Enferm Infecc Microbiol Clin. 2018;36(6):375–381

Enfermedades Infecciosas y Microbiología Clínica

www.elsevier.es/eimc

Review article

Microbiological diagnosis of biofilm-related infections☆

María D. Macià[a,*], José Luis del Pozo[b], María Díez-Aguilar[c], Jesús Guinea[d]

[a] Servicio de Microbiología, Hospital Universitario Son Espases, Instituto de Investigación Sanitaria de Palma (IdISPa) y Red Española de Investigación en Patología Infecciosa-REIPI, Spain
[b] Área de Enfermedades Infecciosas, Servicio de Microbiología, Laboratorio de Biofilms Microbianos, Clínica Universidad de Navarra, Spain
[c] Servicio de Microbiología, Hospital Universitario Ramón y Cajal e Instituto Ramón y Cajal de Investigación Sanitaria-IRYCIS (Madrid) y Red Española de Investigación en Patología Infecciosa-REIPI, Spain
[d] Servicio de Microbiología Clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Universidad Complutense de Madrid, Instituto de Investigación Sanitaria del Hospital Gregorio Marañón y CIBER de Enfermedades Respiratorias (CIBERES CB06/06/0058), Madrid, Spain

ARTICLE INFO

Article history:
Received 11 April 2017
Accepted 17 April 2017
Available online 7 May 2018

Keywords:
Biofilms
Antimicrobial susceptibility studies on biofilms
Microbiological diagnosis of biofilm-related infections

ABSTRACT

Biofilm-related infections represent a serious health problem, accounting for 65–80% of all infections. The infections are generally chronic and characterised by the persistence of the microorganism, due to the increased resistance of biofilms to both the immune system and antimicrobials. Biofilms can be located to almost every human body tissue and on exogenous devices such as catheters, pacemakers, prosthetic material, implants, urinary catheters, etc.

Traditional antimicrobial susceptibility studies in clinical microbiology laboratories have lied on the study of planktonic form of microorganisms. However, this approach might lead to miss the biofilm characteristics and to a treatment failure. Microbiological diagnosis and antimicrobial susceptibility studies of biofilm-related infections are complex and, nowadays, represent a challenge that clinicians and microbiologists have to address as a team in the absence of consensus or standardised protocols.

Diagnóstico microbiológico de las infecciones relacionadas con la formación de biopelículas

Palabras clave:
Biopelículas
Sensibilidad antibiótica en biopelículas
Diagnóstico microbiológico en infecciones por biopelículas

RESUMEN

Las infecciones asociadas a biopelículas suponen un grave problema sanitario ya que representan entre el 65 y el 80% de todas las infecciones. Estas son generalmente crónicas y están caracterizadas por la persistencia del microorganismo debido a su resistencia al sistema inmunitario y a los antimicrobianos. Las biopelículas se pueden localizar tanto en tejidos humanos como sobre dispositivos exógenos tales como catéteres, marcapasos, prótesis, implantes, sondas urinarias, etc.

Tradicionalmente, los laboratorios de microbiología clínica realizan los estudios de sensibilidad sobre microorganismos en crecimiento planctónico. Sin embargo, de esta manera se pierden las características propias de la biopelícula con lo que la antibioterapia basada en estos estudios podría asociarse con fracaso terapéutico o recurrencias. El diagnóstico microbiológico y los estudios de sensibilidad en las infecciones relacionadas con biopelículas son complejos y, hoy por hoy, representan un reto que clínicos y microbiólogos han de abordar en equipo ya que no existe todavía un consenso global ni protocolos estandarizados.

DOI of original article: https://doi.org/10.1016/j.eimc.2017.04.006
☆ Please cite this article as: Macià MD, del Pozo JL, Díez-Aguilar M, Guinea J. Diagnóstico microbiológico de las infecciones relacionadas con la formación de biopelículas. Enferm Infecc Microbiol Clin. 2018;36:375–381.
* Corresponding author.
E-mail address: mariad.macia@ssib.es (M.D. Macià).

Introduction

Biofilms are structured supracellular formations which develop as a survival strategy in hostile environments, supplying the microorganisms embedded in them with resistance to mechanical clearance, the immune system and to antimicrobial agents.[1,2]

Biofilm-related infections, which are typically persistent chronic infections refractory to antimicrobial treatment, represent a significant health problem as they account for 65–80% of all infections. These can be located on almost any tissue of the human body, including chronic skin and soft tissue infections, lung infections in patients with cystic fibrosis (CF) or bronchiectasis, or endocarditis.[2] Biofilms also cause infections related to various biomedical devices. In general, these infections are difficult to diagnose and treat. There is currently much uncertainty with regard to the optimal therapeutic strategy for these patients.[3] Traditionally, clinical microbiology laboratories have focussed on isolating and conducting susceptibility studies on microorganisms in planktonic state. However, releasing microorganisms from biofilms means that the biofilms lose their characteristics, and this can lead to mistakes in the extrapolation of data on antimicrobial susceptibility in planktonic state. This paper addresses both the microbiological diagnosis and susceptibility studies in biofilm-related infections.

