Epidemic meticillin-resistant \textit{Staphylococcus aureus} (EMRSA-15) variants detected in healthy and diseased individuals in India

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This study provides what we believe to be the first report of the presence of EMRSA-15 and its variants isolated from nasal swabs from 13 healthy and diseased individuals in India. The majority of the isolates belonged to staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) type IV and \textit{spa} type t852, whilst four isolates were non-typable and heterotypic for the presence of the \textit{mecA} gene. All non-typable isolates were positive for the \textit{orfX} gene by PCR and belonged to \textit{spa} types t005 and t2986. They may have variant SCC\textit{mec} cassettes indicating genetic changes occurring in the Indian EMRSA-15. All isolates were positive for Panton–Valentine leukocidin and toxic shock syndrome toxin, which is a cause for concern. In addition to soft-tissue infections, the EMRSA-15 isolates from patients were also responsible for meningitis and brain abscesses, which is quite rare.

\textbf{INTRODUCTION}

\textit{Staphylococcus aureus} is a versatile human pathogen causing infections ranging from mild involvement of skin and soft tissue to life-threatening sepsis, pneumonia and toxic shock syndrome. Until recently, meticillin-resistant \textit{S. aureus} (MRSA) was identified mainly as a nosocomial pathogen, but infections attributed to community-acquired MRSA (CA-MRSA) have emerged in patients who did not have established risk factors. Simple skin and soft-tissue infections to serious necrotizing pneumonia, necrotizing fasciitis, and bone and joint infections caused by CA-MRSA are now a serious problem (Feng et al., 2008; Gordon & Lowy, 2008; Chambers & DeLeo, 2009).

Meticillin resistance is conferred on the organism by the presence of a unique mobile genetic element called the staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) carrying the \textit{mecA} gene. The SCC\textit{mec} elements are divided into different types based on nucleotide differences in two essential components, cassette chromosome recombinase (\textit{ccr}) represented by \textit{ccr} genes and \textit{mec} gene complexes, and also by variability in the joining regions (Ito et al., 2001; Okuma et al., 2002). Eight major types of SCC\textit{mec} element have been reported. Hospital-associated (HA)-MRSA isolates contain mainly type I, II and III SCC\textit{mec} cassettes (1B, 2A and 3A in the proposed new nomenclature), whilst CA-MRSA contains type IV and V cassettes (2B and 5C2); the other SCC\textit{mec} elements are type VI (4B), type VII (5C1) and type VIII (4A) (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009).

HA-MRSA isolates are multidrug resistant whilst CA-MRSA tends to be resistant only to \textbeta-lactam antibiotics and sensitive to non-\textbeta-lactam antibiotics (Chambers, 2005). Recent studies indicate that CA-MRSA has been evolving over the last few years with changing antibiotic susceptibility patterns and is increasingly replacing HA-MRSA in hospitals. Many countries have reported that the epidemic MRSA strain EMRSA-15 is replacing HA-MRSA present previously in their hospitals (Amorim et al., 2007; Hsu et al., 2007; Vindel et al., 2009). EMRSA-15 and EMRSA-16 are pandemic clones of MRSA that were first identified in the UK during the early nineties and became predominant HA-MRSA strains. Since then, EMRSA-15 has been detected in many countries and has been characterized as belonging to SCC\textit{mec} type IV and sequence type (ST) 22, although it is mainly associated with hospitals. EMRSA-15 and EMRSA-16 are more successful at surviving, colonizing and spreading in hospitals compared with other EMRSA present in the UK (Moore & Lindsay, 2002). Several countries have also reported region-specific STs indicating changes in CA-MRSA (Ghebremedhin et al., 2007; Aires de Sousa et al., 2008). For example, frequent international travel by large numbers of people has resulted in the spread of isolates and dissemination of USA300, the major US CA-MRSA clone, to Australia, Europe, Japan and South America (Shibuya et al., 2008; Tenover & Goering, 2009). It is no longer possible to

Abbreviations: CA, community-acquired; EMRSA, epidemic MRSA strain; HA, hospital-associated; MLST, multilocus sequence typing; MRSA, meticillin-resistant \textit{S. aureus}; MSSA, meticillin-sensitive \textit{S. aureus}; PVL, Panton–Valentine leukocidin; SCC\textit{mec}, staphylococcal cassette chromosome \textit{mec}; ST, sequence type.
distinguish the CA- and HA-MRSA by antibiotic sensitivity criteria (Popovich et al., 2008). Hence, frequent monitoring and surveillance of isolates in different countries is of the utmost importance.

