REVIEW

Focus on Bioburden Culture Media and Medical Devices

EDITH FILAIRE[1,2,*], VINCENT ROCHETTE[1], ELODIE JAROUSSE[1], and CHRISTIAN POINSOT[1]

[1]Groupe ICARE, Saint Beauzire, France; and [2]ECREIN Team, UMR 1019 INRAE-UCA, UNH (Human Nutrition Unit), University Clermont Auvergne, 63000 Clermont-Ferrand, France

ABSTRACT: Medical devices are a vital part of the global health care system that can have a far-reaching impact on patient treatment. Therefore, they must be sterile to ensure patient safety. The prevalent microorganism's type on a medical device, also known as "bioburden", is a useful indicator of a potential contamination source. Indeed, bioburden is a potential risk to the patient not only because the sterilization process might not be completely effective, but also post-processing because of the possible presence of residual materials. Although bioburden may be confidently killed by destructive sterilization processes, its proliferation before sterilization should be avoided. For the bioburden determination, the culture media and incubation conditions must be carefully selected. The culture medium is of fundamental importance for most microbiological tests: to obtain pure cultures, to grow and count microbial cells, and to cultivate and select microorganisms. A culture medium is essentially composed of basic elements (water, nutrients) to which must be added different growth factors that will be specific to each bacterium and necessary for their growth. Without high-quality media, the possibility of achieving accurate, reproducible, and repeatable microbiological test results is reduced. In ISO 11737-1:2018 "Sterilization of Health Care Products—Microbiological Methods—Part 1: Determination of a Population of Microorganisms on Products", methods for the determination and microbial characterization of bioburden are proposed. However, few guidelines are given as to culture media other than examples and incubation times. Several studies show that other culture media can also be effective, such as Plate Count Agar (PCA). The purpose of this review was to focus on parameters that can have an impact on the bioburden evaluation, specifically the culture medium type for the microorganisms' detection on medical devices. Experimentations conducted in our laboratories showed that PCA appeared to be a medium of primary importance for the detection of bioburden on medical devices; this medium also respects the 3Rs rule.

KEYWORDS: Culture media, Medical devices, Bioburden, Pharmacopeia, Plate Count Agar.

1. Introduction

Medical devices are a vital part of the global health care system that can have a far-reaching impact on patient treatment. Producing safe products is a core goal of all medical device manufacturers, and sterility assurance is a key component in achieving that goal. The U.S. Food and Drug Administration and other regulatory bodies required that the sterilization process must be validated, and these validations typically required a bioburden and sterility testing. Sterility testing and bioburden testing are also performed on devices as part of routine quality control.

* Corresponding Author: Rue Emile Duclaux 63360 Saint Beauzire, France; Telephone: 0033648035198; E-mail: edith.filaire@groupeicare.com
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Bioburden testing determines the microbiological loading of a medical device that is a combination of the microbial content of raw materials, storage conditions, manufacturing environment and cleanliness, manufacturing process steps and staff. However, it is not possible to determine bioburden exactly (1). In fact, as mentioned in ISO 11737-1:2018 "the culture conditions, that is, the culture media and the incubation conditions selected to be used for the determination of the bioburden probably do not allow all potential microorganisms to be detectable". In practice, likely the bioburden is underestimated.

Bioburden estimation is defined as the value established for the number of microorganisms comprising the bioburden by applying to a viable presterilization count and an offset factor for the recovery efficiency. Bioburden estimation is very important because it determines the sterilization dose necessary to achieve the desired sterility assurance level (SAL). To do that, one must be aware of

the limitations of the bioburden test itself in characterizing the bioburden, as well as understand the natural variation in the bioburden from product to product and batch to batch (2).

Beside culture conditions, extraction methods, which can change according to the laboratory (manual agitation, mechanical agitation, ultrasound), may have an impact on the bioburden control. Poinsot and Boucard (3) recently showed that bioburden measurement methods and results varied significantly from laboratory to laboratory and concluded that limiting the examination of the method adopted to the number of media implemented (1, 2, or 3) based on Pharmacopoeia is irrelevant. In this case, the systematic use of several culture media can result in counting microorganisms several times and overestimating the bioburden of a product, and thus call into question the establishment of the sterilization process.

