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Review

MEMS biosensors for detection of methicillin resistant *Staphylococcus aureus*

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ABSTRACT

This review presents the current state of the conventional methods, microfluidic based biosensors, and the commercial products used in the detection of methicillin resistant *Staphylococcus aureus* (MRSA), which is one of the most important threats of nosocomial infections in many parts of the world. The early detection of MRSA in the specimens of the patients is important to enable the appropriate treatment, to decrease morbidity and mortality rates, and to manage control actions in the healthcare units. Thus, rapid and inexpensive diagnostic systems with high sensitivity and specificity are essential to prevent MRSA to be an emerging public health threat. The design and fabrication of new diagnostic systems necessitates working in collaboration between different disciplines to make new challenges in the field of clinical diagnosis and to meet the demands of clinicians. It is certain that in the near future, MEMS and nanotechnology based detection methods will take the place of current methods in clinical diagnosis. The evaluation of new trends for specificity, sensitivity, cost effectiveness, disposability, low weight, ease of use, and facile access should be taken into consideration.

Contents

1. Introduction

Since the early 1990s, there have been a great effort and success towards the development of micro total analysis systems (μTAS or lab-on-a-chip) in fields ranging from health to biosecurity, by using microfluidics and Micro Electro Mechanical Systems (MEMS) technology. Lab-on-a-chip systems have the ability of integration of processes in laboratories and detection of biological analytes at the point of care (Focke et al., 2010a). The advantages of micro scale structures in clinical diagnostics are numerous and include portability, high sensitivity, low cost, and handling samples with small volumes. With these advantages, the interest in developing new methods for detection of biological analytes like bacteria, virus, etc. has increased and this rise is mostly due to the increase in cases of cancer, sexually transmitted diseases, and infectious diseases (Deisingh and Thompson, 2002). Among them, especially the control of infectious diseases is becoming an important danger due to continuous rise in the number of pathogens with antibiotic resistance (Okeke et al., 2005).

One of the most important threats of nosocomial infections in many parts of the world, including Europe, North Africa, USA, and Far East (Klonoski et al., 2010) is methicillin resistant *Staphylococcus*

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aureus (MRSA), whose indicator is *mecA* gene. *Staphylococcus aureus* is naturally found in skin and nose flora and it has the greatest importance because of its ability to adapt different living conditions, intrinsic virulence, and ability to cause a range of illnesses from minor skin infections to life threatening infections (Freeman-Cook and Freeman-Cook, 2006; Lowy, 2003). It has a mechanism to make drugs passive and thus its resistance to a great number of bactericidal antibiotics increases day by day. The prevalence of MRSA, which increased very rapidly in the last two decades, can differ between nationalities, age groups, and specimens taken from blood, wound, and nose (Bordon et al., 2010). For example, in Japan, although the first isolation for MRSA was done in 1980, the prevalence of MRSA in the last decade is 40–70%, which is the highest prevalence of MRSA throughout the world (Kunishima et al., 2010). This is believed to be the result of inappropriate antimicrobial drug consumption and due to the lack of intensive research on infectious diseases.

Early diagnosis and initiation of appropriate treatment are necessary to decrease morbidity and mortality rates related to MRSA based infections (Lindsey et al., 2008). It is evident that patients infected with an antibiotic resistant organism will be ill for a longer period compared to other patients, and need prolonged treatment, which means added financial burden both on clinics and on patients (ETAG, 2006). In the statistical brief of Healthcare Cost and Utilization Project (HCUP) of Agency for Healthcare Research and Quality (U.S. Department of Health and Human Services), it is stated that the number of cases for MRSA infections were increased nearly 10-fold between 1995 and 2005. The approximate cost of hospital stays and duration of hospitalization for MRSA infections were $14,000 and 10.0 days while they were $7600 and 4.6 days for other stays. The cost of treatment of MRSA infections to USA in 2004 was over 4 billion U.S. dollars representing a huge economic burden to healthcare units (Elixhauser and Steiner, 2007). It is obvious that reducing diagnosis time can also reduce the duration of a patient's stay at the hospital and increase the saving costs.

In clinics, conventional culture based methods are mostly employed because of their ease of use and low cost. However, they are time consuming since it is necessary to incubate samples at least 24–72 h to ensure resistance to methicillin after detection of bacterial growth by an automated continuous-monitoring blood culture system (Klonoski et al., 2010). Thus, use of molecular methods is a golden standard for diagnosis not only for its high sensitivity, but also for its promptness. The use of polymerase chain reaction (PCR) based assays has been started in clinical diagnosis in order to increase sensitivity and to reduce time to get the result. However, these assays require expensive equipment and trained personnel.

In order to overcome all of the above-mentioned drawbacks, MEMS based biosensors have been developed by integrating them further to form lab-on-a-chip systems. In the use of this novel technology, it is possible to fabricate wells, reactors, channels, and electrodes in micro scale to perform biological operations like growing cultures, amplification of nucleic acids, and detection of biological analytes; and to work one-to-one with them in these devices. Fabricated surfaces can be activated by using surface chemistry (especially self assembled monolayers (SAMs)) for adsorption of biological samples and responses can be converted into mechanical, optical, or electronic signals by using detection markers that will amplify the signal. It is also possible to detect multiple biological analyte in the same sensor by forming parallel units, which will add on to the advantages of cost and efficiency.

This review covers conventional methods briefly and focuses on MEMS based biosensors, which are currently available for detection of MRSA. First, the basic knowledge on the properties and bacterial function of *S. aureus* are covered. Then, the emergence of antibiotic resistances in *S. aureus* and clinical importance of MRSA are presented. Current detection strategies such as culturing, immunological approaches, and MEMS based biosensors are given. Detection principles of the successfully commercialized products presented for *in vitro* applications are introduced. Finally, future trends and conclusion are discussed.

2. Staphylococcus aureus

S. aureus, which is naturally found in skin and nose flora, is one of the 32 species in *Staphylococcus* genus and is the leading cause of health care associated infections. It was discovered in 1884 by Anton Rosenbach but the origin of *S. aureus* is not known (Deurenberg and Stobberingh, 2008).

"Staphyle" and "cocci" mean "bunch of grapes" and "spherical bacteria" in Greek, respectively. The name "aureus" means "gold" in Latin since bacteria grow in large yellow colonies. Its size is about 1 μm. *S. aureus* is a prokaryote and it has a typical bacterial structure except flagella. The outermost layer (capsule) contains polysaccharides, which play important role in the attachment of *S. aureus* to inert surfaces or to their hosts. Peptidoglycan layer comprises two sugar derivatives (N-acetylglucosamine and N-acetylmuramic acid), and amino acids (L-alanine, D-alanine, D-glutamic acid, glycine and lysine). Its thickness determines the strength of the cell wall (Garcia-Lara et al., 2005; Madigan et al., 2000). In peptidoglycan structure of *S. aureus*, there are about 15 repeating units and, its chain length is 30 peptide units (Dworkin et al., 2006). Since it is involved in bacterial cell division, the prevention of its formation can be bactericidal. The cytoplasmic membrane, which is a device for energy conservation in the cell, prevents the leakage of nutrients into or out of the cells. The membrane is also the site of proteins and enzymes, which are involved in transport of constituents in or out of the cell. Lastly, the cytoplasm consists of ribosomes, nucleoid, and storage granules.

