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Chapter

The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents

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Abstract

Biofilm producer microorganisms cause nosocomial and recurrent infections. Biofilm that is a sticky exopolysaccharide is the main virulence factor causing biofilm-related infections. Biofilm formation begins with attachment of bacteria to biotic surface such as host cell or abiotic surface such as prosthetic devices. After attachment, aggregation of bacteria is started by cell-cell adhesion. Aggregation continues with the maturation of biofilm. Dispersion is started by certain conditions such as phenol-soluble modulins (PSMs). By this way, sessile bacteria turn back into planktonic form. Bacteria embedded in biofilm (sessile form) are more resistant to antimicrobials than planktonic bacteria. So it is hard to treat biofilm-embedded bacteria than planktonic forms. For this reason, it is important to detect biofilm. There are a few biofilm detection and biofilm production methods on prosthetics, methods for screening antibacterial effect of agents against biofilm-embedded microorganism and antibiofilm effect of agents against biofilm production and mature biofilm. The aim of this chapter is to overview direct and indirect methods such as microscopy, fluorescent in situ hybridization, and Congo red agar, tube method, microtiter plate assay, checkerboard assay, plate counting, polymerase chain reaction, mass spectrometry, MALDI-TOF, and biological assays used by antibiofilm researches.

Keywords: mature biofilm, biofilm-embedded bacteria, antibiofilm methods, detection of biofilm gene expression, biofilm detection, MALDI-TOF, CLSM, checkerboard assay, microtiter plate assay, Congo red agar method

1. Introduction

1.1 The structure and pathogenesis of microbial biofilms

Microbial biofilms that are sticky exopolymeric substances (EPS) causing adherence of microorganism to biotic surfaces such as host cells or abiotic surfaces such as medical devices cause antimicrobial resistance, due to its molecular contents such as eDNA and exoenzymes (β-lactamase, toxins, etc.), limited diffusion of antimicrobials through the biofilm matrix, persister cell content, and limited nutrient and oxygen. Surface proteins and polysaccharide intercellular adhesions (PIA) play a role in the biofilm production and development. It is hard to treat

biofilm-embedded bacteria than planktonic forms. Biofilm producer microorganism causes biofilm-related infections such as indwelling and medical device-related infections such as endocarditis, urinary tract infections, septic arthritis, chronic rhinosinusitis, ocular infections, wound infections, etc. The results of biofilm produced on indwelling medical devices are recurrent, untreatable infections and failure of medical device. To overcome chronic and recurrent infections, it is important to detect biofilms of microorganisms, maturation and dispersion, and determine antibiofilm and antibacterial activity of agents against biofilm and bacteria within biofilm, respectively [1]. Identification of genes involved in biofilm formation and measurement of gene expression as a result of antibiofilm and antibacterial activity of agents can be advantageous with carrying out high-throughput screens using microtiter plate assay system.

1.2 Techniques used to study biofilms and biofilm-embedded microorganisms

The standard antimicrobial susceptibility tests such as broth macrodilution and microdilution methods that are routinely used in laboratories and published by Clinical Laboratory Standards Institute (CLSI), National Committee for Clinical Laboratory Standards (NCCLS), and European Committee on Antimicrobial Susceptibility Testing (EUCAST) could never yield accurate results in biofilm producer microorganisms, due to being appropriate for the detection of antimicrobial activity of agents against planktonic microorganism [2].

There are several methods which have been used by clinical microbiologist for detection and measurement of microbial biofilms in response to agents (**Tables 1–3**). Several instruments as model system have been improved such as modified Robbins device, Calgary biofilm device, disk reactor, Centers for Disease Control (CDC) biofilm reactor, perfused biofilm fermenter, and model bladder. Model systems help to define susceptibility of antimicrobial agents against biofilm producer microorganisms by providing information about biofilm mechanisms. Substratums of modified Robbins device, Calgary biofilm device, disk reactor, CDC biofilm reactor, and perfused biofilm fermenter are silastic disks, plastic pegs, Teflon coupons, plastic needleless connectors, and cellulose acetate filters, respectively, whereas substratum of model bladder is urinary catheters (UCs). Medical devices of which dimensions are adjusted to appropriate sizes can also be used as a substratum (abiotic surfaces) for biofilm production by adapting and modifying to related methods by some biofilm researchers. The methods of modified Robbins device and Calgary biofilm device are based on viable counting. In Calgary biofilm device, pegs are sonicated before counting. The methods of disk reactor and CDC biofilm reactor based on direct and viable counting,

Method	Action of application	Aim
Roll plate	Extraluminal biofilm detection	Growth of biofilm-embedded bacteria
Sonication, vortex, and plate counting	Intraluminal and extraluminal biofilm detection	Growth of biofilm-embedded bacteria
Acridine orange staining	Extraluminal biofilm detection	Direct investigation of biofilm produced on catheter by microscopy
Streak plating of alginate swab	Investigation of biofilm produced on indwelling catheter	Growth of biofilm-embedded bacteria

Table 1.
The methods used for detection and measurement of biofilms produced on medical devices.

Method	Aim
Tube method (TM)	Qualitative detection by observing biofilm lined on bottom and walls of tube
Congo red agar (CRA)	Qualitative detection by observing colony color change
Microtiter plate (MtP)	Quantitative detection of biofilm by microplate reader (microELISA)
Real-time PCR	Detection of biofilm genes
Conventional PCR	
Multiplex PCR	

Table 2.
The methods used for detection of biofilm.

