type IVA.

METHODS

Bacterial strains. For analysis of the two subtypes of SCCmec IVA, we used strains cm11 ST72 and cm14 (ST1); these STs have frequently been detected in CA-MRSA strains in South Korea (Park *et al.*, 2007). For the comparative study of the distribution of aminoglycoside-resistance genes and virulence-associated genes, we used 23 isolates of CC1 (ST1, ST493 and ST573) and 22 isolates of the ST72 clone, as reported previously (Park *et al.*, 2007).

Sequencing of the *mecA* complex of SCCmec from CA-MRSA clones. A single PCR method, as described elsewhere (Hisata *et al.*, 2005; Okuma *et al.*, 2002), was performed for comparison of type IVA from strains cm11 and cm14. The class B *mec* complexes of strains cm11 and cm14 were amplified and sequenced with primers as follows: IS5 (5'-AACGCCACTCATAACATATGGAA-3') and mA6 (5'-TATACCAAACCCGACAAC-3') for the IS1272-*mecA* region, mAnew1 (5'-TGGAATTAACGTGGAGACGA-3') and mAnew2 (5'-AACGTTGTAAACACCCCAAG-3') for *mecA*, and mA1 (5'-TGCT-ATCCACCCTCAAACAGG-3') and IS2 (5'-TGAGGTTATTCAGA-TATTTTCGATGT-3') for the *mecA*-IS431*mec* region (Hiramatsu *et al.*, 1992; Katayama *et al.*, 2001; Okuma *et al.*, 2002; Park *et al.*, 2007).

Long-range PCR and primer-walking sequencing. For analysis of the whole sequence of SCCmec from strain cm11, we adopted a long-range PCR-based sequencing method by expanding the long PCR system using an Expand cloning kit (Roche). The primers used were as follows: ILF (5'-TCGTCTTCATCAACTTCA-3'), *ccrAR* (5'-CTCTTAAGGCGTTGACAA-3'), *ccrAF* (5'-GGATCAAGCTTTCG-ACCGACTCAAG-3') and IS1272R (5'-TCCTCGGACAGACATCC-GAGTG-3') designed based on the sequence with GenBank accession number AB245470 in the upstream region; IS5, mAnew2, mAnew1 and IS2 in the *mecA* complex (Katayama *et al.*, 2001; Okuma *et al.*, 2002; Park *et al.*, 2007); and pub1F (5'-AGCTGAATAAGAACGG-TGCTC-3'), pubR (5'-ACATACCAACACTTCAACGCACC-3'), pub2F (5'-CCTTAAGGAACGTACAGACGGCT-3') and orfXR (5'-TCCACATCAAATGATGCGGGTTG-3') designed based on the sequences with GenBank accession numbers D86934 and AP009324 in the downstream region. Gap filling and sequencing of the left and right extremities of SCCmec by DNA walking was carried out with a SpeedUp premix kit (Seegene). Primers 15666F (5'-TGAAATCTG-ATGAAGAAAAGTTGG-3') and 15666R (5'-CAGGACTTGAAGTGCAGAA-3'), designed based on the sequence with GenBank accession number EU272085 [strain 15666 of methicillin-sensitive *S. aureus* (MSSA) ST72], were used for analysis of the insertion site of SCCmec.

Sequence analysis. Sequence data were assembled, and homology searches were performed using BLASTN and BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST>). The ORFs were predicted and translated using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). GenBank accession numbers AB037671, AB037673, AB063172, AB063173, AB097677, AB127982, AB266531, AB266532, AF411935,

AJ810120, AJ810121, AM292304, CP000029, D86934, DQ106887 and EU437549 for *ccrA* were used for phylogenetic analysis of the *ccrA* nucleotide sequence (1350 nt). Multiple sequence alignments were performed with ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2>) (Larkin *et al.*, 2007). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.1 (Kumar *et al.*, 2004) using the neighbour-joining method. The Jukes–Cantor parameter and the γ parameter 1.0 were used for the nucleotide substitution model.

Profiling of virulence-associated genes, aminoglycoside resistance-associated genes and antimicrobial susceptibilities.