Generally, no test or panel of limited tests can detect all types of microorganisms that might be present on a given product, or on a group of products, where selected individual units represent a manufacturing batch. Thus, bioburden on a product can and will vary (2).

The type of microorganisms prevalent on the medical device are a useful indicator of the potential source of the contamination, and the microorganisms' type may affect the sterilization process validation, knowing that there are several sterilization methods utilized by medical device manufacturers. The most common ones are steam sterilization, dry heat sterilization, chemical sterilization using gases like ethylene oxide, and radiation. The choice of sterilization technique will depend on the material composition of the medical device, how it is classified, and its intended use. Sterilization is a process designed to eradicate all viable forms of microbial life, including viruses, bacterial spores, and mycobacteria from the surface of an article or in a fluid (4). All sterilization methods require validation to demonstrate process parameters with a specified load configuration to ensure a minimum SAL of 10^{-6} according to EN 556-1: 2001 "Requirements for Medical Devices to be Designated Sterile—Part 1: Requirements for Terminally Sterilized Medical Devices". Three interdependent elements are important to the sterilization success including 1. intimate and adequate contact between the sterilant and all device surfaces, 2. bioburden minimization (through cleaning), and 3. validated and appropriate sterilant and sterilizing equipment to achieve the correct temperature/sterilant combination.

For ethylene oxide and gas steam, the most popular method to validate the sterilization process is by using an overkill approach with biological indicators having a known resistance to the particular sterilization method under investigation, for example, *Bacillus atrophaeus* for ethylene oxide sterilization and *Geobacillus stearothermophilus* for gas steam. Medical devices terminally sterilized by radiation are parametrically released based on dosimetry results. The additional testing requirement for sterilization validations includes assessing the medical device natural product bioburden for resistance to the sterilization process and biological indicators, also known as a sublethal cycle, in which reduced exposure time is used to challenge the medical device and then a sterility test is performed. This is run in conjunction with the biological indicators as used in the sterilization validation. This testing indicates whether the bioburden contains more sterilization-resistant microorganisms than the biological indicators used in the sterilization validation. As long as the biological indicator remains more resistant than the medical device bioburden, the sterilization validation remains valid (5).

Because the biocontamination of medical devices is a major public health problem, especially for implantable devices, the purpose of this review was to focus on parameters that can have an impact on the bioburden estimate, specifically the selection of product for bioburden test, and the culture media type for the microorganisms' detection on medical devices.

2. Product Selection for Bioburden Test

Bioburden testing is an important part of microbiology for medical devices. Medical products requiring sterilization, in general, are manufactured under conditions that are designed to control product bioburden levels.

This bioburden estimation of a medical device generally consists of four distinct stages including 1. collection of microorganisms from the medical device, 2. enumeration of the collection sample containing recovered microorganisms, 3. bioburden characterization, and 4. application of the correction factor(s) determined during bioburden recovery studies to calculate the bioburden estimate from the raw presterilization count.

Bioburden tests are addressed in ISO 11737-1:2018 and Pharmacopoeia documents such as the United States Pharmacopeia (USP), European Pharmacopoeia (EP),

and Japanese Pharmacopoeia (JP); these latter documents not only addressing medical devices but also drugs.

It is important to note that ISO 11737-1:2018 does not define a single method for determining bioburden due to the wide variety of designs and materials used for medical devices (natural or synthetic materials, number of components, automated or manual assembly), those influencing the variability in the bioburden (2). The type of microorganisms present in each sample can also be varied. This point is very important to consider, even more so if a limited number of samples is evaluated. Moreover, samples of product to be tested should be representative of the production batches; the number of samples required to give a reliable bioburden estimate will depend on the purpose of the bioburden enumeration.

Generally, the practice is to use a sample size of between 3 and 10 samples for routine monitoring of bioburden levels. However, for a new product, the use of more sample items, especially in the initial manufacture of a product, might be useful to detect the magnitude of variability in the bioburden that can be expected for the product (2).

Bioburden testing is commonly performed on solid products (e.g., plastics and metals) by performing an extraction with a water-based solution followed by testing all or a portion of the extraction solution using membrane filtration, pour plating, or spread plating.

For liquid products, bioburden testing is commonly performed by using membrane filtration, pour plating, or spread plating.