Method	Application	Target
Microtiter plate (MtP)	Measurement of biofilm produced on walls of wells in response to agent	Measures the effect of agents against biofilm production
Microtiter plate (MtP) (MBEC)	Measurement of biofilm remained on walls of wells in response to agent and detecting MBEC of agents	Measures the effect of agents against mature biofilm formed on walls of wells
Vortex and plate counting	Plate counting of biofilm-embedded bacteria and detecting bMBC of agents	Screens antimicrobial activity of agents against biofilm-embedded bacteria
Checkerboard assay	Plate counting of biofilm-embedded bacteria and FIC indexes are calculated	Screens antimicrobial activity of combination of agents
Sonication, vortex, and plate counting	Plate counting of biofilm-embedded bacteria and detecting bMBC of agents	Screens antimicrobial activity of agents against biofilm-embedded bacteria
Quantitative PCR	Measurement of specific biofilm gene expression	Monitors expression of biofilm genes in response to agents
Mass spectrometry (MS)	Measurement of exoenzymes located in biofilm matrix	Monitors expression of bacterial proteins in response to agents

Table 3.
The screening methods for antibiofilm and antimicrobial activity of agents against biofilm producer bacteria.

after substratums, are sonicated, vortexed, and homogenized. In perfused biofilm fermenter, viable counting is done, after filters are shaken in sterile distilled water, whereas in model bladder, UCs are examined directly by scanning electron microscopy (SEM) or transmission electron microscopy (TEM) or by chemical analysis [2]. Rate of biofilm formed on model system can be adjusted by parameters such as composition of medium that can contain glucose, iron, antimicrobial agents, multivalent cations such as Ca^{2+} and Mg^{2+} supporting adhesion of bacteria by cross-linking anionic groups on bacteria and substratum, shear force, retention time, flow rate, roughness, and chemistry of substratum and species of organisms (**Table 4**) [2, 3].

The aim of this chapter is to overview certain methods used by biofilm detection and antibacterial and antibiofilm researches such as tube method (TM), Congo red agar (CRA) method, microtiter plate (MtP) assay, plate counting of biofilm-embedded bacteria (sessile bacteria), PCR, mass spectrometry (MS), confocal laser scanning microscopy (CLSM), etc.

Instruments	Culture dynamics	Substratum	Method
Modified Robbins device	Batch culture	Silastic disks	Viable counting
Calgary biofilm device	Batch culture	Plastic polycarbonate pegs	Viable counting, after pegs are sonicated
Disk reactor	Batch culture	Teflon coupons	Direct or viable counting, after coupons are sonicated, vortexed, and homogenized
CDC biofilm reactor	Continuous culture	Plastic connectors	
Perfused biofilm fermenter	Continuous culture	Cellulose-acetate filters	Viable counting, after filters are shaken in sterile distilled water
Model bladder	Continuous culture	Urinary catheters	Examining directly by SEM or TEM or analyzing chemically
Flow cell	Continuous culture	Chambers with transparent surfaces	Examining by confocal laser scanning microscopy

CDC biofilm reactor, Centers for Disease Control biofilm reactor; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Table 4.
Instruments used to produce biofilm and examine biofilm process.

2. Detection of biofilm producer microorganism

2.1 Direct observation

Complexity and dynamics of biofilms can be observed by biofilm imagining optical technology including light microscopy, SEM, TEM, and CLSM. These techniques are used to visualize 3D structure and check the existence of biofilm [4].

2.1.1 Light microscope

Light microscopy is the easiest, cheapest, most simple, convenient and fastest method to quantitatively observe the morphology of microorganisms adhered to surfaces and to semiquantitatively estimate the amount of microorganism attached on surface (exist, absent, abundant, rare, etc.). Microorganisms including *Candida albicans*, *E. coli*, *Pseudomonas*, and *Staphylococcus epidermidis* adhered on acrylic sheets of polymethacrylate films, glass cover slips, and polystyrene petri dishes have been observed by light microscope, respectively. Observation with light microscopy that requires clear, transparent, and planar surfaces on which microorganisms attach does not create 3D vision of biofilm. Dyes can be used such as epifluorescence and fluorescent to enhance image clarity of microorganisms. The observation with the light microscope enables researchers to compare morphologies of sessile form and planktonic form of microorganism required by making smear and centrifuging of sample, respectively [3].

2.1.2 Transmission electron microscope (TEM)

Images of cells and cell structures such as protein and nucleic acid are obtained by electrons at high magnification and resolution. Monitoring of components of cell can be done directly in TEM by negative staining. Due to photons and electrons penetrating cells poorly, thin section of cell cut is stabilized and stained by certain chemicals with the treatment of osmic acid, permanganate, uranium, lanthanum, or lead salts. These