3. Antibiotic resistance and MRSA

Antibiotic resistance is the ability of an organism to resist the effects of an antimicrobial to which it is normally susceptible. The antimicrobial resistance can be in two ways: intrinsic or acquired. In case of intrinsic resistance, bacteria do not have the structure that antibiotic inhibits, it is impermeable to the antibiotic and able to change the antibiotic to passive form (Taylor-Robinson and Bebear, 1997) while acquired resistance is encoded at either the chromosomal or the plasmid level (Madigan et al., 2000; Taylor-Robinson and Bebear, 1997). The resistance is due to the mutations in the chromosome in chromosomal basis. In the plasmid basis, bacteria inactivate the drug by the genes encoding new enzymes (Madigan et al., 2000). Moreover, the bacterial colony can have multi resistant properties because of accumulation of genetic elements with newly developed antibiotics (Nordberg et al., 2005). There are four different mechanisms of resistance in principal: antibiotics can be (1) altered by the enzymes to passive form, (2) kept out from cell entry, (3) inactivated by enzymatic degradation, and (4) ejected from the cell.

Antibiotics can be divided into two groups according to their inhibition sites: cell wall active and non-cell wall active. Cell wall active antibiotics are β-lactams (penicillin, methicillin, etc.), fosfomycin, and glycopeptides (vancomycin) while non-cell wall active antibiotics are protein synthesis inhibitors, aminoglycoside, chloramphenicol, fusidic acid, macrolides, mupirocin, rifamycin, and tetracyclines (Smith and Jarvis, 1999). Among them, β-lactam antibiotics are the mostly used group in medical field due to their specificity and non-toxicity to host cells.

Penicillin G, which is one of the β-lactam antibiotics, is produced by fungus *Penicillium chrysogenum*. Its action is on infections related with gram-positive bacteria. Although it was introduced in

1941, the recognition of penicillin resistant *staphylococci* was as early as 1942 (Lowy, 2003; Smith and Jarvis, 1999). This resistance (around 80%), was due to the production of penicillinase, which is responsible for hydrolysis of β-lactam ring, in penicillin-resistant staphylococcal isolates. Two adjacent regulatory genes, the repressor *blaI* and antirepressor *blaR1* control the activation of *blaZ*, which encodes penicillinase. This enzyme is produced when the regulatory genes are cleaved sequentially. In the presence of penicillin, *blaR1* cleaves itself and activates *blaI* with the cleaved proteins, which leads to penicillinase production (Lowy, 2003; Pantosti et al., 2007).

After the discovery of penicillin resistance, the β-lactam ring was changed in 1960 by the introduction of semisynthetic penicillin, methicillin, in clinical use (Jeljaszewicz et al., 2000). After one year, the first resistance was reported (Berger-Bächi and Rohrer, 2002). The resistance of methicillin in *S. aureus* has the acronym (MRSA) for methicillin resistance *S. aureus*. This acronym is also used for oxacillin resistant *S. aureus* since the mechanism of resistance in both antibiotics is based on the production of penicillin binding protein (PBP2a or PBP2′), which is encoded by chromosomal *mecA* gene, with low affinity to β-lactams (Prere et al., 2006). Production of peptidoglycan layer is catalyzed by PBPs, which are membrane bound enzymes. When methicillin is present, PBP2a replaces with PBPs and enables the bacteria to survive in the presence of antibiotics due to its low affinity to β-lactams (Lowy, 2003). Therefore, methicillin resistance spreads out all β-lactams and their derivatives. Similar to the penicillin resistance, two adjacent regulatory genes, the repressor *mecI* and antirepressor *mecR1* also control the activation of *mecA*, which encodes PBP2a. The response of *mecA* to β-lactams is similar to the regulation of *blaZ* (Pantosti et al., 2007). The methicillin resistance determinant, *mecA* gene is a part of the genomic island and placed on *Staphylococcus* cassette chromosome *mec* (SCC*mec*). The resistance levels are based on the "genetic background of the strain into which SCCmec has entered" (Berger-Bächi, 2002). In healthcare-associated MRSA (HA-MRSA), *mecA* gene is placed on Type I–III SCCmec whereas in community-acquired MRSA (CA-MRSA) it is placed on Type IV SCC*mec* (a and b, AB063172 and AB063173, respectively) (Graves et al., 2010; Hiramatsu et al., 2001). Type IV element is more mobile and shorter than the other types of element and it does not have any other antimicrobial resistance (Hiramatsu et al., 2001; Lowy, 2003).

4. Clinical importance and prevalence

S. aureus belong to the normal bacterial flora of the skin and nose. However, it can cause illnesses from skin infections to sepsis and the severity of illness increases with its methicillin resistant form. Treatment failure due to an inappropriate antibiotic usage and lack of efficacy of anti-MRSA drugs increase morbidity and mortality. Therefore, it is important to prevent transmission of MRSA. Controlling actions like patient isolation, use of disposable gloves etc. are necessary and they increase the burden on patients and healthcare units (Gould, 2005). In some countries like Germany, The Netherlands, and New Zeland, the national guidelines representing the requirements for the control and the prevention of MRSA infection are available. These guidelines include literature review, description of infection, evidence rates, graded or no graded recommendations in major areas like hand hygiene, surgery, decolonization, screening of patients and staff, antibiotic use, and environmental cleaning (Humphreys, 2007).

According to the European Antimicrobial Resistance Surveillance System Report in 2009 (ECDC, 2010), 30,680 nosocomial isolates were collected from 28 European countries and 5965 of them were methicillin resistant. The prevalence of MRSA is different in many parts of the world. It is <1% in northern countries and it increases <50% in southern countries of Europe. Between 2006 and 2009, the trend of prevalence significantly decreased in eight countries including France, Austria, Latvia, Bulgaria, and United Kingdom, while the prevalence in Czech Republic increased 15%. Although a decrease is observed in the collected data of some countries, the MRSA is still a serious health problem all over the world. In USA, the MRSA rate was higher than 50% in 2008 (Bordon et al., 2010). On the other hand, in Asian countries like Singapore and Pakistan the prevalence is 20%, while it is 65% in Taiwan, 56.8% in Hong Kong, 38.1% in The Philippines, 57% in Thailand, 22.6% in India (Reinert et al., 2007; Song et al., 2011). However, it is difficult to compare the prevalence in different countries. Since, these differences may be the outcome of the use of different diagnostic techniques in laboratories and the clonal differences in the genome of the bacteria (Humphreys, 2007).

The above-mentioned control and prevention techniques and the high rates of prevalence in almost all over the world state expressly the importance and the rise in MRSA isolates in human population. Therefore, the rapid and accurate identification is important for timely treatment and the prevention of the spread of MRSA.

5. Strategies for detection

In this section, conventional and MEMS based methods to detect MRSA will be given. Most of the current methods are based on enhancing the expression of resistance in modified cultures, while MEMS based approaches are based on the amplification of signal to enhance detection limits.

