

ORIGINAL ARTICLE

Environmental microbial contamination in a stem cell bank

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Keywords

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Abstract

Aim: The aim of this study was to evaluate the main environmental microbial contaminants of the clean rooms in our stem cell bank.

Methods and Results: We have measured the microbial air contamination by both passive and active air sampling and the microbial monitoring of surfaces by means of Rodac plates. The environmental monitoring tests were carried out in accordance with the guidelines of European Pharmacopeia and US Pharmacopeia. The micro-organisms were identified by means of an automated system (VITEK 2). During the monitoring, the clean rooms are continually under good manufacturing practices specifications. The most frequent contaminants were Gram-positive cocci.

Conclusions: The main contaminants in our stem cell bank were coagulase-negative staphylococci and other opportunistic human pathogens. In order to assure the levels of potential contamination in both embryonic and adult stem cell lines, a continuous sampling of air particles and testing for viable microbiological contamination is necessary.

Significance and Impact of the Study: This study is the first evaluation of the environmental contaminants in stem cell banks and can serve as initial evaluation for these establishments. The introduction of environmental monitoring programmes in the processing of stem cell lines could diminish the risk of contamination in stem cell cultures.

Introduction

The requirement for environmental contamination control has become an important component of stem cell banks. In surgical procedures, contamination control has been an important issue for many years (Dharan and Pitet 2002). However, for processes such as derivation and banking of stem cell lines for clinical use, there are a number of new issues to address.

Maintaining a controlled environment for manufacturing cell products minimizes the potential for introduction of contaminants into the product. Stem cell banks should assure, among other things, the quality and biosafety of these cell products and above all avoid contamination that would be transmitted to recipient patients. This fact is important, because there are several reports in which microbial contamination of stem cell collections have been described (Espinosa *et al.* 1996; Jestice *et al.* 1996). Moreover, in certain cases, the contamination of stem

cells can produce recipient infections by viruses (Clark *et al.* 2006), fungi (Glenn *et al.* 2005; Koldehoff *et al.* 2005) and also bacteria (Arance *et al.* 1997).

At the moment, the functions to ensure the provision of safe and reliable cells and tissues are entrusted to the tissue banks. Although numerous tissue banks in Europe have been accredited by national inspection, there are specific requirements for banks of human stem cell lines. These establishments should be accredited by national organizations (e.g. Medical and Health Care Products Agency) and work under specific international standards (e.g. International Organization for Standardization), directives (European Union 2004, 2006;) and guidelines (United States Food and Drug Administration 2004).

A major function of microbial monitoring is to identify out-of-trend conditions that can indicate a loss of environmental control or a breakdown in aseptic practices (Parenteral Drug Association 2001). Several key

documents describe environmental monitoring programmes for manufacturing sterile pharmaceutical products (European Union 2003). These environmental monitoring programmes comprise a set of procedures and systems, which assure that the cell processing from an environmental point of view is maintained within the established limits for air particles, for microbial air contamination and for contamination of surfaces.

In our bank, we have recently introduced an environmental control programme including the places and the frequency of the sampling, as well as the actions required, like the alert, and action levels for contamination. Such a programme is a part of a global quality control programme that reflects current best practices (International Standard Organization 2000; European Union 2003).

The aim of this study was to evaluate the main environmental contaminants of the clean rooms of our stem cell bank and, additionally, to discuss how the implementation of the environmental control programme should influence the level of contamination of environment and surfaces.

Materials and methods

Clean rooms and particle measuring system description

The clean rooms are the areas of class 100 of approx. 45 m² of surface classified as grade B (European Union 2003) with a system of continuous particle monitoring, which uses an air particle measuring device by means of passing a known volume of air in front of a laser sensor capable of detecting a variable range of particles. An internal microprocessor calculates the number of particles of several sizes by the air volume. In our clean rooms, we installed two aerosol particle sensors (AIRNET 510®; Particle Measuring Systems, Boulder, CO, USA) with two sizing channels of 0.5 and 5 µm and a sample flow rate of 1 cfm. These sensors have a counting efficiency of 50% ± 10% at 0.5 µm. The communication system is by means of Ethernet connector. A facility monitoring system was installed to allow all the particle counters, samplers, environmental sensors to communicate with each other and with a central monitoring station. This monitoring system is controlled by a computer with special software (in our case, Pharmaceutical Net®; Particle Measuring Systems), which allows the operator to observe the system in action. An official calibration of these sensors should be carried out on a yearly basis. In our case, the last calibration was in September 2006.

