

Current molecular approach for diagnosis of MRSA: a meta-narrative review

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ABSTRACT

Introduction: Detection and diagnosis of methicillin-resistant *Staphylococcus aureus* (MRSA) are important in ensuring a correct and effective treatment, further reducing its spread. A wide range of molecular approaches has been used for the diagnosis of antimicrobial resistance (AMR) in MRSA. This review aims to study and appraise widely used molecular diagnostic methods for detecting MRSA.

Methods: This meta-narrative review was performed by searching PubMed using the following search terms: (molecular diagnosis) AND (antimicrobial resistance) AND (methicillin-resistant *Staphylococcus aureus*). Studies using molecular diagnostic techniques for the detection of MRSA were included, while non-English language, duplicates and non-article studies were excluded. After reviewing the libraries and a further manual search, 20 studies were included in this article. RAMESES publication standard for narrative reviews was used for this synthesis.

Results: A total of 20 full papers were reviewed and appraised in this synthesis, consisting of PCR technique (n = 7), deoxyribonucleic acid (DNA) Microarray (n = 1), DNA sequencing (n = 2), Xpert MRSA/SA BC assay (n = 2), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (n = 2), MLST (n = 4), SCCmec typing (n = 1) and GENECUBE (n = 1).

Discussion: Different diagnostic methods used to diagnose MRSA have been studied in this review. This study concludes that PCR has been extensively used due to its higher sensitivity and cost-effectiveness in the past five years

Keywords: Antimicrobial resistance, Molecular diagnosis, MRSA

Introduction

Antimicrobial resistance (AMR) is defined as changes in bacteria that result in the drug being used for its treatment becoming inefficacious (1). *Staphylococcus aureus* is an opportunistic pathogen with a tremendous capacity to

adapt to human hosts and healthcare environments, causing detrimental effects to healthcare-associated infections such as bloodstream infections (2). AMR is reported as the world's biggest 21st-century health threat, and the World Health Organization (WHO) is calling for immediate action. As AMR spreads, common infections are becoming incurable. Reports state that over 700,000 die yearly due to drug-resistant illnesses; by 2050, the number is predicted to rise to 10 million (3).

A major issue pertaining to AMR is the excessive and injudicious use of antibiotics that have led to widespread resistant bacteria and dissemination of their antimicrobial resistant genes (ARGs) (4). It is concerning that the AMR rates are predicted to increase if measures are not taken. One way to overcome this is through early detection, which enables effective management, allowing efficient identification and detection of microbes such that the patient can be treated with the appropriate drug in time.

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Over the years, great leaps have been made in the diagnosis of AMR and diagnostic tests are reported to be an essential tool in early diagnosis, hence it is a robust strategy against AMR (4).

To enhance existing approaches, this review aims to summarize new and current molecular techniques and technologies used to identify AMR using a systematic meta-narrative approach, with a focus on the key benefits and drawbacks. Furthermore, a critical overview of recently developed molecular approaches and an informed assessment of future direction will also be discussed.

Methodology

Study design and inclusion criteria

This systematic review was carried out in a meta-narrative framework. This study qualitatively appraised different molecular methods used in the recent 5 years for the diagnosis of methicillin-resistant *Staphylococcus aureus* (MRSA). This study protocol was created according to the RAMESES (Realist And Meta-narrative Evidence Syntheses: Evolving Standards) meta-narrative review publication guidelines (5). Articles that satisfied the following requirements were considered for the review: (i) original articles written

in English that were published between January 2017 and May 2022, (ii) cross-sectional or cohort studies that assessed the technical performance of molecular methods (sensitivity, specificity, accuracy or concordance) for diagnosing MRSA. Articles were excluded if they were: (i) case reports; (ii) review articles, commentary articles, and short communications.

Search strategies

Articles were searched using PubMed. Search keywords were (((((molecular diagnosis) AND (antimicrobial resistance)) NOT (review [publication type])) NOT (systematic review [publication type])) NOT (meta-analysis [publication type])) AND (methicillin-resistant staphylococcus aureus).

