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Biobanking in microbiology: From sample collection to epidemiology, diagnosis and research

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Abstract

Millions of biological samples, including cells of human, animal or bacterial origin, viruses, serum/plasma or DNA/RNA, are stored every year throughout the world for diagnostics and research. The purpose of this review is to summarize the resources necessary to set up a biobanking facility, the challenges and pitfalls of sample collection, and the most important techniques for separation and storage of samples. Biological samples can be stored for up to 30 years, but specific protocols are required to reduce the damage induced by preservation techniques. Software dedicated to biological banks facilitate sample registration and identification, the cataloguing of sample properties (type of sample/specimen, associated diseases and/or therapeutic protocols, environmental information, etc.), sample tracking, quality assurance and specimen availability. Biobank facilities must adopt good laboratory practices and a stringent quality control system and, when required, comply with ethical issues.

Keywords: Biological bank; Cryopreservation; Freeze–drying; Sample preparation; Quality control

Contents

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1. Introduction: why biobanking

The majority of relevant studies on microbial pathogenesis, infectious disease etiology and epidemiology, and environmental microbiology are based on obtaining biological samples. Biobanking, intended as the process of collecting, treating, and long-term storing biological samples, represents an essential tool for biological, biomedical and industrial research and for laboratory diagnostics. The characteristics of an ideal specimen bank were described by Lee in 1990 [1] as having a secure funding source, a cryogenic storage facility, developed criteria for selection of the best samples to be stored; at the same time each facility must develop an ongoing research to optimize sample collection/processing and storage conditions. Because biobanking has gained an emerging importance in diagnostics, research, and epidemiology, many organizations have now their own biobanking facilities, characterized by different preserving techniques, their own functional protocols, and, ideally, their own bioinformatic procedures. For these reasons, although it requires huge investments in personnel, automation and storing facilities, biobanking is becoming a part of biomedical and environmental national scientific programs.

Millions of human, animal and microbiological samples are stored each year for diagnostic and research purposes, including applications in microbiology and infectious diseases, genetics, oncology, etc. In the microbiological setting, the most important reasons for biobanking could be summarized as follows:

(a) To realize epidemiological studies intended to compare samples of human or animal origin within the same epidemic episode or from episodes occurring at different points in time or at distant locations. Stored samples offer unique opportunities to study the genetic characteristics of microorganisms, to establish transmission modalities in the local or worldwide settings, or to perform additional analysis on old samples when new questions arise or new pathogens are suspected to appear. Infection control plans, including the development of vaccines or of adequate containment procedures are often based on these information.

(b) To ensure progresses in diagnostic procedures by comparing samples from the same individual at different points in time or from different subjects with similar diseases, and by analyzing stored samples with new analytical methods that may increase sensitivity or specificity of infectious disease detection.

(c) To perform research studies requiring large number of samples collected in different geographical locations or requiring multiple parameters to be analyzed in specialized laboratories throughout the world.

(d) To constitute repositories of human or animal cell lines or microorganisms used for diagnostic and research procedures (i.e., isolation of viruses in well characterized cell lines), to set up programs checking the quality in diagnostic and research laboratories or to provide reference (state of the art) reagents for research.

(e) To establish collections of microorganisms aiming at characterizing microbial diversity and microbial evolution in the world. Microorganisms are essential parts of the biosphere; they can be also used in production of drugs, as biocontrol agents, and for many other beneficial purposes. Studying and safeguarding microbial diversity for future use and exploitation is therefore of fundamental importance [2]. Microbial culture collection faces an immense task: for instance, over 1.5 million fungal species are estimated worldwide, but less than 100,000 are described [3]. At the current rate of discovery, it will take 700 years to describe them all. Biological banks may thus be, in the near future, an invaluable tool for accelerated discovery and characterization of microorganisms and for promoting their beneficial uses to mankind.

2. Current situation

Presently, thousands of laboratories of microbiology (including medical, veterinary and environmental microbiology), clinical chemistry, pathology, epidemiology, and genetics have their own ongoing programs for sample collection and biobanking. There are, however, some

public (government) and private non-profit organizations pursuing nation-wide or international programs for biobanking. At national level, the Swedish National Biobank Program (http://www.biobank.se) is a joint national program on functional genomics. The main objectives of this program are to increase the knowledge and the quality of the Swedish biobanking system, to increase usability and availability of stored samples and to increase ethical awareness. Another important nation-wide program, the United Kingdom Biobank (http://www.uk.biobank.ac.uk) aims at building a major resource to support a diverse range of research, which will, in turn, improve the prevention, diagnosis, and treatment of illnesses. Private non-profit reference culture collections were established more than 40 years ago. The World Directory of Collections of Cultures of Microorganisms (http://wdcm.nig.ac.jp), an activity of the World Federation for Culture Collections, holds an excess of 1 million microbial strains, of which 44% are fungi, 43% bacteria, 2% viruses, 1% cells, and 10% others. Two additional important organizations devoted to the acquisition, preservation and distribution of microorganisms and cell lines are the German Collection of Microorganisms and Cell Cultures (DSMZ, http://www.dsmz.de) and the American Type Culture Collection in the United States (ATCC, http://www.atcc.org). Both these organizations constitute invaluable tools, providing state of the art reagents, reference microorganisms and cell lines for research and diagnostic purposes. For an exhaustive list of the major international culture collections the reader is referred to Smith and Ryan [4].

When faced with financial constraints, some academic centers recently decided to transfer their genomic banks to private organizations; this type of collaboration may pose serious problems in the protection of human subjects, in property rights, and in the possible commercial use of future benefits to the community. To avoid these threats, the ''Charitable trust'' was recently suggested as a potentially effective model for genomic biobanks, Because it complies with privacy rules and with benefits to the community, without losing its value in biological research and in epidemiology [5].

