MICROBIOLOGICAL MONITORING OF CLEAN ROOMS IN DEVELOPMENT OF VACCINES

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ABSTRACT

The aim of the present work was to evaluate an environmental monitoring program for clean rooms, or classified environments, involved in the filling and quality control of biological products produced by Butantan Institute, São Paulo, Brazil. This monitoring established the quantification, characterization and seasonality of the microorganisms in air and operators and, moreover, determined the alert and action limits. The total detectable microbial number showed some contrasts in installed air purification systems and in the operational impact on adopted procedures. The typical microbial population consisted of *Staphylococcus sp*, *Micrococcus sp*, *Bacillus sp* and *Penicillium sp*. The highest microorganism concentration occurred during summer and springtime. The established internal alert and action limits supported the operational procedures. Therefore, the environmental monitoring program is recommended for other laboratories involved in the production of vaccines, hyperimmune sera and biopharmaceuticals.

Key words: environmental control, viable particles, clean rooms, alert and action limits

INTRODUCTION

The history of Butantan Institute[1] intermingles with that of public health in Brazil, aiming to assure quality of vaccines, hyperimmune sera and biopharmaceuticals. Butantan Institute performs operational procedures in classified areas according to international regulations such as the International Standards Organization (ISO) (14) and Brazilian National Agency for Sanitary Vigilance (ANVISA) (9,10). In compliance with these guidelines, we have established defined limits regarding the concentration of viable and non-viable particles in suspension. Therefore, Butantan's production currently follows Good Manufacturing Practices (GMP) (13).

In these environments, aseptic procedures are performed, avoiding environmental air contamination throughout complex filter systems, which is composed of thick (G3), and thin filters (F3) and an air terminal unit consisting of high efficiency particulate air (HEPA) filters (4-7). According to ISO regulations, not only the purity class denomination must be evaluated but also the occupational condition of the area. This condition can be as-built, at-rest and dynamic. The global market induces companies' adaptations to international requirements in their production concerning environmental control (13,17,24).

Passive and active air sampling can provide air evaluation for viable particles. In passive air sampling, the environment is monitored by determining the number of microorganisms that settle a culture plate by gravity (15,18,24). In active air sampling, we can quantify microbes by air sample volume.

Humans are considered the main source of contamination in classified areas due to constant endogenous emissions, and the operator who carries out aseptic procedures must be continuously observed and evaluated and must be aware of conduct procedure (15).

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1. Founded in 1901.

Microbiological monitoring of an environment involves microbiological air quality evaluations on operators, the inside of the laboratories and surfaces. Such a program controls the viable microbial load (bioburden), and this monitoring provides data about the air system efficiency in operational procedures.

Alert and internal action at specific microbiological levels can be determined through frequent monitoring of the environment and operators. Therefore, deviations from normal conditions can be detected, allowing corrective procedures before product quality is affected.

The purpose of this work was to introduce an environmental monitoring program for clean rooms or classified environments, designated for the filling and quality control of biological products produced by Butantan Institute.

MATERIALS AND METHODS

Chosen areas

The following areas were selected to be microbiologically monitored at-rest and during dynamic operations, where such areas were susceptible to product contamination risk and to false results in sterility tests. The Filling Section consists of a multi dose vaccine room for diphtheria and tetanus vaccine (dT, DT vaccines), diphtheria, tetanus and pertussis recombinant vaccine (DTP), and hepatitis B vaccine; the places where the samples were collected were the laminar flow hood, bench, dressing room and pass-through (at-rest), and the laminar flow module and bench during starting and ending procedures. Blending Section is a formulation area; the sites where sampling was performed were the laminar flow module, right and left room, dressing room and passthrough (at-rest), and for dynamic, the laminar flow module and bench. Microbiological Control Section handles sterility testing; places where samples were obtained were the laminar flow hood and bench test room, dressing room, room bench and pass-through during the ending procedures (dynamic). Weekly samples were obtained in different places, from May, 2002 to April, 2003.

Microbiological methods

One thousand liters of air sampling were obtained by M-Air T sampler (Millipore) through the active sampling method Split to Agar (24). The plates containing Bacto tryptic soy culture medium (Millipore) were incubated for 48 h at 30ºC - 35ºC and then for 72h at 20ºC - 25ºC. After incubation, the colonies were counted and recorded in specific protocols. The bacteria were identified by methods described elsewhere (16), and fungal genus identification was according to established techniques (1,2).