Selection and appraisal of articles

Two independent reviewers (Lee and Sim) screened the titles and abstracts. Articles with abstracts indicating the use of a molecular approach to diagnose MRSA were read in full. A final consensus was discussed between the two reviewers, and disagreements were resolved with discussion from the third reviewer (SM). EndNote Version 20 was used for article duplicate removal and archives. All the studies reviewed and appraised in this synthesis are summarized in Table I.

TABLE I - Summaries of studies appraised in this review

No	Author	Year	Country	Condition/patients	Sample	Study design	Molecular diagnosis methods	Reference
1	Moutaouakkil et al	2022	China	Children diagnosed with <i>Staphylococcus aureus</i> OAI	Blood cultures, articular fluids, synovial tissues and/or bone fragments	Prospective study	Multiplex polymerase chain reaction	(6)
2	Jin et al	2022	China	1,952 MSSA strains isolated from blood across 17 provinces	MSSA-PENS isolated from invasive BSIs	Retrospective study	Whole-genome sequencing	(2)
3	Senok et al	2021	United Arab Emirates	135 patients with a clinical diagnosis of severe skin and soft-tissue infections	<i>S. aureus</i> isolates associated with SSTI were tested for PVL detection	n/a	DNA microarray assays	(7)
4	Reddy and Whitelaw	2021	South Africa	231 samples	2,822 patients with positive blood cultures exclusively showing GPCC on Gram stain were included	Prospective study	Xpert MRSA/SA BC assay	(8)
5	Choi et al	2021	South Korea	26 children aged <15 years diagnosed with SSSS	Involved area of the skin, the presence of Nikolsky's sign, and the status of desquamation	n/a	PCR	(9)
6	Anafo et al	2021	Ghana	300 diabetes patients and 106 non-diabetic individuals	Anterior nasal swabs	Cross-sectional	PCR	(10)
7	Verdú-Expósito et al	2020	Ethiopia	80 <i>S. aureus</i> strains isolated from human patients with SSTIs	Human samples	n/a	MALDI-TOF and PCR	(11)
8	Tang et al	2020	China	MRSE strains from the dental plaque of a normal, healthy human population	Dental plaque specimens	n/a	PCR	(12)



No	Author	Year	Country	Condition/patients	Sample	Study design	Molecular diagnosis methods	Reference
9	Khawaja et al	2020	Pakistan	105 samples	Human samples	Descriptive cross-sectional study	PCR	(13)
10	Jin et al	2020	China	65-Year-old healthy man with a history of leprosy	Isolate was obtained from the patient's blood, and identified as an ST9-MRSA strain	n/a	Whole-genome sequencing	(14)
11	Geng et al	2020	China	536 neonates	Nasal swabs	Prospective surveillance study	Staphylococcal chromosomal cassette (and) type, <i>spa</i> type, MLST	(15)
12	Crandall et al	2020	USA	357 children with invasive <i>S. aureus</i> infections	Pleural fluid and/or blood	Prospective study	PCR, MLST, SCC <i>mec</i> typing	(16)
13	Bouza et al	2020	Spain	155 adult inpatients diagnosed with skin and soft-tissue infection	Microbiological samples	Prospective study	Gram stain plus GeneXpert® MSSA/MRSA SSTI	(17)
14	Yang et al	2019	China	269 nonduplicate <i>S. aureus</i> clinical isolates were isolated from children	Steril specimens and non-STERIL specimen using VITEK MS system	n/a	MALDI-TOF	(18)
15	Mutonga et al	2019	Kenya	83 adult patients diagnosed with diabetic foot ulcers	Wound swab cultures	Cross-sectional study	Real-time PCR	(19)
16	Latour et al	2019	Belgium	1,447 residents from nursing homes	Pooled sampling of nose, throat and perineum	Cross-sectional prevalence survey	Triplex PCR and MLST	(20)
17	Hida et al	2019	Japan	263 patients suspected of having staphylococcal bacteremia	Fresh and frozen blood culture samples	n/a	GENECUBE <i>mecA</i>	(21)
18	Luo et al	2018	China	275 isolates of <i>S. aureus</i> , including 148 isolates from patients, 127 from ready-to-eat food samples	Secretions, blood, phlegm, cerebrospinal fluid, transudation, urine, fresh meat, meat product, cereal products, fruits and vegetables	n/a	PCR, multiplex PCR	(22)
19	Lin et al	2018	Taiwan	106 hemodialysis patients diagnosed with MRSA	Blood cultures	Retrospective study	PCR and MLST	(23)
20	Yang et al	2017	China	104 children diagnosed with MRSA	Sputum, bronchioalveolar lavage fluid, skin and soft tissues, pus, secretions, secretions of omphalitis, blood, joint effusion, pleural effusion	n/a	MLST	(24)