For convenience, and to improve efficiency, data are displayed on video units to show the status of the areas monitored with alarmed coloured codes and a graphic representation in real time of the individual sensors or of all the sensors.

Microbiological air monitoring

We have measured the microbial air contamination by counting the number of colony forming units per cubic meter [CFU m⁻³ of air by means of an air sampler (Pasquarella *et al.* 2000), to be precise, the perforated atrium sampler (Merck, Darmstadt, Germany), which measures a known air volume. The MAS 100® air sampler is a precise and reliable microbial air sampler that uses standard 100 mm Petri dishes. No external vacuum system is required and the fast sampling is at 100 l min⁻¹. A function control for the anemometer is integrated. A check of calibration should be carried out once per year, as in our case.

Moreover, we have also used the passive air sampling by means of settle plates. Trypticase soy agar plates are placed open and exposed to air for 4 h. In each localization, three plates have been placed (e.g. plate problem, positive control, negative control). The microbes transported by inert particles are deposited on the surface of the agar with an average deposition value of 0.46 cm s⁻¹ (Whyte 1995).

Microbial monitoring of surfaces

In the contact plate, agar (e.g. trypticase soy agar) contained in a specially designed Petri dish, the Rodac plate, is pressed against the surface being sampled. Moreover, the medium contains four neutralizer agents to aim to inactivate the residual disinfectants (e.g. Count-Tact; bio-Mérieux, Marcy l'Etoile, France). Micro-organisms are transferred from the surface to the agar and colonies develop during incubation.

We have estimated the level of contamination of different 'critical' surfaces: the floors of clean rooms, inside biological safety cabinets, under biological safety cabinets, under embryonic microhandler tables, under microscope tables, under CO₂ incubators, under the centrifuges, under the programmable cryopreserver and under the work surfaces. For convenience and consistency, the microbial air monitoring and microbial monitoring of surfaces were carried out in the same location.

Sampling periodicity

The study has been carried out during 1 year and 2 months, from 1 November of 2004 until 31 December of 2005.

For this study, the frequency of sampling was once a week. In two situations, we have carried out an additional environmental control, after a change of use of clean room (different kinds of cell cultures) and after the change of high efficiency particulate air filters. The tests were carried out 'at operational' status.

Incubation conditions

The culture media should be in accordance with the European Pharmacopeia (2004) and/or the United States Pharmacopeia (2004). Both the trypticase soy agar and the blood agar of 90 mm, with incubation temperature of 32°C in air during 7 days were used for the settle plates and active air monitoring testing. For the contact plate testing, the Rodac plate with trypticase soy agar of 55 mm, with incubation temperature of 32°C in air during 7 days was used. Plates were checked daily for visualizing the micro-organisms.

Quality control

Both positive and negative internal quality assurance controls were introduced in order to check that the culture procedures were correct. Unopened plates of trypticase soy agar were incubated as a negative control and other plates (trypticase soy agar and blood agar) were inoculated with selected strains of micro-organisms to act as positive controls. In the same way, a daily calibration of the incubators used for environmental monitoring tests was carried out by means of the Data Logger Thermometer CENTER 305 (CENTER®; Shanghai Electronic Co., Shanghai, China).

The growth promotion test for both the trypticase soy agar and the blood agar was carried out using two micro-organisms, specifically the *Staphylococcus epidermidis* (CECT 4183) and the *Micrococcus luteus* (CECT 244) strains (Spanish Collection of Culture Types, CECT, Valencia, Spain).

Microbial identification

The micro-organisms isolated during the routine environmental monitoring were initially characterized by their cell morphology and staining by Gram's stain. Later, the majority of these micro-organisms were identified by means of an automated system (VITEK 2 compact; Vitek Systems, St Louis, MO, USA). The rest of the micro-organisms were identified by means of manual biochemical test. Moreover, determination of antimicrobial susceptibility of strains was carried out using the same system.