Nevertheless, various techniques of dislodging the viable microorganisms from the samples can be used, including ultrasonication, mechanical shaking, vortex mixing, flushing, and even blending if possible. The choices of extracting fluids may range from sterile water to a sterilized solution of surfactants such as polysorbates. The efficiency of extracting the microbes from the medical device is determined as part of the validation protocol in the bioburden test. Repetitive recovery or repetitive extraction should be conducted to obtain the correction factor that will be used to compensate for the extraction efficiency. Alternatively, if the bioburden count is known or expected to be <10 CFU/device, the inoculation method should be applied to estimate the extraction efficiency and thus the correction factor. It is important to note that repetitive recovery or repetitive extraction are the gold standard; the inoculation method can overestimate the performance of the method. Indeed, microorganisms added to the device are easier to extract than the resident flora.

3. Culture Media for Medical Devices

Once the microorganisms have been extracted into the solution, a few techniques of culturing the microbes can be used; the choice of growth media and incubation conditions will depend on the type of microorganisms to be enumerated. Selective or differential media can be used to exhibit growth of specific microorganism types, knowing that certain types of media or incubation temperatures can result in very specific outcomes.

ISO 11737-1:2018 specified that "the culture conditions must be selected based on knowledge of the manufacturing process of the medical device, the environment, the microorganisms supposed to be present. Moreover, if the specific product conditions indicate that further evaluation is required, the microorganisms counted under typical culture conditions must be compared to those detected under alternative conditions".

Historically, the discovery of culture media allowed the development of microbiology in the nineteenth century (6). The first to have cultured a bacterium in a reproducible way was Louis Pasteur in 1860 thanks to the development of the first so-called artificial culture medium (7). New culture media today mimic the natural environment of bacteria by adding different elements in culture medium to cultivate bacteria that were previously uncultivated (8). Culture media are of fundamental importance to obtain pure cultures, to grow and count microbial cells, and to cultivate and select microorganisms. The medium encourages the support and survival of microorganisms and contains nutrients, growth promoting factors, energy sources, buffer salts, water, minerals, metals, growth factors, and gelling agents (for solid media). Certain biophysical factors such as ambient conditions relating to pH, atmosphere, and temperature must also be considered. Different categories of growth factors are available, among which are purine and pyrimidine bases. They are necessary for the synthesis of nucleic acids. Indeed, some lactic acid bacteria need adenine, guanine, thymine, or uracil for growth. Amino acids and vitamins are also part of growth factors, the latest being coenzymes or precursors of coenzymes. For example, *Faecalibacterium prausnitzii*, which is a fastidious bacterium, requires

many vitamins to grow, such as biotin, folic acid, riboflavin, or vitamin B_{12} (9).

It is also possible to use rumen fluid to promote the growth of certain bacterial species by mimicking their natural environment (10). Adding antioxidants to the culture medium also allows the culture of strict anaerobic bacteria under aerobic conditions (11). This is the case for ascorbic acid and glutathione or uric acid. Dione et al. (11) noted that the addition of these antioxidants to the culture medium and its incubation under aerobic conditions allowed the growth of 135 strict anaerobic bacteria, 12 microaerophilic bacteria, and 22 strict aerobic bacteria.

Certain biophysical factors such as ambient conditions relating to pH, atmosphere, and temperature must also be considered. Moreover, without high-quality media, the possibility of achieving accurate, reproducible, and repeatable microbiological test results is reduced (12).

It has been shown that traditional culture-based tests using microbiological media can recover only 1%–10% of microorganisms present in any environmental sample (13). Using improper or compromised media or failure to incubate the sample properly will further impede maximum recovery. Thus, the quality of the media used for microbiological analyses is very important to assure maximal recovery; many variables affect recovery, including:

1. The colony forming unit or "CFU" is not a true cell count (individual cells are rare in nature, leading to the CFU being an underestimation of the number of microorganisms present
2. Incubation conditions (temperature and time)
3. Nutrition requirement of the organism
4. Physical condition of the organism (stressed or sublethally damaged due to temperature, humidity, high ionic strength, extremes pH, osmotic shock [relating to liquid], residues of antimicrobial)
5. Dilution errors
6. Environmental organisms are unlikely to be recovered when they are in the exponential growth phase

In addition, it is important to keep in mind that microorganisms might be missed because the culture ingredients or conditions are not used (2), inducing partial detection of microorganisms. This is very important, because in the event of a low level of bioburden, the number of microorganisms that might not be detected will be a larger percentage of the total.