The subjects (operators), who had gloved hands during procedures, were monitored by gloved fingerprint in Petri plates containing tryptic casein soy agar, immediately after the procedures. The plates were incubated for 48 h at 30ºC - 35ºC

and then for 72 h at 20ºC - 25ºC, after which the grown colonies were counted, recorded and sent for identification of bacteria and fungi as described above.

Limits for internal alert and action procedures for each integral environment (three monitored sections) were determined by statistical analysis (mean and standard deviation), which were expressed in colony forming units (CFU) per m^3 of air sample. Due to high variability of CFU for single samplings, the following statements were established: Normal work range, mean CFU obtained was increased by two deviations; Alert limit, mean CFU obtained was increased by only one deviation; Action limit, values over the normal range.

RESULTS

The genera and the total bacterial and fungal colony forming units (CFU/m^3) isolated during occupational levels at-rest and dynamic in different environments can be found in Table 1. The microbial population detected in the air consisted mainly of bacteria from the genera *Staphylococcus* sp, *Micrococcus* sp and *Bacillus* sp, and filamentous fungi from the genus *Penicillium sp*, along with six other fungal genera (Table 1). As we looked into performed operations, there was an increase in microbial contamination as can be observed in the results obtained at the end versus the start of the operational procedure.

Several fungal genera were isolated in the Filling Section during the occupational level at-rest. Concerning the occupational level at-rest, the total number of CFU/m^3 in the Filling Section was 350, in the blending area 218, and in the Microbiological Control Section 780. Yet, the numbers at dynamic operations were respectively: total number of UFC/m^3 at filling area 310, Blending Area 1,954, and, finally, at Microbiological Control Section 1,769.

In class A environment laminar flow hoods, we detected low levels of microbes of the genera *Staphylococcus sp*, *Micrococcus sp*, *Bacillus sp*, *and Penicillium sp*, while other filamentous fungi from the genera *Cladosporium sp, Aspergillum sp, Scopulariopsis sp, Fusarium sp, Alternaria sp*, and *Mycelia sterilia* were found only in class D environments (dressing rooms).

The highest level of contamination occurred in the dressing room antechamber. The laminar flow module was free of contamination or had a very low level. Internal alert and action limits for each monitored environment are indicated in Table 2.

Microbial seasonality was evaluated in different environments at-rest and dynamic. Fig. 1A and 1B indicate the total values of CFU/m^3 detected in different seasons: spring (from October to December), summer (from January to March), autumn (from April to June) and winter (from July to

2. In the South Temperate Zone, the seasons are reversed.

Table 1. Total CFU/m^3 for all detected bacteria and fungi for the three monitored areas.

Microbial genus	Filling Section		Blending Section		Microbiological Control	
	A (n=50)	B (n=149)	A (n=43)	B (n=72)	A (n=51)	B (n=155)
Staphylococcus sp	134	247	170	1.063	536	1.388
Micrococcus sp	75	40	43	414	127	207
Bacillus sp	3	15	5	115	18	148
Total bacteria	212	302	218	1,592	681	1.743
Penicillium sp	114	1	Absent	2	27	26
Cladosporium sp	9	3	Absent	Absent	Absent	Absent
Aspergillus sp	5	3	Absent	Absent	Absent	Absent
Scopulariopsis sp	1	Absent	Absent	Absent	Absent	Absent
Fusarium sp	3	1	Absent	Absent	Absent	Absent
Alternaria sp	5	Absent	Absent	Absent	Absent	Absent
Mycela sterilia	1	Absent	Absent	Absent	Absent	Absent
Total fungi	138	8	Absent	2	27	26
Total	350	310	218	1.594	708	1.769

