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Review article

Collection, transport and general processing of clinical specimens in Microbiology laboratory☆

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ABSTRACT

The interpretation and the accuracy of the microbiological results still depend to a great extent on the quality of the samples and their processing within the Microbiology laboratory. The type of specimen, the appropriate time to obtain the sample, the way of sampling, the storage and transport are critical points in the diagnostic process. The availability of new laboratory techniques for unusual pathogens, makes necessary the review and update of all the steps involved in the processing of the samples.

Nowadays, the laboratory automation and the availability of rapid techniques allow the precision and turn-around time necessary to help the clinicians in the decision making. In order to be efficient, it is very important to obtain clinical information to use the best diagnostic tools.

Recogida, transporte y procesamiento general de las muestras en el laboratorio de Microbiología

Palabras clave:
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RESUMEN

La interpretación y el rigor de los resultados microbiológicos siguen dependiendo en gran medida de la calidad de las muestras y el procesamiento de las mismas dentro del Servicio de Microbiología. Conocer el tipo de muestra, el momento adecuado y la manera de obtención, su conservación y transporte determinará la rentabilidad de la misma en el proceso infeccioso. En este sentido, la disponibilidad de nuevas técnicas dentro del laboratorio y el manejo, cada vez menos excepcional, de muestras con sospecha de infección por patógenos no habituales nos obligan a revisar y actualizar todos los pasos implicados en el procesamiento de las muestras.

Hoy día, la automatización del laboratorio y la amplia variedad de técnicas rápidas utilizadas han hecho que el diagnóstico microbiológico tenga la rapidez y precisión necesaria para realizar un diagnóstico de calidad y clínicamente relevante; sin olvidar que, en todos los casos, la información clínica es necesaria y de vital importancia para que el microbiólogo pueda aplicar las técnicas diagnósticas disponibles de la manera más eficiente.

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Introduction

In order for the results provided by the Microbiology Department to be accurate, significant and clinically relevant, it is required that all microbiological samples that arrive there be correctly selected, collected and transported, as this allows for an optimised analysis and interpretation.[1] In recent years, automated equipment has been developed for the seeding of samples, the number of rapid diagnostic techniques of immunological type, and for the detection of antigens has expanded: viral, bacterial, fungal or parasitic, and there has been a deepening in the knowledge of molecular biology techniques and proteomics.

This document is intended to serve as a guide and help in the activities that comprise the pre-analytical phase and the beginning of the analytical phase of the microbiological diagnosis, where the tasks of sampling, transport and registration in the computer system of the laboratory, processing and their preservation are carried out.

General considerations

The interpretation of the microbiological results depends, to a great extent, on the quality of the samples received for study. Therefore, an appropriate management of the samples is necessary to achieve an optimal diagnosis in Microbiology.[2]

The clinical syndrome and the possible aetiological agents involved determine not only the type of sample to be sent, but also the procedure for obtaining, transporting, preserving and processing it. Likewise, clinical information is what allows the laboratory to apply the available diagnostic techniques in a more efficient manner. Therefore, it is essential that there is close communication between the microbiologists and the clinicians responsible for the patient, actively participating in the diagnostic process.

Recommended clinical samples

It is always necessary to choose the biological material in sufficient quantity that best represents the infectious process for which you want to determine the aetiological agent. The analysable substances are all the biological samples available, from sterile fluids, samples from different organs or systems, such as faeces, urine, sputum, bronchoalveolar lavage, aspirates, biopsies and exudates from different locations or superficial or deep lesions, and hospital devices, such as catheters and prostheses. They should be obtained in the acute phase of the disease, preferably before starting any antimicrobial treatment,[3] performing the sampling at the exact site of the lesion with the maximum aseptic conditions that prevent contamination. The sample should not be in contact with disinfectants or antiseptics. It is necessary to avoid contamination with the commensal microbiota to ensure that the sample is representative of the infectious process to be diagnosed and transportation to the laboratory must be as fast as possible.[1]

Containers used

There is a great variety of containers in which microbiological samples can be collected, with a common characteristic to all of them being that they are sterile and with a leak-proof seal. The swabs can be made of different materials[2]: dacron, rayon or nylon, cotton (not recommended for *Chlamydia* spp., *Bordetella* spp. and *Neisseria gonorrhoeae*), calcium alginate (may inhibit PCR techniques and be toxic to viruses) and may have the smooth absorption surface or be flocked; they can be used dry or use Amies transport medium in gel or liquid (recommended for automated work stations).[4] The handle should be made of aluminium or plastic (rigid or flexible). The size and shape of the swab will vary depending on the anatomical location and the type of sample to be taken.