3. Purpose of the review

The purpose of this review is to summarize the resources necessary to set up a biobanking facility; the challenges and pitfalls of sample collection and the most important techniques used for separation and storage of samples will also be presented and discussed. The review will finally deal with principles of electronic data management and of accurate quality control procedures. The factors affecting the quality and the future use of biological samples will be discussed with particular attention to the nature of samples to be stored (i.e., blood, bacteria, fungi, etc.). This review considers some statements that may generally apply to biobanks, but are not for all biobanks. The purposes of the repository (research, diagnosis, epidemiology, industry, etc.), the type of specimen, the availability of personnel and equipment as well as other factors, deeply influence the characteristics of each biobank.

4. Staff and equipment

A functional biobanking facility requires adequate resources in terms of personnel, space, laboratory instrumentation, computers, and of quality system, including protocols and reference reagents. If human samples are stored, ethical issues must be solved. In literature, there are few indications on minimum personnel requirements for a biological bank [6]. The personnel may be a part of the laboratory performing all the routine or research work of the laboratory itself, while in turn holding the biological bank; in alternative, the personnel may be dedicated solely to biobanking, as it may happen in centralized facilities. Although a detailed analysis of the biobanking process and its throughputs is necessary before deciding the type of organization and its needs, two to three laboratory technicians may be considered as the minimum requirement to ensure a continuous biobanking service by processing, aliquoting, storing the samples, and holding the sample archive in all its aspects. Informatics greatly improves the management of a biological bank. Commercially available software packages may be satisfactory in small to medium biobanks, but ideally each facility should develop its own, dedicated software. In this case, a computer engineer or a computer programmer is required to develop and improve the software, in provision of the fact that systems should be amended continuously to meet the changing needs of the laboratory staff [6]. The coordinator of the biobank could be a component of the medical or PhD staff of the lab, who dedicates a part of his/her time to organize the biological bank, ensures the respect of the legal and ethical issues, keeps the contacts with the scientists who request the samples stored in the bank for their own diagnostic or research purposes, and defines with the administration space, personnel and equipment resources necessary to perform biobanking. Laboratory requirements include a processing room with a class II biological safety cabinet, a centrifuge and a microcentrifuge; this room may contain a personal computer with software dedicated to biobanking or a paper-based archive. An additional room is dedicated to the storing facilities. Samples are cryopreserved in freezers at −20 or

−80 °C and/or in liquid nitrogen freezers that incorporate remote alarm systems; freeze–dryers may also be used.

5. Sample collection and processing

The investment in biobanking, in terms of facilities, personnel, and expertise is very important, and a cost-effective rationale is required for its maintenance. A high degree of consistency of the sample, of processing quality and of reproducibility of experimental results obtained with stored specimens can be gained by a centralized biobank facility (i.e., in one department or even in a single institute, university or hospital). When compared to a centralized approach, the distributed organization would require more staff, more hardware and facility space; it has been recently assessed that the cost of a distributed organization was about twice that of the centralised approach (www.ukbiobank.ac.uk). Despite these advantages, usually the need to collect and store different types of specimens (microorganisms, human or animal blood or tissues) for completely different research or diagnostic purposes, and the lack of an institutional-based, rather than a single laboratory-based approach, make centralization of a biobank very difficult to achieve.

Before sample collection, ideally each research or diagnostic staff must define the potential parameters to be studied or, more in general, the study design, so that appropriate collection and processing protocols can be designed. However, samples stored in a biological bank are often used to answer to questions arisen after the initial studies have been completed or to test old questions with new techniques. In order to satisfy future research needs, the original sample may be divided into separate aliquots appropriate for different purposes, such as several vials containing the same material (i.e., several vials containing small aliquots of serum or one bacterial strain) and/or vials containing different materials obtained from a single sample (i.e., intact cells, separated DNA and/or RNA, proteins, etc.). For these reasons, sample collection and processing must be optimized to avoid approaches that may preclude future analysis, and to ensure preservation of the integrity of the whole cell and of its components. Automation procedures can be a tool for the improvement of biobanking throughputs, of quality control and may decrease costs. A great effort has been initially devoted in automating the mechanical aspects of specimen manipulation. In fact, the presence in many laboratories of automated liquid handling and sample dispensing systems constitutes an important aid for appropriate specimen processing and storage [7]. In the last few years process control software supporting the laboratory hardware have greatly improved the automation of biobanking: However, transferring samples between different preparation processes still requires, at least partially, human intervention. Future trends of automation will therefore probably include the integration of a greater number of functions in a single system. In order to produce proper statistical analysis for experimental and epidemiological studies, samples representative of the study protocols should be collected together with adequate controls. Guidelines to collect samples and to evaluate the quality of submitted specimens are therefore the first essential step to set up a biological bank. An appropriate sampling procedure may assure that the sample is correctly identified, that it has been collected and sent to the biobanking facility in order to maximize the recovery of microorganisms or cells and that, if required by the study protocol, the accompanying forms containing the patients' data and that other relevant information have been filled without errors or omissions. For a reliable sample collection, establishing clear communications between researchers and the staff collecting the specimens is essential. After the standardization of the collection step, the researchers may optimize sample processing by running preliminary tests to assess the best conditions for preserving sample integrity. For this purpose, researchers usually compare the effects of different preservation regimens on viability, pathogenicity, morphological or physiological parameters and on the genomic stability of cells or strains before and after preservation [8]. In fact, a major concern of storage protocols regards the stability of biological samples [9]. The factors known to influence stability include: (a) The use and type of preservants, or anticoagulants in the case of blood and urine; (b) temperature range during the time between sample collection and processing, and during short or long-term storage; human and animal cells are quite stable at room temperature for up to 48 h, while viruses and bacteria show very different survival time, according to species-specific characteristics. The appropriate temperature to avoid sample degradation thus depends on the cells or cellular components to be processed and stored; (c) timing before initial processing and storage; this parameter may vary considerably depending on the sample to be stored. Guidelines published by the National committee for clinical laboratory standard [10] stipulate that defined clotting and centrifugation protocols are important to obtain appropriate serum specimens. Furthermore, serum or plasma should be physically separated from blood cells within a two hour maximum from time of collection, while no indications are presently available for human or animal cells or microorganisms. However, in general, chapters considering the effects on recovery of the starting material and of the individual characteristics of bacterial and viral species are contained in laboratory manuals [11,12]; (d) sterility during specimen collection and processing is