5.1. Conventional methods

There are different methods to distinguish methicillin resistance from susceptible strains: agar dilution, E-test (AB Biodisk, Solna, Sweden), breakpoint methods, disk diffusion tests (e.g., cefoxitin, and oxacillin), latex agglutination test which depends on the detection of PBP2a, and molecular methods (e.g., use of radiolabeled DNA probes, and PCR to detect *mecA* gene) (Brown et al., 2005). The expression of resistance in detection of MRSA is strongly based on the selective test agent, NaCl concentration, culture medium, inoculum, incubation temperature, and minimum inhibitory concentration (MIC) breakpoint (Brown, 2001). The selective test agent is oxacillin in almost all culture-based methods since methicillin is no longer being produced. As an alternative to oxacillin, cefoxitin is also used in disk diffusion tests and gives results that are more reliable. Although addition of NaCl increases the expression of resistance in some mediums such as Mueller–Hilton, some strains are adversely affected from high concentration of NaCl. Addition of 2% NaCl into the medium is sufficient to have good results. It is inhibitory to add more than 5% NaCl to the test medium. Temperatures range from 30 to 35 °C is commonly used in incubation. Except for the molecular methods, it is necessary to incubate samples for at least 24 h. Since resistant sub-populations of some strains grow slowly, it may be necessary to incubate samples for 48 h to ensure the resistance. However, further culturing increases the cost and retards to get results (Struelens et al., 2009). MIC breakpoint for oxacillin >2 mg/l indicates resistance for dilution methods and breakpoint methods, while in disk diffusion methods MIC is determined with reduced zones or rings of inhibition around the antibiotic disk. However, optimal conditions for detecting resistance changes with the type of the strains that will also affect the methicillin MIC values. Moreover, heterogeneous MRSA populations and strains with extremely low-level methicillin resistance are susceptible to the most of the non-β-lactam antibiotics, and routine oxacillin tests may lead to false positive results in the use

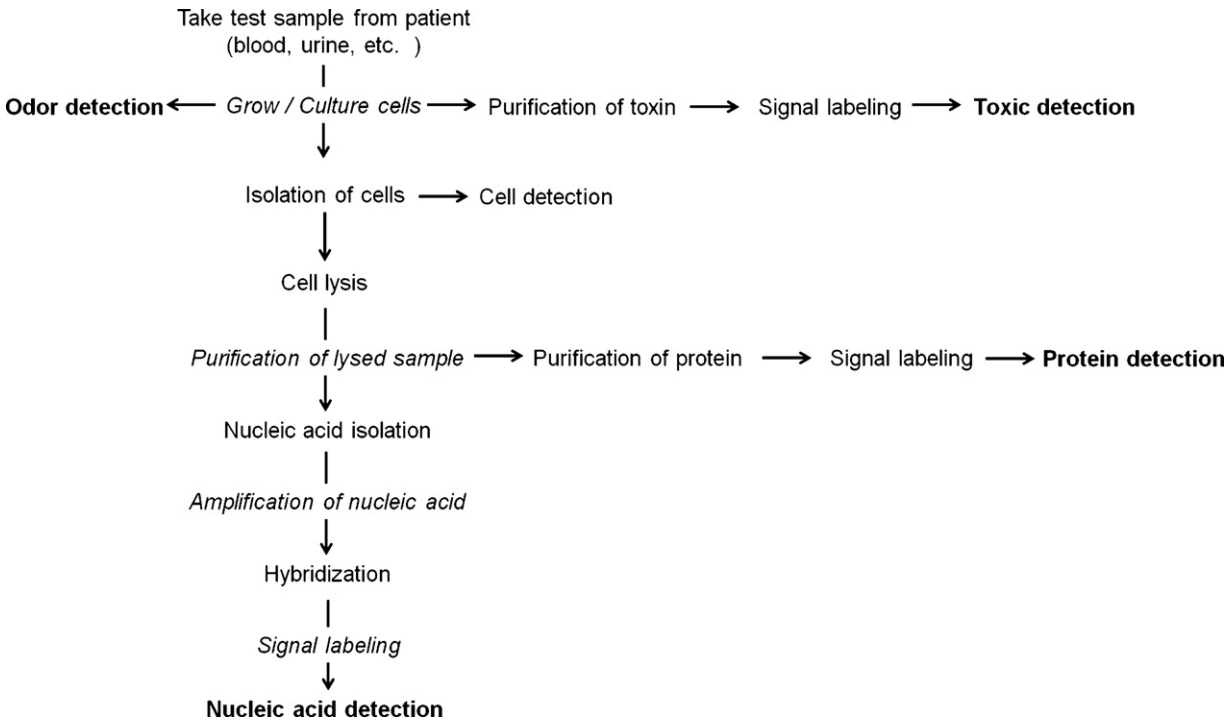

Fig. 1. Main steps of diagnosis with μTAS. (The steps written in italics can be eliminated in μTAS devices.)

of culture based methods (Berger-Bächi and Rohrer, 2002; Felten et al., 2002). Therefore, a reference, which is determined by using a dilution method, is used for comparison in evaluating the performance of the test. The reference method is now being replaced by molecular methods that are regarded as gold standard due to high sensitivity and specificity. The molecular methods generally rely on PCR to detect *S. aureus* (*fem* genes) and *mecA* gene, whose presence directly indicates resistance to methicillin, after primary culturing.

Although the conventional methods maintain their place in clinical diagnosis, MEMS based detection methods are becoming popular due to their numerous benefits.

5.2. MEMS based methods

Lab on a chip, which is also known as micro total analysis system, is an integrated small-scale device (or miniaturization) of the laboratory operations by combining microfluidics and MEMS. Such laboratory operations can be sample preparation units, integrated detection modules, culturing wells, amplification of nucleic acids or cell lysis (Lee and Lee, 2004). There are numerous advantages of these chips, which make them applicable in various biological research areas: low weight, portability, fast response time, fabrication in mass production, high sensitivity, and necessity of low volume samples.

In fact, the main steps for clinical diagnosis in conventional methods are almost the same in μTAS devices except for the number of manual handling steps (Fig. 1). Also, some of the steps (e.g., grow/culture cells, amplification of nucleic acid, etc.) may not be necessary in μTAS devices, since the specificity and selectivity in these devices can be higher than the current methods.

There are mainly four different methods and their combinations in the detection of biological analytes: optical, mechanical, magnetic, and electrochemical. In optical detection, detection can be realized as labeled or label-free (Boedicker et al., 2008; Focke et al., 2010a, 2010b; Guntupalli et al., 2008; House et al., 2010; Lagally et al., 2004; Lutz et al., 2010; Shen et al., 2010; Sista et al., 2008). In labeled strategies, fluorescent dye molecules, quantum dot labels, and metal colloid labels are generally used. The working principle for label based optical detection is that, DNA molecule is conjugated with a dye molecule with a specific excitation and emission wavelength and this excitation is monitored by using optic devices like fluorescence microscope, surface plasmon resonance enhanced fluorescence, fiber optic sensor arrays etc. On the other hand, in label free detection, imaging surface plasmon resonance (SPR) and imaging ellipsometry are commonly used. The major disadvantages in optical detection are necessity of expensive optical devices and an expert to analyze the results. Although the resolutions of these devices are relatively low, they are preferred due to their rapidity (Bally et al., 2006). The working principle of magnetic detection is based on conjugation of paramagnetic and ferromagnetic nanoparticles to biological analytes (Wang et al., 2011a). The dimensions of these particles change between 0.5 μm and 1 μm and can be produced by using different methods. Although producing magnetic nanoparticles is easy and its application is wide, the use of magnetic nanoparticles may not give reproducible results at every point of the sensor and does not have high sensitivity, which is required for some applications such as mutation detection. Moreover, magnetic sensors cannot be used for multiple detections of biological analytes with one sensor, and magnetic particles have the possibility of reacting with the buffer solutions that are used in experiments (Palecek and Fojta, 2007). Mechanical sensors have two different sensing strategies: (1) Determination of change of surface stress, and (2) determination of mass change. Biochemical reaction on the cantilever surface of a mechanical sensor results in a change of stress. Adsorbed biological analytes also results in stress change in cantilever structure. Spring constant, which is one of the design parameters, can be decreased to increase the stress sensitivity. In determining mass change, the cantilever surface is activated by using a complementary probe of the biological target analyte. When the fluid is passed over microcantilever, active sites adsorb the biological analytes and it changes the resonance frequency of the cantilever. Although there are wide research on cantilevers, the sensitivity of cantilever is still questionable since its resonance frequency is affected from factors like environment, fluid velocity, etc.