Infections associated with the formation of biofilms on tissues and devices

Chronic lung infection

Chronic lung infections such as CF, chronic obstructive pulmonary disease and bronchiectasis represent a predisposing factor for chronic infection. The most prevalent microorganisms in this context are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[4–8] Suitable samples for microbiological diagnosis are spontaneous or induced sputum, bronchoalveolar lavage (BAL) or bronchial suction, trying to minimise oropharyngeal contamination while collecting the samples.

Chronic rhinosinusitis

This is an inflammatory condition which affects the mucous membranes of the paranasal sinuses and the nasal passages. It tends to start with a viral infection which, in some cases, progresses and develops into a secondary bacterial superinfection frequently caused by *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. If the viral infection is not resolved, colonisation by anaerobic oropharyngeal microbiota (such as *Fusobacterium nucleatum*, *Prevotella* spp., *Porphyromonas* spp. and *Peptostreptococcus* spp.) and aerobic oropharyngeal microbiota (*P. aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter* spp. and *Escherichia coli*) and *S. aureus* (including methicillin-resistant *S. aureus* [MRSA]),[9] even fungi such as *Aspergillus* spp., generally in elderly and/or immunocompromised patients, predominates. Purulent secretions obtained from the middle meatus or through the cavities of the paranasal sinuses are the preferred samples for microbiological diagnosis.

Chronic otitis media

Otitis media is an infection, which can be acute or chronic, with the presence of exudate in the middle cavity of the ear. The bacteria commonly involved are *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.[10] *P. aeruginosa* and *S. aureus* are the most commonly involved microorganisms in chronic suppurative otitis media and chronic otitis media with cholesteatoma. Microbiological diagnosis is generally reserved for cases which are refractory to treatment. The clinical sample should be obtained by means of aspiration through tympanocentesis, and, if there is spontaneous tympanic perforation, the exudate which flows to the external canal of the middle ear can be used. This sample will be taken with a syringe whenever possible, or, failing that, with a swab.

Chronic wound infection

A chronic wound is considered to be that in which the healing process does not take a normal course and the functional and anatomical integrity of the skin is not achieved after approximately one month. Infection is the main cause of this chronicity.[2,11] All open wounds are colonised by endogenous and exogenous microorganisms, although biofilms are usually composed of only one bacterial species, basically *P. aeruginosa* and *S. aureus*. Anaerobic microorganisms may also be involved (*Bacteroides* spp., *Prevotella* spp., *Porphyromonas* spp. and *Peptostreptococcus* spp.), *Bacillus anthracis*, beta-haemolytic streptococci, *Enterococcus* spp. and Enterobacteriaceae. A culture from the deep tissue biopsy is recommended for the microbiological diagnosis.

Infection in burn patients

Although the surface of the burns is initially sterile, microbial colonisation occurs rapidly: Gram-positive bacteria colonise the wound in the first 48 h, and, after 5–7 days, it may be colonised by other Gram-positive and Gram-negative bacteria, and later on by yeast from normal microbiota. The majority are monomicrobial infections and the most common microorganisms are *P. aeruginosa* and *S. aureus*. Other less common microorganisms are *Acinetobacter baumannii*, *Enterococcus faecalis* (*E. faecalis*), *E. coli*, *K. pneumoniae* and *Enterobacter* spp. The diagnosis is arrived at by clinical suspicion and quantitative culture (10^5 CFU per gram of tissue) of the biopsy material.

Native heart valve infection

Native valve endocarditis is caused by an interaction between the vascular endothelium and circulating microorganisms in the blood which multiply in the lesion forming a biofilm in the form of vegetations. The vegetations can prevent the correct functioning of the valve, generating a continuous source of microorganisms to the bloodstream and a risk of remote septic embolisms. This condition continues to have a high mortality rate and its main causative agents are *S. aureus* (31%), *viridans* group streptococci (17%), *Enterococcus* spp. (11%), coagulase-negative staphylococci (11%), *Streptococcus bovis – S. bovis* (7%), other streptococci (5%), Gram-negative bacilli (2%), fungi (2%), Gram-negative bacilli of the HACEK group (2%, *Haemophilus aphrophilus* [*Aggregatibacter aphrophilus*, *Aggregatibacter paraphrophilus*], *Actinobacillus actinomycetemcomitans* [*Aggregatibacter actinomycetemcomitans*], *Cardiobacterium hominis*; *Eikenella corrodens* and *Kingella kingae*[12]). The diagnosis is based mainly on the positivity of the blood cultures, which should be incubated for more than five days if endocarditis is suspected, and on the echocardiography. Nevertheless, the blood cultures may turn out to be negative (5–30% of cases) due to concomitant antibiotic therapy or in endocarditis caused by fungi or by fastidious microorganisms. Therefore, molecular techniques and serology may be useful. A positive culture from the vegetation is considered a major criterion for the diagnosis of endocarditis. A valve culture is not useful.