Many variants of EMRSA-15 have been reported over the years. The Panton–Valentine leukocidin (PVL) toxin has been detected in some EMRSA-15 strains isolated in The Netherlands, and meticillin-sensitive S. aureus (MSSA) isolates that are closely related to EMRSA-15 have been detected in the UK (Wannet, 2003; Holmes et al., 2005). However, none of the variants reported by O’Neill and colleagues contained the PVL gene (O’Neill et al., 2001; Udo et al., 2006; Wolter et al., 2008).

Several reports of CA-MRSA have appeared in the last couple of years from various parts of India based on antibiotic sensitivity patterns of S. aureus isolated from nasal swabs of healthy adults. So far, the molecular characterization of these CA-MRSA strains has not been reported (Krishna et al., 2004). In our earlier studies on genotyping of HA-MRSA collected from hospitals during 2003–2005, we did not encounter EMRSA-15 (Arakere et al., 2005; Nadig et al., 2006).

The presence of PVL and susceptibility to non-β-lactam antibiotics are considered characteristics of the majority of CA-MRSA isolates. For the past several years, the thinking was that PVL was responsible for the enhanced virulence of S. aureus (Labandeira-Rey et al., 2007). However, recent studies on mortality with rodent models using wild-type and isogenic ΔPVL CA-MRSA strains gave results to the contrary, indicating that PVL may not play an important role in the virulence of S. aureus (Voyich et al., 2006). The role of PVL has assumed importance again from recent studies demonstrating that the expression of PVL by staphylococcal strains confers strong and rapid cytotoxic activity against neutrophils isolated only from human cells and that this action could not be reproduced in murine or Java monkey cells. These results indicate that infection models in mice and non-human primates fail to replicate the pathogenic activity of PVL seen in human cells (Löffler et al., 2010). PVL is also epidemiologically linked to certain types of diseases such as necrotizing pneumonia and severe skin infections. In addition, there is increasing evidence that the effect of virulence factors of S. aureus is host-dependent. Hence, there are wide gaps in our understanding of the role of PVL.

The aim of this study was to determine whether EMRSA-15 is making inroads in India and whether it is present in the community as well as in hospitals. To screen the healthy population, we chose two divergent populations in the community, one a rural and tribal population and the other an outpatient population visiting a state-funded government hospital in Bengaluru. Patient samples comprised disease isolates from soft-tissue infections, meningitis and cerebral abscesses sent to us from three different hospitals in India. To our knowledge, this is the first report of the presence of EMRSA-15 and its variants in healthy and diseased individuals from India.

**METHODS**

**Sample collection**

**Samples from healthy rural and urban populations.** Nasal swabs from two populations were collected: one a rural, tribal population around Saragur about 350 km from Bengaluru, southern India; and the other outpatients who visited the government-run Victoria Hospital, Bengaluru. Nasal swabs from approximately 1500 individuals were collected from rural and tribal areas from all age groups by a domiciliary method among participants with no identified risk factors for MRSA acquisition, which included prior hospitalization, use of antibiotics and surgeries in the past year. Sample collections took place between July 2006 and February 2007. From the other population consisting of outpatients, 400 swabs were collected from May 2007 to April 2008. In both places, swabs were collected after explaining the prepared questionnaire and with the consent of the subjects. The median age of the rural population was 23 and that of the urban outpatients was 32.

**Patient samples.** Six patient isolates, one each from Hinduja Hospital in Mumbai (M37) and Pune (Pune08), and four from the National Institute of Mental Health and Neurological Sciences, a tertiary-care hospital in Bengaluru, were sent to us.

**Patient history.** Isolate M37, sent to us in February 2005, was from a wound swab from a 61-year-old diabetic, hypertensive male who had a coronary bypass 6 months earlier and had developed an infected carbuncle in the right nape of the neck. The carbuncle was surgically debrided and excised. The patient was treated with ciprofloxacin for 10 days and made an uneventful recovery.

Pune08 was isolated in 2008 from a 45-year-old female who had been travelling in the UK for 4 weeks and developed an abscess over the left flank within 72 h of returning from the UK to Pune, India. MRSA was isolated and was resistant to co-trimoxazole, cindamycin, ciprofloxacin and pristinamycin in addition to the antibiotics described in Table 1 and sensitive to teicoplanin, vancomycin and linezolid. The lesion responded to linezolid tablets taken for 15 days.