3.1. Optimal Culture Media for the Bioburden Test

Bioburden testing involves an estimation of the numbers of bacteria and fungi present in a liquid or solid sample using the Total Viable Count (TVC) method, whereby a portion of the material is plated out onto microbial culture media (agar) or mixed with culture media and then incubated for a period to assess microbial growth and microbial numbers. Common TVC methods employed are membrane filtration (in which a portion of the material or a rinse of the material is passed through a microbially retentive membrane filter), pour plate, or spread plate. An alternative method is the Most Probable Number (MPN) method. This method is a serial dilution-based technique and provides an estimate of growth, but not directly of colony forming units (CFU). Moreover, this method, in which multiple species of microorganism can be present, does not readily permit microbial identification because of the risk of one microorganism outgrowing another.

In ISO 11737-1:2018, methods for the determination and microbial characterization of bioburden are proposed. However, few guidelines are given as to culture media other than examples and incubation times. Nevertheless, one point is underlined: "Unless fastidious microorganisms are likely to be present, generic, nonselective culture media and incubation conditions are preferred". Moreover, no combination of culture medium and incubation conditions can allow the growth of all microorganisms; conditions should be chosen that minimize the risk of overestimating the mean bioburden due to the enumeration of the same microorganism in different media.

If aerobic spores are detected in the count of aerobic bacteria and if the number of fungi remains low, it is possible to combine in the same test aerobic bacteria, bacterial spores, and fungi.

In addition to culture media, incubation times and temperatures are key factors for the detection of yeasts and molds.

Synthetically, the suggestions of culture media proposed in ISO 11737-1:2018 include:

1. For facultative aerobic bacteria:

    a. Tryptic soy agar casein-peptone soymeal, Nutrient Agar, Blood Agar Base, Plate count Agar (PCA)

    b. Tryptic Soy broth, Nutrient broth

2. For yeasts and molds:

    a. Sabouraud glucose agar medium, Malt extract agar, Rose Bengal Agar, Chloramphenicol agar, Tryptone Soy Agar, Potato Glucose Agar, PCA

    b. Malt extract broth, Sabouraud glucose broth, Tryptone soy broth, Casein-Peptone Soymeal-Peptone

3. For anaerobic bacteria:

    a. Schaedler blood agar, Columbia agar, Wilkins-Chalgren Anaerobe Agar, blood agar, Reinforced Clostridial medium

    b. Robertson's Cooked Meat Medium, Fluid thioglycolate medium

To our knowledge, studies evaluating the influence of culture media on microorganisms are scarce. Nevertheless, in our laboratories, Faquir (14) focused on this problem and investigated the growth of different types of microorganisms on different media. Six culture media (Plate count Agar-**PCA**; Schaedler Agar-**GSH**; Tryptic Soy agar-**TSA**; Sabouraud Chloramphenicol- **GSC**; Sabouraud media-**Gsab**; and Blood agar**-BD)** on 21 strains (including yeasts, molds, Gram-positive bacilli, and Gram-negative bacilli) were compared. Table I summarizes the strains used.

PCA is a bacteriological nonselective medium used for the determination of the total number of live, aerobic bacteria in a sample. It is used for the enumeration of the total aerobic flora in water, wastewater, food and dairy products, cosmetics, or pharmaceuticals. It is a nutrient medium without inhibitors, the advantage of which is to promote the development at 30°C of all the microorganisms that have been deposited there.

GSH is an extremely nutritious medium used for the nonselective isolation of anaerobes and for the selective isolation of Gram-negative rod anaerobes, *Bacteroides* and *Prevotella* spp., and various other Gram-negative anaerobes.