There are special transport systems, such as bags, vials or tubes with an anaerobic atmosphere, micro-haematocrit capillary tubes (*Trypanosoma* spp.), brushes in liquid transport medium for the papilloma virus, transport medium for universal virus (also valid for *Chlamydia* spp., *Ureaplasma* spp., and *Mycoplasma* spp.) or sterile tubes with fixatives for parasites or with preservative such as boric acid-sodium formate for the culture of urine.

Blood culture bottles, lysis-centrifugation bottles, tubes and bottles with screw closure, vacuum tubes with additives (EDTA, citrate) or separating gel (for serum samples), sterile petri dishes and syringes for obtaining aspirates can also be used.

Sample collection

The consequences of a poorly taken, poorly preserved or poorly transported sample, can result in a failure in the isolation of the aetiological agent or the isolation of possible contaminating microorganisms that can generate unnecessary or inappropriate treatments. Since a large part of the determinations in Microbiology are based on the growth capacity of microorganisms, the conditions of collection and transport must ensure their viability at all times.[1]

The Microbiology laboratory should prepare a clear and concise manual with the rules for collecting and transporting samples[2] which is available to all professionals who may request samples for microbiological study and, if there are doubts about the suitability of the samples or how to obtain them, the Microbiology laboratory must always be contacted before proceeding with the collection.

Collection of samples for study by conventional and automated methods

The collection of samples for microbiological studies can vary depending on whether they are going to be processed using conventional methods or if they will be processed using automated methods. Automated systems can only perform the cultivation from liquid samples directly or in liquid transport media.[4]

The containers depend on the type of sample and sterile containers will be used for abscess samples, catheters, sterile fluids (except blood), prostheses, valves and other devices, tissues and biopsies, faeces, urine, semen, skin scraping, hair and nails, and all respiratory samples. The swab will be used with gel or liquid transport medium (automated seeding and PCR techniques) in all genital exudates, conjunctival exudates, ENT (ear, nose and throat) exudates, wounds, burns and skin ulcers (if aspiration is not possible). Blood cultures and bone marrow will be sent in blood culture bottles (sterile fluids are also possible, except for CSF). Blood smears for parasites in a tube with EDTA, for serological determinations in vacuum tubes with gel and, for nucleic acid detection tests, vacuum tubes with EDTA or specialised tubes. The syringes used for aspiration of the samples can be used as containers.

Sampling for the study of viruses can be done with any type of swab except those made from calcium alginate or wooden stick. The use of transport medium for viruses during the collection of samples depends to a large extent on the sample itself; liquid samples such as blood, CSF, urine and bronchoalveolar lavage fluid do not usually require it, so they must be transported and processed paying special attention to the optimal temperature and storage times.

Finally, there are some particular containers, such as the brush in special transport medium for the papilloma virus or a tube with heparin for the study of *Leishmania* spp. in the bone marrow or the swab without transport medium for antigen detection of *Streptococcus pyogenes*.

Regarding the volume of the sample, it is always recommended to obtain the maximum that can be obtained and from the most purulent area. The minimum volume for inoculating a plate or an enrichment broth is one drop (0.05 ml) and, as a general rule, for bacteriological culture at least 0.5 ml or 0.5 g of material is necessary. As a general rule, in the liquid samples for each type of determination, a minimum of 1–2 ml and a maximum volume between 10 and 20 ml will be requested. The volume for each blood culture bottle is between 8 and 10 ml for adults and 1–3 ml for children and solid faeces >2 g.[1]

The sampling procedure will be done as aseptically as possible and whenever feasible prior to taking antibiotics. There are samples for which peculiar methods are described that must be taken into account and in which the manual of sampling of the Microbiology Department should be consulted, such as the neutralisation of gastric juice with sodium carbonate for the study of mycobacteria.

Collection of samples for study by rapid techniques

The most commonly used rapid tests, excluding fresh examinations and staining are: immunofluorescence, agglutination, immunochromatography (ICT), enzyme immunoassay (EIA) and molecular microbiology techniques (real-time PCR and multiplex PCR). By means of the latter, an isolated or several infectious agents can be detected simultaneously (bacteria, viruses, fungi, parasites), including antibiotic-resistant genes, in a short time, with little manipulation and preparation of the samples and with precise results. These techniques can present specific requirements (including the transport system and sample preservation) that will depend on the technique available in each laboratory. There are, for example, panels marketed for diagnosis of respiratory infections from samples of nasopharyngeal aspirate or exudate or urine, gastrointestinal infections from fresh faeces or in Cary-Blair medium, blood or bone marrow infections or from direct blood with EDTA, serum, special media or positive blood culture bottle, central nervous system infections from CSF or urine, sexually transmitted infections from urethral exudate, endocervical exudate or urine, and skin and mucous infections from skin lesions. PCR techniques are also available for rapid diagnosis of carrier status, such as detection of *Staphylococcus aureus*, *Streptococcus agalactiae* or bacteria that produce carbapenemases in nasal, pharyngeal or rectal exudates.