Enterotoxin and surface protein encoding genes, and aminoglycoside-resistance-associated genes from 55 isolates of CC1 and ST72 were evaluated as described by Choi *et al.* (2003), Jarraud *et al.* (2002) and Vancraeynest *et al.* (2004). Variation in the enterotoxin gene island (*egc*) was determined as described by Thomas *et al.* (2006). Antimicrobial susceptibilities were determined using the disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006).

Nucleotide sequence accession numbers. Nucleotide sequences determined in the present study were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) under accession numbers EU437549 (the complete SCCmec sequence of the ST72 clone) and EU437550 (the *mec* element sequence of the ST1 clone).

RESULTS AND DISCUSSION

Molecular characteristics and antibiotic susceptibility of ST72 and CC1 isolates

Recently, Kim *et al.* (2007) and Park *et al.* (2007) reported that multiplex type IVA was found in ~43 and ~53 % of CA-MRSA isolates in South Korea, respectively. In their studies, type IVA was the most prevalent type in CA-MRSA and was found mainly in clinical isolates belonging to ST72 and CC1 (Kim *et al.*, 2007; Park *et al.*, 2007). Their characteristics including virulence and antibiotic-resistance patterns are summarized in Table 1. Some of the SCCmec PCR typing and antibiotic susceptibility results were included in the findings of a previous study (Park *et al.*, 2007). The profile of the enterotoxins showed that CC1 isolates carried *seh* (>95 %) and ST72 carried an *egc* variant harbouring *seu2*. There were different antibiotics-susceptibility patterns in ST72 and CC1 strains, especially for gentamicin (Table 1); this was probably due to the presence of *aac(6')/aph(2')* in CC1 isolates (Table 1). Although both clones (ST72 and CC1) carried *ant(4')* at the pUB110 locus of SCCmec IVA (Table 1), ANT(4')-Ia encoded by *ant(4')* is not related to gentamicin resistance but is associated with resistance to amikacin, tobramycin, dibekacin, isepamicin and kanamycin (Vakulenko & Mobashery, 2003). However, AAC(6')-Ie-APH(2')-Ia encoded by *aac(6')/aph(2')* confers resistance to virtually all aminoglycosides including gentamicin, but not for streptomycin (Vakulenko & Mobashery, 2003).

Genetic variation of SCCmec type IVA

There were some variations in the PCR results for class B *mec* and *ccrAB* identified in ST72 and CC1 strains (Table 1). We compared the class B *mec* complex and *ccrAB*

Table 1. Molecular characteristics and antibiotic-resistance patterns of major SCCmec IVA clones

MLST	PFGE pattern	SCCmec mutiplex type*	mec class†	ccr type‡	J region‡	agr type§	luk-PV	hlg	Toxin	MSCRAMMs¶	AME#	Antibiotic resistance (%)**				
												GEN	CLI	ERY	TET	TOB
ST72	B	IVA	(B)††	(2)‡‡	IVc, pUB110	I	–	hlg2	egc	icaA-icaD-ebpS-fnbA-eno- clfA-clfB-fib	ant(4')	3.1	3.1	50.0	0	100
CC1§§	D	IVA	B	2	IVc, pUB110	III	–	hlg2	seh	icaA-icaD-ebpS-fnbA- cna -eno- clfA-clfB-fib	ant(4'), aac(6')/aph(2')	100	0	94.7	57.9	100

CLI, clindamycin; ERY, erythromycin; GEN, gentamicin; TET, tetracycline; TOB, tobramycin.

*Multiplex PCR results described by Oliveira & de Lencastre (2002).

†Single PCR results described by Okuma *et al.* (2002).

‡Subtyping of the J region was performed as described by Hisata *et al.* (2005); other characteristics were determined from multiplex PCR results.

§Accessory gene regulator locus (*agr*) group was determined by restriction enzyme *Dra*I RFLP analysis, as described by Papakyriacou *et al.* (2000).

||PVL, *hlg*, *hlg-2* and other toxin-related genes were determined as described by Jarraud *et al.* (2002).

¶Genetic profile of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The bold text indicates the difference between the profiles for ST72 and ST1.

#AME, aminoglycoside-modifying-enzyme-associated gene.