TABLE I
Microorganisms Investigated

Name	Reference
Aspergillus niger	IP 1431-83
Bacillus amyoliquefaciens	CIP 103265 T
Bacillus licheniformis	CIP 52.71 T
Bacillus sphaericus	CIP65 .30 T
Bacillus subtilis	CIP 52.62
Bordetella bronchiseptica	CIP 53.157
Candida albicans	IP 48-72
Cellulomonas hominis	CIP 104574
Enterococcus hirae	CIP 58.55
Escherichia coli	CIP 53.126
Fusarium solani	36031
Micrococcus luteus	CIP 53.45
Mycobacterium smegmatis	CIP 73.26
Pseudomonas aeruginosa	CIP 82.118
Staphylococcus aureus	CIP 4.83
Staphylococcus epidermis	CIP 68.21
Staphylococcus schleiferi	CIP 104370
Streptococcus pneumoniae	CIP 102911 T
Zygosaccharomyces rouxii	CIP 102911 T
Staphylococcus saprophyticus	CIP 60.77 T
Stenotrophomonas maltophilia	CIP 60.77 T

GSC is a medium that allows the growth and isolation of a wide variety of yeasts and molds. The addition of chloramphenicol inhibits the growth of Gram-positive and Gram-negative bacteria.

Gsab is a type of agar growth medium containing peptones. It is used to cultivate types of fungi and can also grow filamentous bacteria.

BD is an enriched medium used to culture those bacteria or microbes that do not grow easily. Such bacteria are called "fastidious" as they demand a special, enriched nutritional environment as compared with the routine bacteria. More precisely, casein is used to grow a wide range of pathogens particularly those that are more difficult to grow such as *Haemophilus influenzae, Streptococcus pneumoniae,* and *Neisseria* spp. It is also required to detect and differentiate hemolytic, especially *Streptococcus*, species.

TSA is a general-purpose, nonselective medium providing enough nutrients to allow for a wide variety of microorganisms to grow.

Results showed that three media, PCA, BD, and TSA, allowed the growth of all strains. Nevertheless, in line with the Directive 2010/63/EU, which states that animal tests must not be conducted if an alternative method is available, PCA seems to be an interesting medium for bioburden evaluation. Indeed, unlike BD, PCA is animal-free.

After this first experimentation, Faquir (14) used these three media to estimate the bioburden on medical devices including perfusion, electrode, and foam adhesive. In the case of medical devices with low contamination, PCA medium was able to detect the most microorganisms (20% more), as compared with the others. One can put forward the hypothesis that the yeast extract in PCA can be the nutrient explaining the different behavior between the media.

3.2. Limits of Detection

The limit of detection refers to the smallest number of microorganisms in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions (15). A bioburden test in which no microbial colonies are recovered will not be reported as "zero CFU", rather the results will be reported as a "less than" statement. Thus, a reflection on the limit of detection is essential because during the next step, which is sterilization, the question to be asked is whether the objective is to eliminate only the microorganisms detected during the bioburden tests or whether the objective is to sterilize all the microorganisms that could be on the medical device.

4. New Directions

Bioburden testing are critical quality control steps in the pharmaceutical production process. Culture media are a key parameter to recover, enumerate, and identify microorganisms.

After stagnation in the development of new culture techniques due to the rapid evolution of new microbiological methods such as metagenomics, bacterial culture is experiencing a new boom. In recent years, culturomics, with the use of new culture media and new culture conditions, have enabled the enrichment of the bacterial repertoire through the isolation of new bacterial specie (8). Numerous studies have been realized to discover specific media for specific microorganisms or for the use of cell cultures linked to pathologies. For example, assuming that the worldwide emergence of multidrug-resistant (MDR) bacteria represents a major public health issue, Bardet and Rolain (16) developed a polyvalent selective culture medium that can isolate both colistin-resistant Gram-negative bacteria, including *Enterobacteriaceae* strains harboring the *mcr-1* gene, and vancomycin-resistant Gram-positive bacteria.

Thus, developing innovative culture media is a real challenge that allows faster and easier detection of bacteria and therefore ensures a better result of the bioburden in medical devices.

5. Conclusion

Microbiological culture media are the most widely used and arguably the most important tools of the pharmaceutical microbiologist. They are the foundation of the bioburden test. The choice of culture medium is very crucial, because it will induce the detection of microorganisms, knowing that traditional culturing cannot detect all the microorganisms that might be present on a medical device (2). Among six culture media that we investigated, PCA appeared to be the medium of primary importance for the detection of bioburden on medical devices; this media also respects the 3Rs rule (17).

Conflict of Interest Declaration

The authors declare that they have no competing interests.

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