BSI = bloodstream infection; DNA = deoxyribonucleic acid; MALDI-TOF = matrix-assisted laser desorption/ionization-time of flight; MLST = multilocus sequence typing; MRSA = methicillin-resistant *Staphylococcus aureus*; MRSE = methicillin-resistant *Staphylococcus epidermidis*; MSSA = methicillin-sensitive *Staphylococcus aureus*; n/a = not available; PCR = polymerase chain reaction; SCC*mec* = staphylococcal cassette chromosome *mec*; *spa* = staphylococcal protein A.

GPCC = Gram positive cocci in clusters; MSSA-PENS = methicillin-sensitive *S. aureus* – penicillin-susceptible; OAI = osteoarticular infections; SSSS = Staphylococcal scalded skin syndrome ; SSTI = skin and soft tissue infections; PVL = Pantone Valentine leukocidin

Results

The dataset includes 20 different authors from Asia (n = 13), Africa (n = 5), Europe (n = 1) and America (n = 1). A total of 20 studies were included in this synthesis: seven

studies employed polymerase chain reaction (PCR) for diagnosing MRSA (6,9,10,12,13,19,22), one study employed deoxyribonucleic acid (DNA) Microarray (7), two studies used DNA sequencing (2,14), Xpert MRSA/SA BC assay (n = 2) (8,17), matrix-assisted laser desorption/ionization-time



