ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

COMPARISON OF PLANT FLORA FOR A NEWLY CONSTRUCTED CLEANROOM AREA BEFORE AND AFTER COMMISSIONING CLEANING AND DISINFECTION

EMİN GÜREL

M.Sc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
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Department:	Faculty of Health Sciences
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Thesis Title:	Comparison Of Plant Flora for a Newly Constructed Cleanroom Area Before and After Commissioning Cleaning and Disinfection
Student's name and Surname:	Emin Gürel
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This is to certify that I have examined this copy of master thesis. I have found that she/he prepared after fulfilling the specified requirements in the associated legislations before the final examining committee whose signatures are below.

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethicel rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

20.06.2023
Emin Gürel

PREFACE AND ACKNOWLEDGEMENT

I would like to thank Mr. Tunç Turgut for his support of our studies.

I would like to begin by expressing my gratitude and thanks to my supervisor, Prof. Dr. Özge Can, for helping and supporting me and my research.

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This MSc program could not have been completed without my wife Filiz Akın Gürel and my son Barış Gürel. They supported me during whole master program and preparing this thesis.

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LIST OF ABBREVIATIONS AND SYMBOLS

AOAC	Association of Official Agricultural Chemists
EN	European Norm
HEPA	High-efficiency Particulate Air
HVAC	Heating, ventilation, and air conditioning
IPA	Isopropyl Alcohol
ISO	International Organization for Standardization
NA	Not Applicable
NF	National Formulary
TSA	Trypticase Soy Agar (Soybean-Casein Digest Agar)
TSA+LTHTh	Trypticase Soy Agar with neutralizers (Lecithin, Polysorbate (Tween®) 80, Histidine, Sodium Thiosulfate)
USP	United States Pharmacopeia

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ÖZET

Yeni İnşa Edilmiş Bir Temiz Oda Alanının Devreye Alma Temizliği ve Dezenfeksiyonu Öncesi ve Sonrası Floralarının Karşılaştırılması

Temiz odalar, partikül boyutlarına ve metreküpteki sayılarına göre sınıflandırılır. Temiz oda sınıflandırma parametreleri ISO 14644-1'de verilmiştir (1). İlaç üretiminde kullanılan temizoda sınıfları çoğunlukla ISO 5, ISO 6, ISO 7 ve ISO 8'dir. Temiz odalar ayrıca belirli sıcaklık aralıklarına, bağıl neme, saat başına hava değişim oranlarına, bitişik temiz odalar arasında belirli basınç farkına sahip olmalıdırlar. Temiz odaların havası ve yüzeyleri de uygun mikrobiyolojik koşullara sahip olmalıdırlar. Yukarıdaki parametreler öncelikle ısıtma, havalandırma ve iklimlendirme (HVAC) üniteleri tarafından kontrol edilir. Temiz odalara temiz hava, HEPA filtreler aracılığıyla verilir ve hava tahliye üniteleri tarafından uzaklaştırılır. Temiz odadaki canlı ve cansız partiküllerin ana kaynağı insanlardır. HVAC üniteleri bu kontaminasyonu ancak kısmen önleyebilir veya ortadan kaldırabilir. Etkili bir kontaminasyon kontrolü için, temiz oda kirleticilerinin fiziksel yollarla uzaklaştırılması gerekir. Bunun için en etkili yol, temiz odalarda uygulanan temizlik ve dezenfeksiyon prosedürleridir. Deterjanlar temiz oda yüzeylerinden leke ve kirlerin fiziksel olarak uzaklaştırılması için kullanılırken, dezenfektanlar mikroorganizmaların inaktivasyonu veya öldürülmesi için kullanılmaktadır. Bir temizleme programının etkinliği mikrobiyolojik testlerle değerlendirilir. Bu değerlendirmede dezenfeksiyon işlemi uygulanmadan önce ve uygulandıktan sonra mikrobiyolojik testlerde izole edilen mikroorganizmaların sayısı ve türleri belirlenir. Bu çalışma, ISO 6, 7 ve 8 temiz odalarına sahip bir ilaç firmasının yeni inşa edilmiş temiz odalarında gerçekleştirilmiştir. Deterjan ve dezenfektanlar uygulanmadan önce ve sonra hava ve yüzeyde mikrobiyolojik testler yapılmıştır. Temizlik prosedürü uygulanmadan önce ve uygulandıktan sonra mikroorganizmaların sayısı, cinsleri ve türleri karşılaştırılır. Bu çalışma ile seçilen deterjan ve dezenfektanın temiz oda yüzeylerine uygulanması ile mikrooganizma cins ve tür sayılarında ayrıca toplam mikroorganizma sayılarında azalma sağladığı gösterilmiştir.