(Fritz et al., 2000). On the other hand, electrochemical detection, which includes amperometric, potentiometric, and conductometric detection, allows one to transfer electronic output signal of a biological reaction to the macroscopic environment, and the magnitude of this signal depends on the electrode surface area and the reaction on the surface, rather than the reaction volume as in the optical detection (Gebala et al., 2011; Wang et al., 2011b). Moreover, it is possible to fabricate the microelectrodes directly in the micro channels by using micro fabrication technology (Verpoorte and De Rooij, 2003).

The classification of the developed biosensors for the identification of MRSA can be done either by the type of detection or by the biological analytes used in the detection. In this study, the classification is made according to the biological analytes for elucidating the sensitivity and specificity of the biosensors in relation to the selected agents. There are five different biological analytes studied by researchers to detect MRSA by using MEMS based devices: odor, cell, modified bacteriophage, toxin gene, and *mecA* gene.

In the detection of volatile organic compounds (odor) produced by MRSA, methicillin sensitive *Staphylococcus aureus* (MSSA), and *S. aureus*, an electronic nose which has an array of 32 carbon black polymer composite resistive sensors is used for analyzing the specimens taken from the infected parts of patients' ear, nose, and throat (Fig. 2a) (Dutta et al., 2005). The electronic nose consists of two parts: A detection system and an analyzing system. Detection is based on the decrease in conductance between carbon sensors, which increases the total resistance, due to the expansion of carbon surfaces when odor is sniffed to the device. Without necessitating culturing before sniffing, the analysis of the signals taken from the array is done by using exploratory techniques like principal component analysis, self-organizing map network, and Fuzzy C Means. Then, three different types of bacteria are analyzed by using artificial neural network classifiers to evaluate the classification performance. Among the neural network classifiers, Radial Basis Function Network (RBF) gives an accuracy of the system, which is above 99%. However, it is very hard to differentiate species, since the acquired data is very complex and requires detailed analysis.

In the study of Boedicker et al., a PDMS based microfluidic chip was developed to form nanoliter droplets to determine MRSA susceptibility to antibiotics (ampicillin, oxacillin, cefoxitin, vancomycin, and erythromycin) and to differentiate MRSA and MSSA from the specimens of human blood plasma (Boedicker et al., 2008). Detection principle is based on the confinement of a single cell, fluorescent dye, and antibiotics into a plug. The increase in fluorescence intensity in these plugs presents the resistance of the bacteria to the antibiotic in the plugs. This "stochastic confinement" based detection allows rapid MRSA detection and antibiotic susceptibility testing without prior incubation of samples. The time to reach the result is directly proportional to the size of the plugs and independent from the initial concentration of the sample and it takes about 2 h for 1 nL plugs with bacterium. The limit of detection is about 10^5 CFU/ml.

In the study of Guntupalli et al., a bacteriophage, which has specific lytic activity to *S. aureus* and MRSA, was used as a bio selective agent (Guntupalli et al., 2008). This bacteriophage was adsorbed on the glass surfaces using Langmuir–Blodgett (LB) technique with a sensitivity of 10^6 CFU/ml to form a monolayer. The advantage of the immobilization technique, which is the reduced non-specific binding, was merged with quantification capability of a light microscope system to accelerate the detection of MRSA. The detection was performed in the aqueous samples with concentrations ranging from 10^6 to 10^9 CFU/ml without labeling and preincubation of bacteria. However, the authors stated that it is necessary to analyze one more analyte (e.g., PBP2a, *mecA* gene) other than the bacteriophage to ensure the specificity of the test.

In detection of genes, it is necessary to lyse the cells, purify the sample and, if the amount of biological analyte in the sample is too low to be detected, it is necessary to amplify either the nucleic acid or the output signal or both of them to have more accurate results. Nucleic acid amplification can be done by using PCR, one cycle of which is composed of template denaturation, primer annealing, and primer extension in a thermal cycler. At each cycle, the amount of the target sequence is doubled. When the fluorescently labeled oligonucleotide probes or DNA stains are used during PCR process, the product of PCR allows quantitative results that can be analyzed in real time (real time PCR). There are also different isothermal nucleic acid amplification techniques like loop mediated amplification (LAMP) (Aryan et al., 2010), transcription mediated amplification (TMA) (Moller et al., 2008), nucleic acid based amplification (NASBA) for selective amplification of RNA (Compton, 1991), rolling circle amplification (RCA) driven by DNA polymerase (Lizardi et al., 1998), and recombinase polymerase amplification (RPA) (Piepenburg et al., 2006).

In the study of House et al., a four well PDMS based PCR chip was used in the detection of MRSA for three different samples, which are purified DNA, crude DNA, and boiled culture (House et al., 2010). Amplification of DNA concentrations as low as 3.73 pg/μl was shown possible with the chip, which was made of PDMS, parylene, and a glass plate to reduce the cost. On the other hand, in the study of Sista et al., the development of electro wetting-based digital microfluidic platform was presented for rapid immunoassays, DNA amplification by using real time PCR, and sample preparation for MRSA detection from whole blood samples (Sista et al., 2008). A cartridge with its chip and cover plate was used in the immunoassays, DNA amplification, and DNA sample preparation. The cartridge had inlet and outlet ports, droplet-dividing units, and an electrode array for moving droplets inside the package, while the cover plate was used to have parallel configuration (Fig. 2b). It was shown possible to perform on-chip immunoassay protocols in less than 10 min and to amplify 400 bp genomic DNA of MRSA in 12 min for 40 cycles by using real time PCR technique with the cartridge.

Integration of microfluidic device with RPA was successfully performed by using a lab on a foil cartridge (Lutz et al., 2010). This cartridge was a product of simple fabrication of a cyclic olefin polymer (COP) and at each cartridge; six identical fluidic devices were fabricated with 30 different reaction chambers (Fig. 3a). It was shown possible to perform different microfluidic unit operations like storage, mixing, and dividing the sample into different fragments. The reagents for RPA were prestored and their delivery to reaction zones is controlled by centrifugal forces, which were generated by a modified type of commercially available thermal cycler for RT-PCR (Rotor-Gene 2000, Corbett Life Sciences, Australia). The analyzed DNA sample had the size of 420 bp as a part of *mecA* gene of *S. aureus* and all reactions are performed at 37 °C. The detection was performed optically by measuring real time fluorescence intensity at the reaction chambers. The detection limit of the cartridge was less than 10 copies with no cross contamination between the reaction zones. Detection time was less than 20 min including the time necessary for loading samples to the cartridge and placing the cartridge into the Rotor-Gene 2000.