Prostate infection

Chronic prostatitis is an infection of the prostate gland over a prolonged period of time, with recurrent urinary tract infections in which the same microorganism from the prostatic secretions is isolated. The main causative agent of this infection is *E. coli*. *E. faecalis*, *P. aeruginosa*, *P. mirabilis*, *Klebsiella* spp. and *Enterobacter* spp. may also be involved.[13] The diagnosis is based on comparative quantitative cultures between (1) urine at the start of urination, (2) mid-stream urine, (3) prostatic secretion obtained after prostate massage (or semen) and (4) urine at the start of urination obtained after prostate massage or after masturbation. It is suggestive of prostatitis when the quantity of CFU/ml is ten times higher in prostatic secretion, semen or post-massage urine than in the first/mid-stream urine.

Vaginosis

Bacterial vaginosis is the most common vaginal disorder in women of childbearing age, which accounts for more than 60% of all vulvovaginal infections. It is accepted that it is an infection associated with biofilms, made up mainly of clusters of *Gardnerella vaginalis* strongly adhered to the vaginal epithelium (clue cells) along with the loss of beneficial bacteria (lactobacilli). Diagnosis is based on the presence of at least three of the Amsel criteria (characteristic vaginal discharge, elevated pH, clue cells, foul smell), as well as the microscopic observation of the vaginal secretion obtained by means of a swab of the clue cells. Vaginal cultures present a very low specificity.

Vascular catheter-related infection

Once a catheter is inserted, the host's proteins cover the internal and external surfaces of the device and serve as an attachment site for certain microorganisms, mainly coagulase-negative staphylococci, *S. aureus* and *Candida* spp. Other less common microorganisms include *Acinetobacter* spp., *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Micrococcus* spp., *Achromobacter* spp., mycobacteria, other yeasts and some filamentous fungi. The microbiological diagnosis is based on the similarity in isolations obtained from the culture from the insertion point or the catheter tip and the blood culture isolates. When the catheter is removed, it should be cultured according to Maki's semi-quantitative method,[14] in which the presence of 15 or more CFU per plaque indicates its colonisation and points to that as the origin of the infection. The culture from a central venous catheter with a subcutaneous reservoir should combine the swabbing of the reservoir chamber, the culture after sonication of the silicone membrane and the culture from the catheter's distal segment.[15]

Infection of prosthetic heart valve, pacemaker and grafts

Depending on the time elapsed from the implantation surgery, endocarditis is classified as early (<12 months) or late. Infections related to electrical stimulation devices can involve the generator, the generator and the electrodes, or the electrodes, and/or present as endocarditis. Vascular graft infection fluctuates around 6% with a mortality rate of 15–50% and an amputation rate of 8–50%. Staphylococci cause between 60% and 80% of these infections, but *viridans* group streptococci and *S. bovis*, *S. pneumoniae*, beta-haemolytic streptococci (mainly *Streptococcus agalactiae*) and *Abiotrophia* spp. and *Granulicatella* spp. can also be involved.[16] The definitive diagnosis of prosthetic valve endocarditis can only be established with certainty by means of a histological and microbiological examination of the vegetations. Duke University's diagnostic criteria are the basis for the diagnosis and positive blood cultures continue to be the cornerstone of the diagnosis (including in the case of infection related to pacemakers and implantable defibrillators). Serology or antigen detection is essential for the diagnosis of pathogens such as: *Coxiella burnetii*, *Brucella* spp., *Bartonella* spp., *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydia* spp. or *Aspergillus* spp. Molecular biology techniques applied to different tissues are also useful. Cultures from the pouch and leads after the extraction of the device are useful in the identification of the causative microorganism. The sensitivity of the biopsy culture from the subcutaneous pouch is higher than that collected from a swab. Percutaneous needle aspiration of the device's pouch should not be performed due to the lack of appropriate diagnostic performance and the theoretical risk of introducing microorganisms.[17]

Mechanical ventilation-associated pneumonia

Mechanical ventilation-associated pneumonia (MVAP) is pneumonia which occurs in patients with endotracheal intubation (or tracheostomy) during the period in which they are intubated. Colonisation of the upper airway, and even of dental plaque, is a predictive factor for the colonisation of the tracheobronchial tube and previous exposure to antibiotics is a predisposing factor for multidrug-resistant microorganisms such as methicillin-resistant *S. aureus* and *P. aeruginosa*. Valid samples for the diagnosis of MVAP are spontaneous or induced sputum, nasotracheal aspiration and the semi-quantitative culture from endotracheal aspirate (option), or samples obtained using a bronchoscopy with the following microbiological criteria: (a) BAL with a threshold of $\geq 10^4$ CFU/ml or $\geq$5% of cells containing intracellular bacteria in the direct microscopic examination; (b) sample obtained using a protected brush with a threshold of $\geq 10^3$ CFU/ml; (c) protected distal aspirate with a threshold of $\geq 10^3$ CFU/ml; (d) quantitative endotracheal aspirate with a threshold of $\geq 10^6$ CFU/ml.