essential if the processing protocols include the isolation of RNA or the culture of cells or viruses from the sample; (e) the presence and activity of endogenous degrading or inhibiting substances in the sample itself. For instance, proteins are sensitive to degradation by proteases, while RNA is very sensitive to degradation by the widely distributed RNAses (see below), so separation protocols must include specific procedures to avoid each type of degradation [8].

Several issues of safety arise when biological samples are manipulated in the laboratory. Therefore special precautions must be taken during collection, processing and storage of the materials used for biobanking. The personnel must be trained in handling infectious materials, and the laboratories must adopt biological safety level II or III, according to the pathogens handled. The description of the safety procedures is beyond the scope of this review and good sources for guidelines related to biosafety issues are available from the Center for Disease Control (Biosafety in Microbiological and Biomedical Laboratories, IV Edition, http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm) and from the World Health Organization (Laboratory Biosafety Manual, II Edition, http://www.who.int, Document WHO/CDS/CSR/LYO/2003.4).

6. Principles of cryopreservation and freeze–drying

Specific conditions are required for the long-term storage of human, animal, and microbial whole cells or for separated cellular components such as nucleic acids. The most widely used systems for storage include cryopreservation and freeze–drying. Cryopreservation means that materials are stored at low (from −20 to −80 °C in freezers) or ultra-low temperatures (−150 °C in liquid nitrogen containers); in the latter case, cryopreservation takes place in the liquid or in the vapor phase of nitrogen. Liquid nitrogen devices permit storage at quite constant temperatures, while mechanical freezers are subjected to temperature variations that may compromise the quality of samples stored in the upper compartments of upright front-loading freezers [13]. Because of their increased storage capacities liquid nitrogen devices are more suitable for banking facilities with thousands of stored samples, while −80 °C freezers support small and medium-sized biological bank facilities. Due to the possibility of cross contamination of non-infected samples with stored infectious materials harboring hepatitis B or other pathogenic viruses [14], modern nitrogen freezing systems are based on vapor-phase, rather than on liquid-phase cryopreservation.

The scientific rationale of using ultra-low temperatures is that, below −139 °C for pure water or below −130 °C for culture media, molecules do not move, thus preventing any chemical reaction and reducing cell damage, occurring during cryogenic procedures [9]. In fact, a major drawback of cryopreservation consists in severe damage of the cells exposed to freezing. Cryoinjury is a lethal event, depending on the formation of intracellular ice, on the efflux of water outside the cell, and on an increase in the concentration of intracellular salts in the solution. The most critical range at which thermal shock occurs is between +15 and 0 °C, while minor events occur at temperatures below 0 °C [15]. The modern era of cryobiology started with the discovery that glycerol protects eukaryotic cells against freeze damage; after this initial observation, several cryoprotectants were discovered and are now widely used to cryopreserve cells, including dimethylsulfoxide (Me_2SO), glycerol, blood serum, ethylene glycol, methanol, skimmed milk, yeast extracts, tripticase soy, etc. (for an excellent review on cryoprotectants, see [16]). A recent survey showed that dimethylsulfoxide was used in 314, glycerol in 308, and blood components in 238 scientific papers involving cryopreservation of microorganisms [16]. These agents may provide protection from cryoinjury by binding intracellular water, thus preventing the formation of ice crystals and the excessive cellular dehydration, or by reducing the concentration of intracellular salts. Non-permeable cryopreservants (polysaccharides, proteins, dextrans) are particularly suitable for microorganisms, because they adsorb on the microbial surface and form a viscous layer that is highly effective in protecting microbial cell walls and membranes [16]. The cooling rate of the sample plays also a major role in cellular preservation after freezing, with different cell types having different survival rates depending on the rate used for their cooling. Generally, a slow cooling rate, in the order of 5–10 °C/min, does not allow intracellular crystallization and results in a high cellular viability after storage. A recent paper suggested that ultra high cooling rates (>5000 °C/min) may also result in high viability [17], but this solution is technically demanding and not available in the majority of biobanking facilities. Freezers with computer-driven programmed cooling/thawing rates are available on the market, allowing a strict and effective control and optimization of the cooling process. After storage, and immediately before the desired use, gentle thawing at 37 °C in a water bath can rapidly reawaken cells. Automation is increasing also in the field of cryopreservation and covers everything from normal lab freezers to large integrated archives. Industrial companies have developed fully automated storage and retrieval systems, which operate in low temperature freezers (−80 °C) and contain computer-driven robotic systems that scan and sort cryogenic vials or microplates from the freezers [7]. At the moment, due to their extreme sensitivity to minimal temperature variations, peripheral blood and tissue cells processing is not feasible with automated storage/

retrieval systems. Cryopreservation is the most widely used method for the long-term storage of cells and cellular products; major disadvantages are the relative high costs of equipment and reagents and, in the case of liquid nitrogen, the need of a constant supply, as any interruption in flux causes the loss of stored samples.