Each molecular detection test can be performed on specific samples, presenting specific requirements that will differ depending on the technique used, so that the collection system and storage conditions may vary depending on the manufacturer's specifications.

Preservation of the sample until its processing

Available media, temperature and time

In general, samples collected for microbiological studies should be sent as quickly as possible to the Microbiology laboratory. They can be transported at room temperature if their shipment is not delayed after they are obtained, although some will require ice for transport. In the event that they cannot be transported immediately, the following general recommendations may be followed, although with exceptions[1,3,5]:

a. for studies of molecular microbiology, viruses or mycobacteria, refrigerate the samples (2–8 °C);
b. samples with request for parasites will be stored at room temperature, except the blood tube with EDTA that if not processed in 1 h will be stored in the refrigerator;
c. for the rest of the studies, samples of urine, faeces, catheters, abscesses, wounds, burns, biopsies, tissues, recto-vaginal exudate of pregnant women, gastric aspirate, external ear, samples of respiratory origin and some types of exudates and biological fluids (according to the request) will be kept refrigerated if the processing is not done in the first 2 h;
d. and samples of blood, bone marrow, CSF, genital samples with suspected sexually transmitted bacterial infection, conjunctival and pharyngeal exudate, corneal scraping, vitreous humour, inner ear, nasopharyngeal aspirate, skin, hair and nails will be stored at room temperature.

There are bacteria that are especially sensitive to environmental conditions: *Shigella* spp., *N. gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and anaerobic bacteria; reliable detection of these species requires immediate processing.

Transport

The transport of samples of biological material within a hospital or centre, from a health centre to a hospital, from one laboratory to another, from one hospital to another within the same city or to another city must be managed by the hospital itself, by the health service or by any transport organisation or agency that has been authorised. Spain has adopted the recommendations described by the United Nations regarding the transport of infectious substances.[6]

Regulations for the international transport of infectious substances

International standards for the transport of infectious substances through any means of transport are based on the Recommendations of the United Nations Committee of Experts on the Transport of Dangerous Goods (UNCETDG). These recommendations are reflected in international legislation through the following international transport regulations.

- For air transport, the legally binding international regulations are the Technical Instructions for the Safe Transport of Dangerous Goods by Air, published by the International Civil Aviation Organization (ICAO). The International Air Transport Association publishes dangerous goods regulations (DGR) that incorporate the ICAO provisions and may add additional restrictions.
- For rail transport in countries of Europe, the Middle East and North Africa the Regulation concerning the International Carriage of Dangerous Goods by Rail is applied.
- For road transport, the European Agreement concerning the International Carriage of Dangerous Goods by Road is applied in 48 countries.
- For maritime transport, the International Maritime Dangerous Goods Code published by the International Maritime Organization is mandatory for all contracting parties to the international convention for the Safety of Life at Sea (SOLAS).
- For postal transport, the letter post manual published by the Universal Postal Union reflects the recommendations of the United Nations using the ICAO provisions as a basis for shipments.
- Local and national regulations for the transport of infectious substances.

The principles on which safe transport is based at the national or local level are the same as those applied for international transport and the purpose is that the sample has no possibility of leaving the container under normal transport circumstances. In Spain, Royal

Decree 65/2006, of 30 January, establishes the requirements for the import and export of biological samples and Order SAS/3166/2009, of 16 November, replaces the annexes of the Royal Decree of 2006.

When the laboratory is required to send samples to an external centre that is within the European Union (EU), current regulations state that the shipment must only comply with the regulations reflected for collection, packaging, labelling and shipping procedures, and no document is necessary for the package to leave the country.

If the laboratory is outside the EU, the sponsor has two options: request voluntary registration in the register of importers and exporters of biological samples or apply for a one-off import and export licence for biological samples.

Classification of infectious substances for transport

Dangerous goods are assigned UN numbers and, based on their classification of danger and their composition, they are assigned the corresponding official transport designations. Infectious substances are classified in accordance with division 6.2 and are assigned to numbers UN 2814, UN 2900, UN 3291 or UN 3373, as applicable. Thus, infectious substances are divided into the following categories.

Category A: an infectious substance that is transported in a way that, when exposed to it, is capable of causing permanent disability, is life-threatening or constitutes a deadly disease for previously healthy humans or animals. Therefore, the pathogens belonging to risk group 4 fall under this category, including the agents identified as new or emerging pathogens or the substances on which there are doubts about whether or not they meet the criteria for inclusion in this category. All substances that belong to category A and may cause disease in humans and animals will be assigned to UN 2814. *Category B:* infectious substance that does not meet the criteria for inclusion in category A. Infectious substances in category B will be assigned to UN 3373.

