Chapter 9

Microbial Quality Concerns for Biopharmaceuticals

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Additional information is available at the end of the chapter

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1. Introduction

Finding an appropriate definition or a clear classification for biologically occurring pharmaceutical products is a complicated task because of overlapping borders and consequent misconceptions in this area. Indeed, numerous definitions and classifications for this category of products have been proposed so far, and different points of view for this concept can be found in research literature, business, industry, and even the general public [1, 2].

To obtain a better view of biopharmaceutical concept, first, it is necessary to know the present definitions for the main constituents of the word, that is, pharmaceutical product and biological product.

According to the WHO, a finished pharmaceutical product (FPP) is “A finished dosage form of a pharmaceutical product, which has undergone all stages of manufacture, including packaging in its final container and labeling." [3]

An active pharmaceutical ingredient can be defined as “A substance used in a finished pharmaceutical product (FPP), intended to furnish pharmacological activity or to otherwise have direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to have direct effect in restoring, correcting or modifying physiological functions in human beings." [3] Hence, in brief, it can be said that any material, regardless of its origin or structure, with treatment, diagnosis, or prevention applications and passing regulatory requirements, is a pharmaceutical product.

On the other hand, the FDA definition for biological products is as follows: “Biological products or biologics are medical products made from a variety of natural sources (human, animal or microorganism). Like drugs, some biologics are intended to treat diseases and

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medical conditions or to prevent or diagnose diseases.” [4] Consequently, any product of biological origin with treatment, diagnosis, or prevention applications is a biological product.

A biotechnology-derived product is another concept that should be taken into consideration. It is defined by Walsh as “any pharmaceutical product used for a therapeutic or in vivo diagnostic purpose, which is produced in full or in part by either traditional or modern biotechnological means.” [5]

On comparing the definitions for a biotechnology-derived product and a biological product, both of which should be of biological origin, it is obvious that the key element in the former definition is the application of biotechnological means for production.

In practice, the regulatory requirements needed for a biotechnology-derived product and a biological product are methodologically different from the pharmaceutical product due to their biological essence. For example, the determination of adventitious agents such as viruses, transmitting spongiform encephalopathy (TSE), and mycoplasma are included in most of the related guidelines and pharmacopeias for a biotechnology-derived product and a biological product.

Finally, a biopharmaceutical is defined by Walsh as “A protein or nucleic acid based pharmaceutical substance used for therapeutic or in vivo diagnostic purposes, which is produced by means other than direct extraction from a native (non-engineered) biological source.” This definition that will be used in the present chapter for biopharmaceuticals includes all pharmaceutical products produced by modern biotechnology techniques as well as nucleic acid (DNA or RNA) based pharmaceutical products for gene therapy. Hence, the overlapping area between biotechnology-derived products and biopharmaceuticals is the application of modern biotechnological means in their production. However, the differentiating area can be the application of traditional biotechnological means for the production of biotechnology-derived products. In addition, nucleic acid-based pharmaceutical products that are categorized as biopharmaceuticals are not biotechnology-derived products [6]. Figure 1 illustrates these overlapping and differentiating areas. Moreover, some examples of products in these categories are shown in Table1.

	Biotechnology-derived product by:		
Biological products	**Traditional technology**	**Modern technology**	**Biopharmaceuticals**
Blood and blood products	Therapeutic proteins from natural sources	Recombinant proteins	Recombinant proteins
Human cells and tissues	Antibiotics fully or partially from microorganisms	Monoclonal antibody produced by hybridoma technology	Nucleic acid-based pharmaceutical products for gene therapy

Table 1. Some examples of products related to biological, biotechnology-derived, and biopharmaceutical products.

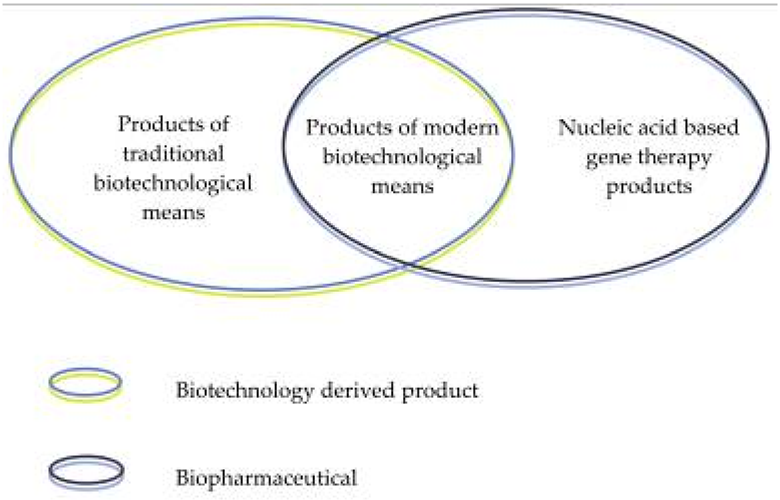

Figure 1. The schematic representation for biotechnology-derived products and biopharmaceutical categories and the overlapping areas between them.

2. Methods for biopharmaceutical production

2.1. Production of an original recombinant system

2.1.1. Recombinant DNA technology

The main category of biopharmaceuticals is manufactured via recombinant DNA technology. Indeed, recombinant DNA technologies are enabling techniques that manipulate and engineer different gene fragments and which have been introduced less than 50 years ago by the revolutionary invention of Polymerase Chain Reaction (PCR) by Kary Mullis [7].