Freeze–drying, also called lyophilization, is an alternative method for the long-term storage of biobanked samples. Freeze–drying is a three-step process. In the first step, the material is frozen to convert the water into ice. In the second step, the ice formed after freezing is removed by conversion from solid to vapor form. In the third step, the additional water left in the sample because of its strong bound to the solid components of the sample is converted into vapor and removed. The lyophilized material is then stored in sealed vacuum ampoules that are used throughout the entire freeze–drying process. Before utilization, the samples can be restored by opening the ampoules and by the addition of sterile water. Freeze–drying causes a form of damage that is termed lyoinjury. Intracellular proteins compensate the loss of hydrogen bonding with water by protein–protein interactions that lead to denaturation and loss of their activity. In membranes, water loss causes phase transitions from the biologically active fluid to gel phase, with possible alteration of their functions [18]. Bacteria and yeasts are quite tolerant to lyophilization and therefore freeze–drying of these cells can be carried out satisfactorily, while cells with higher structural complexity are less suitable for lyophilization [19,20]. Freeze–drying requires a special apparatus (a freeze–dryer), but, after the process, the samples can be kept at temperatures >5 °C, so freezers are not necessary. Although the majority of biological banks rely on cryopreserved samples, freeze–drying in general ensures a satisfactory long-term viability, avoids contamination during storage (ampoules are tightly sealed) and requires simple storage and distribution. Major disadvantages of lyophilization are: it is a time consuming process not suitable for all cell types (i.e., some eukaryotic cells do not tolerate lyophilization) and it requires detailed definition and optimization of specific protocols for individual cell types or microbial species.

7. Storage of viable microorganisms

In most laboratories, bacterial or fungal strains, parasites and viruses need to be maintained for epidemiological purposes, quality control, teaching, and research. In the last few years, a special scientific requirement has arisen regarding the long-term viability and the retention of selected properties of genetically modified microorganisms.

7.1. Storage of bacteria

Bacteria are recovered from clinical, environmental or from other specimens by incubation in media routinely used for their isolation. Cultures are allowed to mature to late growth or stationary phase before being harvested [21]. After confluence, bacterial cultures are suspended in liquid media and processed for storage. Because of their cellular organization and regrowth capacities, bacterial prokaryotic cells can be either cryopreserved or freeze–dried. The majority of bacterial stock isolates can be easily kept at −80 °C, although the supporting material, the concentration of the initial inoculum, and the type of cryopreservative used may have a significant impact on bacterial survival and regrowth capacities [22–24]. Small glass or plastic beads can also be used before freezing; the bacteria will coat the beads and then individual beads can be removed after storage, avoiding thawing of the entire sample. After storage, a rapid thawing of cultures and a quick transfer of bacteria to an appropriate growth medium is recommended. In general, bacteria can be stored at −20 °C for one to three years, at −70 °C for one to ten years, while freezing in liquid nitrogen preserves bacteria for up to 30 years [21]. It has been shown that, after 12–18 months, viability of common pathogenic bacterial species stored without preservants dropped down to <20% of the initial inoculum, while the addition of these substances raised viability to >80–90% [16,22]. Experimental infections using stored bacteria have shown that virulence properties are usually maintained after lyophilization or freezing at −70 °C [25]. After storage and thawing, however, some ''fastidious'' bacterial species may pose special problems in terms of viability and stability of antigenic, molecular and biochemical properties (Table 1). *Neisseria gonorrhoeae* and *Helicobacter pylori* are prototypic ''difficult to grow'' bacterial species, whose characteristics have been studied after preservation [26,27]. Harbec et al. [26] showed that *N. gonorrhoeae* strains could be stored at −20 °C or at −70 °C by using specific cryogenic protocols. After 18 months, strains were viable and retained their original antimicrobial susceptibilities, making both methods suitable for epidemiological purposes (i.e., comparison of antibiotic resistance profiles) or

Table 1
Bacterial species characterized by a potentially reduced viability and/or stability of antigenic properties after storage

Bacterial species	References
Neisseria gonorrheae	[26]
Helicobacter pylori	[27]
Haemophilus influenzae	[24]
Chlamidiae	[29]
Mycoplasmas	[28]

for research protocols. *Helicobacter pylori* is another bacterial species that is very sensitive to storage conditions. To adequately preserve the viability and the genetic and physiological characteristics of helicobacters, Shahamat et al. [27] suggested that, when cryopreserving *Helicobacter pylori*, the initial inoculum should be greater than 5×10^6 cells/ml and at least 90% of the cells should be vegetative, i.e., spiral shaped cells at microscopic observation. They also tested the effects of different cryopreservative media for bacterial storage at −70 °C or in liquid nitrogen. Although most of the strains did recover within 24 months, longer periods of storage significantly lowered the percentage of viable strains after thawing (around 60%). These data confirm that selected bacterial species are particularly fragile after long-term storage and that specific protocols aiming at improving their survival are still required. Mycoplasmas and chlamydiae differ from the bacteria commonly encountered in biological and environmental specimens on account of their cellular composition, their lack of staining with the Gram stain, and their special in vivo and in vitro growth characteristics. A few scientific papers have demonstrated that, despite their peculiar growth and metabolic requirements, mycoplasmas and chlamydiae can be successfully stored in cryopreserved or lyophilized vials for up to ten years [28,29]. However, a loss of 95% in infectious elementary bodies of *Chlamydia* species may significantly reduce recovery after storage, suggesting that cryopreserving these species may require adjustment in the initial inoculum and optimization of the freezing protocols [29]. The survival rate of bacteria after freeze–drying has been measured by Miyamoto-Shinohara et al. [30]. They showed that the survival rate after ten years of storage varied significantly according to the species tested; in fact, the survival rate of gram-positive bacteria was generally higher than that of gram-negative bacteria, probably because of their greater resistance to drying due to the structure of their surface components.