A – occupational condition "at-rest"; B – occupational condition "dynamic"; n – number of samples.
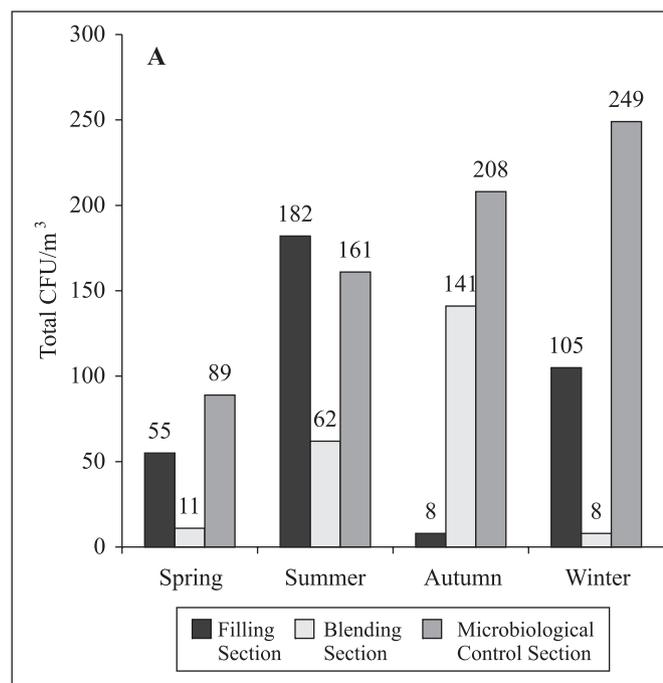

Figure 1A. Isolated microorganisms (total CFU) in the occupational condition "at-rest", at Filling Section, Blending Section and Microbiological Control Section, from May, 2002 to April, 2003.
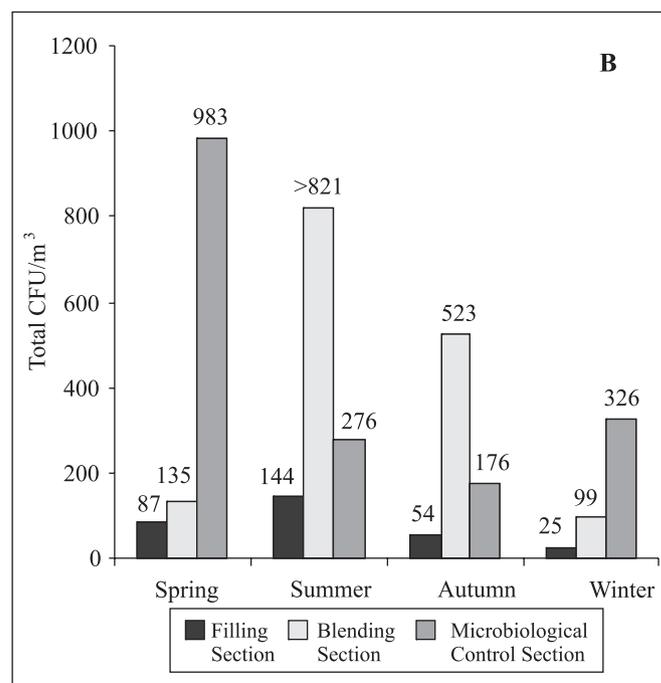
Figure 1B. Isolated microorganisms (total CFU) in the occupational condition "dynamic", at Filling Section, Blending Section and Microbiological Control Section, from May, 2002 to April, 2003.

Table 2. Normal working range and internal alert and action limits (CFU/m^3), for each monitored area.

Environment	Normal working range	Alert limit	Action limit
Filling Section – at-rest			
Laminar flow module (Class A)	0	0	0
Bench (Class C)	0	0	0
Pass-through (Class C)	0 – 0.44	0.33	> 0.44
Dressing-room (Class D)	0 – 2.18	0 – 0.44	> 2.18
Exiting dressing room (Class D)	0 – 1.01	0.57	> 1.01
Dressing room hall	0 – 25.33	15.56	> 25.33
Filling Section – dynamic			
Laminar flow module – procedure starting	0 – 0.47	0.25	> 0.47
Laminar flow module – procedure ending	0 – 0.48	0.26	> 0.48
Bench - procedure starting	0 – 1.90	1.11	> 1.90
Procedure ending	0 – 3.82	8.13	> 13.82
Blending Section – at-rest			
Laminar flow module (Class A)	0	0	0
Bench (Class C)	0 – 9.18	5.3	> 9.18
Pass-through (Class C)	0 – 4.45	2.64	> 4.45
Dressing room (Class C)	0 – 6.12	3.60	> 6.12
Exiting dressing room (Class C)	0 – 15.46	8.84	> 15.46
Blending Section – dynamic			
Laminar flow module – procedure starting	0 – 0.61	0.33	> 0.61
Procedure ending	0 – 0.62	0.45	> 0.62
Bench - procedure starting	0 – 55.72	33.50	> 55.72
Procedure ending	0 – 47.27	29.49	> 47.27
Microbiological Control Section – at-rest			
Laminar flow hood (Class A)	0	0	0
Bench (Class C)	0 – 8.28	5.23	> 9.28
Pass-through (Class D)	0 – 6.19	3.69	> 6.19
Entrance dressing room (Class C)	0 – 46.45	27.67	> 46.45
Dressing room (Class D)	0 – 8.87	5.66	> 8.87
Exiting dressing room (Class D)	0 – 21.63	13.12	> 21.63
Microbiological Control Section – dynamic			
Laminar flow module – procedure ending	0	0	0
Bench - procedure ending	0 – 55.50	33.17	> 53.50
Pass-through	0 – 7.16	4.29	> 7.16