Results

Compliance with the guidelines and particulate counts

Each manufacturing operation requires an appropriate environmental cleanliness level in the operational state in order to minimize the risks of particle or microbial contamination. Clean areas for the manufacture of aseptic products (e.g. stem cell lines) are classified according to the required characteristics of the environment. This classi-

fication has been previously published in the European guidelines of good manufacturing practice (GMP) (European Union 2003). With respect to microbiological sampling, the recommended limits for microbiological monitoring of clean areas are also published in the European guidelines (European Union 2003).

During the environmental and microbiological monitoring of our clean rooms 'in operation' state, these clean rooms have continually been under GMP regulation and both the pressure levels within these rooms and the differential pressure within the corridors were normal.

During the operation state, the number of particles in our clean rooms ranged from 2000 to 30 000 particles m⁻³ for particles of 0.5 µm; for particles of 5 µm, the number of particles ranged from 5 to 200 particles m⁻³ in the operation state. In clean room 2, the number of particles usually was greater than in clean room 1, probably because of the greater activity. All these data are saved in the software of the monitoring system. During the monitoring, for grade B classification, the maximum number of particles of 0.5 µm is 350 000 particles m⁻³ and of 5 µm is 2000 particles m⁻³, both in operation state (European Union 2003).

Monitoring from surfaces

The number of overall isolates with the contact plates method was 54 micro-organisms in the period of the study. The most frequent isolates were Gram-positive cocci (52), followed by Gram-positive bacilli (1) and 1 Gram-negative bacilli. Contaminants isolated from surfaces of clean rooms are shown in Table 1. The frequency

Table 1 Micro-organisms isolated from the clean room surfaces

Micro-organisms	Number of isolated/percentage
Coagulase-negative staphylococci	22/40.7
<i>Staphylococcus epidermidis</i>	12
<i>Staphylococcus hominis</i>	3
<i>Staphylococcus warneri</i>	2
<i>Staphylococcus capitis</i>	2
<i>Staphylococcus xylosum</i>	1
<i>Staphylococcus intermedius</i>	1
<i>Staphylococcus lentus</i>	1
<i>Micrococcus luteus</i>	15/27.7
<i>Granulicatella adiacens</i>	5/9.2
<i>Enterococcus casseliflavus</i>	2/3.7
<i>Leuconostoc mesenteroides</i>	2/3.7
<i>Globicatella sanguinis</i>	2/3.7
<i>Staphylococcus aureus</i>	1/1.8
<i>Erysipelothrix rhusiopathiae</i>	1/1.8
<i>Gemella bergeri</i>	1/1.8
<i>Rhizobium radiobacter</i>	1/1.8
<i>Kytococcus sedentarius</i>	1/1.8
<i>Alloiococcus otitidis</i>	1/1.8

Micro-organisms (total number)	Number of isolations	Specific location/clean room
<i>Staphylococcus epidermidis</i> (12)	2	Centrifuge/clean room 1
	1	Work table/clean room 1
	1	Embryos micro-handler/clean room 1
	1	CO ₂ incubator/clean room 1
	3	CO ₂ incubator/clean room 2
	3	Centrifuge/clean room 2
	1	Wall 1/clean room 2
<i>Micrococcus luteus</i> (15)	2	Embryos micro-handler/clean room 1
	2	Wall 3/clean room 1
	5	CO ₂ incubator/clean room 2
	2	Wall 2/clean room 2
	1	Cryopreserver/clean room 2
	2	Centrifuge/clean room 2
	1	Horizontal cabinet/clean room 2
<i>Granulicatella adiacens</i> (5)	1	Work table/clean room 1
	1	Wall 1/clean room 2
	2	Cryopreserver/clean room 2
	1	Centrifuge/clean room 2
<i>Staphylococcus hominis</i> (3)	1	Work table/clean room 1
	1	Centrifuge/clean room 2
	1	Wall 4/clean room 2
<i>Staphylococcus warneri</i> (2)	1	Wall 2/clean room 1
	1	CO ₂ incubator/clean room 1
<i>Staphylococcus capitis</i> (2)	1	Wall 3/clean room 1
	1	Cryopreserver/clean room 2
<i>Enterococcus casseliflavus</i> (2)	1	Centrifuge/clean room 1
	1	Centrifuge/clean room 2
<i>Leuconostoc mesenteroides</i> (2)	1	Wall 2/clean room 1
	1	Cryopreserver/clean room 2
<i>Globicatella sanguinis</i> (2)	1	CO ₂ incubator/clean room 1
	1	Cryopreserver/clean room 2
<i>Staphylococcus aureus</i> (1)	1	Centrifuge/clean room 1
<i>Staphylococcus xylosus</i> (1)	1	Wall 1/clean room 1
<i>Staphylococcus intermedius</i> (1)	1	Wall 2/clean room 1
<i>Staphylococcus lentus</i> (1)	1	Wall 2/clean room 1
<i>Erysipelothrix rhusiopathiae</i> (1)	1	Wall 1/clean room 1
<i>Gemella bergeri</i> (1)	1	Centrifuge/clean room 1
<i>Rhizobium radiobacter</i> (1)	1	Work table/clean room 1
<i>Kytococcus sedentarius</i> (1)	1	Centrifuge/clean room 1
<i>Alloiococcus otitidis</i> (1)	1	Embryos micro-handler/clean room 1