The remaining four patient isolates, which caused neurological diseases, were isolated in 2008. NMB was from a blood culture from a 15-year-old boy who was previously healthy and was diagnosed with septic cavernous sinus thrombosis and meningitis. MRSA was also isolated from his cerebrospinal fluid. Treatment included intravenous vancomycin and oral rifampicin with other supportive treatments for 4 weeks. The patient was discharged after 2 months in hospital, and 10 months into the follow-up, he had right ocu-lomotor nerve palsy with mild left hemiparesis (Veenakumari et al., 2010).

NP113 and NP115, from a 1-year-old girl, were isolated from pus from a right frontal and parietal cerebral abscess with an interval of 15 days. Both isolates were sensitive to linezolid, vancomycin, lincomycin, chloramphenicol and tetracycline. NP114, from a 32-year-old male, was isolated from pus from a cerebral abscess and was sensitive to linezolid, vancomycin, rifampicin, lincomycin, chloramphenicol and tetracycline.

**Phenotyping and genotyping of S. aureus.** The identification of S. aureus using phenotypic tests and determination of MIGs for different antibiotics have been described in an earlier publication (Arakere et al., 2005). Antibiotic sensitivity testing by disc diffusion was carried out according to standard procedures on Mueller–Hinton agar plates for all the antibiotics listed, except for oxacillin, meticillin and...
accomplished this by molecular characterization of S. aureus isolates collected from healthy subjects in a remote rural and tribal community and from an urban outpatient community and disease isolates from three different hospitals in India.

This study demonstrated that all three categories, which are dealt with individually in detail, contained EMRSA-15 isolates, and four variant patterns of EMRSA-15 by PFGE were present. All isolates studied were urease-negative, PVL-positive and TSST-1-positive and contained the staphylococcal enterotoxin D gene (etd) by PCR. They also amplified variant primers for the clonal complex 22 sauIhsdS1 region characteristic of ST22 (Cockfield et al., 2007). The agr locus is a strong indicator of genetic background and all isolates belonged to agr type I.

**RESULTS AND DISCUSSION**

The aim of this study was to investigate whether EMRSA-15, which is a major epidemic strain in several parts of the world, has now made an appearance in India. We dealt with individually in detail, contained EMRSA-15 isolates, and four variant patterns of EMRSA-15 by PFGE were present. All isolates studied were urease-negative, PVL-positive and TSST-1-positive and contained the staphylococcal enterotoxin D gene (etd) by PCR. They also amplified variant primers for the clonal complex 22 sauIhsdS1 region characteristic of ST22 (Cockfield et al., 2007). The agr locus is a strong indicator of genetic background and all isolates belonged to agr type I.

**Urban outpatient population**

A total of 400 nasal swabs were collected from the outpatient department of the government-run Victoria Hospital from which 52 MRSA isolates and five isolates having characteristics of EMRSA-15 were obtained. Table 1 shows the molecular characteristics of these five urban Indian isolates, VH115, VH165, VH170, VH267 and VH391. The antibiotic resistance patterns are shown in Table 1. All five urban isolates were resistant to meticillin, oxacillin and co-trimoxazole. In addition, VH165, VH170 and VH391 were also resistant to gentamicin and erythromycin; VH115, VH165 and VH170 were resistant to ofloxacin; and VH115 and VH170 were resistant to tetracycline. The MIC for oxacillin of most of these isolates was low except for VH170. When cefoxitin resistance was checked with a 4% NaCl agar plates containing 4% NaCl.
and the variant CRG1250 from the USA. All Indian isolates were grouped in one PFGE pattern as the differences between them were within one to three bands and they differed from classical EMRSA-15 and CRG1250 by three to four bands. All five urban isolates had the same PFGE pattern, which was similar to pattern B4 reported by O’Neill et al. (2001) lacking the sec gene, although none of those isolates contained PVL, TSST-1 or etd.