Joint prosthesis-related infection

This infection has a mean incidence in Spain of 3–4%, which is probably underestimated. Gram-positive microorganisms (coagulase-negative staphylococci, *S. aureus*, enterococci and streptococci) are involved in around 65% of cases. Aerobic Gram-negative bacilli (*E. coli*, *P. mirabilis* and *P. aeruginosa*) represent 5–10%, and anaerobic Gram-negative bacilli (including *Propionibacterium acnes* and *Finegoldia magna*) account for 1–4%. Fungi, atypical mycobacteria, *Brucella* spp., etc. represent 1% of the total number of cases. Twenty per cent (20%) of cases are polymicrobial infections, and no microorganisms are isolated in 7% of cases.[18] Diagnosis of joint prosthesis-related infection continues to be a challenge. The criteria for chronic late infection are considered to be the presence of at least one of the following: (a) isolation of the same microorganism in two cultures from joint aspirate or from samples of periprosthetic tissue collected during the intervention; (b) presence of signs of acute inflammation in the histological examination of periprosthetic tissue; (c) fistulous tract in contact with the prosthesis; (d) purulent material in the joint space.

Synovial fluid should be sent for culture and a Gram stain should be performed, although it has limited sensitivity (<26%). The samples of periprosthetic material collected during the revision surgery of prostheses are the most cost-effective, although it is necessary to obtain a sufficient number of samples (5/6), which increases the sensitivity of the cultures and which facilitates discrimination between contaminating microorganisms and pathogens. Cultures from chronic fistula exudates, as well as cultures from swabs collected in the intraoperative period must be avoided.[18]

Due to the presence of biofilms, diagnosis using conventional techniques is difficult, and it is sometimes advisable to take a direct culture from the prosthesis. The prosthesis should be subjected

beforehand to a sonication process in order to detach the bacteria adhered to the removed implant. Cultures from samples obtained using sonication are more sensitive than conventional cultures from periprosthetic tissue and also than cultures from synovial fluid for the diagnosis of knee or hip prosthesis infection, especially in those patients who have received antibiotic treatment in the 14 days prior to surgery. It is important to perform aerobic and anaerobic cultures from the fluid resulting from sonication as 5% and 11% of cases, respectively, will be positive only in one of the two culture media.

Urinary catheter-related infection

Bacteriuria associated with urinary catheterisation is the most common nosocomial infection in our setting (up to 40%) and it is associated with an increase in mortality.[19] The microorganisms involved normally come from the patient's microbiota and they tend to be monomicrobial infections caused by *E. coli* or other Enterobacteriaceae, and sometimes by *P. aeruginosa*, enterococci or *Candida* spp. When the duration of catheterisation is extended, the infection may be polymicrobial, and, if patients are also receiving antibiotics, the isolation of multidrug-resistant Gram-negative bacilli is relatively common. Urinary catheter-related infection is defined as the presence of signs or symptoms compatible with urinary tract infection with no other possible focus, along with pyuria and a count $>10^3$ CFU/ml of a single bacterial species in a sample of urine obtained through the catheter or from a patient in whom the catheter was removed in the previous 48 hours. The finding in the urine culture of more than one microorganism should be interpreted with caution, as an infection in a catheter is often polymicrobial. Gram staining of a urine sample may be especially useful in seriously ill patients. The urine culture has to be collected by puncturing the catheter. In patients with permanent catheterisation, replacing the catheter and subsequently performing the urine culture is recommended to prevent contamination. Urine samples should not be collected systematically in asymptomatic patients.

Infection associated with other types of biomedical devices

Breast implant-associated infection is a significant cause of morbidity, with an incidence of up to 35% in cancer patients. Gram-positive microorganisms (*S. aureus* and streptococci) and some Gram-negative microorganisms are the most common in early infections, while coagulase-negative staphylococci, propionibacteria and atypical mycobacteria predominate in late infections (one month after surgery).[17]

Mesh-related infections have an incidence of approximately 1–2%. Once placed, the mesh is permeated with plasma and interstitial fluid, which contain proteins that act as receptors for certain bacterial adhesins, thereby initiating the development of the biofilm. The microorganisms most commonly involved in these types of infection are *S. aureus*, coagulase-negative staphylococci and *E. coli*.[17]

Infection is the most serious complication after a penile implant (2–3% in primary implants and up to 30% in re-interventions), given that this can cause multiple local complications that give rise to surgical interventions, prolonged hospitalisations, loss of functionality of the implant and psychological harm, in addition to generating a significant financial cost. Coagulase-negative staphylococci (in particular *Staphylococcus epidermidis*) are the most common microorganisms, followed by *S. aureus*, *E. coli*, *P. aeruginosa*, *Candida* spp. and even *Neisseria gonorrhoeae*.[17]

The incidence of infection associated with neurostimulation devices is highly variable, ranging between 0.6% and 12%. Staphylococci (*S. aureus* and *S. epidermidis*) are the most common microorganisms, although *P. acnes*, Gram-negative bacilli (such as *P. aeruginosa*) and atypical mycobacteria (such as *Mycobacterium fortuitum*) are sometimes found.[17]