The integration of magnetic bead based detection with LAMP process was studied recently by Wang et al. (2011a). A microfluidic chip, which consists of three structural layers made of glass, PDMS, and metals, was presented for the specific detection of MRSA directly from three types of specimens, which are sputum, serum, and milk. Protein A (spa) gene was used for identification of *S. aureus,* and *mecA* gene was used for the detection of methicillin resistance in the identification of MRSA. Hybridization was conducted by using magnetic bead conjugated complementary probes. Cell lysis, DNA isolation, and amplification by using LAMP technique were performed totally in the microfluidic device. The amplified

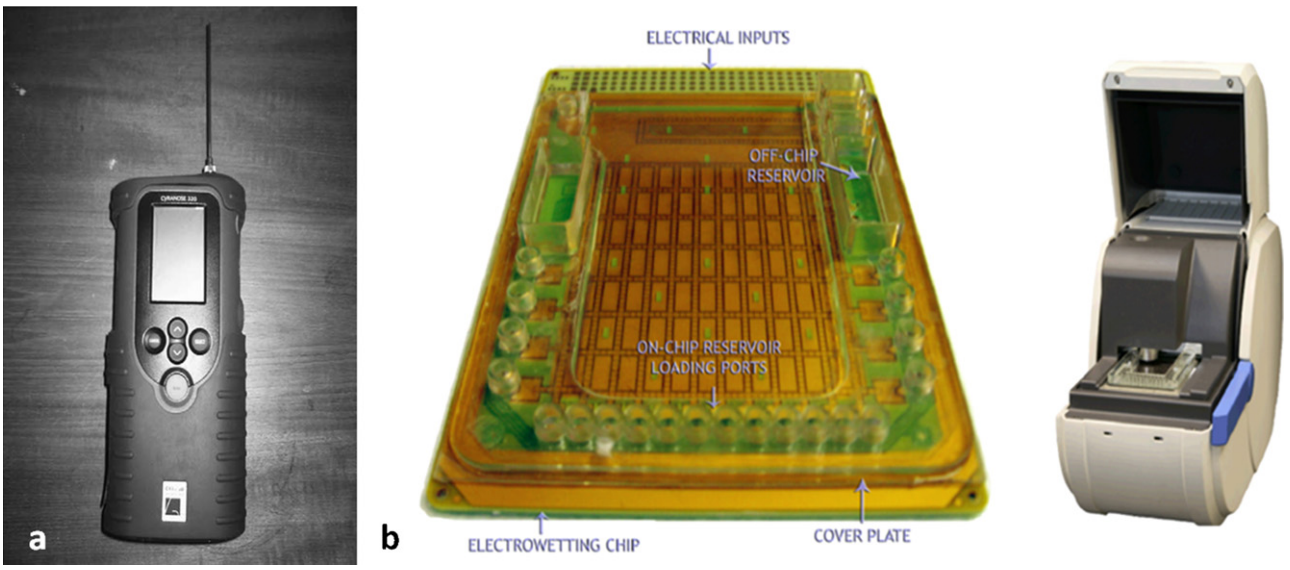

Fig. 2. (a) E-nose used in the odor based detection of MRSA. (b) Multiwell plate cartridge and its control instrument. Reprinted with the permissions of Elsevier and RSC, respectively.

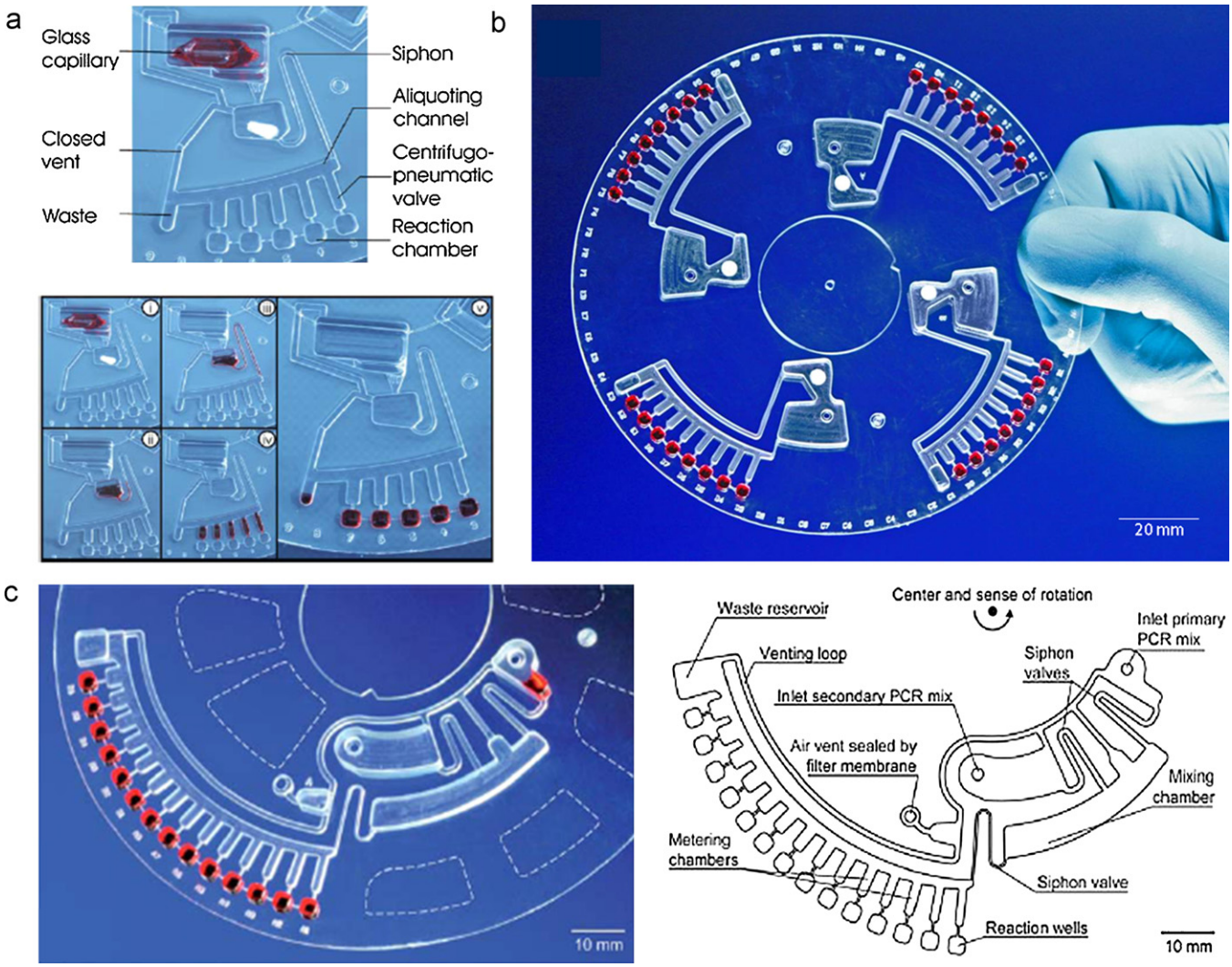

Fig. 3. Microfluidic lab-on-a-chip foil cartridge for (a) RPA, (b) RT-PCR, (c) primary amplification and secondary RT-PCR, based nucleic acid amplification. Reprinted with the permission of RSC.

products were analyzed by using either gel electrophoresis or spectrophotometer, which implies the integration ability of the device with automatic systems. The high specificity of the detection procedure was confirmed by analyzing ten different strains of MRSA and MSSA, and seven types of Gram-negative and Gram-positive bacteria. The limit of detection is determined as 10 fg/μl of DNA with a turnaround time of approximately 60 min.