The following are samples that are not subject to regulations on transport of dangerous goods: those that do not contain infectious substances or that are not likely to cause diseases in humans or animals, those that contain microorganisms that are not pathogenic in humans or animals, those in which the pathogens present have been neutralised or inactivated in such a way that they do not pose health risks, environmental samples (including food and water samples) that are considered not to present appreciable risks of infection, drops of dried blood or samples for the detection of blood in faecal matter, blood collected for transfusions or for the preparation of blood products, tissues and organs destined for transplantation and samples from patients presenting a minimum risk of containing pathogens if they are transported in a packaging/container designed to avoid any leak and on which the indication "Exempt human specimen" appears.

Preparation of shipments for transport

The requirements for packaging, labelling and documentation of infectious samples currently in force are determined by the UNCETDG and are included in packing instructions P620 and P650. These requirements are established according to whether the infectious sample belongs to category A (UN 2814) or category B (UN 3373), respectively.

To transport all infectious substances, the basic triple packaging system must be used. This transport system comprises three layers:

1. Primary container. It is the primary leak-proof and watertight container that contains the sample. This container should be wrapped in absorbent material with the capacity to absorb all the fluid in case of breakage or leakage.
2. Secondary container. Resistant, watertight, leak-proof container that encloses and protects the primary container. Several wrapped primary containers can be placed in a secondary container, but sufficient absorbent material must be used to absorb all the fluid in case of breakage or leakage.
3. Outer container. The secondary containers are placed in outer transport packages provided with a suitable cushioning material. The outer containers protect the contents from the external elements, such as physical damage, while the package is in transit. None of the faces of the outer container shall have dimensions less than 10 cm × 10 cm.

Each package prepared for transport must be correctly marked and labelled and accompanied by the relevant shipping documents.

Packaging, marking, labelling and documentation corresponding to category A infectious substances

Category A infectious substances can only be transported in containers that meet the specifications corresponding to UN class 6.2 and Packing Instruction P620. In the packages containing the material to be transported, marks are shown that provide information about their content, the nature of the danger they pose, as well as the packaging standards applied.

The marks of the containers shall be located in such a way that they are clearly visible and not covered by any other label or mark. Category A infectious substances have the number UN 2018 "*Infectious substances affecting humans*" on the outer packaging. There are two types of labels: hazard labels and handling labels. Those commonly used in these packages include: hazard label for category A infectious substances and for microorganisms and genetically modified organisms that meet the definition of category A infectious substance; hazard label for certain microorganisms and non-infectious genetically modified organisms (UN 3245) and for solid carbon dioxide (dry ice) (UN 1845); hazard label for liquid nitrogen; handling label for cryogenic liquids (liquefied gases at very low temperatures) in air transport or orientation label to indicate the position of closures of primary containers, for the air transport of category A liquid infectious substances in quantities exceeding 50 ml per primary container. Regarding the documentation, a declaration of dangerous goods, a packing list (or shipping list) or pro forma invoice are required by the sender indicating the address of the recipient, the number of packages and a description of the contents, indicating their weight and value, and an import or export permit or declaration (or both); and a document on knowledge of air shipment, for air transport, or equivalent documents, for shipments by road, train and sea. In addition to the documents indicated, a list of the contents between the secondary packaging and the outer packaging shall be included in the goods assigned to numbers UN 2814 and UN 2900.

Packaging, marking, labelling and documentation corresponding to category B infectious substances

The basic triple packaging system is applied, including for local surface transport, which must fully comply with the requirements of Instruction P650. The official designation "Biological substance, category B" must be shown next to the diamond mark for category B infectious substances. For this type of transport, no dangerous goods documents are required, only for international shipments, a packing (or shipping) list or pro forma invoice, an import or export permit or declaration and a document on knowledge of air

transport, or equivalent documents for shipments by road, train and sea are required.

Receipt in the Microbiology laboratory

Each Microbiology laboratory must have standards for the receipt and acceptance of samples for microbiological diagnosis that the requesting doctors must know. Given that all clinical samples are unique, every effort will be made to avoid rejection, trying to resolve in the section of recording and receipt of samples the problems that could be a cause for rejection. Each sample must be accompanied by the corresponding request (application form or electronic request).