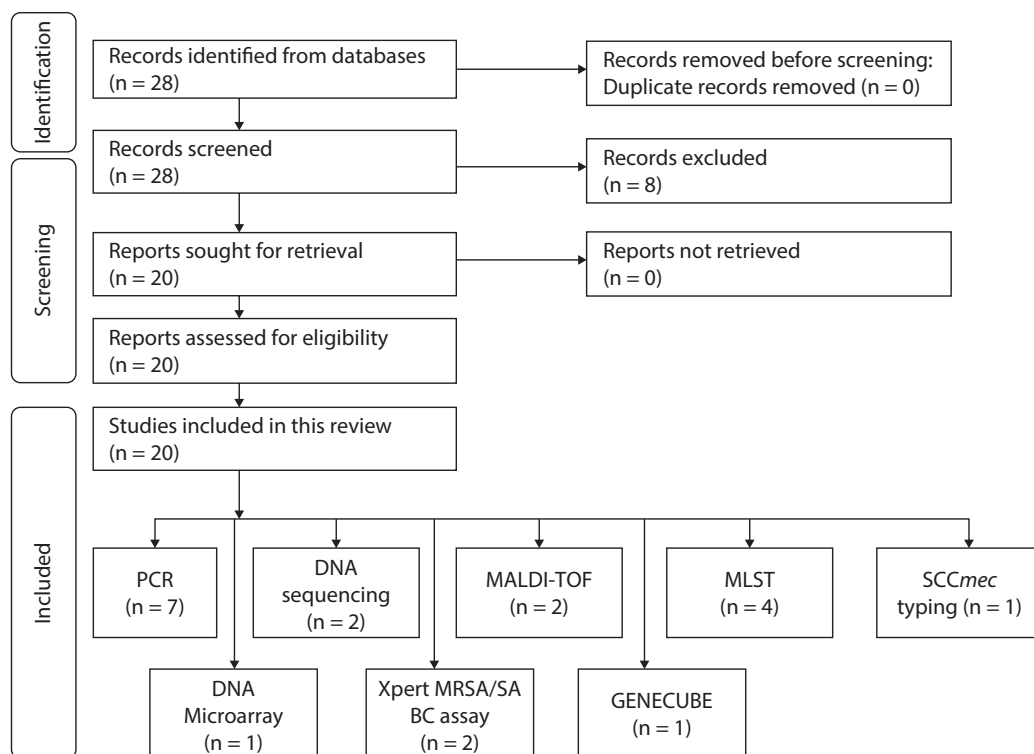


Fig. 1 - Diagrammatic flow of the study selection and list of techniques appraised in this review.

of flight (MALDI-TOF; $n = 2$) (11,18), multilocus sequence typing (MLST; $n = 4$) (15,20,23,24), GENEUCUBE ($n = 1$) (21) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing ($n = 1$) (16). Figure 1 is the diagrammatic flow of the study selection and list of techniques appraised in this review.

Recent molecular methods for diagnosis of MRSA

Polymerase chain reaction

PCR approaches have been commonly used for the effective diagnosis of MRSA, and the rapid emergence of MRSA has led to a series of PCR approaches that have been developed for the identification of MRSA (25). PCR approach identifies *S. aureus* based on a single-base-pair mismatch in the staphylococcal 16S ribosomal RNA gene sequence (26). Recent researchers have also cited the use of the PCR approach for *mecA* gene detection as the gold standard method for the detection and identification of the prevalence of MRSA (27,28). In this synthesis, a total of seven studies have employed PCR for the detection and diagnosis of MRSA. A study conducted by Moutaouakkil and colleagues among patients suspected of *S. aureus* hospitalized in pediatric orthopedic clinic reported the detection of *mecA* using PCR (6). This study also utilized different biological samples such as blood cultures, articular fluids, synovial tissues and bone fragments for the detection of MRSA. Another study showed that the fluorescence signal of real-time (RT)-PCR could display the quantity of products formed

and increases exponentially, enabling a user-friendly diagnostic (29). Furthermore, Mutonga and colleagues (2019) have demonstrated that the sensitivity of RT-PCR for MRSA is 100% (19).

Multiplex PCR amplifies multiple DNA sequences simultaneously, which gives an advantage over conventional PCR (30). The detection of target sequences, such as the *nuc* and *coaA* or elements necessary for methicillin resistance, such as *femA*, or *femB*, has provided the basis for PCR identification of *S. aureus*. It uses two pairs of primers specific to the staphylococcal *nuc* and *mecA* for PCR amplification of a 280-bp *nuc*-based fragment and a 533-bp *mecA*-based fragment (31). Tsai and colleagues (2019) reported *mecA* gene (*mecA*-F and *mecA*-R) is amplified and can be used to diagnose MRSA (32). Chikkala and colleagues showed that it exhibits 97% of specificity and 90% sensitivity (33).

DNA sequencing

DNA sequencing allows the detection of single-nucleotide polymorphisms (SNPs) and known resistance-associated genes and their variations (34). The availability of bacterial genomes in public databases facilitates the use of whole-genome sequencing for MRSA detection. It enables high-resolution characterization of antibiotic resistance (35). Whole-genome sequencing has a definite edge over conventional Sanger sequencing because it may produce millions of reads that are roughly 35 to 700 bp in length (36). There is growing evidence on the effectiveness of bacterial

whole-genome sequencing in controlling outbreaks. Whole-genome analysis, such as DNA microarray, simultaneously identifies relative concentration of different nucleic acid sequences (37). It allows a bulk number of nucleic acid sequences in a mixture to be tested and analyzed. The study by Jin and colleagues (2,14) used StaphyType DNA microarray (Abbott [Alere Technologies GmbH], Jena, Germany) and the INTER-ARRAY Genotyping Kit *S. aureus* (Inter-Array GmbH, Bad Langensalza, Germany) for the detection of MRSA. The study by Senok and colleagues (2021) also reported that DNA microarray exhibited 100% specificity and sensitivity (7). In a study done by Ma and fellow colleagues, Illumina's Nextera DNA library preparation kit was used to create whole-genome sequencing libraries, which were then sequenced on an Illumina MiSeq using the 500 cycle V2 protocol (38).