**All isolates showed resistance to oxacillin and penicillin, but no isolate showed resistance to arbekacin, ciprofloxacin, rifampicin, penicillin or vancomycin.

††Increased size of class B *mec*.

‡‡The *ccrA2* gene shared 96 % identity with the other *ccrA2* gene.

§§CC1: 21/23 ST1, 1/23 ST493 and 1/23 ST573.

Fig. 1. Illustrative comparison of the class B *mec* element of SCCmec type IVA from the ST1 and ST72 MRSA clones. The GenBank accession numbers were EU437549 and EU437550 (this study) for the nucleotide sequence of the class B *mec* element of the ST72 and ST1 clones, respectively, and AB425823 for strain JCSC6668.

variations in cm11 (ST72) and cm14 (ST1) strains. The overlapping single SCCmec PCR products were sequenced to compare the differences in more detail (Fig. 1). Variations were found in the class B *mec* complex of strain cm11: a transposase 20 family gene (*tnp20*, ORF C017; Table 2) was inserted, and the hypervariable region (HVR) and *ugpQ* were deleted, when compared with other class B *mec* elements (Fig. 1). *tnp20* was almost identical (~99.6%) to SCC*pbp4*, commonly reported in *Staphylococcus epidermidis* strains; it has also recently been reported in a Swedish MRSA strain (JCSC6668; Berglund *et al.*, 2009). The class B.3 *mec*, of strain JCSC6668, was similar to that of strain cm11, except for

deletion of the HVR and *ugpQ* (Fig. 1). The deletion of the HVR was similar to the class B *mec* element of type IA (Fig. 1).

In contrast, strain cm14 (ST1) carried a typical class B *mec* (IS1272– Δ *mecR1*–*mecA*–HVR–IS431), when compared with other SCCmec IV types (Fig. 1). It had the *mecA* upstream region, similar to SCCmec type IVc, but had linearized plasmid pUB110 in the downstream region, which was different from that of IVc (Table 1).

ccr allotyping has been useful for examination of the phylogenetic relationships among SCCmec types (Oliveira *et al.*, 2006b, 2008). Therefore, we investigated the genetic

Table 2. List of ORFs of SCCmec type IVA from the ST72 clone

ORF	Position (nt)	Identity (%)*	Homologues and other characteristics
IR-L	3238–3263	–	Inverted complementary repeat (IR-L) of SCCmec
C007	3303–4103	99	Hypothetical protein (<i>S. aureus</i> MR108, SCCmec IVc), abortive phage resistance protein (<i>S. epidermidis</i> ATCC 12228)
C008	4133–5287	100	Hypothetical protein (<i>S. aureus</i> MR108), abi-alpha protein (<i>S. epidermidis</i> ATCC 12228), COG2865
C009	5742–6788	100	Hypothetical protein (<i>S. aureus</i> MR108), hypothetical protein (<i>S. epidermidis</i> ATCC 12228)
C010	7277–9070	99	Hypothetical protein (<i>S. aureus</i> MR108), pfam06048 (DUF927)
ccrA2	9304–10653	96	Cassette chromosome recombinase A type 2 (<i>S. aureus</i> MR108)
ccrB2	10675–12303	100	Cassette chromosome recombinase B type 2 (<i>S. aureus</i> MR108)
C013	12825–13175	100	Hypothetical protein (<i>S. aureus</i> MR108, SCCmec IVc)
C014	13262–13573	100	Hypothetical protein (<i>S. aureus</i> MR108, SCCmec IVc)
C015	13585–14094	100	Hypothetical protein (<i>S. aureus</i> MR108, SCCmec IVc), COG4333
IS1272	14230–15741	98	Truncated transposase of IS1272, COG3666 (transposase 11)
C017	15838–16851	99	Transposase (<i>S. epidermidis</i> ATCC 12228), pfam02371 (transposase 20)
Δ <i>mecR1</i>	17536–18504	100	Truncated signal transducer protein MecR1
<i>mecA</i>	18622–20628	100	Penicillin-binding protein 2'
IS431	21035–21709	100	Transposase for IS-like element (IS431 <i>mec</i>), COG3316
C021	21960–22673	99*	Kanamycin nucleotidyl transferase (pUB110)
C022	22896–23294	100	Bleomycin resistance protein (BRP), pfam00903 (glyoxalase)
C023	23801–25063	99	Plasmid recombination enzyme (plasmid pUB110), pfam01076
C024	25287–26081	100	Replication protein (plasmid pUB110), COG5655
IS431	26382–27056	100	Transposase for IS-like element (IS431 <i>mec</i>), COG3316
C026	27088–27327	100	Hypothetical protein SAV0026 (<i>S. aureus</i> Mu50)
C027	27742–29037	100	Hypothetical protein SAV0025 (<i>S. aureus</i> Mu50)
IR-R	29310–29335	–	Inverted complementary repeat (IR-R) of SCCmec
orfX	29320–29806	–	ORF X partial