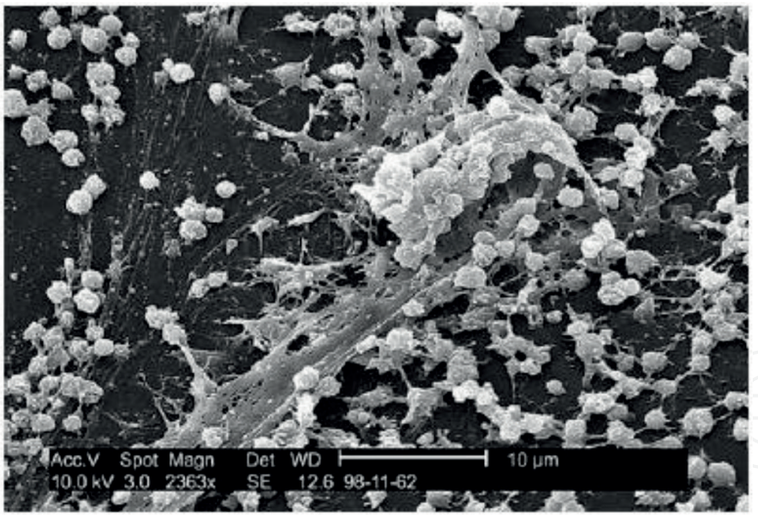


Figure 1.
The SEM image of S. aureus embedded in biofilm colonized on intravenous catheter [6].

stains contain high atomic weight. Due to stains having high atomic weight, contrast is accelerated by electron dispersion from sample. If observation of outer structure of cells will be done, it is not important whether the section of cell is thin or thick.

Due to inadequate stabilization of polysaccharides is done by the conventional fixatives such as aldehydes, glutaraldehyde, paraformaldehyde, and osmium tetroxide, water content of biofilm is eliminated by graded dehydration with alcohol after this postfixation step. After sample is infiltrated with resin, sample is embedded in gelatin capsule and headed for polymerization. Then, thin section taken is poststained with uranyl acetate and lead citrate.

Exopolysaccharide constituents are not observed with its own electron dense and staining poststains such as uranyl acetate and lead citrate with TEM, due to not only having high electron translucent, but also contrast is not developed by conventional poststains. According to the studies, glycocalyx of *Staphylococcus hominis* and *Staphylococcus epidermidis* can be stabilized by the usage of certain cationic reagent combinations including ruthenium red, alcian blue, lysine, lysine monohydrochloride, or lysine acetate and paraformaldehyde [5]. After all these steps are done, sample is observed in TEM (**Figure 1**).

2.1.3 Scanning electron microscope (SEM)

To visualize 3D images of cell sample is coated with heavy metals such as gold. Electrons released from metal coating of sample are caught by SEM for image

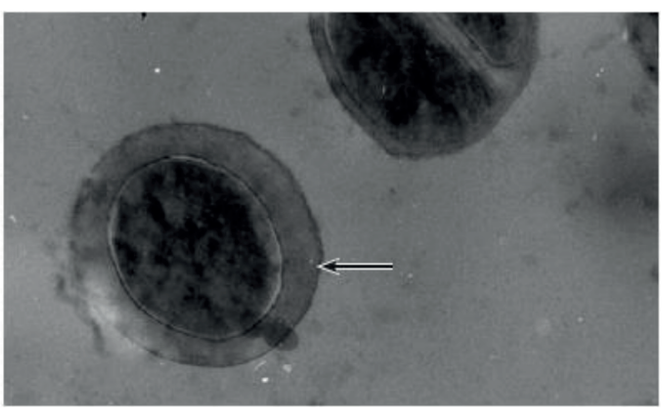


Figure 2.
The TEM image of Staphylococcus spp. surrounded by glycocalyx [6].

production. The procedure of SEM is similar to TEM except for some additional chemicals (gold), lacking infiltration, embedment in resin, polymerization, and thin section staining with lead citrate and uranyl acetate [5]. As in the steps of TEM method, postfixation and dehydration steps of SEM are similar to TEM. The step is applied after dehydration step is drying and coating sample with gold in the processing for SEM, rather than infiltration with resin, embedment in gelatin capsule, and staining with lead citrate and uranyl acetate in the processing for TEM. After dehydration process with graded alcohol, sample is dried and coated with gold palladium [5]. After all these steps are done, sample is observed in SEM (**Figure 2**).

2.1.4 Florescent tagging of biofilm

2.1.4.1 Confocal laser scanning microscopy (CLSM)

Biofilms formed on flow cells of which surface are transparent can be observed by confocal laser screening microscopy (CLSM). Three-dimensional (3D) morphology and physiology of biofilms can be screened by CLSM [2]. Thick samples such as biofilms and microorganisms localized in the depth such as biofilm-embedded microorganisms need to be observed by CLSM (**Figure 3**).

For observation of biofilm with confocal microscopy and related methods, biofilm must be fluorescent as a result of fluorescent molecules such as green fluorescent protein (GFP) that is fluorescent protein expressed by biofilm producer microorganism within biofilm (gene of cell interested is tagged by gene cassette encoding GFP) or staining components of heterogeneous mass of biofilm with fluorescence or fluorescence-labeled dyes [2]. Stains such as lectins target extracellular matrix, whereas certain fluorophores target extracellular DNA (eDNA) to visualize eDNA content of biofilm matrix [2, 7].

By scanning laser light across the sample, deep penetration of excitated energy is provided. As a result of fluorescence of biomolecules such as GFP or chlorophyll that are intrinsic fluorophores or molecules signed by exogenous probes such as fluorescent-labeled antibodies detected by photomultiplier, 3D digital image is formed. Observation of biofilms that are multilayered and have complex 3D structures requires additional resolution [2]. Images of each layers of biofilm obtained are combined by computer for construction of digital 3D images of whole biofilm.