The incidence of *cochlear implant-related infection* is low, between 1.6 and 10%. The surgical wound is most commonly affected, followed by otitis media and derived complications such as mastoiditis or meningitis. *S. aureus* is the most common cause of surgical wound infection and *S. pneumoniae* and *H. influenzae* of secondary infections. Unfortunately, there are no guidelines or recommendations regarding the microbiological diagnosis of these infections. In general, if peri-implant collections/exudates are observed, a puncture-aspiration can be performed, or a sample of tissue can be extracted when appropriate and the material can be sent to the microbiology laboratory for microscopic observation and culture. Blood cultures should be extracted from those patients who present with a fever or symptoms of sepsis or suggestive of bacteraemia. If it is decided to remove the implant, this should be placed in a sterile bottle and sent to the microbiology laboratory in order to perform a culture after sonication.[17]

Microbiological diagnosis of biofilm-related infections

The diagnosis of biofilm-related infections is complex and should combine an overall and multidisciplinary perspective which considers clinical aspects and microbiological findings. Therefore, it will require close and fluid communication between clinicians, microbiologists, nursing personnel and laboratory technicians.

Collection, transport and storage of samples

The samples will depend on whether the infection is located on living tissue or on a biodevice. Whenever possible, samples should be obtained before antimicrobial therapy is started, in the best aseptic conditions. Proper disinfection of the skin should be carried out beforehand (where applicable), and there should be minimal handling of the sample from when it is obtained until it is processed in the laboratory. They should be placed in suitable sterile containers without formalin or other preservatives and the use of transport media for anaerobic organisms should be avoided when a fungal or mycobacterial infection is suspected. The samples will be processed as quickly as possible and may be stored in a refrigerator at 2–8 °C for a maximum of 24 h, until processed. The samples in the transport medium for anaerobic organisms should be kept at room temperature. Whenever possible, a portion of frozen sample should be kept for the subsequent conduct of molecular studies.

Processing of samples

Where possible, it is recommended to study the samples microscopically, for example with routine staining methods such as the Gram stain, in order to view the biofilms and/or the presence of inflammatory cells (polymorphonuclear leukocytes), which reveal that there is an ongoing infectious process. The following liquid or semi-liquid samples do not need prior processing: blood for blood culture (in native or prosthetic heart valve infection, intravascular catheter-related infection, infection associated with pacemakers and implantable defibrillators, and infections in which it is necessary), urine (including that obtained through the urinary catheter), semen, prostatic secretion and joint fluid. However, the following semi-liquid samples do require prior processing: bronchial secretions in chronic lung infection, purulent secretions in rhinosinusitis and respiratory secretions in MVAP. In general, respiratory samples (especially sputum) present an elevated consistency and should be submitted to a prior homogenisation process with mucolytic agents (N-acetylcysteine) or dithiothreitol, thereby preventing prolonged treatment which may inhibit or delay microbial growth, or

a mechanical method (gentle sonication). Depending on their viscosity, it may be necessary to perform prior sampling in samples of pus in otitis, as in the case of respiratory secretions, stirring with saline solution or sonicating gently.

Biopsies

This will include deep tissues in chronic wound infection and burn patients, bone biopsies and periprosthetic tissue in joint prosthesis-related infection, vegetations in native or prosthetic heart valve infection when there is a valve replacement or post-mortem and biopsies of the subcutaneous pouch in the removal of pacemakers. Biopsy samples should be cut and homogenised in a stomacher homogeniser or in a sterile mortar with a small quantity of saline solution or brain heart infusion (BHI) broth, before being inoculated into the culture media. The smearing for stains should be performed using an imprint on the slide or from the homogenised sample. The inoculation should be performed with an inoculating loop or sterile pipette, transferring the homogenised sample to the culture media.

If molecular techniques are available, from tissues adhered to prosthetic heart valves or extracorporeal devices, one part of the sample will be separated for culturing and another part to perform a universal 16S rRNA polymerase chain reaction (PCR) technique and, if possible, a third part of the sample will be stored in an archive at −70 °C.

Prostheses, removed implants (processing using sonication)

This group will include pacemakers and implantable defibrillators, joint prostheses and implants (breast, penile, cochlear, etc.). When the prosthetic material is completely or partially removed, the tissues adhered to the material are also removed, and they should be processed separately as biopsies. For electrical stimulation devices, the sonication of the generator and leads is recommended, with the latter having shown a greater diagnostic performance. The processing of explanted devices should be performed using a sonication or agitation technique which makes it possible to separate the biofilm. If a sonicator is not available, the same procedure will be followed, performing only vortex agitation. If molecular techniques are available, from the product obtained from sonication or agitation of the prosthetic heart valves and heart electrical stimulation devices, two 1-ml aliquots will be separated: one for PCR and the other for archiving. A cut-off point has not been established, as in the case of joint prosthesis-related infection, to interpret the results obtained from the culture of the "sonicate", and the results should be interpreted with great caution. Immersing the devices in enrichment broth to then subculture, or the use of swabs which sample the surface of the device, are not recommended.[17,20]