In a latter study, the suitability of microfluidic foil cartridges for real time PCR was demonstrated by Focke et al. (2010a). Micro thermoforming by soft lithography (μTSL) was used in the fabrication of microfluidic cartridges. The design of the cartridge was different from the one used in the study of Lutz et al., to distribute sample evenly to the reaction chambers, to facilitate efficient heating during real time PCR and to align it in the Rotor-Gene 2000 (Lutz et al., 2010). The cartridge had four identical microfluidic devices with 32 reaction chambers (Fig. 3b). In the system, Exfoliatin Toxin A (*ExfA*) gene of MRSA was studied with pre-stored dry reagents and probes and the microfluidic flow was controlled by centrifugal forces. The limit of detection was less than 10 copies of DNA and process time for detection was 110 min with a high specificity, which was controlled by using seven different genetic subtypes of MRSA.

Preamplification can be done to increase the sensitivity of real time PCR for low quantity genes. In the study of Focke et al., primary amplification and secondary real time PCR were integrated with a centrifugal lab on a foil disk to detect *ExfA* gene (Focke et al., 2010b). The disk contained two identical devices with 14 different reaction chambers, mixing chambers and valves at each device (Fig. 3c). The efficiency of amplification was found as 85% with no cross contamination over the wells for 10 set of thermocycles. The limit of detection was as low as seven copies of DNA with great reproducibility. Moreover, the applicability of the microfluidic foil cartridge for the preamplification of multiplex PCR, in which amplifications of different nucleic acid targets were conducted by using multiple primers and reagents, was demonstrated by using four target genes. In the study of Shen et al., multiplex PCR was performed for 20 different genes of four species (MRSA, MSSA, *Candida albicans*, *P. aeruginosa*, and *E. Coli*) by using SlipChip platforms which had either 40 or 384 wells (Shen et al., 2010). The dry reagents were prestored in each well without cross contamination avoiding any need of pumps to divide the reagents. The fluid was controlled by sliding two plates, the well geometries of which were different, relative to each other with the help of a program.

The detection of amplified samples can also be done by using capillary electrophoresis. The integration of PCR amplification with capillary electrophoresis and MRSA detection in a portable device was performed in 2004 (Lagally et al., 2004). The presence of MRSA in blood samples was analyzed by detecting 219 bp part of *femA*, whose presence indicates the species of *S. aureus*, and 310 bp part of *mecA* genes. The system consisted of portable PCR-CE microchip, detection unit, and a computer to analyze the results. Precultured and diluted samples with a concentration of 10,000 cells/μl were used for 30 cycles of PCR amplification in less than 20 min. In the study of Naikare et al., multiplexed isothermal amplification of products of bacterial cell lysis was followed by detection of *femA* and *mecA* genes through using cycling probe reaction and capillary electrophoresis and without necessity of purification of lysed cell sample (Naikare et al., 2009). The limit of detection was around 10^4 CFU/ml with a time to result of 3 h without culturing the bacteria.

In the above techniques, the hybridization reaction was carried out in micro reactors. On the other hand, it is also possible to detect biological analytes on modified surfaces such as thin film biosensors. In the study of Tombelli et al., two different immobilization techniques (usage of either biotinylated or thiol derivatised synthetic probes) were studied on active surface of piezoelectric device for detection of 27mer specific region of *mecA* gene of methicillin resistant strain *S. aureus* subspecies *aureus* Rosenbach (ATCC 700699) for piezoelectric biosensor (Tombelli et al., 2006). It was observed that the frequency change for the biotinylated surface is six times higher than the change for thiol modified surface. In the study of Lindsey et al., a thin film biosensor was developed to detect visually *mecA*, *femB,* and *tuf* genes of *S. aureus* directly from positive blood culture with a detection limit of 10 fM in 90 min of time to result (Lindsey et al., 2008). The detection strategy was based on the surface activation by using capture probes and hybridization with the target sequence. After that detector probe was hybridized to the target sequence and antibody/horseradish peroxidase (HRP) conjugate was bound to the probe for signal amplification. The substrate, 3,3,5,5-tetramethylbenzidine (TMB) was reacted with HRP to produce precipitate, whose color was visually detectable when its thickness increases at least 20 Å, and, the limit of detection with the use of charge-coupled device was quantified as10 fM (Jenison et al., 2001). This method is advantageous since it does not necessitate nucleic acid amplification. Therefore it eliminates the cross contamination due to amplification samples and it does not necessitate optical instrument for detection. Although the limit of detection is good enough, the detection limits and time to result can be lowered to have more rapid and accurate detection. In the study of Klonoski et al., the fastest signal amplification method was presented to detect *mecA*, *femB*, and *tuf* genes of *S. aureus* directly from positive blood culture (Klonoski et al., 2010). The activation of surfaces by immobilizing 5′-I modified capture probes was done and after that, target sequences were hybridized to capture biotin modified signal probe. Two signal detection protocols were compared: Standard detection and polymer enhanced detection (PED) protocols. In standard detection strategy, biotin labeled probe was detected directly by anti-biotin antibody/HRP that was used to enhance the signal by TMB reaction. On the other hand, in PED, the probe was detected by streptavidin/polymer complex, which increases the adsorbed number of HRP molecules. The detection limit in standard detection was enhanced by 25-fold in PED protocol (detection limit is 1 fM) within 25 min. Gebala et al. used a SAM modified microelectrode array, which was composed of 32 individually addressable gold electrodes to detect MRSA by electrochemical detection (Fig. 4) (Gebala et al., 2011). Gold electrodes (70 μm × 70 μm) were modified by using thiolated capture probe DNA and 11-mercapto-1-undecanol and, hybridization occurred with the complementary target strand, which was pre-modified with ferrocene units at its 5′ end. Intercalation of biotinylated intercalator and avidin/alkaline phosphatase (AP) conjugate was done with further modification. With the enzyme amplification, the limit of detection was as low as 10 pM. In a recent study by Wang et al., label free detection of 30mer DNA of specific part of MRSA on graphene oxide modified glassy carbon electrode with a diameter of 3 mm was studied (Wang et al., 2011b). Electrochemical impedance spectroscopy (EIS) was used to analyze the electron transfer and diffusion processes during DNA adsorption on the modified surface with different concentrations of target DNA. Although it is label free detection, the achieved detection limit is 100 fM, which is quite good.

5.3. Commercialized products

There are several products approved by U.S. Food and Drug Administration (FDA) for *in vitro* diagnostics of MRSA (Table 1) (FDA, 2011). The products are allowed for prescription use in clinics as a clinical aid in conjunction with other laboratory tests that are used in the identification and diagnosis of MRSA from infected patients' samples. These products can be classified in four categories: Culture media, nucleic acid amplification test systems, test kits/assays, and antimicrobial susceptibility test systems. In the

Table 1
Commercial FDA approved products as a clinical aid to identify MRSA.