DNA and RNA extraction from different cell types, cutting DNA fragments using restriction endonucleases, joining DNA fragments by DNA ligases, PCR to amplify gene fragments, cloning of the gene fragments into different vectors, introduction of recombinant constructs into proper hosts, protein expression, extraction, and purification are some of the most widely used means in recombinant protein production.

Figure 2 schematically represents the summarized process of production of a recombinant protein. As can be seen from the chart, first, the gene of interest should be isolated and amplified from the original cell. According to the type of the cell, it can be done through direct total DNA extraction followed by a PCR using proper primers to obtain the gene in prokaryotes. On the other hand, in eukaryotes, due to the existence of introns and some modifications that occur in the transcribed mRNA, the process is considerably complicated. Introns are non-coding sequences which are removed after transcription versus coding sequences that are called *exons*. In addition, mRNA is more modified by the addition of a methylated guanine (CAP) on its 5′end and a poly-adenine tail on its 3′end. After these modifications, mature mRNA is exported to the cytoplasm in order to start the translation process. Consequently, to obtain a gene of interest in eukaryotes, the mature mRNA should be extracted from the cell, and the complementary DNA should be synthesized followed by amplification of the gene by PCR using proper primers. However, in both cases (prokaryotes and eukaryotes), the short genes can be obtained by a solid-phase synthesis process.

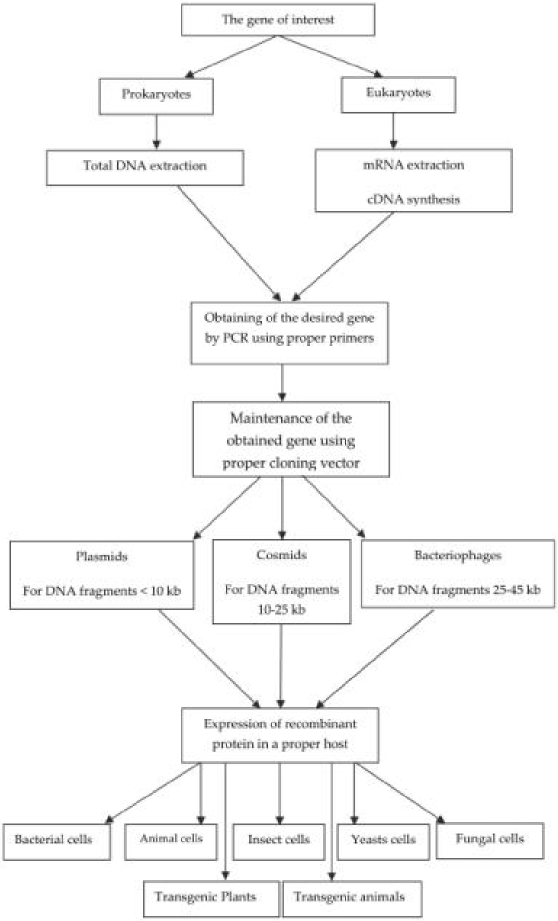

Figure 2. Schematic representation of recombinant protein production process

Based on their sizes, the obtained genes can now be introduced into a proper vector for maintenance, replication, or expression purposes. Plasmids, cosmids, and bacteriophages are the most important cloning vectors that are classified according to the size of the DNA fragment that can be inserted into them. Ultimately, the new recombinant construct should be introduced into an expression system for production. Different classes of expression systems with their certain merits and disadvantages are available and range from cell systems such as bacterial, animal, fungal, and yeast cells to transgenic systems such as transgenic plants and animals [7, 8].

2.1.2. Monoclonal antibody production

2.1.2.1. Classical hybridoma technology

Each specific antibody is secreted by a specific B cell and could recognize a specific region on the antigen that is called *epitope*. Each antibody-secreting B cell could be used as a source of an antibody of interest if it is isolated and cultured in vitro. Nevertheless, B cells are not considered a satisfactory source, as they are not immortalized and cannot survive for a long time. The main approach for overcoming this problem is cell hybridization, which includes the fusion of antibody-secreting B cells with tumor cells (such as mouse myeloma cells) proliferating ever more. The classical hybridoma technology was first introduced by Georges Kohler and Cesar Milstein in the mid 1970s for the generation of immortalized hybridoma cells that could grow in cell culture for a long time and produce the desired monoclonal antibodies [9, 10]. The basic process (Figure 3) includes the immunization of a mouse with the desired antigen. The mouse was then sacrificed, and B lymphocytes secreting antibodies that were selective for the specific epitope on the antigen were isolated from the spleen. The spleen is considered the most ready source for antigen-specific lymphocytes that provides access to a large number of antibody-secreting cells [1]. The isolated B cells were subsequently fused with immortal mouse myeloma cells. The resultant hybridoma cells were then separated from the unfused cells by culturing in specific cell culture media. The cell culture media for the hybridoma growth and production of monoclonal antibodies have been reviewed in detail by Bols et al. [11].

In general, for the successful fusion of hybridomas, the cells are grown in HAT selection medium. The selection medium is called *HAT*, as it has Hypoxanthine, Aminopterin, and Thymidine. This is because a mutation in either the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or the thymidine kinase (TK) gene of the cells would cause their death in the HAT medium. Generally, a TK-deficient cell (TK-negative mutant) is resistant to bromodeoxyuridine (BrdU), and an HGPRT-deficient cell (HGPRT-negative mutant) is resistant to 6-thioguanine (6-TG) and 8-azaguanine. Hence, in order to make myeloma cells sensitive to HAT (unable to grow in HAT media), they are treated with one of these drugs before their passage to HAT media.