Anahtar Sözcükler: Temiz oda, ISO, Deterjan, Dezenfektan, HVAC.

ABSTRACT

Comparison of Plant Flora for a Newly Constructed Cleanroom Area Before and After Commissioning Cleaning and Disinfection

Cleanrooms are classified according to particle sizes and numbers in cubic meter. The cleanroom classification parameters are given in ISO 14644-1 (1). The cleanroom classes used in pharmaceutical manufacturing are mostly ISO 5, ISO 6, ISO 7 and ISO 8. Cleanrooms in addition must have definite temperature ranges, relative humidity, air exchange rates per hour, differential pressure between adjacent cleanrooms. The air and surfaces of clean rooms should also have appropriate microbiological requirements. The above parameters are controlled primarily by heating, ventilation and air conditioning (HVAC) units. Clean air is supplied into the cleanrooms through HEPA filters and removed by air removal units. The main source of viable and non-viable particles in the clean room is personnel. HVAC units can only partly prevent or remove this contamination. For effective contamination control, cleanroom contaminants must be physically removed. The most effective way for this is the cleaning and disinfection procedures applied in clean rooms. While detergents are used for physical removal of soil and dirt from cleanroom surfaces, disinfectants are used for the inactivation or destroying the microorganisms. The effectiveness of a cleaning program is evaluated by means of microbiological tests. In this evaluation the number and types of microorganisms isolated in microbiological tests are counted before and after applying disinfection procedure. This study is performed in newly constructed cleanrooms of a pharmaceutical company having ISO 6, 7 and 8 cleanrooms. Microbiological tests were performed in the air and on the surfaces before and after applying detergents and disinfectants. The number, genus and species of microorganisms prior to and after applying cleaning procedure are compared. With this study, it has been shown that the chosen detergent and disinfectant reduces the number of micro-organism genus and species, as well as the total number of microorganisms.

Keywords: Cleanroom, ISO, Detergent, Disinfectant, HVAC.

1 INTRODUCTION AND AIM

Severeal chemical compounds are used as disinfectants in the industry. According to USP (**3**), the major chemical compounds used in pharmaceutical industry are as follows:

Table 1. General Classification of Antiseptics, Disinfectants and Sporicidal Agents

Chemical Entity	**Classification**	**Example**
Aldehydes	Sporicidal agent	2% Glutaraldehyde
Alcohols	General purpose disinfectant, antiseptic, antiviral agent	70% Isopropyl alcohol, 70% alcohol
Chlorine and sodium hypochlorite	Sporicidal agent	0.5% Sodium hypochlorite
Phenolics	General purpose disinfectant	500 µg per g Chlorocresol, 500 µg per g chloroxylenol
Ozone	Sporicidal agent	8% Gas by weight
Hydrogen peroxide	Vapor phase sterilant, liquid sporicidal agent, antiseptic	4 µg per g H_2O_2 vapor, 10%-25% solution, 3% solution
Substituted diguanides	Antiseptic agent	0.5% Chlorhexidine gluconate
Peracetic acid	Liquid sterilant, vapor phase sterilant	0.2% Peracetic acid, 1 µg per g peracetic acid
Ethylene oxide	Vapor phase sterilant	600 µg per g Ethylene oxide
Quaternary ammonium compounds	General purpose disinfectant, antiseptic	Concentration dependent on application, Benzalkonium chloride
β-Propiolactone	Sporicidal agent	100 µg per g β-Propiolactone

"The efficacy of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected (glass, plastic, vinyl, epoxy, stainless steel, ceramic, terazzo, etc.), the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present" (4).

2 BACKGROUND

Microorganisms present in the cleanroom environment generally travel by attaching to airborne particles. Most of the microorganisms which are attached to airborne particles can be removed by air exchange process within the cleanroom. Not all microorganisms are suspended in the air, but some of them reside on the surfaces of the equipments, floor and the wall. Air removal for these microorganisms which are attached to cleanroom surfaces is not possible. In this case the microorganisms on the surfaces can be either killed or inactivated by using disinfectants. The disinfectants are applied mechanically or sprayed.