September)*3. The largest concentration of microorganisms occurred during the summer and spring.

Operators' gloved hands evaluated after operational procedures in each monitored environment displayed mainly bacteria from the genera *Staphylococcus sp, Bacillus sp, Micrococcus sp* and the filamentous fungus *Penicillium sp*. The majority of samplings on the operators' hands resulted in the lack of CFU or the presence of only one CFU. Other microorganisms such as *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Alcaligens xylosidans* were detected in sampling from distinct operators (Table 3).

Table 3. Collection number grouped according to detected CFU/m^3 and microbial genera on Operator's gloved hands for each monitored area.

Area	Collection number				Total
	0 CFU	1 CFU	2 CFU	≥ 3 UFC	
Filling Section	129	18	03	12	162
Blending Section	153	05	01	01	160
Microbiological Control Section	167	23	09	10	209

Area	Microbial Genera (CFU – %)				
	Staphylococcus sp	*Bacillus sp*	*Micrococcus sp*	*Pseudomonas sp*	*Penicillium sp*
Filling Section	111 CFU (52.11%)	75 CFU (35.21%)	23 CFU (10.8%)	4 UFC (1.88%)	0
Blending Section	52 CFU (49.06%)	14 CFU (13.21%)	14 CFU (13.21%)	0	26 UFC (24.53%)
Microbiological Control Section	41 CFU (33.61%)	52 CFU (42.62%)	25 CFU (20.50%)	2 UFC (1.64%)	2 UFC (1.64%)

DISCUSSION

Environmental monitoring programs, although developed in other institutions, have limited detailed published results. Therefore, it is difficult to make a comparison between the data from this study and those of others. Besides, in Brazil, only three large institutions are dedicated to immunobiologicals production, Butantan Institute (São Paulo), Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro) and Paraná Technology Institute (TECPAR, Paraná). These institutes have only recently started the implementation of this program (SBCC, 2002) (19).

The total viable microorganisms detected in the occupational condition at-rest revealed some existing differences in the air purification systems. The multi-dose vaccine filling room had 350 CFU, the blending room 218 CFU, and the sterility test room I, 708 CFU. These results suggest a lower efficiency in the air system, since the cleaning procedures are the same for each evaluated area.

Total viable microorganisms detected in the occupational condition dynamic reflected the impact of the operational procedures. Comparing both occupational conditions, for the Filling Section, there was no difference in its microbiological characteristics: CFU number for at-rest condition was 12.9%, lower than for dynamic condition. On the other hand, for the Blending Section and for Microbiological Control Section, there were greater differences between observed CFU in the two conditions. Blending Section had an increase of 631.19% for the dynamic operational condition, probably due to the intense circulation in the area in manipulating large equipment such as tanks used in vaccine formulation. The Microbiological Section showed a difference of 149.85% between the operational conditions. This value may indicate that the air purification system is not able to achieve an efficient auto-regeneration, since the procedures performed were with small equipment for testing sterility. Comparing the results before and after procedures, in the Filling Section, there was no effect on the room microbiological characteristics when the operational procedures were performed under the laminar flow module, but when they were performed on the bench there was an increase in CFU number. This finding indicates that the operator's work impacted directly the results.

At the Blending Section, both operational procedures under the laminar flow module or on the bench did not show differences in microbial characteristics of the environment, which indicates that the operator's work did not affect the results.