Urinary catheters and intravascular catheters

Culturing after sonication of the catheter has proven to be more sensitive than the urine culture[19] by ensuring the detection of the biofilm after isolation of only the adherent bacteria. Currently, there are no standardised protocols for inoculation after sonication or with counts and subsequent interpretations of cultures. Therefore, the urine culture obtained via the catheter cannot be disregarded.[19] Regarding the processing of catheters, it is not known whether it is necessary to perform sonication prior to inoculation or if intraluminal colonisation should be determined. The Maki semi-quantitative technique is simple and is accepted as a reference technique. However, it has limitations (unknown sensitivity, low specificity and impossibility to diagnose endoluminal infections).[17] The techniques which enable intra- and extraluminal colonisation to be determined (washing the inside of the catheter and sonication) do not reach a sensitivity greater than 58% separately. The method described by Liñares et al.[14] (which combines the Cleri and Maki methods) presents a sensitivity of 100%, although it is difficult to implement in the laboratory due to its labour intensity. Therefore, the Maki technique continues to be the reference technique in most clinical microbiology laboratories.[14,17]

Microbiological culture

In contrast to microscopic observation, the culture requires prior release of the microorganisms from the biofilm by means of physical procedures (sonication, crushing and/or agitation). These infections tend to result in a low load of microorganisms, which makes it necessary to use enrichment media. At the same time, taking samples is susceptible to contamination. It is therefore key to distinguish between the microorganisms present in the biofilm and those that are part of the area's normal microbiota. This is complicated by the fact that the microorganisms in the normal microbiota can contribute to the biofilm (for example, *S. epidermidis*). The ideal situation is that both the cultures and the microscopic observation are quantitative or semi-quantitative and, whenever possible, that multiple samples are taken to increase the sensitivity. In these types of infection, some microorganisms may be viable and microscopically detectable, but it is not possible to culture them in conventional media. This means that it is necessary to resort to special media and/or molecular techniques.

Direct inoculation of the homogenised samples from biopsies, osteoarticular prostheses and intra- and extravascular biomedical devices should be performed in conventional media for aerobic bacteria (blood agar, chocolate agar) and selective medium for Gram-negative bacilli (MacConkey agar or similar), anaerobic bacteria (*Brucella* agar or Schaedler agar) and streptococci (nalidixic acid blood agar, CNA agar). Furthermore, an enrichment liquid medium such as BHI broth, tryptic soy broth (TSB) or thioglycolate will be inoculated. If a mycobacterial infection is suspected, specific media (such as plated Middlebrook 7H10) will be inoculated. If *Neisseria gonorrhoeae* is suspected in osteoarticular infection, a plate of Thayer-Martin medium incubated in a 5% CO_2 atmosphere will be inoculated. The blood agar, chocolate agar and CNA (colistin nalidixic acid) agar plates will be incubated at 35–37 °C with 5% CO_2. The *Brucella* agar and Schaedler agar plates for the culture of anaerobic organisms will be incubated at 35–37 °C in anaerobiosis. The broths will be incubated at 35–37 °C.

The incubation time for the plates will be 2–7 days and 7–10 days for the enrichment broths. If slow-growing microorganisms (*Brucella* spp., mycobacteria or fungi) are suspected, this period will be extended appropriately. The plates and liquid media will be examined daily to detect the presence of microorganisms.

Molecular techniques

These techniques enable the identification of microorganisms in the event of infection when the culture is negative, either by the presence of microorganisms which are difficult to culture or because there was prior antibiotic therapy which inhibited microbial growth. They can be performed on most samples. Currently, the techniques which are used most frequently are universal or specific PCR based on the detection of DNA or RNA. Universal PCR is based on the amplification of the gene which encodes for 16S ribosomal DNA (or ITS for fungi) and subsequent sequencing, comparison of sequences with databases and identification of the microorganism. Specific PCR allows for the detection of the presence of specific "non-cultivable" microorganisms, such as *Tropheryma whipplei*, *C. burnetii* or *Bartonella* spp. The infections caused by these microorganisms usually have negative blood cultures. Both formats can be designed quantitatively. Molecular techniques can be more sensitive than culturing in some cases, but they should complement it rather than replace it. More studies are necessary to define their role in the diagnosis of biofilm-related

infections, as well as protocols which describe their use, their application and make their use mainstream in clinical laboratories.

Antimicrobial susceptibility studies on biofilms

Microorganisms which form part of the biofilm are significantly more resistant to antimicrobials than those that grow planktonically. Cut-off points for these forms of growth have not yet been established, as at present the classic antimicrobial susceptibility studies are still performed with planktonic-state bacteria. Therefore, in general, it may not be possible to extrapolate the results of classic susceptibility tests to biofilm-related infections. In recent years, *in vitro* biofilm formation models have been implemented and tested in different species of bacteria and yeasts.[21] However, the current lack of standardisation of the methods, parameters and interpretation of the results limits the application of the data obtained in the clinical setting, including the comparison of different treatment strategies.[21,22]