7.2. Storage of fungi

Unusual fungal infections are emerging as important morbidity and mortality causes in immunocompromised human hosts and pose special problems to diagnostic laboratories; in addition, fungi possess an enormous potential to provide solutions in agriculture, environment, human and veterinary medicine. These reasons explain the growing interest in the characterization and preservation of these microorganisms. Many protocols have been suggested to be suitable for preservation of fungi, although no individual preservation technique has been successfully applied to all fungi [4,31–33]. As first step to preservation, fungal cultures are obtained by conventional sampling techniques and are further grown on Sabouraud agar or on other media widely described in literature [11]. Usually, a cellular amount sufficient to establish a biobank for yeasts is obtained after a 48–72-h incubation in aerobic atmosphere, while molds may require 7–12 days of incubation. Although some cryoprotective agents have been shown to facilitate the dispersal of fungi, the difficulty in obtaining homogeneous suspensions after culture may compromise the possibility to prepare a fungal suspension adequate for freezing [32]. Cryopreservation in liquid nitrogen and freeze–drying (lyophilization) are the methods recommended by the American Type Culture Collection for fungal repositories [34]. Other techniques may be appropriate for organisms that cannot be cryopreserved or lyophilized, such as storage in soil, water or mineral oil [4,35]. Cryopreservation at temperatures <−140 °C is the preferred technique in most microbial biological banks because cultures can remain stable for long periods due to little metabolic activity occurring at that temperature. The technique of freeze–drying is most suitable for members of the Ascomycota, Zigomycota and some Basidimomycota, with survival in excess of 30 years for some isolates [4]. The main advantage of this technique is that sealed ampoules offer a consistent protection against air dispersal of fungi during storage or during packaging and shipping to distant laboratories. The main disadvantage of freeze–drying is that lyoinjury and genetic damage may occur during the cooling and drying stages [36,37]. Because both cryopreserved and lyophilized fungal cultures present problems of viability after reconstitution, it is very important to check viability before and after preservation independently of the technique used. Experimental protocols on stored fungal cultures established that acceptable viability for fungi is the germination and development >75% of propagules/cells [4]. Although the majority of fungi can be preserved with the above-mentioned techniques, some "preservation recalcitrant fungi" cannot be. These fungi often include those that do not sporulate in culture (Oomycota, Basidiomycota) and others, which are difficult to maintain in culture (*Diplocarpon*) or are facultative pathogens (Table 2) [4].

Table 2
Preservation "recalcitrant" fungi

Species	Subphylum	Site of isolation	Notes
Saprolegnia spp.	Oomycota	Water	Fish pathogen
Aphanomyces spp.	Oomycota	Water, vegetables	Fish pathogen
Serpula lacrymans	Basidiomycota		Human alveolitis
Diplocarpon		Vegetables, flowers	

7.3. Storage of parasites

Cryopreservation at ultra low temperatures represents an acceptable choice for the long-term storage of parasites; however, the majority of the conventional methods for the propagation and preservation of parasites have some limitations, including the length of the procedures used, difficulties in initial isolation, the loss of strains, bacterial and fungal contamination during manipulations and changes in the original biological and metabolic characteristics [38]. The technical procedures used to cryopreserve parasites do not differ significantly from those used for other microorganisms. In general, glycerol and dimethylsulfoxide have the highest cryoprotective effects, but glycerol permeates the cell more slowly than dimethylsulfoxide and requires a period of equilibration [38]. The optimal cooling rate is highly variable depending on the parasitic species, while faster thawing rates produce usually best infectivity or motility [39]. Few other important peculiarities of parasitic cryopreservation have been described. Firstly, it has been shown that blood protozoa can be frozen without cryoprotectants or a precise control of the cooling rate [38]; secondly, developmental stages within the same parasitic species may be selectively affected by different freezing protocols [40]. Additional information is available on the cryopreservation of selected parasitic species. Morphological studies on Trypanosoma species documented ultrastructural changes in frozen cells (membrane injuries, denaturation of chromatin, occurrence of large vacuoles), while functional experiments demonstrated that the infectivity of Trypanosomes [41] and of *Strongyloides stercoralis* was partially maintained [42]. Fayer et al. [43] demonstrated that oocysts of *Cryptosporidium parvum* can retain viability and infectivity at temperatures between −10 and −20 °C. Vaccinology and diagnostic immunology constitute important fields taking advantage of the capacity of long-term storing techniques to provide parasitic products that keep their stability through time. In fact, the preservation of *Toxoplasma gondii* bradyzoites has been successfully applied to make a live bradyzoite-based vaccine [44], while cryopreserved plasmodia were a source for the preparation of parasitic antigens to be used in diagnostic or research immunoassays [45]. Preliminary experiments have demonstrated that also the lyophilisation technique might be useful for the preservation of apicomplexan parasites, especially those with a potential for being used as live vaccines, such as *Babesia* and *Plasmodium* [46].