Device name (Company)	Target specimen	Inoculation	Test methodology	Target sequence	Turnaround time	Special instrument requirement	Clinical sensitivity/specificity	LoD	510 (k) Number
Culture media									
MRSA*Select* (Bio-Rad)	Skin and soft-tissue wound	Direct from specimen collection devices	Selective chromogenic agar	NA	18–28 h	NA	91.7%/99.4%	10^3 CFU/ml	K100589
Remel Spectra™ MRSA (REMEL, Inc. as a part of Thermo Fisher Scientific)	Nasal swab	Positive blood culture	Selective and differential chromogenic prepared culture medium	NA	24 h	NA	NA/NA	NA	K092407
Remel Spectra™ MRSA (REMEL, Inc. as a part of Thermo Fisher Scientific)	Nasal swab	Anterior nares swab	Selective and differential chromogenic prepared culture medium	NA	24 h	NA	NA/NA	NA	K073027
BBL™CHROMagar™ MRSA II (Becton Dickinson & CO.)	Anterior nares	Direct from specimen collection devices	Selective and differential culture media	NA	18–28 h	NA	NA/NA	NA	K092767
BBL™ CHROMagar™ MRSA (Becton Dickinson & CO.)	Anterior nares swab	Direct anterior nares specimens	Selective chromogenic agar	NA	24–48 h	NA	NA/NA	NA	K042812
chromID™ MRSA Agar (BIOMERIEUX, Inc.)	Anterior nares swab	Direct from specimen collection devices	Selective and differential chromogenic prepared culture medium	NA	24 h	NA	NA/NA	NA	K091024
MRSA Screen Agar (Mueller–Hilton W/NACL & Oxacil.) (HARDY MEDIA)									K870649
Nucleic acid amplification test systems									
Xpert® MRSA/SA Blood Culture Assay (CEPHEID)		Positive blood culture	Real-time PCR	Sequence incorporating the insertion site (attBsse) of *Staphylococcal* mec (SCCmec) for detection of MRSA Sequence specific to methicillin/Oxacillin resistance (mecA gene)	50 min	GeneXpert® Dx System (GX-4 or GX-16 instruments, and the GeneXpert® Dx System Software 1.6b)		250 CFU/test	K101879
Xpert® MRSA/SA Nasal Assay (CEPHEID)									K100822
LightCycler® MRSA Advanced Test (ROCHE Molecular Systems, Inc.)	Nasal swab	Direct from specimen collection devices	Real-time PCR and melting peak analysis	Sequence incorporating the insertion site of the *SCCmec* in the *S. aureus orfX*gene	2 h	LightCycler 2.0 Instrument	NA/NA	240 CFU/swab	K091409

IDI-MRSA™ Assay (Infection diagnostic (I.D.I.) Inc.)	Nasal swab	Direct from specimen	PCR	Presence of SCCmec cassette (genetic element that carries the mecA gene) at orflX junction (specific to *S. aureus*)	60–75 min	Smart Cycler® Instrument	NA/NA	325 CFU/swab	K042357
BD GeneOhm™ MRSA ACP Assay (BD Diagnostics (GENEOHM SCIENCES, Inc.)	Nasal swab	Direct from specimen collection devices	Real-time PCR and molecular beacon technology	Mec Right Extremity Junction genotypes i, ii, iii, iv, v and vii	60–75 min	Cepheid SmartCycler II System with Dx Software version 1.7b.	95%/95%	Approximately 1250 CFU/swab for Type i, ii, iii, iv, and vii Approximately 625 CFU/swab for Type v	K093346
Xpert™ MRSA/SA SSTI Assay (CEPHEID)	Skin and soft tissue infection swabs	Direct from skin and soft tissue infection swabs	Real-time PCR	Staphylococcal protein A (spa), for methi-cillin/oxacillin resistance (mecA), and for the staphylococcal chromosomal cassette (SCCmec) insertion event into the *Staphylococcus aureus* chromosomal attB site	Approximately 50 min	GeneXpert® Dx System (GX-4 or GX-16 instruments, and the GeneXpert® Dx System Software 1.6b)	NA/NA		K080837
Xpert™ MRSA (CEPHEID)	Nasal swab	Direct from specimen collection devices	Real-time PCR	Sequence incorporating the insertion site (attBsc) of Staphylococcal Chromosome Cassette mec (SCC mec)	75 min	Gene Xpert® Dx System	NA/NA	80 CFU/swab	K070462
Test Kits									
MASTALEX™-MRSA RST 501 (MAST GROUP Ltd.)			Slide latex agglutination	PBP2′ (penicillin binding protein 2′)					K062864
MRSA-Screen (DENKA SEIKEN'S)			Slide latex agglutination	PBP2′ (penicillin binding protein 2′)					K011400
Velogene™ Genomic Identification Assay for MRSA with one-step detection (ID Biomedical CORP.)			Cycling Probe Technology	mecA gene	90 min after primary isolation				K010858

Table 1 (*Continued*)

Device name (Company)	Target specimen	Inoculation	Test methodology	Target sequence	Turnaround time	Special instrument requirement	Clinical sensitivity/specificity	LoD	510 (k) Number
Velogene™ Rapid MRSA Identification Assay (ID Biomedical CORP.)			Cycling Probe Technology	mecA gene	90 min after primary isolation				K990640
Antimicrobial Susceptibility test systems									
BBL Crystal MRSA ID System (Becton Dickinson Microbiology Systems)									K941997
BBL Crystal MRSA ID System (Becton Dickinson Microbiology Systems)									K926294

LoD = Limit of detection, NA = not applicable

identification of MRSA, the mostly used target specimens are nasal swab, and skin-and-soft tissue wounds.

In the list of culture media products, the mediums are selective chromogenic for qualitative detection of MRSA. The medium contains selective antibiotics (e.g., cefoxitin) and antifungal substrates to damage the growth of the yeasts, Gram-negative and Gram-positive bacteria (especially *Staphylococcus epidermis*) except MRSA. The differentiation of MRSA from the other colonies are done by the split of a chromogenic agent, which results in the growth of colored colonies, in the presence of a specific enzyme of *S. aureus*. The growth of colored (e.g., strong pink, blue, denim-blue, mauve, and green) colonies on the agar plate are the indications of positive MRSA. There is no need of any special instrument in the analysis since it is done manually. Time to result in the usage of culture media for identification of MRSA is ranging from 18 h to 48 h. Malhotra-Kumar et al. compared five chromogenic media (Brilliance MRSA (REMEL Inc.), ChromID (BIOMERIEUX), MRSA*Select* (Bio-Rad), CHROMagar (CHROMagar Microbiology), BBL™-CHROMagar™ (BD Diagnostics)) for 24 h incubation period of pure isolates and pre-enriched nasal and groin swabs (Malhotra-Kumar et al., 2010). It is stated that, BBL™-CHROMagar™ (BD Diagnostics) gave the best results for specificity, sensitivity, and positive predictive value among the five types of media. However, cross reactivity with other staphylococcal species were observed on the chromogenic media. Therefore, it is necessary to incubate the media for further 24 h to increase the sensitivity of all types of the media and an accurate identification method is necessary to confirm the results. The use of nucleic acid detection systems may be useful to settle any query (Rajan et al., 2007).

The basic detection principle of nucleic acid amplification test systems is based on four main steps: isolation of nucleic acid, amplification, hybridization, and detection. RT-PCR or PCR is used to amplify the target sequence(s) of the specimen. After completion of amplification, a melting curve analysis or analysis of molecular beacons with different fluorescence properties is performed to detect hybridization. *mecA* gene, which is the indication of methicillin/oxacillin resistance, *orfX* gene, which is specific to *S. aureus* staphylococcal protein A (spa), and chromosomal attB site for the staphylococcal chromosomal cassette (SCCmec) insertion event into the *S. aureus* are used as target sequences to detect MRSA. The specified turnaround times for nucleic acid amplification systems are ranging from 50 min to 2 h although the tests are performed from the direct specimen collection devices. The analyses of results are performed automatically by using special instruments and software specific to these instruments and a report of the results can be generated. The limit of detection is also different for the products. Xpert™ MRSA (CEPHEID) has 80CFU/swab, while BD GeneOhm™ MRSA ACP Assay (BD Diagnostics (GENEOHM Sciences, Inc.)) has approximately 1250 CFU/swab for Mec Right Extremity Junction Genotypes i, ii, iii, iv, and vii, and approximately 625 CFU/swab for Type v with a clinical sensitivity and specificity, of both 95%. In the aspect of test kits/assays, target analytes for identification of MRSA are PBP2′ and *mecA* gene. Slide latex agglutination or cyclic probe technology are used in different products as a test methodology. Moreover, there are antimicrobial susceptibility test systems, in which the analysis of the results is done manually. The identification principle of BBL Crystal MRSA ID system depends on the fluorescence detection of a fluorescent indicator, which becomes active when dissolved oxygen in the medium is consumed by bacteria. The resistance to methicillin/oxacillin can be detected by adding oxacillin into the medium (Knapp et al., 1994).