In the process of receiving the clinical sample, the following is required:

- A correct identification of the sample: this fact is essential to be able to establish an unequivocal correspondence between the container in which the received sample is found, the sample it contains and the patient to which it belongs.
- That the type of sample received is appropriate for the studies requested.
- That the volume or quantity of sample is what is necessary in order to carry out the planned microbiological studies.
- That the sample has been transported in the appropriate container and in the transport and preservation conditions necessary so that its viability is not affected.

Any incident that appears in relation to the non-compliance of any of the necessary requirements (incorrect identification, spilled samples, or inadequate transport/preservation) for the acceptance of a sample must be recorded.[2]

In all cases, the requesting doctor will be contacted informing him or her of the incident in the receipt of the sample to see if it can be solved or, if this is not possible, a new sample will be requested. Depending on the importance of it, it is possible to opt to process it before the resolution of the incident, so that the sample does not deteriorate.

Devices and materials

Some of the usual conventional equipment required in the processing of a sample for microbiological study are: biosafety cabinets (several levels depending on type of laboratory), stoves (different temperatures and atmospheres), freezers, refrigerators and centrifuges. Periodically (preferably daily), the temperature (and humidity/CO_2 pressure if necessary) of each stove/refrigerator/freezer should be monitored at the beginning of the working day with a thermometer permanently located in the centre of each piece of equipment. The reviews, as well as any type of incident, of the devices or material must be detailed in the equipment maintenance sheet within the laboratory's quality plan and must be strictly complied with.

After the automation of serological processing, new instruments of partial automation (inoculation and seeding) emerged and recently marketing has begun of "all-in-one" automatic processors (labelling, stirring, opening and closing of the container, inoculation, seeding, identification and antibiogram) that can connect with incubators in various conditions, work in stations connected to a central processor and offer high resolution image identification. Most automated systems process liquid samples including swabs in liquid transport medium, exudates, aspirates, sterile liquids and urine. Overall, they prevent labelling errors, work accidents, save time and make it possible to reorganise the work of laboratory technicians.[7]

Currently, there are three automated platforms[4,8]: WASP (WalkAway Specimen Processor) by Copan Diagnostic, InoqulA by Becton Dickinson Kiestra and FMLA (Full Microbiology Laboratory Automation) from bioMérieux (has ceased its distribution in Spain). The characteristics shared by the first two include: a central nodule of inoculation, an automated system for the opening and closing of the container, stirring/vortex of the sample, the possibility of making smears for Gram stain, inoculating broth in tube and preparation of plates for mass spectrometry (MALDI-TOF). Some of the main differences are that the method of inoculation is automatic by reusable metal loop (WASP) or automatic and manual with magnetic ball (InoqulA).

Culture media and reagents

In general, most samples are inoculated on sheep blood agar medium. In addition, for normally sterile samples from the respiratory tract and the genital tract, ear, conjunctival exudate, superficial, deep wounds and abscesses, a chocolate agar plate is added. A MacConkey agar plate is added to samples from non-sterile sources.[2] There are specific media for certain pathogens such as BCYE medium *(Legionella* spp.), Granada Agar (*S. agalactiae*), BCSA medium (*Burkholderia cepacia* complex), Helicobacter Agar (*Helicobacter pylori*), among others, and also selective media can be used, especially useful for isolating certain pathogens from the rest of the microbiota, such as Columbia CNA (colistin-nalidixic acid) agar that selects gram-positive microorganisms by inhibiting gram-negative microbiota, or selective media for stool culture containing inhibitory compounds for normal, non-pathogenic microbiota. Most of these selective media use organic compounds and dyes as selective chemical toxins on certain bacteria. Likewise, the use of antibiotics included in the medium, alone or in combinations, facilitates the suppression of the growth of a type of bacteria or several, for example: Thayer-Martin medium (*N. gonorrhoeae*). Some components allow the differentiation of pathogens in a specific way according to metabolic reactions that are expressed as pH changes and, in other cases, these reactions produce colour changes in the colonies grown in chromogenic media. There is a wide variety of chromogenic media marketed for the isolation of different microorganisms (*Acinetobacter* spp., *Candida* spp., *Escherichia coli*, *E. coli* O157:H7, Enterobacteriaceae producing extended-spectrum beta-lactamases or carbapenem-resistant Enterobacteriaceae, vancomycin-resistant enterococci, *Listeria* spp., *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp., methicillin-resistant *S. aureus*, *S. agalactiae* and *Vibrio* spp.).

Liquid enrichment media, such as thioglycollate broth or brain heart infusion broth, will be used in sterile samples, superficial wounds, deep wounds and abscesses. Media for anaerobes such as blood agar for anaerobes *(Brucella* agar), laked blood with kanamycin and vancomycin agar or *Bacteroides* bile esculin (BBE) agar will be used in abscesses, biopsies, wounds and sterile liquids (except CSF).