2.1 Microbial Sources in a Pharmaceutical Cleanroom Environment

Microorganisms are ubiquitous in the environment. The main source of microorganisms in pharmaceutical industry are operators (humans). Humans continuously shed particles as well as microorganisms. Therefore, although strict physical measures are present to prevent microbial contamination, humans frequently act against these measures. Water, materials, air flow and ventilation system are other microbial sources. Each these different sources bring about to have different microbial genus and species in the cleanrooms. According to the study of Dr. Tim Sandle (2022) (5), about 70% of microorganisms in a cleanroom are human origin. Human skin and breath continuously shed microorganisms. About 10% of microorganisms are waterborne. Water is used for the manufacturing operations and for the cleaning and disinfection. About 10% of microorganisms are originated from ventilation system. Dust and solid particles are accumulated on the filters in a air ventilation system. Any failure or damage in these filters causes distribution of particles accumulated on them into a cleanroom. About 5% of microorganisms are material origin. This means equipment and machinery in the room, raw materials used in the production, cleaning utensils may be a source of microbial contamination.

The type of a microorganism present in a cleanroom may indicate the possible source of that microorganism.

2.1.1 Human origin microorganisms in a cleanroom

Human origin microorganisms in a cleanroom belong generally to genera *Micrococci* and *Staphylococci* (6). Human skin and hair are main sources.

2.1.2 Waterborne microorganisms in a cleanroom

Waterborne microorganisms are mostly gram-negative rods. Residual water in cleanroom sinks or basins are the main sources of gram-negative rods.

2.1.3 Airborne microorganisms in a cleanroom

Airborne microorganisms are generally gram-positive cocci and gram-positive rods. These microorganisms originate from operators in cleanrooms and from poor and faulty venilation system.

2.1.4 Material origin microorganisms in a cleanroom

Gram-positive rods are the major type of microorganisms transported into a cleanroom by means of incoming materials and equipments and also footwear (6).

2.2 How Microorganisms are Isolated in a Cleanroom

Some instruments or microbiological sampling techniques are used to isolate and count the microbial colonies. According to USP chapter <1116> Microbiological Control and Monitoring of Aseptic Processing Environments, environmental sampling consist of;

- Active air sampling
- Settle plates
- Surface tests

2.2.1 Active air sampling

Active air sampling can be done using different types of instruments. Among them, sieve impactor is the most common. “The principle of the sieve impaction systems is based on the Anderson impaction principle. Air is aspirated through a perforated lid. A radial fan, controlled by a flow sensor, accurately regulates air flow real time at 100 liters/min. The air is impacted onto the surface of growth media (usually TSA, a general-purpose non-selective medium) in a 90-100 mm Petri dish or 55-60 mm Contact Plate” (7). After the sampling is performed, the nutrient agar plate is incubated for the appropriate time and temperature. The colonies formed are counted.

Figure 1. Millipore MAS-100 NT® Ex

Picture taken from: (https://www.merckmillipore.com/TR/tr/product/MAS-100-NT-and-MAS-100-NT-Ex,MM_NF-C143563?CatalogCategoryID=)

2.2.2 Settle plates

Settle plates are widely used as a simple and inexpensive way to qualitatively assess the environment (8). The settle plate usually contains TSA medium. They are exposed to the environment up to 4 hours. After the sampling is terminated, the nutrient agar plate is incubated for the appropriate time and temperature. The colonies formed are counted.

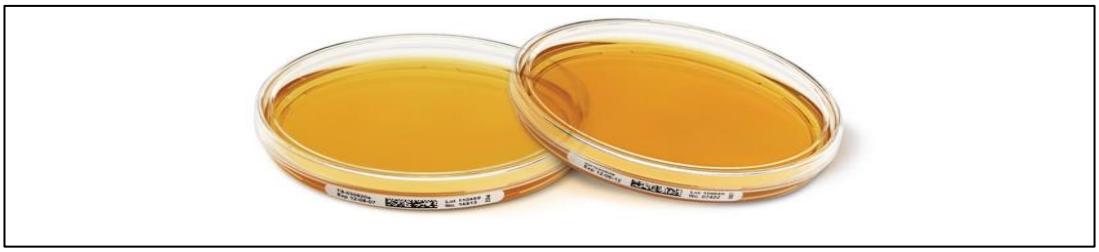

Figure 2. Millipore TSA 90 mm settle plate

Picture taken from: (https://www.merckmillipore.com/TR/tr/search/-?SearchTerm=*&SingleResultDisplay=SFProductSearch&SearchContextCategoryUUIDs=Rd2b.qB.EDcAAAFAWAI.1Zwo)