Biofilm producer microorganisms can be manipulated genetically by tagging of microbial gene of interest by gene cassette encoding GFP as a reporter gene

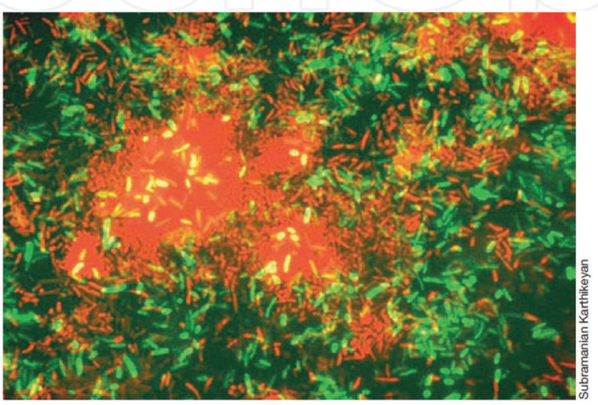


Figure 3.
Bacterial community embedded in a biofilm matrix visualized by CLSM. Each bacterium observed with a distinct color located at different depths of biofilm [11].

(*gfp* genes) to monitor gene expression and metabolic physiology activity in biofilms and determine location of microorganism within biofilm [2, 8, 9].

Idea about gene activity in biofilms is given by confocal microscopy applied to 3D localization of nonenzyme reporter systems such as GFP. Growth phase and activity of bacteria embedded in biofilms can be defined by promoter-reporter systems that is designed and fluoresced just in living dividing cells. In situ cellular growth activity of bacteria embedded in biofilms is determined by measuring ribosome-hybridization-signal intensity, due to synthesis rate and content of ribosome correlated with the growth rate (especially in exponential phase). Expression cassette that is active only in growing cells, labeled by GFP and controlled by rRNA promoter, can be constructed to monitor growth phase and activity of bacteria within biofilm [2, 10].

2.1.4.2 Fluorescent in situ hybridization (FISH)

Specific microorganisms present in a heterogeneous biofilm community can be identified by the probes of fluorescent in situ hybridization (FISH) method. GFP that is translated enables procedure not to require fixation or staining. Fluorescent-labeled microorganism within biofilm can also be examined by FISH. DNA probes designed to hybridize 16S rRNA of microorganism integrated to either fluorescent dye such as FITC or Rhodamine or enzyme such as horseradish peroxidase. The advantage of probes conjugated with horseradish peroxidase is not to destroy microorganism within biofilm. The growth rate of microorganism within biofilm can be determined by FISH method, due to the amounts of ribosomes existing in a microorganism that is directly proportional to growth activity of microorganism. Probe must be designed to label conserved region of only a single species (**Figure 4**) [2, 12].

2.2 Indirect observation

2.2.1 Roll plate method

Roll plate method is applied for the detection of possible microbial colonization having a potential to develop indwelling device-associated infection on the outer surface of cylindrical materials such as catheters and vascular grafts. Microorganism colonize on external surface of catheter is detected by roll plate method, instead of microorganism colonize on intraluminal site of catheter. Material is touched and rolled on the surface of medium [3].



Figure 4.
Fluorescence image of 28 distinct E. coli strains labeled by fluorophore-conjugated oligonucleotides complementary to 16S rRNA of E. coli [11].

2.2.2 Congo red agar (CRA) method

Congo red agar (CRA) method that is a qualitative assay for detection of biofilm producer microorganism, as a result of color change of colonies inoculated on CRA medium, is described by Freeman et al. The CRA medium is constructed by mixing 0.8 g of Congo red and 36 g of sucrose to 37 g/L of Brain heart infusion (BHI) agar. After incubation period that was 24 h at 37°C, morphology of colonies that undergone to different colors is differentiated as biofilm producers or not. Black colonies with a dry crystalline consistency indicate biofilm producers, whereas colonies retained pink are non-biofilm producers (**Figure 5**) [13].

2.2.3 Tube method (TM)

Tube method (TM) that is a qualitative assay for detection of biofilm producer microorganism, as a result of the occurrence of visible film, is described by Christensen et al. [14]. Isolates are inoculated in polystyrene test tube which contained TSB and incubated at 24 h at 37°C. The sessile isolates of which biofilms formed on the walls of polystyrene test tube are stained with safranine for 1 h, after planktonic cells are discharged by rinsing twice with phosphate-buffered saline (PBS). Then, safranine-stained polystyrene test tube is rinsed twice with PBS to discharge stain. After air drying of test tube process, the occurrence of visible film lined the walls, and the bottom of the tube indicates biofilm production (**Figure 6**) [14].

2.2.4 Detection of biofilm production by microtiter plate assay

Microtiter plate (MtP) assay is a quantitative method to determine biofilm production by microplate reader. Bacterial suspension is prepared in MHB supplemented with 1% glucose and adjusted to 0.5 McFarland (1.10^8 cfu/ml). This bacterial suspension is 20-fold (1/20) diluted to reach 5.10^6 cfu/ml. Then 180 μl of Mueller-Hinton Broth (MHB) supplemented with 1% glucose [15] and 20 μl of bacterial suspensions are inoculated into 96-well flat-bottomed sterile polystyrene microplate to obtain 5.10^5 cfu/ml as a final concentration (tenfold dilution (1/10)). Microplates are incubated at 24 h at 37°C. The sessile isolates of which biofilms formed on the walls of wells of microplate are stained with only 150 μl of safranine for 15 min, after planktonic cells in wells of microplate are discharged by washing twice with phosphate-buffered saline (PBS) (pH 7.2) and wells are dried at 60°C for