Normal cells can synthesize the required nucleotides in two pathways: (1) the main one or de novo biosynthetic pathway, and (2) the alternative one or the salvage pathway (when the main pathway is blocked).

Aminopterin (a folic acid analog that inhibits dihydrofolate reductase) blocks the activation of tetrahydrofolate, which is required for the synthesis of nucleotides via the de novo synthetic pathway, and, therefore, the main pathway is blocked. Thus, in aminopterin-treated cells ($HGPRT^+$ and TK^+), the synthesis of nucleotides shifts to the salvage pathway only if hypoxanthine and thymidine are supplied in the medium. HGPRT and TK, the two enzymes, are required for the salvage pathway, and they catalyze the synthesis of purine and thymidylate from hypoxanthine and thymidine substrates, respectively.

Since unfused myeloma cells lack HGPRT or TK, they cannot use the salvage pathway. Thus, the unfused myeloma cells get killed in the HAT medium, as both biosynthetic pathways are blocked. Normal unfused B cells die in the HAT medium, as they are not immortalized and cannot grow for a long time. Nevertheless, the fusion of normal B cells with the $HGPRT^-$or TK^-myeloma cells allows the hybridoma cells to grow in HAT medium, as the B cells provide the necessary enzymes for growth of the hybridoma cells.

Hence, the HAT selection medium offers an ideal environment for the isolation of fused myeloma and B cells (hybridoma cells) from unfused myeloma cells and unfused B cells, as this medium allows only the hybridoma cells to survive in the culture.

The production of monoclonal antibodies could be accomplished by ascites (ascitic fluid) production (in vivo) or by cell culture (in vitro) methods. In the in vivo method, hybridoma cells are injected intraperitoneally into mice. The peritoneum serves as a growth chamber for the injected cells. These cells could secrete a high-titered solution of desired antibodies as they grow in the cavity. Finally, the produced antibodies are extracted from the ascitic fluid accumulated in the peritoneal cavity [6]. The antibody concentrations typically range between 1 and 15 mg/ml. The in vivo method offers a very high concentration of monoclonal antibody that often does not need more concentration procedures. Nevertheless, monoclonal antibodies produced by this technique may be contaminated by considerable levels of mouse proteins and other contaminants that might require more complicated, subsequent downstream purifications. The other disadvantage of the ascites production is related to animal welfare issues, as these could cause distress in mice.

Currently, more than 90% of monoclonal antibodies are produced by in vitro techniques [12] that use large-scale manufacturing plants containing several 10,000-L or larger culture bioreactors [13]. The in vitro method of monoclonal antibody production decreases the use of mice and also avoids the need for experienced personnel for animal handling. Regardless of the privileges and importance of the in vitro methods of antibody production, there are some situations in which this method is not applicable; for instance:

1. Hybridoma cells do not adapt well to in vitro conditions.
2. Downstream purification methods cause protein denaturation or decreased antibody activity.
3. The cell line cannot maintain the production of monoclonal antibodies.
4. When hybridoma cells are contaminated with infectious agents (such as yeasts or fungi), the cells must often be passed through mice. Since removal of the organisms cannot be accomplished by current antimicrobial drugs, thus the in vivo method may save a valuable hybridoma.

5. When in vitro methods result in monoclonal antibodies that are glycosylated at positions different from those harvested from mouse ascites, they affect antigen-binding capacity as well as biological functions [12].

Taken together, the cell culture technique is a method of choice for large-scale monoclonal antibody production due to the simplicity of the cell culture and financial considerations without ethical concerns that are related to animal use.

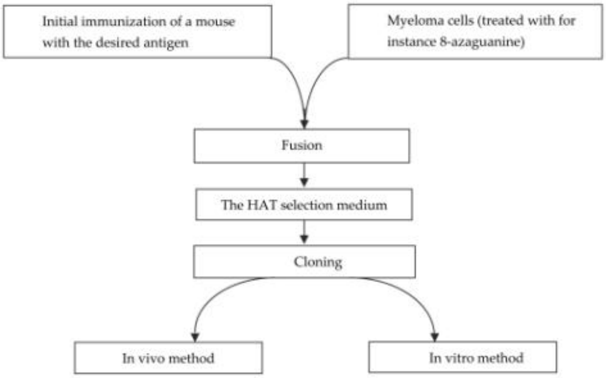

Figure 3. The diagram of the monoclonal antibody production via classical hybridoma technology

2.1.2.2. Chimeric and humanized antibodies

In 1986, about 10 years after the conception of monoclonal antibody technology, Orthoclone OKT3 was approved by the FDA for use in patients with acute rejection of a transplanted kidney [14]. Unfortunately, early clinical applications of murine monoclonal antibodies were disappointing. This was due to the fact that monoclonal antibodies produced via the classical method are of murine origin and are, therefore, immunogenic to human subjects. In general, patients receiving an antibody exhibit HAMA responses (human anti-mouse antibodies) within two weeks. Multiple infusions of murine monoclonal antibodies significantly enhance the HAMA reactions [6]. In addition, the immune system eliminates the murine monoclonal antibody molecule. Thus, murine monoclonal antibodies demonstrate short serum half lives after administration to humans. Furthermore, the other main difficulty related to murine monoclonal antibodies is the poor recognition of the Fc region by human effector systems of complement and Fc receptors.