2.2.3 Surface tests

Surface sampling is another technique of microbiological environmental monitoring. The surfaces of the cleanroom (floors, walls) and surfaces of the equipment can be sampled using this method. Contact plates (special petri plates filled completely with nutrient agar medium usually containing neutralizer) and swab sticks are used for surface sampling. Medium with neutralizer is used to neutralise any disinfectant residue left on the surfaces. Because disinfectant residues may prevent microorganisms to grow. Contact plates are used to sample flat surfaces. Swab sticks are used for sampling irregular or non-flat surfaces. After sampling, swab sticks are either applied directly onto agar plate to transfer microorgansims collected or put in a suitable solution for further membrane filtration of the solution to recover microorgansims. The nutrient agar plate is incubated for the appropriate time and temperature. The colonies formed are counted.

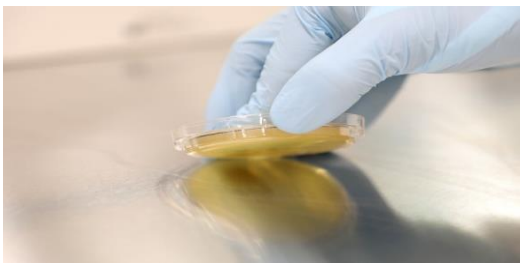

Figure 3. Contact plate to sample flat surfaces

Picture taken from: (https://eagleanalytical.com/product/65mm-tsa-plate/)

Figure 4. Surface sampling using swab stick

Picture taken from: (https://www.newfoodmagazine.com/article/118437/sars-cov-2/)

2.3 Use of Disinfectants in a Cleanroom

If complete kill or removal of microorganisms is desired, the method which is used is sterilization. Sterilization can be achieved using autoclaves, applying gamma irradiation, filtration through 0.2µ pore size filters, applying chemical compounds like hydrogen peroxide or ethylene oxide in gaseous form. The method which will be chosen depends on the material type. On the other hand, it is not possible to sterilize a cleanroom surface or a big equipment in a cleanroom by autoclaves and gamma irradiation. Although gas sterilization can be applied, it is not easy to apply in big cleanrooms and for the inside of equipments. The contact time and the amount of chemical gas sprayed must be validated correctly. In this sense it is time consuming and expensive. The easisest and widely used method for controlling the number of microorganisms in a cleanroom is the disinfection.

2.3.1 Disinfection

By definition, “the disinfection is defined as the treatment of surfaces/equipment using physical or chemical means such that the amount of microorganisms present is reduced to an acceptable level” (9).

Before using a disinfectant in a GMP regulated facility, some requirements must be fulfilled. The efficacy of the disinfectant must be demonstrated on each surface type available by reducing the number of microorganisms to a accepted level. The effective concentration of the disinfectant must be established and used. The disinfectant must be compatible also with the surfaces applied. It must not cause any corrosion or deterioration on the surfaces. It must not leave residues on the surfaces applied to prevent cross-contamination. It must be also compatible with other disinfectants used rotationally. Disinfectant manufacturers perform most of these studies and tests before launching a product into market. Apart from manufacturers each GMP regulated facility must perform its own studies and tests to be able to use effectively and safely the disinfectant he has chosen. These studies and tests are performed using a disinfectant efficacy test protocol. Acceptance criteria in this protocol must be fulfilled. The test protocols are prepared according to EN and ISO standards. The USP chapter <1072>, AOAC and European Standards define how to validate a disinfectant. European Approach (10) is followed in this study. This approach consists of three tests performed separately.