7.4. Storage of viruses

The methods by which viruses may be stored are similar to those employed for the other microorganisms and the majority of literature using viral repositories store viruses by cryopreservation or freeze–drying. Their infectious and pathogenic nature and the absence of therapeutic options able to eradicate many viral infections, require special safety precautions for handling biological samples. First, the laboratories handling viruses must adopt biosafety level II or biosafety level III facilities, depending on the characteristics of the viruses handled; secondly, biobanks having viral stocks require experienced personnel adopting written, internationally accepted guidelines for biosafety. The recent episodes of fatal infections during the laboratory manipulation of SARS coronavirus has evidenced that in many laboratories safety precautions are less than optimal [47]. Exhaustive biosafety manuals are found in the WHO and CDC web sites (see Section 5). The appropriate selection of the specimen represents an important issue in virology. The specimen or the collection site must permit recovery of the virus under study, avoiding contamination with undesired microorganisms, and it must represent the process to be investigated by diagnostic or research protocols. Several papers have demonstrated that this goal may be sometimes difficult to achieve. A prototypic example includes the poor association between the detection of human papillomavirus DNA in oral exfoliated cells as compared to biopsies of cancers of the oral tract and the presence of an oral tumor, indicating that exfoliated cells contain a mixed human papillomavirus population that does not accurately reflect the viral population involved in the development of the tumor [48].

An excellent state of the art article published in 1999 [49] punctualizes some important general rules necessary for an adequate preservation of viruses: (a) Freeze–dried viral preparations can be maintained for decades at 4 °C; (b) viruses stored in liquid nitrogen retain their pathogenic properties. It must be kept in mind that viral stocks stored in liquid nitrogen containers may cross-contaminate other samples archived in the same container and, therefore, vapor phase containers are best indicated. It has been suggested that including individual vials containing viral preparations in a second, heat shrinkable tube may protect from cross contamination and from accidental ruptures in liquid nitrogen containers [49]. Storing conditions may vary according to the final destination of banked materials: if retention of virus infectivity is not essential, for instance when the sample is used to obtain antigen preparations, it can be safely stored at −20 °C; (c) proteins added to biobanked viral stocks provide protection of virus infectivity in stored samples by mechanisms that are not completely understood. One hypothesis suggested that proteins provide buffering capacities against pH changes, and reduce processes that damage nucleic acids; (d) virus infectivity is best maintained when samples are preserved in small volumes, because freezing and thawing

the samples occur much quicker and viruses are much less damaged when preparations contain high viral titres. Viruses are quite stable at low temperatures, although the genetic material of RNA viruses may present a certain degree of fragility when inappropriately handled. The small size of the virus and the absence of free water are largely responsible for this stability; viruses with lipid envelopes are often less stable than non-enveloped viruses at room temperatures, although this difference is less evident at low or ultra low temperatures [49]. The effects of multiple freezing and thawing of serum specimens on TT virus and Hepatitis B virus stability has been evaluated by Durmaz et al. [50]; they showed, by using semi-nested PCR and a hybrid capture system, that viral DNAs continue to be positive until the seventh cycle of freezing and thawing, but these qualitative results do not allow any conclusion on the possible quantitative effects on the viral load of repeated freezing and thawing. More detailed quantitative studies on HIV RNA have demonstrated that RNA levels in plasma stored for up to one year at −70 °C are quite stable [51,52]. In these studies, the stability of nucleic acids was ensured by the fact that the protocol for plasma handling included an additional centrifugation step to eliminate contaminating blood cells that contained RNases. Our quality control data on plasma EBV-DNA, however, suggest that a certain loss of viral DNA occurs after two to three years of cryopreservation (P. De Paoli, MT Bortolin, unpublished data).

7.5. Storage of genetically modified microorganisms

Very few studies have monitored viability and plasmid retention in frozen recombinant microorganisms. These cultures are widely used for industrial and research purposes, like production of enzymes, nucleic acids, controls, etc. Recently, Koenig [53] studied the long-term viability and plasmid retention of recombinant *Escherichia coli* strains. She demonstrated that frozen cultures showed stable viability and high plasmid retention rates over periods of up to 11 years; in few cases lower viability and/or plasmid retention were due to an inappropriate selection of initial colonies. Although this study did not address plasmid structural stability, it showed no changes in the restriction patterns of the strains, indicating the absence of gross structural instability of transfected sequences. Similar results were obtained by investigating storage stability in liquid nitrogen and post-thaw productivity of a recombinant strain of *Saccharomyces cerevisiae* expressing the human factor XIIIa protein [54]. Storage stability and optimal product expression after thawing are essential for the proper use of cryopreserved recombinant microorganisms.

8. Storage of human and animal cells or cell lines

Human or animal cells or cell lines may be stored for further detailed phenotypic, genomic or functional studies in living cells, to recover virions or viral nucleic acids and to enhance the availability of nucleic acids from the study subjects (i.e., cultured lymphoblastoid cell lines allow harvesting virtually unlimited amounts of DNA from single subjects). Cryopreservation for transfusion or for bone marrow transplantation is beyond the scope of this review and will not be included. The different response of human and animal cell components to freezing conditions and the existence of cell-to-cell contacts or mutual relationships between cells are major problems when storing these cell types. Human and animal peripheral blood mononuclear cells (PBMCs) can be easily obtained by density centrifugation through gradients and are the most frequently stored cell types. The generally used cryoprotectants include proteins (fetal bovine serum) and dimethylsulfoxide (DMSO). Factors influencing the quality of stored blood cells include the anticoagulant used and the storage temperature. For instance, disadvantages of heparin used as blood anticoagulant include its influence on T cell proliferation and problems in inhibiting the polymerase chain reaction assays [55,56], which have not been confirmed in other studies [9]. Cryopreservation at −80 °C or in liquid nitrogen is the only established method to preserve PBMCs samples valuable for future experiments, and published guidelines establish that PBMCs may be stored for a minimum of 24–48 h at −80 °C and then transferred to liquid nitrogen for long-term storage [12]. One major problem of cryopreservation is the significant reduction in the number of viable cells after thawing [57,58]. In PBMCs, cryoinjury appears to be mediated also by the specific activation of caspases and the induction of mitochondrial membrane permeability [57]. In HIV-infected subjects, the immune function, as measured by in vitro assays, improves significantly in response to antiretroviral therapies [59]. As a result, in vitro measurements of lymphocyte proliferation and cytokine production are being explored as a substitute for clinical endpoints in therapeutic clinical trials [60]. However, due to the damage induced by the HIV itself, by additional viral infections, and by antiretroviral therapies, lymphocytes from HIV infected subjects may pose special problems of cryopreservation. Therefore, extensive studies investigated the effects of cryopreservation on the immune functions of HIV-infected patients. The results of these studies indicated that the median of cells recovered after several years of preservation was around 50% of the initial number, and that viability greater than or equal to 70% was necessary to obtain reproducible and affordable results of the in vitro testing [61,62]. These data imply that storing at least 10^7 PBMCs per vial yields sufficient viable cells