Each laboratory should monitor the quality of the culture media it uses, both those prepared in the laboratory itself and those commercially purchased preferably using collection strains (American type culture collection [ATCC], Spanish Type Culture Collection [CECT]).

The reagents used in the seeding phase of the samples include those that are necessary to perform the fresh examinations such as distilled water, saline solution or Indian ink; those used in the different stains performed on the samples such as Gram stain (although there are currently automated staining machines available on the market), the systems generating different incubation atmospheres and the commercial reagents necessary for the realisation of the rapid techniques used in the laboratory.

Processing of samples

The laboratory must establish a system of prioritisation in the processing of samples, based on previously defined criteria (request of the clinician, care burden and continued care), although it is obvious to say that any sample required urgently has to be processed immediately, and that all samples must be processed in biosafety cabinets.[9]

In general, CSF, fluid samples and sterile cavities, surgical specimens and rapid antigen detection tests should always be processed first (within 20 min after receipt). This group is followed by samples with a limited processing time, such as the culture of mycobacteria or tests for nucleic acid detection, tissue samples or recent aspirates (within 1 h after receipt) and respiratory samples (can remain 1 h at room temperature and 2 h if they are kept at 4 °C without the microorganisms losing viability, except bronchoalveolar lavage, which must be processed in the first 20 min after receipt). Stool samples should be sent in transport medium if the processing cannot be done in a period of time between 30 min and 1 h. Finally, urine samples (they can be kept up to 8 h at 4 °C) and the swabs with transport medium will be seeded. Blood cultures can be maintained at room temperature up to 4 h after receipt.

Conventional processing: cultivation

Within the conventional processing of the samples that arrive at the Microbiology laboratory, four well differentiated phases can be established that must be carried out following this order:

1. *Pre-treatment phase:* when this is necessary, there are different techniques such as centrifugation (sterile fluids), homogenisation (biopsies and tissues) or sonication (prosthesis), which will be applied depending on the type of sample and the request made.
2. *Inoculation of culture media:* manual seeding will be done using the isolation technique for most samples and the counting technique for those that need a colony count (e.g. urine). It is necessary to first process the samples for anaerobes and it is advisable to first inoculate the less selective media to avoid carrying any inhibitory substance to another medium. The media will be selected according to the type of sample and the diagnostic suspicion of the possible causal agent to be detected[1] (bacteria, anaerobes, fungi, etc.).
3. *Preparing the smears:* it is necessary to make smears for Gram staining of most samples, including respiratory samples, wound exudates, abscesses, samples from normally sterile origins and, upon request, urine and genital samples. In the case of sterile fluids, especially CSF, it is recommended to cytocentrifuge the sample to prepare the smears; in case of insufficient sample, culturing will be prioritised over staining.
4. Finally, *the incubation* that can be done in different *atmospheres*: aerobiosis, for the majority of microorganisms and blood agar and MacConkey agar media, enriched with 5–7% of CO_2 for the chocolate agar and Thayer-Martin media, microaerophilic for the isolation of *Campylobacter* spp. and *H. pylori* and anaerobiosis to search for anaerobes and *Brucella* agar media, laked blood with kanamycin and vancomycin (LKV) agar and *Bacteroides* bile esculin (BBE) agar.

As for temperatures, although most bacterial cultures are incubated at 35–37 °C (temperature set in automated equipment). Some exceptions are the samples in which *Mycobacterium marinum*, *Mycobacterium ulcerans*, *Mycobacterium chelonae* and *Mycobacterium haemophilum* are suspected which grow between 35° and 33 °C. For the isolation of *Campylobacter* spp. an incubation of 42 °C is needed and 35 °C (*Campylobacter fetus*), and fungi are usually incubated at 30 °C.[10]

The *incubation time* will depend in large part on the different requests (bacteria, mycobacteria, fungi, viruses), since the recovery times of the different microorganisms may be different. In general, for a routine bacteriological culture, most samples in the laboratory must be maintained for at least 48 h, except urine samples, which are usually incubated for only 24 h. Some samples of wounds, sterile fluids or prosthetic material must be kept in incubation for up to 7–10 days to recover slow-growing microorganisms such as *Propionibacterium* spp., and even 12 weeks if *M. ulcerans* is suspected. In the case of fungal cultures, the duration will vary depending on the clinical suspicion, when this is for the most common fungi such as *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp. or *Fusarium* spp., they are maintained for seven days, but some dermatophyte and dimorphic fungi (*Histoplasma* spp.) require longer periods and are incubated up to 3–4 weeks.