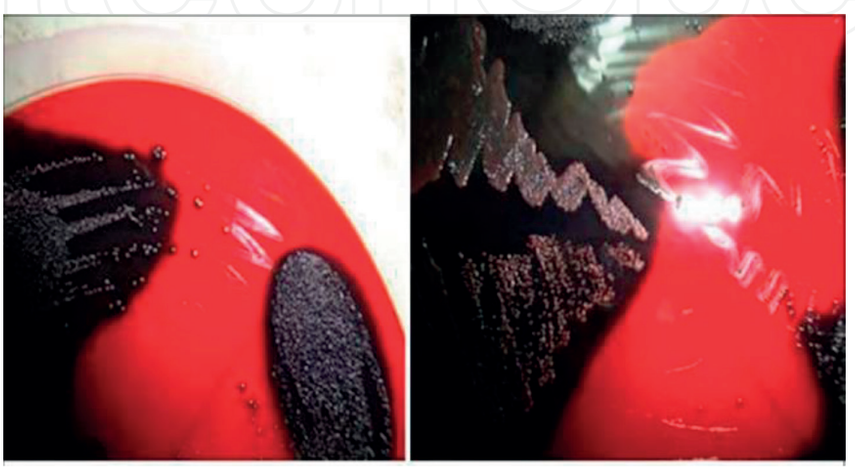


Figure 5.
CRA method applied on CRA medium. Black crystalline colonies of biofilm producer cell and pinkish-red colonies of biofilm nonproducer cell.

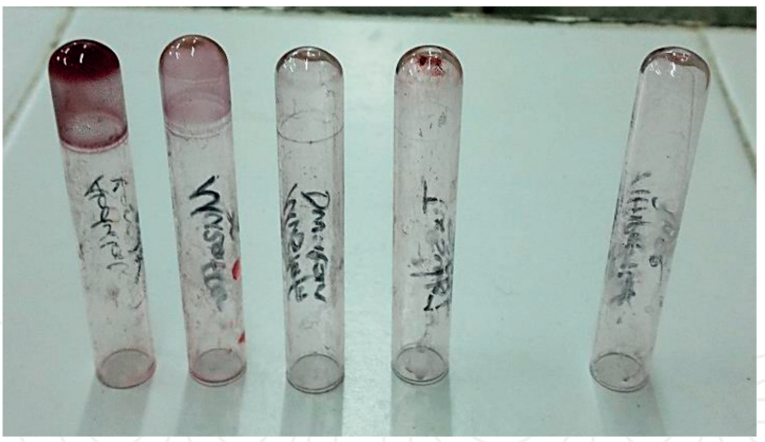


Figure 6.
Tube method. The first two polystyrene test tubes from the left indicate biofilm production. Other test tubes rather than the first two polystyrene test tubes from the left indicate lacking of biofilm production.

1 h [14]. Before staining with safranine, fixation of biofilms can be done by either subjecting to 150 μl of methanol for 20 min or drying at 60°C for 1 h. Then safranine-stained wells of microplates are washed twice with PBS to discharge safranine stain. After air drying process of wells of microplate, dye of biofilms that lined the walls of the microplate is resolubilized by 150 μl of 95% ethanol or 33% glacial acetic acid or methanol. Then microplate is measured spectrophotometrically at 570 nm by a microplate reader [15, 16]. The studies are repeated in triplicates. Uninoculated wells containing sterile MHB supplemented with 1% glucose that are considered to be the negative controls are used as blanks. The blank absorbance values are used to identify whether biofilm formation of isolates exists or not. The wells of isolates of which OD values are higher than blank well are considered to be biofilm producers. Cut off value (ODc) can provide categorization of isolates as biofilm producer or not.

$$\text{Odc: Average OD of negative control} + (3 \times \text{standard deviation (SD) of negative control}) \quad (1)$$

$$\text{OD}_{\text{isolate}}\text{: Average OD of isolate} - \text{ODc} \quad (2)$$

Negative value obtained from this formula and represented as zero indicates lack of biofilm production, whereas positive value indicates biofilm production (**Figure 7**).

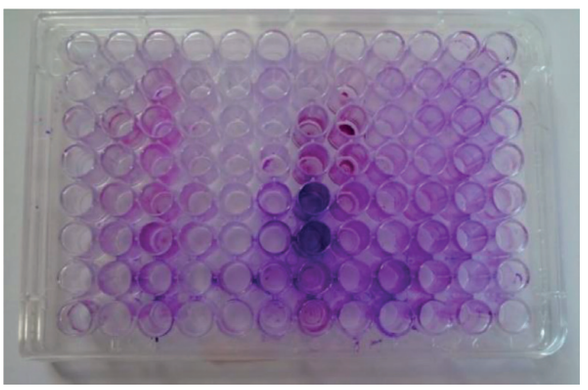


Figure 7.
Microtiter plate assay indicating biofilm production.

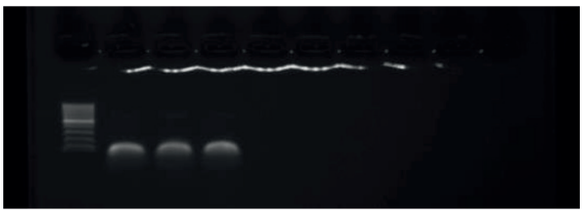


Figure 8.
Image of mecA gene on agarose gel. First sample is DNA size marker (ranging from 250 to 10,000 kb), second sample is ATCC 43300 methicillin-resistant Staphylococcus aureus (MRSA) positive control, third and fourth samples are mecA gene-positive isolates, and fifth sample is ATCC 29213 methicillin-sensitive Staphylococcus aureus (MSSA) as a negative control.