Rural and tribal population

The prevalence of *S. aureus* in the rural and tribal community was very low (3 %) and that of MRSA was only 0.8 %. These data need to be corroborated with analysis from other rural and tribal communities. Two *S. aureus* isolates (Sa559 and Sa871) from this milieu had PFGE patterns of two EMRSA-15 variants and belonged to ST22 and spa type t005. Both isolates were non-typable but amplified the *orfX* gene, which is common for all SCCmec types. Sa871 was heterogeneous for the *mecA* gene when DNA from several single colonies was tested and was positive for the presence of *ccrC* by PCR. The purified PCR product of *ccrC* was sequenced and was identical to the *ccrC* of type V SCCmec isolates (data not shown). EMRSA-15 isolates from a non-SCCmec type IV background have not been reported until now and they may have evolved independently. Our data on the genotyping of CA-MRSA indicated a much higher percentage of SCCmec type V isolates than type IV, and horizontal transfer could be taking place with some SCCmec type V isolates. Sequencing of the SCCmec region of Sa871 would clarify whether this is a composite cassette of various elements.

Both isolates were resistant to oxacillin, meticillin, gentamicin and tetracycline and were sensitive to cefoxitin in the disc diffusion test in the presence of 4 % NaCl. Sa871 was also resistant to erythromycin and co-trimoxazole.

These two isolates had two different variant patterns of EMRSA-15. The PFGE pattern of Sa559 (Fig. 1, lane 7) was different from that of classical EMRSA-15 and the other Indian isolates with the 208 kb band in EMRSA-15 being replaced by two bands very close to each other at approximately 190 and 195 kb. The third variant pattern was for Sa871 (Fig. 1, lane 16), where a band of around 260 kb was missing and the rest of the pattern was identical to that of the first two variants.

Disease isolates from Indian hospitals

The six disease isolates (M37, Pune08, NMB08, NP113, NP114 and NP115) from pus and blood sent from three different hospitals were characterized as MRSA with SCCmec type IV, *spa* type t852 and ST22 from MLST performed on a representative isolate. Two other urban isolates, VH115 and VH165, had *spa* type t852 and ST22 from their MLST pattern.

The antibiotic resistance patterns of all isolates except for M37 were very similar. M37 was resistant to oxacillin, meticillin, gentamicin and cefoxitin. All the other disease isolates were, in addition, resistant to erythromycin, levofloxacin, ofloxacin and co-trimoxazole. Pune08 was sensitive only to linezolid, teicoplanin, vancomycin and, surprisingly, to cefoxitin (30 μg disc). Although the four non-typable urban isolates belonged to *spa* types t005 and t2986, the PFGE pattern of five of the disease isolates was identical to that of the five urban isolates. Only Pune08 (Fig. 1, lane 9) had an additional band around 80 kb, resembling the classical EMRSA-15 in the lower molecular mass bands and differing from the other variants.

Antibiotic sensitivities

The majority of Indian isolates were resistant not only to β-lactam antibiotics but also to other classes of antibiotics.

![PFGE patterns of Smal restriction digests of MRSA isolates. Lanes: 1, NCTC 8325; 2, classical EMRSA-15; 3, CRG1250; 4–6, 14 and 15, urban isolates VH115, VH165, VH170, VH267 and VH391; 7 and 16, rural isolates Sa559 and Sa871; 8, M37; 9, Pune08; 10, NMB; 11, NP113; 12, NP-115; 13, NP-114.](image)
although β-lactam antibiotic resistance was very low even in the presence of the mecA gene. The very low resistance to β-lactams in these isolates might lead to erroneous treatment. Cefoxitin resistance is a clear indication of the presence of mecA according to large surveys, which is contrary to our findings (Broekema et al., 2009). The MICs of oxacillin and cefoxitin (≤6 μg ml⁻¹) were very low for most of our EMRSA-15 isolates. CA-MRSA strains have a different genetic background with much smaller SCCmec cassettes than those of HA-MRSA. Recent studies implicate the involvement of genes other than the known effectors of meticillin resistance such as PBP4 and the vraS/vraR two-component regulatory system in CA-MRSA (Llarrull et al., 2009). Antibiotic usage in India, which includes widespread suboptimal use of different antibiotics, may also play an important role in the resistance patterns of EMRSA-15 isolates studied here.