Thus, new strategies that are used for producing humanized mouse antibodies that are less immunogenic have been discovered. The first strategy includes the production of functional specific recombinant IgG molecules consisting of mouse variable regions and human constant regions; these are known as chimeric antibodies. Taken together, in the chimeric antibody, 8 out of 12 domains are of human origin (constant regions of the heavy and light chains) (C_H and C_L) [15]. Chimeric antibodies exhibit reduced HAMA responses compared with mouse antibodies, but the affinity and the selectivity are the same. Furthermore, since the Fc region contains human sequences, the activation of Fc-mediated immune effector functions is allowed.

To further minimize the antigenicity of murine antibodies, humanized antibodies were developed. For their generation, hyper-variable complementarity-determining regions (CDRs) of the specific murine antibody are transferred to a fully human framework. In comparison with the mouse antibodies, humanized antibodies suggest a lower occurrence of HAMA responses.

Further efforts have been invested in the development of technologies that generate fully human monoclonal antibodies. One of the approaches entails the development of transgenic mice, in which a repertoire of human immunoglobulin germline gene segments is inserted into the mouse genome. After the immunization of these mice, they produce fully human antibodies, which can subsequently be separated with the classical hybridoma technology [15].

Figure 4 illustrates the schematic structures of mouse, chimeric, humanized, and human antibodies.

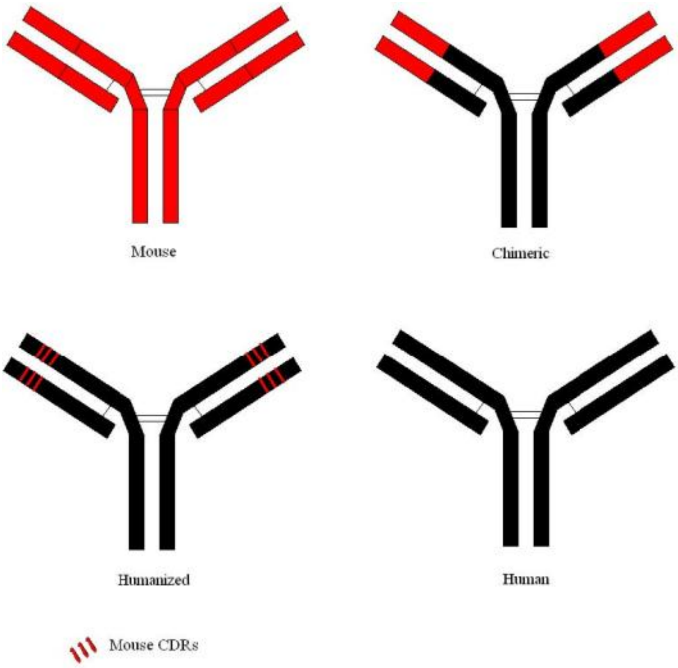

Figure 4. The structure of mouse (shown in red color), chimeric, humanized (shown in black color), and human antibodies. Chimeric antibodies comprise mouse variable regions and human constant regions. Humanized antibodies consist of murine hyper-variable complementarity-determining regions (CDRs) that are grafted to fully human framework.

2.2. Mass production of a recombinant product

Mass production of recombinant products can be achieved in a process that is divided into two main sections called *upstream* and *downstream* processing, as schematically depicted in Figure 5.

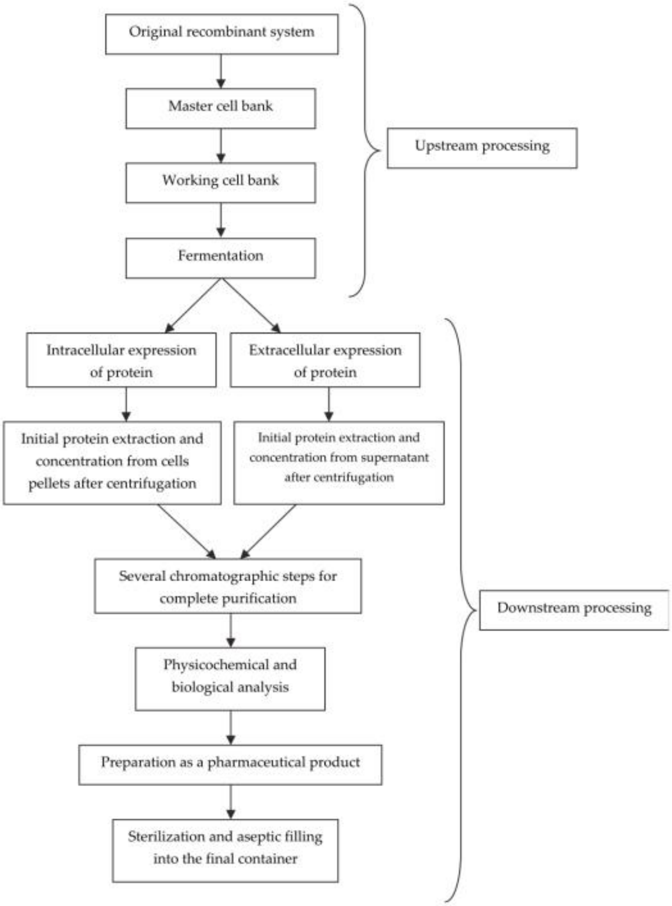

Figure 5. Mass production of a recombinant product.