The characteristic microbial population identified in these monitored areas was composed of the bacteria *Staphylococcus* sp, *Micrococcus* sp, *Bacillus* sp, and the fungus *Penicillium* sp, with a predominance of *Staphylococcus* sp in all areas. These three bacterial genera have a ubiquitous distribution. They have been isolated from the environment or on human skin and mucous membranes. Members from the genus *Staphylococcus sp* can cause a large variety of illnesses, such as folliculitis and abscess, whereas the *S. aureus* is the most important pathogen from this genus (26). Fungi from the genus *Penicillium sp*, Deuteromycetes, commonly found in soil, are the main components of atmospheric microbiota (1,2). Therefore, the presence of these microorganisms in classified areas is due

to a constant endogenous release from the operators, which indicates that they are one of the most important causes of environmental contamination.

The low incidence of contaminants in Class A environments is characterized by microorganisms from *Staphylococcus sp* and *Bacillus sp* genera, while in Class C and Class D, *Staphylococcus* sp, *Micrococcus* sp, *Bacillus* sp and *Penicillium* sp were isolated. Other filamentous fungi such as *Cladosporium* sp, *Aspergillus* sp, *Scopulariopsis* sp, *Fusarium* sp, *Alternaria* sp and *Mycelia sterilia* were found in low concentrations and predominantly in class D environments.

There are no recommendations about maximum number of viable microorganisms per cubic meter for the at-rest operational condition in the current Brazilian national guidelines. In Class A environments there were no contaminants. The working range was from 0 to 9.18 CFU in Class C. Class D environment, consisting of entrance dressing-rooms, showed a range from 0 to 46.45 CFU, dressing-room from 0 to 8.87 CFU, and exiting dressing-room from 0 to 21.63 CFU. The values reflected the operator's flow. A gradual reduction was observed from the dressing-room to the room with laminar flow, where critical procedures were performed.

Dynamic operational procedures, being the most critical moment of the operation, had for Class A environments, a normal range from 0 to 0.62 CFU and Class C environments, from 0 to 53.50 CFU. These results were below the maximum recommended in the guidelines (9,10) for each environment. Furthermore, these values demonstrate that there was no impact of environmental contamination on the final product quality, since after the sterility test the products were approved.

Seasonality and microbial population distribution could only be evaluated in the dynamic operational condition, because the areas have stable and controlled conditions and because they are not influenced by external conditions such as humidity and temperature. The presence of different operators and the constant release of endogenous contaminants led to an increase in CFU in the three evaluated areas after October. The highest contamination was observed in spring and summer and the lowest during the autumn and winter periods.

Microbial load at the operator's gloved hands reflected the same microbial genera as those from the air contamination, indicating the operator as the main source of contamination . The predominance of *Staphylococcus* sp reveals that the operators, even dressing in complete uniforms, need an extensive training in aseptic techniques.

The results obtained show that the operational procedures can affect the microbiological characteristics of the areas evaluated , which are related to the kind of activity and the air system efficiency. Furthermore, the working conditions at the Blending Section, Filling Section and Microbiological Control Section were satisfactory, assuring high product quality and can be recommended for other producers of immunobiologicals

and biopharmaceuticals. The normal working ranges and internal alert and action limits allow deviations to be controlled before product quality is affected.

RESUMO

Monitoramento Microbiológico de Áreas Classificadas em Desenvolvimento de Vacinas

O presente trabalho teve por objetivo avaliar um programa de monitoramento microbiológico ambiental para áreas limpas, ou ambientes classificados, envolvidas na produção, envasamento e controle dos imunobiológicos produzidos pelo Instituto Butantan. Este monitoramento permitiu a quantificação, a caracterização e a sazonalidade da população microbiana presente no ar e nos operadores, e a determinação dos limites de alerta e ação. O número total de bactérias detectáveis revelou diferenças nos sistemas de purificação de ar instalados e o impacto operacional ocasionado pelos procedimentos realizados. A população microbiana característica foi composta por bactérias dos gêneros *Staphylococcus sp*, *Micrococcus sp*, *Bacillus sp* e por fungos filamentosos do gênero *Penicillium sp*. A maior concentração de microrganismos ocorreu nos períodos de verão e primavera. Os limites internos de alerta e ação estabelecidos asseguram os procedimentos operacionais, recomendando o monitoramento microbiológico ambiental a outros laboratórios envolvidos na produção de vacinas, soros hiperimunes e imunobiológicos.

Palavras-chave: controle ambiental, partículas viáveis, áreas limpas, limites de alerta e ação

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