for phenotypic and/or functional analysis. Phytohemoagglutinin assay and cytokine production are useful in vitro indicators of PBMC functions; both these assays demonstrated that cryopreserved cells maintain a consistent proportion of their functional activities even after ten years of storage [60,62]. Granulocytes are seldom used in biological banks; these cells are particularly sensitive to storing procedures, although their yield after storage is >80%. Granulocytes are mainly used as a source of DNA, but simpler methods, using peripheral blood buffy coat or whole blood as a DNA source, have been recently employed [8,9]. These methods require minimal sample manipulation; therefore they are less expensive and less time consuming.

It has been demonstrated that the presence of cell-cell and cell-matrix attachments may alter the cryobiological properties of cells [63], suggesting that special methods are necessary to store adherent cells or cell lines. Pegg [64] recently overcame these limitations developing a method for the cryopreservation of a human endothelial cell line as a single suspension or as a confluent layer on microcarrier beads. Furthermore, a recent report [65] described a simple method of freezing human or animal cells while still attached to the substratum of a multiwell plate; these authors demonstrated an effective storage of adherent mammalian cells up to three months at −80 °C. This system offers consistent savings of time and effort and may be very useful when screening large panels of cell clones.

9. Storage of serum/plasma

Plasma and serum can be used to measure antibodies against selected pathogens, to measure immune related substances like cytokines or vitamins and other metabolites. In addition, these blood products may permit to obtain nucleic acids from blood borne viruses or from bacteria during bacteriemic diseases [11,12,66]; finally, when a cellular source is not readily available, they can be also used to extract small quantities of DNA of human or animal origin [67]. Plasma can be obtained from anti-coagulated blood samples through separation of cellular components, while serum recovery can be optimized by gel separators and clot activators [68]. Plasma is more suitable for nucleic acid and cytokine detection, although the choice of the anticoagulant may hamper the possibility to subsequently test some specific analytes, while serum specimens are ideal to measure antibody levels. Quality control programs have shown that, due to the appreciable stability of immunoglobulins during time, serum/plasma specimens for antibody assays can be stored for years at −20 °C [68,69]. Cytokines have short half-lives in vivo and may be further degraded in vitro. On the other hand ex vivo induction of cytokines can occur during blood processing. Therefore plasma or serum samples for cytokine assays must be collected and processed almost simultaneously. The stability of cytokines after storage has been studied by Kenis et al. [70]. They showed that serum samples for the determination of Interleukin-6, soluble IL-6 and cytokine inhibitors can be stored at −20 °C for several years, while for Interleukin-10 determinations storage at −70 °C is recommended.

The quality control program for storage of blood specimens in the Malmoe Diet and Cancer Study demonstrated, by the use of bioassays based on the measurement of endogenous oxygen metabolism, that plasma and serum can be preserved without any degradation for at least up to 140 weeks [71].

10. DNA and RNA extraction and storage

Sample processing may be quite simple, for example separation and freezing of serum or plasma or freezing of bacterial cells. More complex sample processing includes the extraction and purification of nucleic acids. The quantity and quality of nucleic acids obtained for biobanking purposes depends from several factors: (a) the quality of the original biological sample; (b) the extraction method used; and (c) the storage conditions [72]. DNA can be extracted from nucleated cells, including whole blood, buffy coats, mucosal swabs, and from fresh or formalin-fixed tissues. DNA and RNA can be also extracted from serum/plasma or other fluids, from which low yields can be obtained.

The quality of nucleic acids obtained from biological samples depends from the quality of the original sample, as mentioned in the chapter ''Sample collection and processing''. The quality of extraction and the conditions used for storage are the other factors of paramount importance when handling DNA or RNA. DNA can be extracted from biological samples using phenol-chloroform extraction [73], while RNA is extracted by the guanidinium isothiocyanate plus ethanol technique [74]. New methods, including commercially available kits (Quiagen, etc.) may include the use of resins or affinity gels [75–77]. As compared with new systems, traditional methods have generally a higher yield of nucleic acids, although the presence of toxic chemical reactants may discourage their use. The presence of bacterial and fungal integuments may require the use of stronger extraction systems, like those based on alkali wash and heat lysis [78].