Automated processing

Currently, there are several models of automatic seeding machines capable of inoculating the sample and seeding it, of uncovering and covering the containers, of labelling the plates to be inoculated, of inoculating enrichment media and of preparing the smears to be stained. The advantages of using these processors are in the improvement of traceability, the reduction of human errors in the labelling, less risk of accidents when handling samples, saving time of laboratory technicians who can engage in other activities, saving analysis-result time, economic savings and advantages when obtaining greater speed in the aetiological diagnosis and in the antibiotic susceptibility report for patients.[11]

In the available models, the differences between both systems may be related to the mode of inoculation; WASP uses a loop to inoculate the sample and seed it and the InoqulA system collects the sample with a pipette tip and seeds with a magnetic ball. In both systems, the advantages of seeding the urine culture compared to the conventional method have been evaluated and confirmed, when the counts are high.[12]

The seeding machine InoqulA BD has the largest capacity, 612 plates, and can load 12 different types of media. It is capable of processing different types of sample containers with automated opening and closing. The system of seeding by magnetic ball makes it possible to seed up to 400 plates in 1 h and to seed five plates simultaneously. It is capable of incubating plates in anaerobiosis and has an internal HEPA filter to eliminate air particles that may contaminate the plates and samples.

The WASP (Copan) seeding machine consists of two robots that work independently to receive the sample, select the media to seed each sample, shake, open the container and inoculate and re-close and transfer the sample and the inoculated medium to a row of "processed" samples. It also has an internal HEPA filter. It uses two metallic loops, that while one inoculates and seeds, the other one is incinerated to be reused, and in this way it reduces costs without risk of cross-contamination. The presence of a carousel where the inoculated liquid media are stored and four dispensers for antibiogram provides added versatility and functionality.

Rapid techniques

Stains: the fresh test and stains (Gram, Ziehl-Neelsen, auramine, Giemsa, Field, etc.) are the first rapid diagnostic tools used in Microbiology. Gram staining is probably the most used and, although it raises problems of sensitivity and specificity, it is very useful when performed by expert professionals and in certain samples (CSF).

Immunochromatography and EIA: the immunological techniques of antigen detection are based on the antigen-antibody affinity

and enable the detection of the presence of microorganisms or fragments thereof in clinical samples. They are especially useful in those cases in which the causative organism grows slowly or does not grow in the culture media.[13] Among the immunochromatographic techniques available: the detection of the capsular polysaccharide antigen of *S. pneumoniae* and of the soluble antigen of serogroup 1 of *Legionella pneumophila* in urine, the simultaneous qualitative detection techniques of rotavirus, adenovirus, astrovirus and norovirus, the techniques for the detection of *Giardia* spp. and *Cryptosporidium* spp. in faeces, the detection of group A antigen of *S. pyogenes*, the detection test of the fusion (F) protein of the respiratory syncytial virus (RSV), the detection of *H. pylori*, the qualitative detection test of circulating antigens of *Plasmodium* spp. in venous blood,[14] centred on the antigen of the specific histidine-rich protein II of *Plasmodium falciparum* and a pan-malarial antigen common to *P. falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* (does not detect *Plasmodium knowlesi*), among others, and the Dermatophyte test strip, which uses specific monoclonal antibodies against the polysaccharide of the cell wall of dermatophytes.[15]

In addition, there are currently EIA and ICT techniques for influenza A and B viruses, for respiratory syncytial virus and adenovirus. The results obtained by ICT are comparable with those obtained with EIA techniques in sensitivity and specificity; however, the sensitivity of these techniques with respect to immunofluorescence is lower. At present, rapid membrane EIAs (soap-type) are available for the diagnosis of infections caused by *Clostridium difficile*, detecting glutamate dehydrogenase antigen only or simultaneously with toxins A and B in faecal samples.

Techniques based on nucleic acid detection: molecular techniques are currently playing a major role in the detection and identification of pathogenic microorganisms. They are being marketed in closed, automated formats, easy to use, close to the patient (point-of-care) and with very fast issuance of results. The menus of these platforms, which include the detection of multiple agents and which lead to an improvement in performance, response times and the cost-effectiveness of the application of this technology in Microbiology laboratories, are increasingly being extended.[16]

At the same time, other additional molecular methods, such as next-generation sequencing and proteomics-based tests, have been introduced in microbiological diagnostics and are accelerating the transition between conventional diagnosis and molecular diagnosis. The molecular detection of gastrointestinal pathogens or identification by sequencing or epidemiology by molecular typing, are examples of the acceleration that is taking place in this field.

Among molecular microbiology techniques, rapid detection techniques have been developed for the main pathogens causing serious infections such as bacteraemia or meningitis. Thus, there are nucleic acid hybridisation tests that, using probes, identify the microorganisms present in blood cultures, such as the Nanosphere Verigene system panel.