In vitro *models*

Depending on the continuous or static release of nutrients (and other characteristics such as if it is necessary to stop experiments to obtain structural data or CFU counts), the models can be classified as closed or static (batch cultures) and as open or continuous (continuous cultures). Closed models have the advantage of being simple, feasible, reproducible, inexpensive and not very susceptible to contamination. This means that they can be easily implemented into a microbiology laboratory's routine. Moreover, small modifications to protocols can also allow for feasibility studies. The major disadvantage is that the thickness of biofilms does not exceed 50 μm, and this limits structural studies. Examples of these models are susceptibility studies in multi-well plates or in the Calgary model.[23] Open models replicate the dynamics of the *in vivo* formation of biofilms more accurately, as they control parameters such as the flow of the medium, the arrival of nutrients and the temperature. Constant environmental characteristics are maintained via a continuous flow, controlling the temperature and the arrival of the culture medium. These models also allow for the implementation of PK/PD models and the observation under the microscope of biofilm formation, as well as the determination of structural parameters such as biomass or thickness. With these systems, planktonic cells are eliminated with the flow. Therefore, the analysis of cells which form part of the biofilm is ensured. Susceptibility studies in the flow cell system, bioreactors (such as CDC) or the *BioFlux* system have been performed.

Pharmacokinetic/pharmacodynamic parameters of antibiotic activity

Thanks to the susceptibility studies performed on these models, it has been possible to define pharmacokinetic/pharmacodynamic (PK/PD) parameters which quantify the activity of antimicrobials on biofilms, such as the minimum inhibitory concentration of the biofilm (MICB: lowest concentration of antibiotic which results in a difference of DO650 nm $\leq$10% of the mean of two readings of positive control wells), the biofilm bactericidal concentration (BBC: lowest concentration which eliminates 99.9% of cells recovered from a biofilm culture in comparison with the control growth), the minimum concentration for biofilm eradication (MCBE: lowest concentration of antibiotic required to eradicate the biofilm or, in other words, the lowest concentration of antimicrobial agent which prevents visible growth in the recovery medium of the biofilm cells), or the biofilm prevention concentration (BPC: parameter which could be used in order to reduce cellular density to prevent biofilm formation).

In vitro *studies and correlation with clinical trials*

In almost all cases, the parameters described have been defined using the Calgary system,[23] or related systems. These parameters have been used to compare the activity of antibiotics on bacteria in planktonic growth and in biofilms. Many of the first studies were carried out on biofilms formed by *P. aeruginosa*. In these studies, most of the antibiotics presented an MICB two or more dilutions greater than the MIC. The same occurred with the MCBE or BBC vs minimal bactericidal concentration (MBC). However, studies with other models and other microorganisms have demonstrated the complexity of the interaction of antibiotics on biofilms and that, unfortunately, few general practical conclusions can be drawn as the results are more linked to the design of the experiments or the model employed rather than the antibiotic used. In fact, based on the analysis of the two clinical trials comparing the treatment of *P. aeruginosa* chronic lung infection in CF, in accordance with standard susceptibility studies versus susceptibility studies on biofilms, current evidence is insufficient to recommend the selection of antibiotics based on antimicrobial susceptibility testing on biofilms in this context.[24]

Reporting of the results

The microbiological report in a biofilm-related infection should contain results of the microscopic observation, the culture and the susceptibility studies, where applicable. Although the Gram stains should be performed and reported as soon as possible, and even preliminarily, the findings of the different methodologies used should be interpreted jointly for the final result.

In the microscopic observation, the complete smear(s) should be viewed, and its result will not rule out the culturing of the sample. The observation of polymorphonuclear leukocytes and/or if a microbial biofilm is detected will be assessed and reported, using descriptive terms such as "Gram-negative bacilli arranged in aggregates are observed in the Gram stain along with the presence of polymorphonuclear leukocytes suggestive of a biofilm-related infection".

Microorganisms in cultures are found in planktonic growth. Therefore, their interpretation is not modified. The consistency between the microscopic observation of the biofilm and a pure culture of the presumed microorganism is highly suggestive of it being an infection related to the microorganism in biofilm form. In this scenario, it would make sense to perform the susceptibility study on this microorganism using a growth model on biofilm. When there is no consistency, the microbiologist should be able to give a reasonable interpretation and provide a useful result to the clinician. Therefore, a positive microscopic observation could be accompanied by a negative culture when the patient has received antibiotic treatment, when the bacteria cannot be cultured or if the sample processing may have altered the results. If the laboratory has access to molecular techniques, it would be very useful to apply them to the sample. The opposite situation (negative microscopic observation with positive cultures) creates greater complexity as, depending on the type of infection, contamination of the sample while it is being collected or processed cannot be ruled out. Thus, in many infections (such as those related to exogenous devices), clinical value should be given to microorganisms that in other circumstances would be considered normal skin microbiota, particularly when biotype and susceptibility profile match, or when they are found in more than one patient sample. Likewise, the mixed cultures should be interpreted according to the type of

infection, microscopic observation and repeated isolation in different samples, before deciding whether to perform conventional susceptibility studies or studies on the biofilm. Stains or negative cultures do not rule out a biofilm-related infection, and it is recommended to keep the direct inoculation plates, as well as a portion of the samples, in case it is necessary to perform additional tests.