DNA is the most stable component in biological samples. In fact, isolated DNA is generally stored at 4 °C for several weeks, at −20 °C for several months and at −80 °C for several years [8]. RNA is very sensitive to degradation by RNAses and its integrity is guaranteed with RNAse-free handling and by the addition of

RNAse inhibitors. Furthermore, the integrity of RNA is maintained at very low temperature and must be stored at −80 °C [8]. The structural integrity of nucleic acids can be measured by the determination of the size of extracted DNA using agarose gel or SDS-PAGE electrophoresis, while functional integrity can be assessed by specific amplification procedures [72]. To address this issue, Jerome et al. [79] performed quantitative Real time PCR for herpes simplex virus DNA in a large series of fresh specimens and again after 16 months of storage. They demonstrated that viral DNA remains quantitatively stable for at least 16 months when frozen at −20 °C. Similarly, Walther et al. [80] revealed that closed circular plasmid forms, which are sought for efficient gene transfer procedures, are preserved after long-term storage. Although these conclusions may apply for other microbial DNAs, there are presently no additional experimental evidences. RNA integrity can be measured by reverse transcription PCR or by a commercial system using RNA chip that is able to determine purity, degradation and ribosomal RNA contamination of separated RNA preparations [81].

Filter paper represents an attractive alternative to freezing for the long-term storage of DNA. Filter paper methods require less equipment, less technical expertise and are less expensive. Samples stored with this method have been used to amplify human, viral and bacterial DNAs [72,82,83].

11. Documentation and data bases

Informatics can facilitate sample registration and unique identification, the cataloguing of sample properties (type of sample, type of specimen, presence of associated diseases and/or therapeutic protocols, environmental information, experimental conditions, etc.), sample tracking, quality assurance and specimen availability [6,81]. The components of a banking inventory system must permit data entry, queries across groups to facilitate any combination of sample identification/retrieval per trial or project, and the preparation of periodic reports; furthermore, data must be available for a long period of time, maintained in a standardized format and combined from collaborative sources [81].

Studies that store identified or identifiable specimens must maintain confidentiality. Anonymous and anonymized biological materials are impossible to link to their sources, while identified and identifiable materials can be linked. In the latter cases, although national regulations may differ, coding of the biological materials and for the code key to be stored separately is usually recommended [85].

Software for biobanks have now been designed based on cooperative study networks [6,81] or tailored on an individual basis [84]. Management software integrating sample preparation, processing and storage are also present on the market (CryoByo System, Paris, France). Biological banks storing microorganisms may require additional information that partially differ from those required for biological samples of human or animal origin; for instance, the software used in the laboratory of medical microbiology at the Centro di Riferimento Oncologico, Aviano, includes specific fields for each bacterial strain stored in the bank, indicating the pertaining microbial genus and species and the biological material from which the microorganism has been cultured. Specific queries for bacteria include the identification in the repository of microorganisms of the same species, or isolated from the same disease group, or having serum or DNA samples obtained from the same patient on the same date, etc.

12. Quality control

The Swedish Medical Research Council [85] recommends that all biobanks containing biological materials and associated information must have an organization with explicit procedures for quality assurance, including systems for storage, coding and registration. Biobanking facilities must adopt general laboratory standards, such as Good Laboratory Practices or ISO series and participate in an external proficiency program reflecting the specialty of the laboratory. In addition, there are four levels of quality control applying more specifically to biological bank facilities: (a) training and certification of personnel and assignment of bankers' responsibilities; (b) control of instrument variability; (c) verification of yield and purity of processed materials; (d) long-term control of stored samples. Briefly, the quality control of instrument or equipment performance includes the documentation of function checks, as specified by the manufacturers, the documentation of preventive maintenance, and the maintenance of records for the life of the instruments [86]. Yield and purity of the materials depend on general quality rules in microbiology [11,12,86], but the specific biological or molecular assays to test these parameters in samples containing microorganisms, animal, and human cells or nucleic acids have been mentioned in the previous sections dealing with each material. Storage quality control includes the periodic verification of alterations induced in the samples during storage and the documentation of freeze-thaw cycles of each sample. Quality control should also specify the limits of acceptability of recovery, purity, structural and/or functional integrity of the samples after storage, and the number of freeze-thawing cycles, which permit the distribution and further use of the samples for epidemiological, research or diagnostic purposes. Finally, an audit consisting in the control of the application of the quality system is recommended [87].

13. Ethical issues

Regulations pertaining to the collection and storage of samples of human origin, including tissues, cells and nucleic acids, and of the related information are at the beginning stage in most European countries and in the U.S.A. The first issue to be regulated regards the identification types that may be employed to store the samples, while a second issue includes the requests for banking: the American society of human genetics stated that "a DNA bank or a DNA diagnostic laboratory should accept samples only in response to requests from health-care professionals and not in response to requests from individuals ... " [88]. National or international regulations must ideally also define the security and regulations of banking facilities. Extensive reviews of the existing professional guidelines, legal frameworks, ethical issues and other documents related to the data storage and DNA banking practices in public and private sectors have been recently published [85,86]. Presently, ethical issues and regulations do not apply to microorganisms stored in biological banks.

14. Concluding remarks

This review presented the state of the art in staff and equipment necessary to set up a biobanking facility, in the problems related to sample collection and processing of various cell types or cellular components, and in data management and quality control. Microbiology laboratories rely even more frequently on biological bank specimens for diagnostics and research. To ensure quality, a biobanking facility must organize the space, equipment and personnel, adopt strict protocols for proper handling of the specimens, set up a quality program including periodic controls of the instrumentation, training of the laboratory staff, and quality control of stored specimens. Because biological banks constitute an invaluable resource for research and diagnostics, it is conceivable that they will require, in the near future, additional investments in terms of human and material resources. Single laboratories may be unable to support their own biobanking facility and institutional or multi-laboratory approaches will be the future of biobanking. The Commission of the European Community has already recognized the need to dedicate a financial contribution for the implementation of European quality standards for biobanking, aiming at supporting large scientific projects involving common diseases. It is conceivable that similar examples may allow the creation of reliable biobanking facilities providing materials that support research projects and thus improve our knowledge in biological sciences.

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