The first step, the upstream processing step, is the mass production of a recombinant protein via the fermentation process. The original recombinant system that is used for the expression of the recombinant protein (i.e. in the form of a recombinant plasmid maintained in a suitable host cell) forms the cell deposit in a cell banking system. There are two levels of cell deposits in the cell banking system: The first line is called *master cell bank*, which is prepared directly from a culture of the original recombinant system that includes several hundred stored ampoules.

The second line that is referred to as the *working cell bank* is produced from a single master cell bank ampoule. Each ampoule from the working cell bank is thawed and used to seed the fermentor for the production of a batch. Obviously, the fermentation process for various recombinant systems, such as bacterial fermenting systems or animal cell systems, is quite different and needs its own certain requirements.

On the other hand, *downstream processing*, which is the next step, refers to the purification of the mass produced protein. The first step in downstream processing is the initial extraction and concentration of the product, which depends on the situation of the expressed protein; that is, it should be extracted from the pellet cells for intracellular proteins or from the supernatant for extracellular proteins after centrifugation.

The second stage entails (1) several chromatographic steps that complete the purification of the product; (2) the potency test; (3) the addition of suitable excipients; (4) sterilization; (5) filling of the product in its final form (liquid or solid) into the final container before sealing; and labeling [6].

3. Sources of biological contamination of biopharmaceuticals

In line with conventional pharmaceutical products, the main sources of biological contamination in biopharmaceuticals can be related to raw materials and the production environment. Indeed, the biological contaminant content of any pharmaceutical product is a representative of their starting materials and the production environment flora.

3.1. Raw materials

Animal origin materials, such as cell culture media, sera, and supplements that are extensively used in biopharmaceutical production, are of high contamination risk. These materials can be considered the main source for the contamination of biopharmaceuticals with adventitious agents such as TSEs, viruses, and mycoplasmas. Therefore, they should be supplied from reliable resources, and special attention should be paid to their quality control procedure. It should be ensured that all raw materials, especially those of high risk, gain quality specifications for current good manufacturing practice.

Standard methods for sterilization of cell culture media, sera, and supplements should be established according to the properties of the materials. Due to the heat-labile nature of the majority of materials used in biopharmaceutical production, autoclaving is usually replaced with alternative strategies such as filter-sterilization or less frequently high-temperature, short-time treatment strategies. In spite of the routine filter-sterilization procedure that uses 0.22 μm, it is usually performed with 0.1-μm membrane filters due to the risk of contamination with adventitious agents.

Furthermore, high-temperature, short-time treatment strategies are sometimes employed for the elimination of biological contaminants from small solutes such as vitamins and amino acids [16].

Another important raw material that is used in the production of any pharmaceutical product, including biopharmaceuticals, is water, which can be considered an important source for contamination, with water-borne bacteria such as Pseudomonas spp., Alcaligenes spp., Flavobacterium spp., Chromobacter spp., and Serratia spp. Water for pharmaceutical purposes is discussed in detail in the USP [17]. Due to the fact that the intended administration of biopharmaceuticals in the majority of cases is via injection, Water for Injection (WFI) which is sterile and apyrogen is routinely used in this area.

3.2. Production environment

Pharmaceutical products' contamination may occur from the transformation of microorganisms from the production environment to the product. The production environment includes air, surfaces, instruments, equipments, and personnel.

The main groups of microorganisms that are isolated from air are the spore-forming bacteria (Bacillus spp. and Clostridium spp., the non-sporing bacteria Staphylococcus spp., Streptococcus spp., and Corynebacterium spp.), the molds (Penicillium spp., Cladosporium spp., Aspergillus spp., and Mucor spp.), and the yeast (Rhodotorula spp.). These contaminants may be air borne or can be initiated from process equipment or personnel [16]. Consequently, environmental monitoring programs in a production environment are essential actions. Furthermore, the critical operations in biopharmaceutical production should be performed in controlled environments or clean rooms. A clean room is a place with high control of the entrance of particles via the establishment of some air filters called *high-efficiency particulate air (HEPA) filters*. HEPA filters made from a microglass material with a pleated construction system provide a large surface area that efficiently filters the incoming air and generates a constant air motion. Based on the permitted quantity of viable microorganisms and particulates, various classes of clean rooms can be established using HEPA filters with required efficiencies.

According to the *EC Guide to Good Manufacturing Practice for Medicinal Products* (EC GGMP), four grades for clean rooms are available, such as grade A, B, C, or D, based on the number of viable microorganisms and particulates (Table 2).

Clean room grade	Maximum permitted number of particles/m $_3$	Maximum permitted CFU of viable microorganisms/m $_3$ in air sample
A	3500	<1
B	3500	10
C	350000	100
D	3500000	200

Table 2. Clean room grades according to the number of viable microorganisms and the number of particulates

Critical operations such as inoculum preparation and aseptic filling are generally performed in the highest air grade (A); however, less critical operations can be performed in lower grades or even non-classified air.

In addition to the establishment of suitable filters in the clean rooms, special attention should be paid to the position, type, and texture of surfaces, floors, and fixtures. They should be made from smooth and chemically stable materials. In addition, a distinct transfer lock area should exist before entry to the clean room for sanitization of materials and personnel or garment changing. Furthermore, all doors should be interlocking [6].