Systems that use real-time PCR include: the BD Max system (BD diagnostics) for detecting bacteria such as *Bordetella* spp., gonococcus or *C. difficile*, parasites such as *Trichomonas vaginalis*, fungi such as *Pneumocystis jirovecii* or respiratory and gastrointestinal viruses and the GeneXpert system (Cepheid) for detection of *Mycobacterium tuberculosis*, methicillin-sensitive and methicillin-resistant *S. aureus*, detection of toxigenic/O27 *C. difficile* strains in faeces, or the presence of enterobacteriaceae producing different types of carbapenemases.

The FilmArray system (BioFire), is a two-stage nested PCR that presents qualitative nucleic acid diagnostic panels for multiple bacteria and yeasts, which allows the identification of the causative agents of bacteraemia from a positive blood culture, as well as the targets that encode resistance mechanisms. They also present a panel for diagnosis (detection and simultaneous identification) of specific agents of meningitis or encephalitis in CSF, a panel for the detection of gastrointestinal infections (bacteria, viruses and parasites) and a panel for the detection of agents that cause respiratory infection (viral and bacterial).

There are fully automated systems, such as Cobas Amp (Roche) and M2000 (Abbot), based on a multiple PCR plus detection by fluorescent probes; and finally, PNA-FISH (AdvanDX), ribosomal RNA hybridisation technique of specific microorganisms with peptide probes of nucleic acids with *in situ* detection with fluorescence microscopy.

Storage of samples after processing

The storage of processed samples must include sufficient time to resolve any type of complaint or to be able to extend any request by the responsible clinician or the microbiologist who is processing the sample. Most samples (sterile and non-sterile) should be stored at 4 °C, leaving the sterile samples a minimum of seven days and with storage between two and four days being sufficient for the rest of the samples. Biopsies for determination of *H. pylori* must be stored at −80 °C and samples destined for PCR or virus culture should be stored preferentially at −70° or −80 °C and, as a second option, at −20 °C.

Collection and transport of samples with suspected microorganisms of great relevance in public health

Microorganisms with great interest and repercussion for public health are considered to be those aetiological agents of emerging diseases such as Ebola virus, Zika virus, or those that can potentially be used in bioterrorist attacks (*Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis, Burkholderia pseudomallei*), that, besides being a threat to society, constitute a diagnostic challenge for Microbiology laboratories.[17]

The management of these samples will be carried out according to the biosafety level of the laboratory, with safety in their management being a fundamental priority.[18] The handling of the samples should only be carried out by trained and qualified personnel, who should also be in charge of their safekeeping and organisation of the transportation. To respond to these biological events, the clinical microbiologist must be trained and know the level of biosafety of his or her own laboratory, the principles of sample collection, preservation and transport of these pathogens, the criteria to recognise or suspect emerging diseases or bioterrorism attempts, the levels of safety for the different pathogens that may be involved in these situations, the knowledge of the consensus protocols and, fundamentally, of the chain of safekeeping of these samples in his/her own institution.

The biosafety conditions must be the highest of those available to the laboratory or the hospital and, as a minimum, must include facilities with negative pressure, rooms or class II biological safety cabinets and adequate personal protective equipment (rear opening lab coat and hood or jumpsuit with hood, boot swabs, face shield, FFP3 or FFP2 mask and double gloves),[19] which would correspond to type 2 biosafety laboratories, to which most of the microbiology laboratories of the hospitals in Spain belong.

Prior to the extraction of the samples, the microbiologist must be notified in advance to prepare all the necessary materials and move to the immediate vicinity of the restricted access area, since a pneumatic tube system should never be used to send these samples and he or she will also be the guarantor to verify that the primary tube is aseptically introduced into the secondary and tertiary packaging for shipment to laboratories with a higher level of biosafety, complying with the specifications corresponding to UN class 6.2 and the Packing Instruction P620.[6]

The obtainment of samples will be done in the usual way, maintaining the aseptic conditions in a rigorous way and the transport must be immediate. In the case of bacteria and blood samples of the Ebola virus,[20] it can be done at room temperature, for the rest of viruses and bacteria if they are going to be processed after a period longer than 2 h it will be done between 2° and 8 °C.

The tests to be performed in the laboratory should be limited to the minimum necessary and the microbiological technique to be used will be scrupulous, avoiding risks of aerosolisation, spills or splashes. In case of manual centrifugation, it will be necessary to have hermetically sealed cuvettes or rotors, which, once the centrifugation is finished, will be opened inside the biosafety cabinet. The handler should be calm, with no pressure, and supervised by a person with technical knowledge. Samples of confirmed cases of this type of infection, at the time of its elimination, should be considered as sanitary waste of group III.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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