Xpert MRSA/SA BC assay

Xpert MRSA/SA Blood Culture is an in vitro diagnostic test for *S. aureus* and MRSA. The targeted DNA is amplified using automated RT-PCR and Fluorogenic target-specific hybridization, providing real-time detection of specific genes of MRSA and *S. aureus*. A study by Buchan and colleagues (39) reported the use of blood cultures for the detection of *Staphylococcus* protein A (*spa*) sequences, gene that encodes for methicillin resistance (*mecA*) and *SCCmec*. A study by Reddy and colleagues has shown the performance of the Xpert MRSA/SA BC assay to be 100% in specificity and sensitivity. It shows a failure rate for an interpretable result of just 1.7% (8). However, it is notable that the microbiological sampling should be of high quality to ensure rapid and accurate results, despite the significance of Xpert MRSA system.

MALDI-TOF

MALDI-TOF mass spectrometry (MS) has become a widely used technique for the rapid and accurate identification of bacteria (40). Despite the efficiency and sensitivity of MALDI-TOF, this method's limitation is that new isolates can only be detected if the spectral database contains peptide mass fingerprints (PMFs) of the type strains of specific genera/species/subspecies/strains. This method identifies microbes by comparing the PMF of unknown organisms with the PMFs deposited in the database or matching the masses of biomarkers with the proteome database. A recent study by Tang and colleagues (41) reported that MALDI-TOF MS on intact bacteria combined with a refined analysis framework allows accurate classification of methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA. Esener and colleagues showed that MALDI-TOF has a sensitivity of $99.93\% \pm 0.25\%$, specificity of $95.04\% \pm 3.83\%$, and accuracy = $97.54\% \pm 1.91\%$ (42). MALDI-TOF is low in cost, and analysis can be conducted within a short time, allowing rapid microbial resistance to be detected. Latour and colleagues employed MALDI BioTyper database for bacterial identification of suspected colonies (20). A study by Chen and colleagues has shown that MLST has been used for the past decades for MRSA epidemiological

typing (43). However, it is only based on the sequences of seven house-keeping genes' internal fragments to identify individual isolate lineages.

MLST

MLST is a technique that distinguishes between isolates of bacteria species by utilizing sequences of internal fragment house-keeping genes (44). The strands are sequenced on both side by using an automated DNA sequencer. Different sequences of house-keeping genes found in bacterial species are characterized as distinct alleles. In contrast, seven loci alleles address each isolate's allelic profile or sequence type. Hence, species isolates are unambiguously characterized by a series of seven integers which label the alleles at the seven house-keeping genes. The seven house-keeping genes used in MLST for *S. aureus* are the Carbamate kinase (*arcC*), Shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), Guanylate kinase (*gmk*), Phosphate acetyltransferase (*pta*), Triosephosphate isomerase (*tpi*), acetyl coenzyme A acetyltransferase (18,24,45).

SPA typing

Spa is an important gene virulence factor that allows *S. aureus* to avoid host immune responses (46). It codes for protein A, which is found in the cell wall of *S. aureus* (47). SPA genes were replicated using PCR followed by DNA sequencing (48). This method identifies the polymorphic X region of the protein A gene (*spa*). Based Upon Repeat Pattern (BURP) algorithm was used, and *spa* types with more than five repeats were clustered into different groups, with the calculated cost between group members being less than or equal to 6 (49). Spa typing is evidently reproducible and provides interchangeable information. However, a disadvantage of this method is that it requires additional targets such as *SCCmec*, lineage-specific virulence or resistance genes or alternative polymorphic regions of the *S. aureus* chromosome. Studies included in this synthesis employed Ridom Staph Database and SPA typer tool (<http://spatyper.fortinbras.us/>) (24,50,51). Reports cited that *spa* type of t437 was more prevalent in MRSA (24). A study by Luo and colleagues showed that the most prominent *spa* type was t030, reported to be 15.64% (43/275) (22).