*Result from BLASTN.

variation and relationship of *ccrAB* of type IVA. The nucleotide sequence of the *ccrAB* locus in strain cm14 was almost identical (>99%) to that of the type IVc variant. The *ccrB2* sequence from strain cm11 was identical (100%) to that of IVc (MR108); however, *ccrA2* was slightly different, with a 96% identity determined by BLASTP. Comparison of type IVA and other *ccrA* allotypes showed that most of the type IV subtypes clustered in one closely related group distinct from those of type II, with the exception of type IVb (Fig. 2). The *ccrA2* of type IVA (cm11) clustered with type IV subtypes and was much closer to that of SCCmec IVg (Fig. 2). In the nucleotide sequence analysis of *ccrA2*, type IVA should be phylogenetically closer to type IV than to type II.

SCCmec type IVA of strain cm11 (ST72) could provide evidence of exchange and recombination of SCC DNA in staphylococci inter- and intra-species

We examined the whole SCCmec structure of strain cm11 (ST72), which appeared to be similar to SCCmec type 2B.N.2 (Fig. 3a). Twenty-one ORFs in SCCmec were identified by ORF Finder and BLASTP; most had >98% identity with previously identified staphylococcal genes, except for *ccrA2* (Table 2). The dispersed ORFs of the left extremity region were almost identical to those of type IVc (MR108) and SCCp_{bp4} (*S. epidermidis* ATCC 12228) (Fig. 3b, Table 2). The J3 region with linearized pUB110 was almost identical to that of type IA and II (Table 2). The organization of the class B variant and the J3 region in this structure may be more similar to that of type IA than other types, but the *ccr* type and other J regions seemed to be derived from type IV (Fig. 3a). Analysis of type IVA showed a unique structure composed of the recombination of a variant class B *mec*

element and the type 2 *ccrAB* with the J regions derived from type IA or II (J3) and IVc or SCCp_{bp4} (J1 and J2) (Fig. 3a). It has been suggested that exchange and recombination of SCC DNA probably occurs in staphylococci inter- and intra-species (Hanssen & Sollid, 2007; Hanssen *et al.*, 2004). The genetic structure of SCCmec type IVA, from strain cm11, could provide evidence supporting both inter- and intra-species recombination.

Analysis of the left boundary of SCCmec IVA indicates SCCmec acquisition by non-CcrAB-mediated insertion

The integration sequence and the inverted repeats at the right and left boundaries of SCCmec were identical to SCCmec type IVc (*S. aureus* MR108). However, the outermost sequence of the SCCmec attachment site (SCCmec *attB*) of the left boundary was very different from that of IVc (MR108) (Fig. 3c). The results of primer-walking studies showed that the 15 bp core sequence (SCCmec *attB*) and the SCCmec acquisition site of strain 15666 (MSSA ST72) were conserved surrounding the sequences at the left-end extremity of SCCmec IVA (cm11) (Fig. 3a, c). Noto *et al.* (2008) suggested that there could be two mechanisms for the acquisition of SCCmec; CcrAB-mediated and non-CcrAB-mediated insertion mechanisms (other integrase mechanisms or homologous recombination). For CcrAB-mediated acquisition, a specific sequence should be necessary at the surrounding boundary of the left-end extremity. However, at the left-end extremity of type IVA, there was no CcrAB-mediated specific sequence (Fig. 3c). In addition, there were no lost or gained sequences at the SCCmec acquisition site, compared with strain 15666 (Fig. 3c). These findings suggest that MSSA ST72 strains could acquire SCCmec type IVA by non-CcrAB-mediated insertion.