To interpret results, categorization can be done as no biofilm production (0), weak (+ or 1), moderate (++ or 2), and strong biofilm production (+++ or 3) by the calculation of cutoff value (ODc) shown below [15]:

$OD \leq ODc$ no biofilm production

$ODc < OD \leq 2 \times ODc$ weak biofilm production

$2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm production

$4 \times ODc <$ OD strong biofilm production.

2.2.5 Detection of biofilm-associated genes by PCR

PCR techniques is used for not only identification of pathogens by amplifying species-specific nucleic acid sequences but also detection of virulence factors by amplifying target virulence genes such as biofilm genes with the usage of gene-specific primers, even in the uncultured pathogen present in the sample.

Forward and reverse primers of biofilm-associated gene are designed. Firstly, multiple alignments were done in the NCBI to find oligonucleotide sequences specific to the species. Then primer pair of biofilm-associated gene is designed by using Primer3Plus verified by FASTA analysis checking specificity of primers for microbial sequences in the database [17, 18].

Genomic DNA of microorganism is extracted by extraction kits of which protocols can vary according to species and Gram-positivity or Gram-negativity of microorganisms. DNA of microorganism is measured spectrophotometrically by microplate spectrophotometry reader to determine the amount of DNA extracted as microgram per microliter.

Biofilm-associated gene is amplified by PCR such as qualitative real-time PCR, multiplex and conventional PCR that is used to detect whether biofilm-associated gene is present or not in microorganism. If conventional and multiplex PCR protocols are applied to detect biofilm gene, rather than qualitative real-time PCR, PCR product isolated is visualized on an agarose gel containing a DNA-intercalating dye such as ethidium bromide to confirm the presence of amplified gene (**Figure 8**). Only in qualitative real-time PCR, the amplicon is detected by fluorescence using a pair of specific hybridization probes labeled with fluorescence dye [11].

3. Detection of antimicrobial efficacy of agents against biofilm production

3.1 Detection by microtiter plate assay

Microtiter plate (MtP) assay is a qualitative assay to detect efficacy of agent against biofilm production by microplate reader.

Bacterial suspension is prepared in MHB supplemented with 1% glucose and adjusted to 0.5 McFarland (1.10^8 cfu/ml). This bacterial suspension is 20-fold (1/20) diluted to reach 5.10^6 cfu/ml.

A 180 μl of agent doses and 20 μl of bacterial suspension are dispersed to each well of microplate to obtain 5.10^5 cfu/ml as a final concentration (tenfold dilution (1/10)). After incubation at 37°C for 24 h, ongoing processes are done according to MtP assay as mentioned previously for the determination of effect of agent against biofilm production [14, 16].

4. Detection of antimicrobial efficacy of agents against mature biofilms

4.1 Determination of MBEC by microtiter plate assay

Biofilms remained after eradication by agent are measured by this technique. Biofilms of bacteria that line the walls of wells are formed according to MtP method.

After the content of microplate is discharged, 200 μl of each dose of agents is dispersed to each well of microplate of which the walls are lined with biofilm. A 200 μl of distilled water is added into a well of microplate of which the walls are lined with biofilm as a control. Then the effect of agent against mature biofilm is determined according to MtP assay as mentioned previously. Minimum concentration of agent eradicating mature biofilm that is named as minimum biofilm eradication concentration (MBEC) can be determined by this modified plate assay. MBEC50 and MBEC90 indicate the minimum concentrations of agents inhibiting 50 and 90% of mature biofilm formed.

5. Detection of antimicrobial efficacy of agents against biofilm-embedded bacteria

5.1 Determination of bMIC and bMBC by plate counting

In summary, biofilm formation process on abiotic surfaces by bacteria is done. Quantification of sessile biofilm-embedded bacteria lined on abiotic surface and sessile biofilm-embedded bacteria remained on abiotic surface after addition of agent on abiotic surface on which mature biofilms formed is determined by plate counting. Bacterial suspension is prepared and adjusted to 0.5 McFarland (1.10^8 cfu/mL) in Mueller-Hinton Broth (MHB) supplemented with 1% glucose [15]. This bacterial suspension is 200-fold (1/200) diluted to gain 5.10^5 cfu/mL. Kirschner wire orthopedic implants are placed into each test tube containing 5.10^5 cfu/mL isolate and incubated at 37°C for 24 h to lead bacteria to produce biofilm on Kirschner wire. After incubation, Kirschner wires on which biofilms are produced are discharged and rinsed with PBS (pH 7.2) and then transferred into each test tubes containing agent concentrations. After incubation at 37°C for 24 h, Kirschner wires are discharged and placed into test tubes containing 1 mL of sterile MHB and sonicated at 42 kHz for 2 min after vortexed for 5 min. Then 100 μl samples of each test tube sonicated and vortexed are inoculated on Mueller-Hinton agar (MHA) and incubated at 37°C for 24 h [19].