GENECUBE assays

GENECUBE (TOYOBO Co., Ltd., Osaka, Japan) is a fully automated genetic analyzer that uses PCR to amplify a target gene (21). This tool can evaluate up to eight samples simultaneously. The target DNA is amplified, and fluorescently labeled oligonucleotides are used to hybridize targets based on fluorescence intensity changes (52). Data are automatically obtained on the GENECUBE monitor after completion of the assay. The advantage of this assay is that it is time efficient and easy to prepare. GENECUBE tests are anticipated to be clinically valuable for effectively identifying MRSA. Studies have reported the sensitivity and specificity



of the GENECUBE to be 100% (33). The system is accurate, rapid (52 minutes), and reliable; however, it does not detect the *mecC* gene (21).

SCCmec typing

SCCmec is a diagnostic method that divides SCCmec elements into groups based on their structural variations (53). The *mec* complex, which comprises the *mec* gene, its regulatory genes, the *mecl* and *mecR1* genes, and several insertion sequences, confers methicillin resistance (54,55). The specific SCCmec type is determined by combining the *ccr* gene complex and the *mec* gene class. SCCmec typing provides valuable information about the resistance of genes to methicillin and identifies the origin of strains. A recent study by Chongtrakool et al (56) typed SCCmec of methicillin-resistant *S. aureus* strains isolated in 11 Asian countries. Another study showed that 610 of 615 (99.2%) MRSA strains could be classified into four SCCmec elements: type 3A, 370 strains; type 2A, 207; type 2B, 32; type 1B, 1 strain. This study on pandemic MRSA clones in Asia reported the ST59-SCCmecIVa as the most prevalent MRSA clone (15). A study by Chen and colleagues that used the web-based SCCmecFinder reported that this technique is efficient for detecting MRSA (43). SCCmecFinder is a web-based tool for SCCmec typing using whole-genome sequences (<https://cge.cbs.dtu.dk/services/SCCmecFinder/>, accessed on January 11, 2023). The SCCmecFinder website uses read data for whole-genome sequencing or preassembled genome/contigs to determine homology to the complete cassette in prediction of SCCmec types, *mec* complex and J regions (57).

Discussion

This meta-narrative review reports the commonly used molecular methods for the detection of MRSA in the past 5 years. This review has also summarized the advantages and disadvantages of each technique included in this synthesis.

S. aureus is a common cause of community and hospital-acquired infection (58,59). The WHO has regarded it as one of the primary clinical concerns, due to the global recognition of MRSA as a public health issue and the antibiotic resistance pattern of MRSA (60). The primary issue with MRSA is the incidence of multidrug resistance, which remains high (61).

The *mecA* encodes penicillin-binding protein 2a (PBP2a), which is an enzyme responsible for crosslinking peptidoglycans in the bacterial cell wall (62). The low affinity of PBP2a for β -lactams leads to resistance to β -lactam antibiotics, including penicillins, cephalosporins (except ceftaroline and ceftobiprole) and carbapenems (63). Recent reports have reported growing resistance to clindamycin and levofloxacin, necessitating an effective treatment.

The virulence factor of *S. aureus* is multifactorial and depends on a variety of toxins, adhesion, immune evasion and other virulence characteristics (64). Evaluation of the virulence factor is an effective method of predicting how these bacteria would behave in the host, enabling prediction of the onset and progression of an infection. The first stage of staphylococcal infection is when the bacterial cells

connect to the host's tissues. The surface-exposed proteins, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), are made by *S. aureus*, which functions to attach to one or more host extracellular matrix (ECM) components, such as laminin, elastin, fibrinogen, fibronectin and collagen (65,66). The extracellular adherence protein (Eap) produced by *S. aureus* is a member of the SERAMs (secretable expanded repertoire adhesive molecules) family, binds to ECM glycoproteins, including fibronectin, fibrinogen, sialoprotein and collagens (67). This protein is involved in the internalization of bacteria and the adherence of *S. aureus* to fibroblasts. Proteases are crucial virulence factors for *S. aureus* and can cleave host proteins to enable MRSA cells to change from an adhesive to an invasive phenotype.