Table 2 Frequency of isolation and specific locations of micro-organisms in clean room surfaces

of isolation and the specific location in each clean room are showed in Table 2.

Monitoring from air of clean rooms

The number of overall isolates with the trypticase soy agar and blood agar plates was 80 micro-organisms in the period of the study. In this testing, the most frequent isolates were also Gram-positive cocci (78) followed by 2 cases of Gram-positive bacilli and yeast, respectively. The results of this testing are shown in Table 3. The results of susceptibility testing of all coagulase-negative staphylococci are shown in Table 4.

Moreover, the contamination rate of environment and surfaces and its relevant microbial agents percentage is shown in Fig. 1.

Discussion

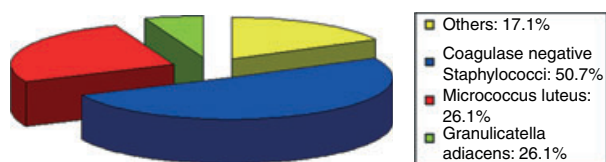
There are four major sources of microbiological contamination in controlled areas, such as surgical 'operating theatres' and clean rooms for manufacturing of aseptic products: air, water, materials and people. From an environmental point of view, more than half of the pathogens that cause contamination in these areas, originate from the normal skin flora of the

Table 3 Micro-organisms isolated from the clean room air

Micro-organisms	Number of isolated/percentage
Coagulase-negative staphylococci	46/57.5
<i>Staphylococcus epidermidis</i>	19
<i>Staphylococcus hominis</i>	14
<i>Staphylococcus intermedius</i>	4
<i>Staphylococcus warneri</i>	3
<i>Staphylococcus cohnii</i>	2
<i>Staphylococcus lentus</i>	1
<i>Staphylococcus capitis</i>	1
<i>Staphylococcus lugdunensis</i>	1
<i>Staphylococcus haemolyticus</i>	1
<i>Micrococcus luteus</i>	20/25
<i>Alloicoccus otitis</i>	4/5
<i>Granulicatella adiacens</i>	3/3.7
<i>Rhodotorulla glutinis</i>	1/1.2
<i>Kytococcus sedentarius</i>	1/1.2
<i>Gemella bergeri</i>	1/1.2
<i>Aerococcus viridans</i>	1/1.2
<i>Streptococcus pneumoniae</i>	1/1.2
<i>Ochrobactrum anthropi</i>	1/1.2
<i>Leuconostoc mesenteriodes</i>	1/1.2

Table 4 Antimicrobial resistances from coagulase-negative staphylococci strains

Antibiotic	Resistance/percentage
Oxacillin	27/39.7
Gentamicin	3/4.4
Ciprofloxacin	3/4.4
Erythromycin	39/57.3
Vancomycin	0
Linezolid	3/4.4
Quinupristin/dalophopristin	0

**Figure 1** Microbial agents percentage obtained from the environmental monitoring tests.

personnel who work in these areas (Bitkover *et al.* 2000).