The lowest concentration of agent in which bacterial growth is below or equal to control is determined as biofilm minimum inhibitory concentration (bMIC) for biofilm. bMIC50 and bMIC90 indicate the minimum concentrations of agent inhibiting 50 and 90% of biofilm-embedded bacteria. After incubation, the lowest concentration of agent in which colonies of biofilm-embedded bacteria are not grown is determined as biofilm minimum bactericidal concentration (bMBC) of agent for biofilm [19].

5.2 Determination of combination effects of agents against biofilm

Checkerboard assay is used for the determination of combination effects of two different agents. A 250 μl twofold dilutions of each agent from the stock solutions are dispersed to each row and column to obtain final varying concentrations by starting at fourfold of zero MIC for each isolate. So each well contains distinct combination of concentrations of two agents. First wells of rows and columns are left behind for sole treatments of each dose of agents. One well is used for bacterial control (**Figure 9**). Kirschner wires on which bacterial biofilm is produced are dispersed to each well. This microplate is incubated at 37°C for 24 h. After incubation, Kirschner wires are discharged and sonicated at 42 kHz for 2 min after vortexed for 5 min. The lowest concentration of agent in which bacterial growth that is not observed is determined as biofilm minimum inhibitory concentration (bMIC) of agent for biofilm. Then 100 μl samples of each test tube sonicated and vortexed are inoculated on MHA and incubated at 37°C for 24 h. After incubation, the lowest concentration of agent in which colonies of biofilm-embedded bacteria are not grown is determined as biofilm minimum bactericidal concentration (bMBC) of agent for biofilm. For the determination of whether the synergism is present between agents or not, fractional inhibitory concentrations (FICs) index that are calculated for each agent are summed up according to formula written below:

$$\text{FIC (A): MIC A (A in the presence of B (A + B))/MIC A (A alone)} \quad (3)$$

$$\text{FIC (B): MIC B (B in the presence of A (A + B))/MIC B (B alone)} \quad (4)$$

$$\Sigma\text{FIC: FIC (A) + FIC (B)} \quad (5)$$

When ∑FIC is equal and lesser than 0.5, between 0.5 and 1, equal to 1, higher than 1 and equal and lesser than 4, and higher than 4, it is interpreted that the effect between agents in combination is synergistic, partial synergistic, additive, indifferent, and antagonistic, respectively [20].

The wells having the highest synergy rates of two agents that constitute the combinations are determined by taking the average and standard deviation of FIC indexes calculated of the wells with the lowest drug combination without bacterial growth in each row and column (**Figure 9**).

5.3 Measurement of biofilm-associated gene expression by quantitative real-time PCR

Measurements of biofilm genes repressed or induced by agent are done by quantitative real-time PCR (qPCR). So efficacy of agent against biofilm-associated genes can be detected by qPCR.

Complementary DNA (cDNA) is copied from RNA by enzyme reverse transcriptase. Gene expression in pathogen is monitored by qPCR copying cDNA from RNA of target gene. Amplified cDNA probed for identification. Fluorescent probes such as dye SYBR Green are used to indicate double-stranded DNA, consequently amplification. Accumulation of PCR amplicons labeled fluorescently is monitored through the qPCR processes (**Figure 10**). Visualization of amplicon on agarose gel is not needed to confirm amplification in qPCR [11].

Bacterial control	Agent X Dose 1	Agent X Dose 2	Agent X Dose 3	Agent X Dose 4	Agent X Dose 5	Agent X Dose 6	Agent X Dose 7
Agent Y Dose 1							
Agent Y Dose 2							
Agent Y Dose 3							
Agent Y Dose 4							
Agent Y Dose 5							
Agent Y Dose 6							
Agent Y Dose 7							

Figure 9.
The schematization of checkerboard assay.