Fig. 2. Phylogenetic tree of the complete nucleotide sequence of *ccrA* (1350 nt), constructed using the neighbour-joining method based on the Jukes–Cantor parameter and γ parameter 1.0. GenBank accession numbers are indicated. Bar, 0.1 nucleotide substitutions per site.

Epidemiological features of SCCmec type IVA strains prevalent in South Korea

The genetic diversity of CA-MRSA strains is probably due to the successful conversion of diverse MSSA lineages to MRSA by the transfer of SCCmec in the community. In particular, SCCmec type IV, the smallest structural and most diverse type, predominates among the diverse CA-MRSA strains worldwide (Robinson & Enright, 2003; Vandenesch *et al.*, 2003). However, instead of type IV, SCCmec IVA types are the most prevalent CA-MRSA strains found in South Korea, compared with other countries (Cha *et al.*, 2005; Kim *et al.*, 2007; Park *et al.*, 2007, 2008). The emergence of SCCmec type IVA strains is a major concern in the public health setting and for infection control in hospitals, and these strains have recently been shown to be spreading (Kim *et al.*, 2007; Park *et al.*, 2007, 2009).

Epidemiological studies of *S. aureus* and antibiotic use in South Korea have reported that ST72 (major SCCmec IVA

Fig. 3. Schematic analysis of ST72 SCCmec type IVA. (a) The genetic structure of IVA is based on the nucleotide sequences deposited in GenBank under accession numbers EU437549 (SCCmec of ST72 clone) and EU272085 (MSSA ST72 15666 clone). ORFs are indicated by arrows. The bars indicate homology to previously described SCCmec elements. (b) The homologous left-extremity *ccr* (L-C) region of SCC elements. ORFs of SCCmec IVA are indicated by boxes. Each homologous ORF of IVc and SCC*pbp4* is described below the boxes. The nucleotide sequences with GenBank accession numbers EU437549 (cm11 SCCmec IVA), AE016744 (*S. epidermidis* ATCC 12228 SCC*pbp4*) and AB096217 (strain MR108 SCCmec type IVc) were used. (c) Comparison of the chromosome-SCCmec junction sequences of *S. aureus* strain cm11. The nucleotide sequences around the left (i) and right (ii) boundaries were aligned with those of strains MR108 (type IVc, AB096217), M03-68 (type IVg, DQ106887), N315 (type II, D86934) and the SCCmec insertion site of MSSA strain 15666 (EU272085). The dotted arrows indicate inverted repeats of IR-L and IR-R at both extremities of the SCCmec elements. The box indicates the SCCmec *attB* site.

clonal lineage) MSSA and MRSA strains were not frequently isolated until 2005 in South Korea (Kim *et al.*, 2008). This suggests that IVA strains have emerged recently in South Korea. In addition, it has been reported that the antimicrobial use density of aminoglycosides was one of the highest in a survey of antimicrobial use during 2004–2007, which corresponded with the results of the Health Insurance Review and Assessment Service (<http://www.hira.or.kr>) in South Korea (Yoon *et al.*, 2008). The finding that *ant(4')* was carried on the genetic island of type IVA by both SCCmec IVA strains (ST72 and CC1) and that *aac(6')/aph(2')* was carried by CC1 strains (Table 1) suggests that exposure to aminoglycosides might provide a favourable environment for the spread of SCCmec IVA strains rather than IV strains.

In conclusion, we have described two variants of SCCmec IVA, each of which showed genetic differences in the class B *mec* complex. In particular, the genetic organization of type IVA in ST72 strains appeared to be more related structurally to types IA (class B *mec* complex variation and J2 region) and IV (*ccr* type, J1 and J2 region) than the other types. The genetic relationship of ORFs on SCCmec type IVA suggests that the development of type IVA appears to result from the dynamic genetic exchange and recombination of SCC DNA inter- and intra-species. In the future, it will be necessary to investigate the genetic evolutionary relationships of other SCC DNAs, as well as their epidemiology, to determine how these types disseminate in community and hospital settings.

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