4. Hazards of biological contamination of biopharmaceuticals

Similar to other pharmaceuticals, biological contamination of biopharmaceuticals may perhaps cause product spoilage. It may result in product metabolization by microorganisms, and, therefore, lead to a decrease in biopharmaceutical potency. The product spoilage may also provide a potential health hazard to patients and lead to outbreaks of infections that may cause additional complications. In addition, microbial-derived agents secreted in products such as endotoxins can be hazardous to a patient's health.

5. Determination of biological contaminants

5.1. Bacteria and fungi

Bacteria and fungi can be considered important contamination sources for all kinds ofpharmaceutical products, including biopharmaceuticals; hence, the control of them is of critical importance. The control of both bacteria and fungi is considered to be worthy of mandatory tests for nearly all kinds of pharmaceuticals in pharmacopoeias. All the related tests and procedures are covered in detail in the major pharmacopoeias such as USP and EP [17, 18].

Since almost all the biopharmaceuticals are administered intravenously, general sterility testing must be carried out for these products. Basically, sterility testing can be defined as "a test that evaluates whether a sterilized pharmaceutical product is free of contaminating microorganisms." The European Pharmacopoeia (2002) proposes two media for sterility testing:

(1) fluid mercaptoacetate medium (also known as *fluid thioglycollate medium*), which is mainly appropriate for the culture of anaerobic organisms at 30–35°C; and (2) soyabean casein digest medium, which is used for the culture of both aerobic bacteria at 30–35°C and fungi at 20–25°C.

Two main methods are used for sterility tests: (1) direct inoculation of the test samples in the media mentioned earlier; or (2) filtration of the test material through a sterile membrane filter with a pore size of 0.45 μm; then, the filter containing any microorganism present in the fluids is divided aseptically, and portions are transferred to the media.

The eradication of bacteria and fungi from the products is generally carried out via inactivation and sterile filtration.

5.2. Endotoxins

Since most of the biopharmaceuticals are administered intravenously, finished-product biopharmaceuticals must be sterile and free from pyrogenic substances. The endotoxin limit for the intravenous administration of pharmaceutical and biological products is 5 endotoxin units (EU)/kg of body weight/hour by all pharmacopoeias [19]. Hence, the detection and removal of pyrogenic substances, especially endotoxins (lipopolysaccharides in the cell wall of gram-negative bacteria), are necessary to ensure safety of biopharmaceutical products. Currently available methods for endotoxin detection include the U.S. Pharmacopeia rabbit test and the Limulus amebocyte lysate (LAL) test [7].

The rabbit pyrogen test entails measurements of the rise in body temperature of rabbits after an intravenous injection of a test substance. The presence of pyrogens of all kinds can be tested using this method. However, this method suffers from a number of disadvantages and limitations: (1) Endotoxin tolerance occurs after repeated use of rabbits; (2) variations in the response depending on sex, age, and species; (3) differences between the responses of rabbits and humans to various pyrogen types; and (4) the rabbit pyrogen test is inadequate for sera, radiopharmaceuticals, chemotherapeutics, analgesics, cytokines, immunosuppressive agents, and others [20].

Accordingly, the use of the rabbit pyrogen test has been reduced. Nowadays, the most widely used endotoxin detection systems are based on the highly sensitive LAL test. It is based on the coagulation cascade of the blood of a horseshoe crab, Limulus polyphemus, which is induced by lipopolysaccharide. The currently known methods for lipopolysaccharide detection entail (1) gel-clot assay, (2) turbidimetric LAL technique, and (3) the chromogenic LAL technique.

The gel-clot assay is a limit test that provides simple positive or negative results. The LAL reagent is introduced to a sample, and the test material is considered endotoxin positive if a gel is formed via a clotting reaction.

The turbidimetric and the chromogenic LAL techniques are quantitative tests. The former is based on the fact that turbidity increases as a result of the precipitation of the clottable protein that is related to endotoxin concentration in the sample. The optical density is read by a spectrophotometer at either a fixed time (for the end-point method) or progressively (for the kinetic assay) as turbidity develops.

The chromogenic LAL technique makes use of a synthetic substrate which contains an amino acid sequence similar to that of the clottable protein, coagulogen, in order to detect endotoxin. The enzyme cleaves a yellow-colored substance from the chromogenic substrate, and the color intensity produced is proportional to the amount of endotoxin present in the sample.

Endotoxins are temperature and pH stable, and, therefore, their removal is one of the most challenging issues. Numerous techniques are used to reduce endotoxin contamination of biopharmaceuticals, including ion-exchange chromatography, sucrose gradient centrifugation, gel filtration chromatography [19], affinity adsorption [21], charged membrane/depth filtration, and ultrafiltration [22].

5.3. Viruses

Owing to the risks of transmission of adventitious agents to patients, the different cell levels should be studied for the absence of these agents. Among the adventitious agents, special attention should be paid to viruses that are capable of contaminating the original species. Generally, the virological safety of biopharmaceuticals includes several levels of control at various manufacturing stages, including 1 - rigorous screening of cell banks (both master cell bank and working cell bank) for viruses; 2 - screening of each cell culture harvest for adventitious agents; and 3 - a demonstration that the purification process can clear potential adventitious agents [15].