HainLifescience (Germany) developed four different products for MRSA detection of swabs taken from nose, throat, skin, and wounds for clinical and research use in Europe. The products present different times to get result from the specimen depending on the use of cultured or noncultured specimen. Times to result

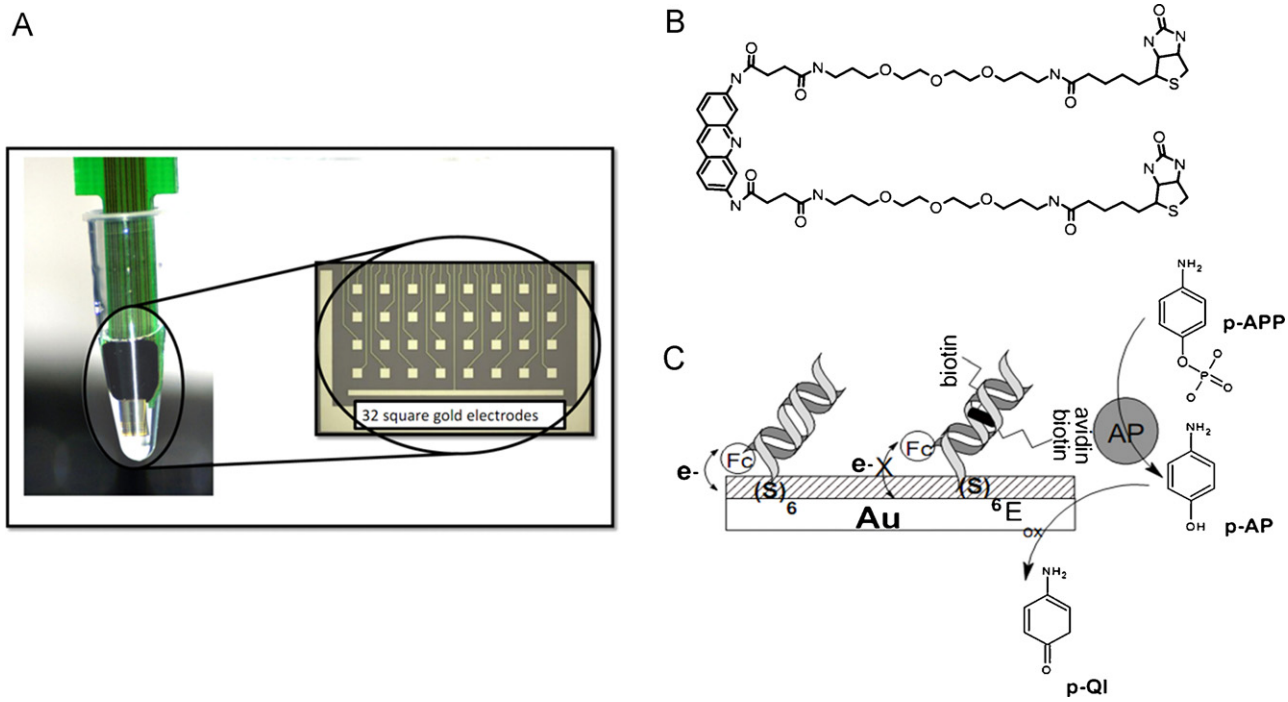

Fig. 4. (a) The dipstick-type biosensor which contains 32 square gold microelectrodes. (b) Structure of dibiotinylated proflavine derivative. (c) Schematic representation of the assay strategy.
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for GeneQuick® MRSA and GenoType® MRSA Direct are 2.5 h and 4 h, respectively, and these products do not necessitate culturing of the specimen. The other two products, GenoType® MRSA and GenoType® *Staphylococcus* require primary culture and the time to result is 4 h for both. The principle of detection for all products is based on the DNA-STRIP® technology, except GeneQuick® MRSA that is based on GenoQuick® technology. In both technologies, three steps are necessary for detection of *mecA* gene: DNA isolation, DNA amplification, and hybridization (Prere et al., 2006). Following DNA isolation with enzymatic lysis, amplification is performed by using PCR. In DNA-STRIP® technology, hybridization is done on strips, which are further modified with a streptavidin conjugated AP and its substrate. Then, hybridization is made visible with a colorimetric reaction, which can be observed manually other than automatically. On the other hand, in GenoQuick® technology, hybridization is performed with specific probes that have a gold binding site. After hybridization, gold particles are adsorbed from the test strip and gold particle conjugated hybrid is adsorbed to the specific sites on the strip while moving in a lateral flow of buffer. This results in a specific visible band for the specimen. In the study of (Bischof et al., 2009; van Hal et al., 2007), it was stated that the IDI-MRSA™ Assay (Infectio Diagnostic (I.D.I.) Inc.) is the most sensitive product when it is compared with CHROMagar MRSA (BD Diagnostics, Sparks, MD), Genotype® MRSA Direct PCR assay (Hain-Lifescience (Germany)), MRSA ID (Biomerieux Inc.), and MRSA*Select* (Bio-Rad). The sensitivities for the target specimens of nasal, groin and axilla, and overall are 94%, 80%, and 90%, respectively. On the other hand, LightCycler® MRSA Advanced Test demonstrated equivalent sensitivity and specificity with BD GeneOhm™ MRSA Assay (Peterson et al., 2010).

6. Conclusion

In conclusion, high sensitivity and specificity, and short turnaround time are mainly desired properties of *in vitro* diagnostics for the identification of MRSA since the aim is rapid identification of target analyte with accurate positive test results for early and appropriate treatment. In this review, standard and MEMS based methods, and commercial products for the detection of MRSA have been considered for specimens taken from infected patients' samples (e.g., blood, wound, nose, and throat). Present conventional methods are mostly limited by the incubation time of media to grow culture for at least 24 h to increase the specificity and sensitivity (Brown et al., 2005). Therefore, there is a potential that the lab-on-a-chip devices will replace the current methods by which patients are diagnosed. Thus, the use of integration of molecular methods and MEMS, at which *mecA* gene is used as an indicator of methicillin resistance, can be a common approach in identification of MRSA.

There are different micro devices for micro culturing, cell lysis, PCR, capillary electrophoresis, etc. which are used individually or in combination. However, to the best of our knowledge, there is not yet a compact micro device reported to handle sample directly taken from the patient and analyze it on a chip to identify the MRSA without the need of any macro device. This is because the novel technology to combine all of the advantages of MEMS into a device is not yet fully developed. Currently, knowledge of theory behind the microfluidics and nanofludics, the surface chemistry, and transport of the samples in the microfluidic channels and reservoirs affect the detection limits. The design limitations of the MEMS based devices can be numerous and it is essential to take care of them at the beginning stage of the development. Therefore, it is necessary to work in collaboration with different disciplines to tackle challenges in the field of clinical diagnosis and to meet the demands of clinicians.

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