Early diagnostic and therapeutic intervention in patients with MRSA infection risk factors is essential (68). Treatment with empiric antibiotics against MRSA should not be delayed in the event that MRSA infection is diagnosed. Molecular diagnostic tests can robustly identify staphylococcal species in clinical samples, thus improving antimicrobial stewardship (69).

In this review, multiple molecular methods such as PCR, DNA sequencing, Xpert MRSA/SA BC array, MALDI-TOF, MLST, SPA typing and SCCmec typing, have been appraised. This review summarizes that PCR technique has been widely used for the diagnosis of MRSA within the last 5 years (2017-2022).

PCR technique is frequently and commonly used to detect *S. aureus* and it identifies a single-base-pair mismatch in the staphylococcal 16S ribosomal RNA gene sequence for detection (26). PCR assay is cost and labor effective and can be conducted within a short period of time (70,71). However, studies have reported that different target genes may impact the specificity and sensitivity of PCR for diagnosis. The *nuc* gene has a 100% success rate (25,72). Several PCR techniques such as multiplex PCR, RT-PCR and isothermal identification have been developed to identify MRSA as a result of its rapid emergence. The *mecA* and *nuc* genes are being used due to their 100% sensitivity and 97% specificity respectively with a shorter turnaround time of 48 hours (73,74).

The second commonly used molecular techniques are SCCmec typing and MLST, respectively. Over the years, the structures of novel SCCmec have been identified and verified by molecular cloning and traditional sequencing (75). In a study by Singh-Moodley and colleagues (76), SCCmec typing method was used to replace multiplex PCR and was employed to classify additional un-typeable SCCmec elements based on *ccr* and *mec* gene complex combinations. However, this technique has been deemed highly complex because the SCCmec region is variable and newer types are permanently being developed. Another possible reason for using SCCmec typing could be its potential as a benchmark for testing for the *ccr* gene and *mecA* gene compared to other methods.

MLST is well-established and assigns alleles at multiple house-keeping loci directly by DNA sequencing. Sequence type is obtained based on the alleles identified at each of the seven loci using the SA MLST database. MLST detection of MRSA is based on the sequencing of the seven house-keeping conserved genes in the bacterial chromosome (77). MLST is also widely used due to its straightforward procedure for characterizing isolates of bacterial species (78). Due to



numerous alleles in each of the seven loci, it is unlikely that two isolates will have the same allelic profile. Instead, isolates with the same allelic profile can be identified as belonging to the exact clone. MLST has several advantages: (1) it uses sequence data to detect changes at the DNA level; (2) it is readily reproduced and does not require specialized reagents or training; (3) it does not require high-quality genomic DNA; and (4) the data generated are fully portable (79). The disadvantage of MLST is that it only uses seven genes, limiting its ability.

DNA microarray and Xpert MRSA/SA BC assay are the least used in the last 5 years. DNA microarray contains covalently immobilized probes specific for about 180 genes and 300 alleles of *S. aureus* (80). It allows simultaneous detection of the presence of numerous genomic loci. Studies have reported that DNA microarray may serve as an alternate molecular typing method, offering complementary characterization of the MRSA strains. However, this technique is labor and cost extensive and a single experiment could significantly increase the budget of the experiment. Subsequently, many probe designs are based on a sequence of relatively low specificity, sensitivity and accuracy (81).

Conclusion

This meta-narrative review has appraised and summarized molecular diagnostic methods frequently used to detect MRSA in the last 5 years (2017-2022), thus concluding that PCR technique is the most frequently used technique due to its high specificity, low cost and labor effectiveness.

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Disclosure

Conflict of interest: All authors declare no conflict of interest.

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