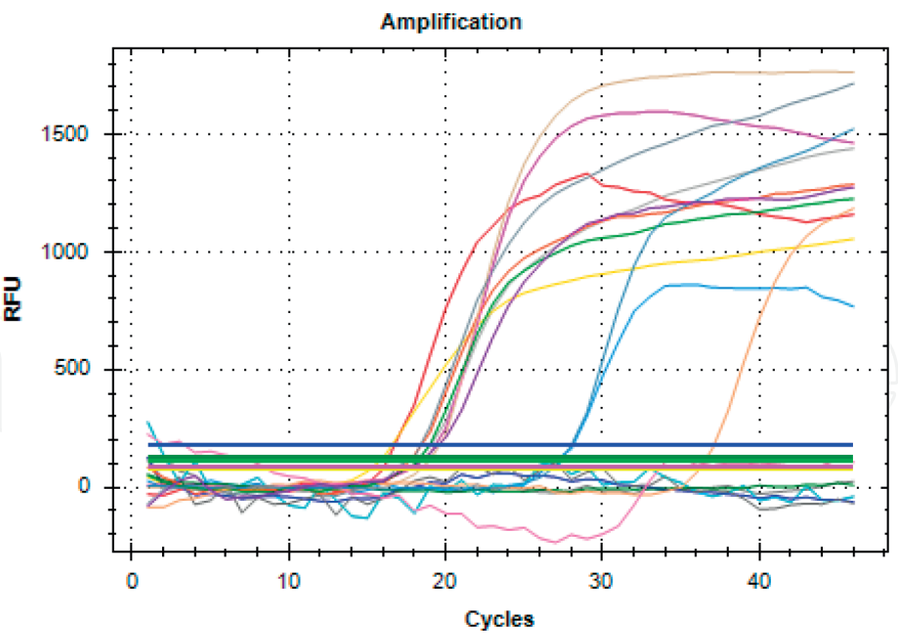


Figure 10.
Quantitative real-time polymerase chain reaction (qPCR) of atl (light blue), 16S RNA (red), mecA (purple), and nuc (light pink) genes of sample; icaA (gray), icaD (plato), atl (pink), mecA (blue), and nuc (green) genes of ATCC 43300 methicillin-resistant Staphylococcus aureus (MRSA); and icaA (orange), 16S RNA (yellow), and nuc (grayish blue) genes of ATCC 29213 methicillin-sensitive Staphylococcus aureus (MSSA). Expressions of all these genes are monitored by qPCR except nuc (light pink) gene sample. Lines below threshold monitored by qPCR indicate the negativity of genes such as icaA and icaD genes samples and icaD (turquoise), atl (plato), and mecA (light pink) genes of ATCC 29213 MSSA. RFI, relative fluorescence intensity.

Total RNA of microorganism is isolated according to protocols of RNA isolation kits. Kit protocols can vary according to the species of microorganism. Total RNA of microorganism is measured spectrophotometrically by microplate spectrophotometry reader to determine the amount of total RNA isolated as microgram per microliter. Then cDNA is synthesized from total RNA with qPCR using primer pair of the biofilm-associated gene, which is designed using Primer3 and verified by FASTA analysis, which controls the specificity of the primers for microbial sequences in the data system, after multiple alignments were done in the NCBI to find oligonucleotide sequences specific to the species [17, 18].

5.4 Mass spectrometry

Extracellular polymeric substances (EPS) not only contain polysaccharides but also contain proteins such as extracellular enzymes. These expressed proteins located in the matrix of EPS can be detected and characterized by mass spectrometry (MS) [1]. Large biologic molecules can be also detected and characterized in complex biologic structures such as EPS by MS. Chemicals involved in biofilm process are examined in detail by MS. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the types of MS [2]. In time-of-flight (TOF) mass spectrometer, mass is analyzed by ions desorbed in vacuum chamber. These two technics are combined and called MALDI-TOF.

Sample is ionized and vaporized by laser. Ions generated from sample by laser pass through the column of MALDI-TOF device toward TOF detector by an electric field. Depending on the mass/charge ratio of molecule, measurements are done by TOF. If this ratio is smaller, ions move faster (**Figure 11**).

Bacteria are identified, expression of bacterial proteins such as surface proteins and exoenzymes like β-lactamase in response to antimicrobials can be monitored, and growth of bacteria is measured by applications of MALDI. MS has high sensitivity and requires minimum amount of sample [2].

5.5 Biological assay

Biofilm-embedded bacteria can be estimated by biologic assays that is an indirect assay. Biological assays that measure production of microbial product give an opinion about estimation of the number of microorganism within biofilm. Amount of biologic product is correlated with biofilm-embedded microorganism producing the product by standardization of planktonic microorganism. Biologic products produced by planktonic microorganism are similar to biologic products produced

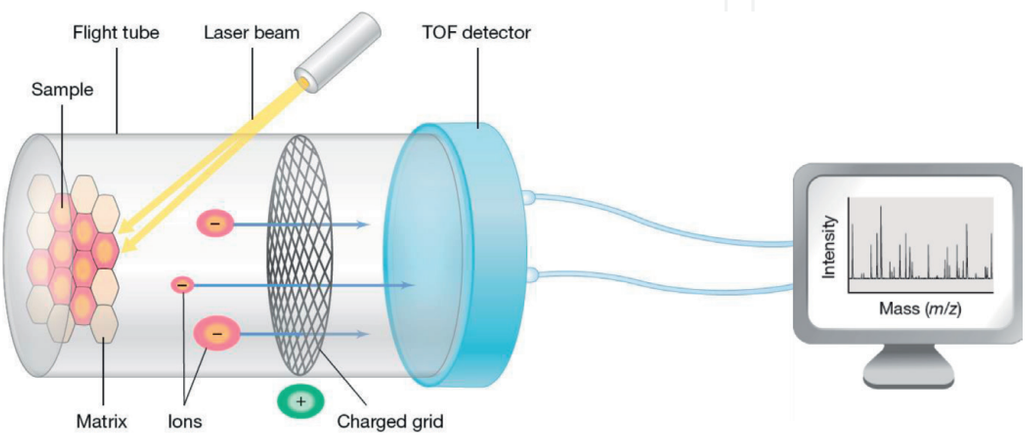


Figure 11.
MALDI-TOF mass spectrometry device [11].

by biofilm-embedded bacteria. Standardization curves of each microorganism tested need to be formed. Measurement of total protein at the absorbance is 550 or 950 nm; tryptophan fluorescence, endotoxin [2], ATP production via bioluminescence caused by luciferin and luciferase, urease production to estimate number of attached microorganism, and electron transport via the production of formazan are done by biological assays [3].

6. Conclusion

Biofilms cause resistance to many antimicrobial agents. The results of biofilm produced on indwelling medical devices are recurrent, untreatable infections and failure of medical device. To overcome chronic and recurrent infections, it is important to detect biofilms of microorganisms, determine antibiofilm activity of agents against biofilm, and determine antibacterial activity of agents against biofilm-embedded microorganism with the appropriate methods by clinical microbiologist and biofilm researcher microbiologist. Identification of genes involved in biofilm formation and measurement of gene expression as a result of antibiofilm and antibacterial activity of agents can be advantageous in biofilm studies.

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