**spa types**

Other than the four non-typable isolates, all isolates responsible for soft-tissue infections and cerebral abscesses belonged to spa type t852, making this the predominant spa type among urban outpatient and disease isolates. spa type t005 is present in a large number of isolates belonging to ST22, but there are only 36 isolates with records of spa type t852 in the Ridom SpaServer database (http://www3.ridom.de/spa-server/). The first MSSA isolate with spa type t852 was reported from Portugal, but MRSA isolates with the same spa type have now been reported from Denmark, Germany, New Zealand, Norway and Sweden. spa type t852 is a single repeat variant of t005 where the first repeat r25 is replaced by r07 in t852. spa type t2986 has one less repeat unit than t005 and t852 where r17 is replaced by r16 and r25 is absent. Two other isolates with t2986 have been reported, one from the UK and one from Slovenia. Even with these differences in spa type, the urban isolates belonging to t852, t005 and t2986 had the same PFGE pattern, whilst the two rural isolates with spa type t005 had two variant EMRSA-15 patterns. It has been reported recently that USA300 isolates with two different spa types, t008 and t024, have identical PFGE patterns, and the two may have evolved independently (Larsen et al., 2009).

**PFGE patterns and EMRSA-15 variants**

We have reported four variants of classical EMRSA-15 by PFGE patterns. All the Indian isolates carried the sed, tst and PVL genes. Most EMRSA-15 isolates are known to contain sec. As these toxins are on mobile genetic elements, the Indian isolates may have acquired new enterotoxins and shed others. All isolates were positive for tst by PCR, which is also not typical of EMRSA-15. As the PVL gene is phage-encoded and tst is on a mobile genetic element, there may be specific advantages to the organism in acquiring them. Recently, Wolter et al. (2008) reported an EMRSA-15 variant from the USA which was negative for sec and the PVL gene but positive for tst. These changes indicate significant evolutionary differences. EMRSA-15 could be emerging from a different genetic background, as indicated by these isolates.

**Presence of PVL**

Few EMRSA-15 isolates containing the PVL gene have been reported (Wannet, 2003; Udo et al., 2006). All of our isolates from three different populations were PVL gene-positive. Data from our laboratory show that a high percentage of Indian MSSA and MRSA isolates are positive for the PVL gene and hence it may be easily acquired by horizontal transfer (unpublished data).

**Similarities between urban and disease isolates**

Even in the small number of samples screened, the majority of urban and disease isolates from healthy individuals and those with skin and soft-tissue infections and brain abscesses belonged to spa type t852. The majority of urban outpatients belonged to a lower socioeconomic class and lived in high-density slums. Their nasal swabs contained a higher percentage of MRSA isolates resembling the disease isolates rather than the rural isolates.

**EMRSA-15 in tropical countries**

Most information on invasive S. aureus infections comes from temperate countries. Little is known about the antibiotic resistance patterns of community-acquired organisms that circulate in developing countries where antimicrobials are available from pharmacies without prior consultation with a physician. There are considerable gaps in knowledge of the epidemiology, treatment, drug resistance and outcome of invasive S. aureus infection in the tropics. EMRSA-15 causing cavernous sinus thrombosis and meningitis and cerebral abscess is a rare phenomenon according to a recent survey (Naesens et al., 2009). That the Indian disease isolates responsible for these rare nervous system disorders are similar in characteristics to urban EMRSA-15 isolates is troubling. These are the first Indian variants of EMRSA-15 identified by molecular characterization from healthy and diseased individuals. The fact that all were PVL gene- and tst-positive is a cause of great concern and points to the need for regular surveillance of MRSA in India, especially with increasing international travel.

**EMRSA-15 spread**

EMRSA-15 has been reported as a dominant clonal type replacing HA-MRSA in the Czech Republic, Spain, Singapore and Portugal (Melter et al., 2006; Amorim et al., 2007; Hsu et al., 2007; Vindel et al., 2009). Our CA-MRSA sample numbers were not large enough to assess whether EMRSA-15 is replacing HA-MRSA in Indian hospitals. However, prior to 2005, when we collected HA-
MRSA isolates from eight hospitals in India, we had not encountered CA-MRSA or EMRSA-15 in hospitals. The first EMRSA-15 isolate was sent to us from Hinduja Hospital in Mumbai in February 2005. Most of the other disease isolates were collected in 2008, although the rural and urban nasal isolates were collected in 2006 and 2007.

We have reported here what we believe to be the first characterization of Indian EMRSA-15 isolates whose PFGE patterns indicate four variants of classical EMRSA-15, and four isolates that are non-typable and may have evolved independently. This is also the first report of Indian EMRSA-15 isolates causing nervous system disorders such as brain abscesses.

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Indian EMRSA-15 variants in health and disease