In a clean room where the air supply is free of bacteria and the room pressurized against the access of outside bacterial contamination, the major source of bacteria is people. People disperse hundreds and sometimes thousands of bacteria per minute into the air (Whyte and Bailey 1985). These bacteria are dispersed on skin cells, or parts of skin cells. The size distribution of bacteria-carrying particles in the air of a hospital has been determined

(Noble *et al.* 1963) and an average deposition rate of 0.46 cm s^{-1} has been reported (Whyte 1995).

Although there are a few reports of contamination in clean rooms for pharmaceutical production, the most frequently isolated micro-organisms in controlled areas used for aseptic processing are bacteria from the human skin (e.g. *Staphylococcus* spp., *Micrococcus* spp., *Corynebacterium* spp.), airborne bacterial spores (e.g. *Bacillus* spp.); occasionally, airborne fungal spores (e.g. *Aspergillus niger*, *Penicillium* spp.), and most infrequently Gram-negative bacteria (e.g. *Enterobacter cloacae*, *Burkholderia cepacia*) (Halls 2004; Owers *et al.* 2004).

In our study, we can observe that the majority of the micro-organisms recovered in both situations are opportunistic human pathogens that are inhabitants of normal human skin, mucosal, oropharynx and also in the environment. Most of them do not cause serious diseases, but occasionally could produce illnesses like bacteraemia (Goenaga Sanchez *et al.* 2003), septicaemia (Anatoliotak *et al.* 2003), otitis media (Kalcioğlu *et al.* 2002) and meningitis (Hay and Lo 1999).

Moreover, with the particle level counts and the micro-organism locations results, we can observe that the most critical locations inside the clean rooms were the CO₂ incubator and centrifuge areas of clean room 2. This analysis was mainly carried out, taking into account the two main micro-organisms isolated, *Staph. epidermidis* and *M. luteus*. These locations coincide with the greater work level and major presence of personnel in these areas. The place of less risk of contamination was the biological safety cabinet, because of both the presence of laminar flow and the exhaustive cleanliness of this instrument, in application of good laboratory practices.

A control and trend analysis to avoid the contamination of stem cell cultures and their possible inoculation in humans is necessary.

Traditionally, the identification of isolated micro-organisms is not usually carried out. However, at present, it is recommended to keep records of the number and type of isolated micro-organisms. The isolation frequency and the microbial counts are used to establish trends and to demonstrate a continuous level of environment control (e.g. repeated isolation of a common species of micro-organisms in different areas, repeated isolation of a single species in the same location). Furthermore, the change in the supposed typical microflora found in the clean rooms and other areas under control should be monitored, as it can indicate a possible decrease in the level of environmental control in these areas. Furthermore, we consider that a routine identification of the isolated micro-organisms had to be carried out not only in terms of the genus but also in terms of the level of species, and that it may be wise to carry out sensitivity tests on the locally avail-

lable antimicrobials in each case. This identification is important and necessary to carry out research where products have been contaminated, as part of the validation of the new installations, observation of resistance to disinfectants, in research into epidemics and validation of change in products and procedures. The identification of the micro-organisms recovered can also determine possible sources of infection and methods of control.

Microbial air contamination is one of the main problems in clean rooms for manufacturing aseptic products or for stem cell line culture. Environmental monitoring is a documented programme, implemented through standard operating procedures that describe the procedures and methods used for monitoring particles as well as micro-organisms in controlled environments in detail (United States Pharmacopeia 1998). Environmental monitoring programmes to ensure that the cells are manufactured in an environment under control are one of the many ways to decrease the chance that products are contaminated during manufacturing. Other ways by which product contamination can be minimized include facility design, materials and personnel flow, process controls and staff training and competency.

The majority of laboratories that carry out routine processing and cryopreservation of haematopoietic stem cells do not present formal environmental monitoring programmes. However, in cell processing carried out in centres banking stem cell lines and other procedures for the production of other cellular materials for cell therapy, the cells are cultured, banked and processed for a potentially

large number of patients over decades. Regular monitoring of micro-organisms and airborne particles using an environmental monitoring programme will be important in order to demonstrate to coordinators of future clinical trials, and their inspectors, that the cellular material was produced aseptically in an environment that minimized the risk of contamination according to current best practice at the time.