- Basic suspension test
- Quantitative suspension test
- Surface test

2.3.1.1 Basic suspension test

The test uses EN 1275 and EN 1040 standards. “This test evaluates the activity of a disinfectant against a range of microorganisms under conditions which simulate use. After challenging the disinfectant solution with a microbial population the mixture is plated out, after the required contact time, and the surviving microorganisms enumerated.” (10)

2.3.1.2 Quantitative suspension test

This test uses bactericidal suspension test (EN 1276) and fungicidal suspension test (EN 1650). “The purpose of the test is to evaluate the activity of a disinfectant against a range of microorganisms under conditions which more closely simulate practical use. The test consists of adding a test suspension of bacteria or fungi to a prepared sample of the disinfectant under test in simulated ‘clean’ and ‘dirty’ conditions. After a simulated contact time an aliquot is taken and the bactericidal/fungicidal action is immediately neutralized by the addition of a proven neutralizer. Following this, the number of surviving microorganisms in each sample is determined and the reduction in viable counts is calculated and expressed in logarithms to base 10 (10).

2.3.1.3 Surface test

Surface test uses EN 13713, EN 13697 and AOAC Hard surface carrier test method.

“Representative manufacturing room and equipment surface samples of approximately 2x2 cm coupons are inoculated with a selection of microbial challenge microrganisms. The selected disinfectant is applied to the inoculated surfaces and exposed for a predetermined contact time after which the surviving microorganisms are recovered using a qualified disinfectant-neutralizing broth and test method (surface rinse, contact plate or swab). The number of challenge microorganisms recovered from the test samples (exposed to a disinfectant) is compared to the number of challenge microorganisms recovered from the corresponding control sample (not exposed to a disinfectant) to determine the ability of the disinfectant to reduce the microbial bioburden. A 4 log decrease for bacteria and a 3 log decrease for fungi is required. Successful completion of the validation qualifies the disinfectant evaluated for use” (10).

2.3.2 Commissioning cleaning

There is no one and correct way to perform a commissining cleaning. The first step for the commissioning cleaning is to remove all soil and debris from the surfaces of a cleanroom. This is done mechanically using brush or mops and using water or an ordinary detergent. One can repeat this cleaning a couple of times until a visible dirth is not seen. After this first treatment of the cleanroom surfaces, a neutral detergent solution (Klercide^{TM} Neutral Detergent Unit Dose Concentrate) is applied to surfaces to further clear the surface from non-visible dirth and sticky materials. With the application of the neutral disinfectant the surface will be ready for the appliction of the disinfectant. At this stage a bactericidal or sporicidal disinfectant can be used depending on the floral microorganims present and depending of surface types. A sporicidal disinfectant as its name implies is used against bacterial and fungal spores. It is expected to kill all vegetative microorganisms also (11). A bactericidal disinfectant is effective on most bacterial strains. After the sporicidal application rinsing with water is often recommended because of the corrosive effect of the sporicidals on most cleanroom surfaces.

In this study a sporicidal disinfectant (Klercide^{TM} Sporicidal Active Chlorine Unit Dose Concentrate (5000ppm)) is chosen for its broad spectrum activity on bacterial and fungal spores as well as vegetative bacteria.

3 MATERIALS AND METHODS

In this study surface tests and active air sampling is performed to collect and isolate the cleanroom microorganisms both before and after the commissioning cleaning. Although active air samples are not direct indicator of surface cleanliness, they are included in this study, because microorganisms settled down on cleanroom floors may lift off with the movement of personnel and with the air flow. All sampling is done at rest conditions, meaning there was no opeartors working.

3.1 Equipments Used For The Tests

The equipments used are listed in table 2.

Table 2. Equipments used

Equipment	Brand	Model
20-25°C incubator	Thermo Scientific	51031565 – IMP400
30-35°C incubator	Thermo Scientific	51029334 – IGS750
Colony Counter	VWR	STC-100
Air sampler	Millipore	MAS-100 NT® Ex
Identification System	Biomérieux	VITEK2 Compact

3.2 Materials Used For The Test

The materials used are listed in table 3.

Table 3. The materials used

Material	Brand	Catalog no.
TSA	Merck	105458
TSA+LTHTh	Merck	146554
Color Gram 2 (4x240 ML)	Biomérieux	55542
Gram Negative identification card	Biomérieux	21341
Gram Positive identification card	Biomérieux	21342
Bacilli identification card	Biomérieux	21345
DENSICHEK Calibration Standard	Biomérieux	93059
DENSICHEK Plus Standart kits	Biomérieux	21255

Table 3. The materials used (continued)

Material	Brand	Catalog no.
Sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0)	Biomérieux	V1204
12x75 mm clean and clear plastic (polystyrene) disposable test tube	-	-
Sterile inoculating loop or swab stick	Copan	0519C
Klercide™ Neutral Detergent Unit Dose Concentrate	Ecolab	3078810
Klercide™ Sporicidal Active Chlorine Unit Dose Concentrate (5000ppm)	Ecolab	3082590

3.3 Formulations of the Neutral Detergent and Sporicidal Disinfectant

The formulations are given in table 4.