The detection of viruses in cell lines can be carried out via various techniques. The commonly used methods of detecting viral infections include

- co-cultivation assays (specific in vitro tests),
- in vivo assays,
- antibody production in animals (MAPs, RAPs, or HAPs),
- immunoassays for viral specific proteins,
- Transmission Electron Microscopy (TEM),
- Polymerase Chain Reaction (PCR).

For the co-cultivation assays (specific in vitro tests), the cells used for production, or culture supernatant, or the final product are incubated with the detector cells. The detector cell lines are susceptible to different viruses and are used to detect desired viruses via monitoring subsequent cytopathic effects, hemadsorption, morphological changes, or other signs of viral infection. The detector cells usually contain humans, primates, and cells from the same species.

The in vivo assay can be performed by the inoculation of cells or cell lysates into animals, including newborn and adult mice, guinea pigs, rabbits, or embryonated chicken eggs to detect viruses. The animals are consequently monitored for any abnormality.

Species-specific viruses potentially present in rodent cell lines can be examined using assays for antibody production in the animals. The MAP, RAP, and HAP (mouse, rat, and hamster antibody production assays, respectively) tests involve an injection of the test article into the animals. The inoculated animals are bled after four weeks, and the sera are tested for the presence of the antibodies against the specific viral antigens. For instance, Hantaan virus, Lactic Dehydrogenase virus, and Sendai virus have been screened using MAP.

An immunoassay for viral-specific proteins can be undertaken through production of the relevant antibodies after an injection of a virus of interest into animals. Currently commercially available immunoassays are able to detect various viruses.

Another method that is used for virus detection is TEM (Transmission Electron Microscopy). TEM is a quantitative assay that is based on the visualization and morphological identification of virus particles in samples [23].

Nevertheless, more sensitive methods, such as the PCR identification methods, can be employed for the detection of sequences of the viruses [16].

Since the biopharmaceuticals can be originated from mammalian cell lines with a high risk of endogenous retroviruses, on one hand, and these products may be infected with adventitious viruses through processing, on the other hand, virus inactivation and removal steps in the purification process are required [24]. These entail gamma irradiation, low pH treatment, or virus filtration.

Indeed, ensuring the absence of virus contamination in biopharmaceuticals is challenging. For instance, a limited number of commercial poultry vaccines were contaminated by avian leukosis virus even after routine quality assurance procedures. In addition, reovirus was found as a contaminant in urokinase. On the whole, sourcing and testing alone cannot guarantee the virological safety of biopharmaceuticals owing to some limitations: the limit of sensitivity for cell culture and PCR tests and also due to the fact that cell culture or in vivo tests are not able to detect all known kinds of potential contaminants [25]. Thus, practical methods are required for the virological safety of biopharmaceuticals, which involve the inclusion of risk assessment as well as management policies.

5.4. Mycoplasma

Mycoplasmas are the smallest free-living and self-replicating organisms in nature that are sized between 50 and 500nm. They lack a rigid cell wall and, consequently, are highly pleomorphic from round to filamentous. They are filterable and penicillin-resistant forms. Furthermore, their membrane contains sterol and due to this, mycoplasmas require the addition of serum or cholesterol to the growth medium. They grow on special media in aerobic or anaerobic conditions with optimum growth at 37°C and pH 7.0 and form with a “fried egg” morphology on agar media (Figure 6).

Mycoplasma contamination of cell culture systems for the production of mycoplasmas is a critical problem due to its effect on various parameters within the cell culture system. Mycoplasma contaminates cell cultures approximately without any sign, and it persists for a long time. Indeed, mycoplasma-positive cell cultures can be considered the major source of biopharmaceutical infection, and they should be discarded or effectively decontaminated. Taken together, mycoplasma-positive cell cultures pose a serious problem and should be effectively detected and eradicated [26].

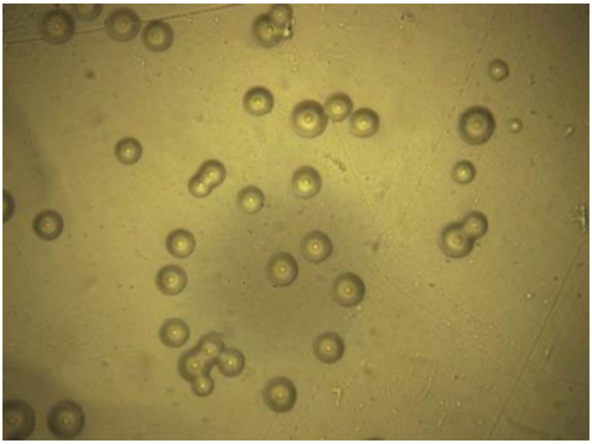

Figure 6. Mycoplasma colonies with fried egg morphology on mycoplasma agar medium. Picture was taken at Mycoplasma Reference laboratory, Razi vaccine and serum research institute, Iran

Different methods are used in international pharmacopoeias and guidance for detecting mycoplasma in biological test samples, mainly categorizing them as direct assay by microbiological culture, indirect assay by indicator mammalian cell culture, and PCR.

Direct assay by microbiological culture: The principle of detection is based on the growth of mycoplasma on supporting agar and liquid media (broth). First, the test sample is introduced into a special broth culture, is incubated for an appropriate time, and, consequently, it is sub-passaged to plate agar. After the required incubation period, the presence of mycoplasma colonies is observed microscopically in the agar plates (Figure 6).