In some biofilm-related infections, especially when these are a focal point for systemic infection (for example, those associated with intravascular devices or urinary catheters, chronic lung infection exacerbations), classic susceptibility studies can be implemented successfully. However, clinicians should be informed that a recurrence of the infection may occur due to the biofilm site if this is not eradicated. Unfortunately, there are no recommendations on how to report these results and, furthermore, no clinical advantage has been found in patients treated according to the results of biofilm susceptibility studies compared to conventional studies. Parameters such as the MICB should be recorded for the different antibiotics and microorganisms without providing an interpretation (S, susceptible; I, intermediate and R, resistant) in the absence of cut-off points defined by official agencies.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgement

To Dr Antonio Oliver of the Microbiology Department of the Hospital Universitario Son Espases for his valuable help in the drafting and review of this manuscript.

References

1. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284:1318–22.
2. Bjarnsholt T. The role of bacterial biofilms in chronic infections. APMIS Suppl. 2013;136:1–51.
3. Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, et al., ESCMID Study Group for Biofilms and Consulting External Expert Werner Zimmerli. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect. 2015;21 Suppl. 1:S1–25.
4. Oliver A, Canton R, Campo P, Baquero F, Blazquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science. 2000;288:1251–4.
5. Macià MD, Blanquer D, Togores B, Sauleda J, Perez JL, Oliver A. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob Agents Chemother. 2005;49:3382–6.
6. Luján AM, Maciá MD, Yang L, Molin S, Oliver A, Smania AM. Evolution and adaptation in *Pseudomonas aeruginosa* biofilms driven by mismatch repair system-deficient mutators. PLoS ONE. 2011;6:e27842.
7. Macià MD, Pérez JL, Molin S, Oliver A. Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of *Pseudomonas aeruginosa* biofilm treatment. Antimicrob Agents Chemother. 2011;55:5230–7.
8. Doring G, Flume P, Heijerman H, Elborn JS. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. J Cyst Fibros. 2012;11:461–79.
9. Ramakrishnan Y, Shields RC, Elbadawey MR, Awilson J. Biofilms in chronic rhinosinusitis: what is new and where next. J Laryngol Otol. 2015;129:744–51.
10. Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA. 2002;287:1710–5.
11. Oates A, Bowling FL, Boulton AK, Bowler PG, Metcalf DG, McBain AJ. The visualization of biofilms in chronic diabetic foot wounds using routine diagnostic microscopy methods. J Diabetes Res. 1535;2014:86.
12. Murdoch DR, Corey GR, Hoen B, Miró JM, Fowler VG, Bayer AS, et al. Clinical presentation, etiology, and outcome of infective endocarditis in the 21st century: the International Collaboration on Endocarditis-Prospective Cohort Study. Arch Intern Med. 2009;169:463–73.
13. Nickel JC, Costerton JW. Bacterial localization in antibiotic-refractory chronic bacterial prostatitis. Prostate. 1993;23:107–14.
14. Maki DG, Weise CE, Sarafin HW. A semiquantitative culture method for identifying intravenous-catheter-related infection. N Engl J Med. 1977;296:1305–9.
15. Liñares J, Sitges-Serra A, Garau J, Pérez JL, Martín R. Pathogenesis of catheter sepsis: a prospective study with quantitative and semiquantitative cultures of catheter hub and segments. J Clin Microbiol. 1985;21:357–60.
16. Klug D, Balde M, Pavin D, Hidden-Lucet F, Clementy J, Sadoul N, et al., PEOPLE Study Group. Risk factors related to infections of implanted pacemakers and cardioverter-defibrillators: results of a large prospective study. Circulation. 2007;116:1349–55.
17. De Cueto-López M, del Pozo-León JL, Franco-Álvarez de Luna F, Marin-Arriaza M. Diagnóstico microbiológico de las infecciones asociadas a dispositivos biomédicos. Enferm Infecc Microbiol Clin. 2016;34:655–60.
18. Del Pozo JL, Patel R. Clinical practice Infection associated with prosthetic joints. New Engl J Med. 2009;361:787–94.
19. Hola V, Ruzicka F, Horka M. Microbial diversity in biofilm infections of the urinary tract with the use of sonication techniques. FEMS Immunol Med Microbiol. 2010;59:525–8.
20. Trampuz A, Piper KE, Jacobson MJ, Hanssen AD, Unni KK, Osmon DR, et al. Sonication of removed hip and knee prostheses for diagnosis of infection. New Engl J Med. 2007;357:654–63.
21. Macià MD, Rojo-Molinero E, Oliver A. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clin Microbiol Infect. 2014;20:981–90.
22. Malone M, Goeres DM, Gosbell I, Vickery K, Jensen S, Stoodley P. Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. Expert Rev Antiinfect Ther. 2016;15:147–56.
23. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J Clin Microbiol. 2004;42:1915–22.
24. Waters V, Ratjen F. Standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis. Cochrane Database of Systematic Reviews. 2015;5:CD009528.