Table 4. Formulation of neutral detergent and sporicidal disinfectant (12, 13)

Material	Content	pH	Sterilization method
KlercideTM Neutral Detergent Unit Dose Concentrate	Deionized water, fatty alcohol ethyloxylate	5.5-7.5	0.2 micron filtered and gamma irradiated at no less than 25 kGy
KlercideTM Sporicidal Active Chlorine Unit Dose Concentrate (5000ppm)	Active ingredient in 100 g is 50 g Troclosene sodium	-	Filled in ISO Class 8 and gamma irradiated at no less than 25 kGy

3.4 Collection of Surface Samples

Surface samples are collected according to facility internal procedure prepared according to USP<1116>. Since the sampled surfaces are flat, only contact plates were used. Swab sticks were not used. Totally 32 cleanrooms are sampled. One floor and one wall sample is taken from each room. Totally 64 surfaces are sampled. Sampling is performed both prior to and after the commissioning cleaning. Before commissioning cleaning the cleanrooms were not classified according to ISO 14644-1. The HVAC units were not functioning also. This means each room has the same environmental conditions as the others. Same type of microorganisms were expected to be isolated across each room.

After the rooms were classified the same surfaces were sampled as before commissionning cleaning.

The sampling technique is to press agar surface onto sampling site about 10 seconds. The property of a contact plate is, it is full of nutrient media so that agar surface can easily touch the sampling surface. 10 second of contact time is required to be able to recover as many microoganisms as possible. The medium used is 55 mm TSA+LTHTh contact plate.

3.5 Collection of Air Samples

Air samples are collected according to facility internal procedure prepared according to USP<1116>. Totally 32 cleanrooms were sampled. One sample from each room was taken. Sampling is performed both prior to and after the commissioning cleaning as in surface sampling.

The samples were taken using Millipore MAS-100 NT® Ex. Total sampling volume for each sample is 1000 L. With the flow rate of 100 L/min. it takes 10 minutes to take a sample. The medium used is 90 mm TSA plate.

3.6 Incubation of the Collected Samples

Incubation of both surface and air samples were performed at the same temperature and for the same duration. The plates were first incubated at 30-35°C for 2 days, the same petri plates were then incubated at 20-25°C for 3 days. The reason the incubation was performed first at high temperature is that, fungal colonies grow best at 20-25°C and may cover whole agar surface making difficult for bacterial count. The incubation was done at aerobic conditions.

After the incubation terminates, the colonies on the agar medium were counted and recorded.

3.7 Number of Rooms for Each ISO Class

ISO Class	No of Rooms Present
B	9
C	17
D	6

4 RESULTS

The test results of the samples both before and after cleaning are given in table 5.

Table 5. Floor surface test results for each cleanroom

Room no.	ISO class	Test results before commissioning cleaning and room classification (cfu/plate)	Test results after commissioning cleaning and room classification (cfu/plate)
1	B	45	0
2	B	28 (4 molds)	1
3	B	82 (2 molds)	0
4	B	23	0
5	B	29	0
6	B	57 (3 molds)	0
7	B	88	0
8	B	61	0
9	B	57 (1 mold)	1
10	C	116 (1 mold)	0
11	C	46 (2 molds)	0
12	C	46 (1 mold)	0
13	C	57 (3 molds)	1
14	C	61	0
15	C	49	0
16	C	53 (3 molds)	2
17	C	41 (1 mold)	0
18	C	59	0
19	C	28 (1 mold)	0
20	C	42 (3 molds)	0
21	C	70 (2 molds)	1
22	C	37	1
23	C	78	0
24	C	46 (1 mold)	0
25	C	41	11
26	C	121 (1 mold)	0
27	D	62 (3 molds)	0
28	D	27	5
29	D	24 (3 molds)	0
30	D	18	0
31	D	37	2
32	D	32 (1 mold)	0
Total		**1661 (36 molds)**	**25 (0 mold)**