Indirect assay by indicator mammalian cell culture: The indirect method requires the co-cultivation of the test sample with an indicator cell line for two to three days. Typically, VERO cells with a large cytoplasm area around the nucleus were used. Consequently, the cells were stained using a DNA binding stain (such as Hoechst stain) that binds specifically to DNA and is observed via fluorescent microscopy. Due to the affinity of mycoplasmas for the mammalian cell membrane, mycoplasmas appear as granules surrounding the nucleus.

Mycoplasma PCR: In this method, detection is carried out using specific oligonucleotide primers for the amplification of mycoplasma DNA. This method is specially recommended for detecting contamination with the non-cultivable strains of *M. hyorhinis* [27].

On the whole, it is advisable to use two different methods in the detection of mycoplasmas in order to allow for the differentiation between false-positive and false-negative results.

5.5. DNA

The importance of DNA contamination detection in biopharmaceuticals is related to the fact that the DNA from some sources such as hybridoma cell lines in monoclonal antibody production may act as active oncogenes. These kinds of DNA contaminants can be introduced and expressed in human cells and result in the initiation of cancer cells. According to guidelines, the acceptable level of residual DNA in recombinant products is 10 pg per therapeutic dose. DNA hybridization studies that use radiolabeled DNA probes with a specific nucleic acid sequence constitute one of the most widely used methods for the detection of DNA contaminants in the product to a nanogram (ng) range [6]. The important steps involved in DNA hybridization are shown in Figure 7.

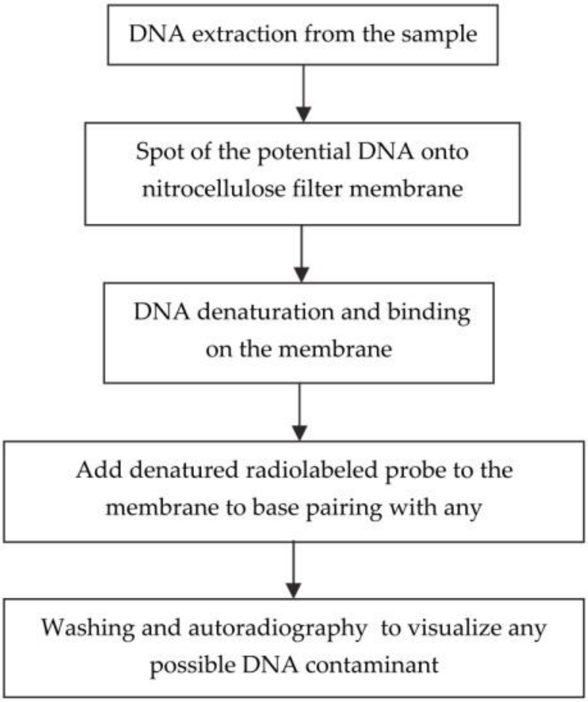

Figure 7. The main steps of the DNA hybridization procedure

5.6. Cross-contamination

Cell cultures may be infected with other cell types due to the use of contaminated items or operator mistakes. Also fail of the sterilization process can be another reason. The detection of cross-contamination is very challenging due to the fact that macroscopic and microscopic properties of the original and contaminant cells are commonly the same. Cross-contamination in the production of biopharmaceuticals would prove to be disastrous and terrible.

Various tests for detecting cross-contaminations can be applied; however, a product-specific identity test will be the best choice [16].

6. Summary

Microbial quality control plays a prominent role in the manufacture of safe and effective biopharmaceuticals. The main sources of microbial contamination can be related to raw materials and the production environment. The main categories of raw materials that are involved in the manufacturing of biopharmaceuticals with a high risk of contamination are those of animal origin such as cell culture media, sera, and supplements. The production environment includes air, surfaces, instruments, equipments, and personnel. All these can be considered the main source for the contamination of biopharmaceuticals with adventitious agents such as viruses, bacteria, fungi, transmitting spongiform encephalopathy, and mycoplasma. The use of contaminated biopharmaceuticals causes product spoilage, which may lead to (i) metabolization of the therapeutic agents by microorganisms, thus bringing about a decrease in the potency of the therapeutic agent; (ii) a potential health hazard to patients as a result of either infectious diseases or microbial-derived agents such as endotoxins that are secreted into products.

Various methods are used for detecting and eliminating different biological contaminants that are used in the manufacturing of biopharmaceuticals. Generally, bacteria and fungi can be detected by standard sterility testing or macroscopic and microscopic characteristics, as well as biochemical tests. In addition, viruses can be detected via a number of methods such as co-cultivation assays (specific in vitro tests), in vivo assays, antibody production in animals (MAPs, RAPs, or HAPs), immunoassays for viral specific proteins, TEM, or PCR. The detection of endotoxin can be carried out using the pharmacopeial rabbit test or LAL test. The available approaches for the detection of mycoplasma include direct assay using special culture media, indirect assay by mammalian cells, and DNA staining, as well as PCR. Furthermore, DNA hybridization is a widely used approach for the detection of DNA contaminants in biopharmaceuticals. The best method for cross-contamination detection includes a product-specific identity test.

With regard to the collection of tests for biological quality control of biopharmaceuticals summarized in this chapter, it is obvious that various sets of methods are available in different guidelines and pharmacopeias which are complicated and problematic. The development and compilation of harmonized guidelines for biological quality control of biopharmaceuticals is a critical necessity that can facilitate the control of the safety of these ever-increasing products.

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