Regulatory agencies require environmental monitoring programmes for aseptic manufacturing facilities to be defined and documented with respect to where and how often samples should be taken. In Europe, proposals were put forward by the Parenteral Society (Parenteral Society 1990) and in the United States, Agalloco (1996) published general recommendations implicitly considering facilities in frequent or constant use. Both sources may be used as guidance to establish an environmental monitoring programme, but they cannot be seen as final. Each aseptic manufacturing facility is unique (in technology, manning, design and in use).

The environmental testing programme should operate within a well-documented quality assurance system that establishes the standards relevant to the work, implemented through standard operating procedures that describe the methods and procedures used to carry out the monitoring in detail. These programmes should essentially incorporate the control of three fundamental components: air particle sampling, microbiological air sampling and surface sampling. Other valuable controls to be applied include the periodic monitoring of the personnel

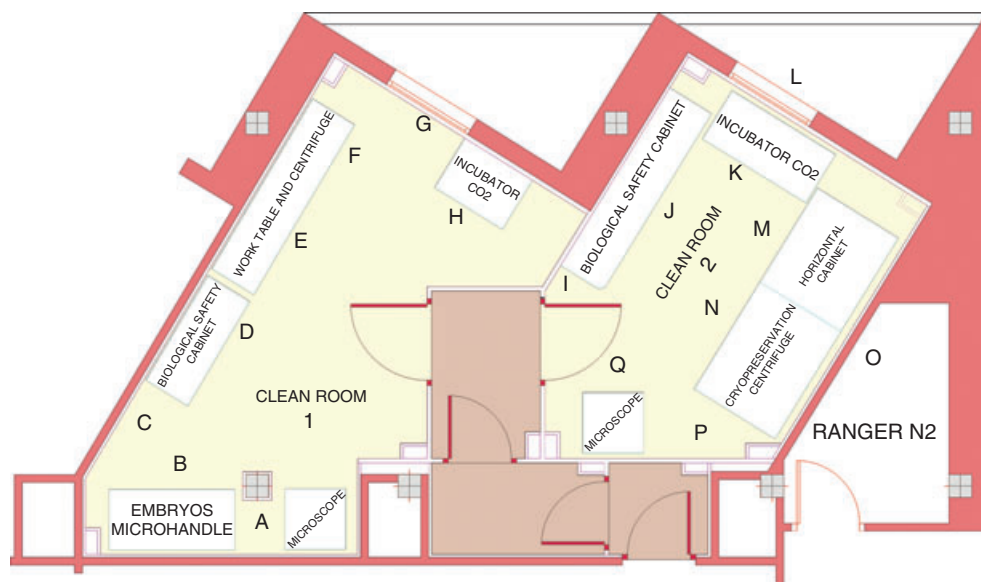


Figure 2 Diagram of clean rooms and sampling points. Clean room 1: A, wall 1; B, embryos micro-handler; C, wall 2; D, biological safety cabinet; E, work table; F, centrifuge; G, wall 3; H, CO₂ incubator. Clean room 2: I, wall 1; J, biological safety cabinet; K, CO₂ incubator; L, wall 2; M, horizontal cabinet; N, cryopreserver system; O, centrifuge; P, wall 3; Q, microscope.

who work in these installations and quality control of cell culture raw materials and intermediates. The introduction of these programmes should also include aspects like frequency and localization of the samples using localization maps (Fig. 2), the duration/volume of the sampling, establishment of 'alert' and 'action' levels, a plan to study/resolve the results obtained above those limits and the resources used for each type of monitoring.

To conclude, after the construction and validation of a controlled environment, the design and implementation of an environmental monitoring programme is vital to assure the maintenance of acceptable conditions in the clean rooms throughout all routine activities (Reich *et al.* 2003). Rapid and continuous microbiological testing methods are required and will be especially important where cell therapy products cannot be cryopreserved. Environmental monitoring should not be overburdening and testing should not be so rigorous as to potentially interfere with cell processing through excessive 'in process' interventions and procedures that may well put the quality of the final product at risk.

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