Table 6. Wall surface test results for each cleanroom

Room no.	ISO class	Test results before commissioning cleaning and room classification (cfu/plate)	Test results after commissioning cleaning and room classification (cfu/plate)
1	B	7	0
2	B	4	0
3	B	4	0
4	B	0	0
5	B	7 (1 mold)	0
6	B	0	0
7	B	1	0
8	B	7	0
9	B	3 (2 molds)	1
10	C	4	0
11	C	0	0
12	C	2	4
13	C	5 (3 molds)	0
14	C	45	1
15	C	7 (1 mold)	0
16	C	53 (3 molds)	0
17	C	1	0
18	C	4 (1 mold)	0
19	C	1	0
20	C	8 (1 mold)	6
21	C	6 (4 molds)	0
22	C	7	0
23	C	3 (1 mold)	0
24	C	340 (4 molds)	0
25	C	12 (3 molds)	0
26	C	16 (1 mold)	0
27	D	3 (1 mold)	0
28	D	22	7
29	D	6	0
30	D	6 (2 molds)	0
31	D	6 (2 molds)	0
32	D	1	0
Total		**591 (30 molds)**	**19 (0 mold)**

Table 7. Active air test results for each cleanroom

Room no.	ISO class	Test results before commissioning cleaning and room classification (cfu/m^3)	Test results after commissioning cleaning and room classification (cfu/m^3)
1	B	54	0
2	B	61	0
3	B	77	0
4	B	81	0
5	B	96 (6 molds)	0
6	B	101 (2 molds)	0
7	B	110	0
8	B	193	0
9	B	245	0
10	C	35	0
11	C	74 (1 mold)	2
12	C	79 (1 mold)	5
13	C	85 (1 mold)	5
14	C	89 (1 mold)	9
15	C	97 (3 molds)	4
16	C	100	2
17	C	105	2
18	C	105 (1 mold)	4
19	C	110 (1 mold)	0
20	C	123 (3 molds)	14
21	C	125 (1 mold)	9
22	C	127 (2 molds)	2
23	C	136	1
24	C	146	1
25	C	149	3
26	C	160	1
27	D	101	0
28	D	103 (3 molds)	23
29	D	122 (3 molds)	17
30	D	125 (1 mold)	8
31	D	139 (4 molds)	3
32	D	174	5
Total		**3627 (34 molds)**	**120 (0 mold)**

4.1 Identification of Microorganisms

All isolated colonies from the surface and active air tests are classified according to their morphology. One single colony is selected from each morphology to be identified to genus and species level. The selected colonies are subcultured on TSA plates and incubated about 24 hours. Identification is performed on these subcultured colonies. Identification to genus and species level is performed using VITEK® 2 Compact Identification System.

4.1.1 VITEK® 2 compact identification system working principle (15)

VITEK 2 Compact is an automated system used for the identification of microorganisms. It uses growth-based technology. Gram-positive spore forming bacilli, yeast, Gram-positive and gram-negative microoganisms can be identified to genus and species level using this system.

The system uses Gram-positive (GP), gram-negative (GN), Bacillus (BCL) and yeast (YST) cards. These cards have 64 wells that contain an individual test substrate. Substrates measure various metabolic activities of the microorganisms such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. “A transmittance optical system allows interpretation of test reactions using different wavelengths in the visible spectrum. During incubation, each test reaction is read every 15 minutes to measure either turbidity or colored products of substrate metabolism.

Calculations are performed on raw data and compared to thresholds to determine reactions for each test. On the VITEK 2 Compact, test reaction results appear as “+” or “–”. An unknown biopattern is compared to the database of reactions for each taxon, and a numerical probability calculation is performed. Various qualitative levels of identification are assigned based on the numerical probability calculation.” (15)

5 DISCUSSION

In this study we evaluted the effectiveness of the chosen disinfectants for the commissioning cleaning of pharmaceutical cleanrooms. The cleanrooms are ISO Class B, C and D.

Samples taken before commissioning cleaning represent the dirty state of the cleanrooms. The cleanrooms are not classified at this stage. HVAC units are not functioning also. High microbial counts are expected from the samples taken before cleaning.

The test results before cleaning conformed to expectation. High microbial counts were obtained in the surface and air samples.

Samples taken after commissioning cleaning represent the clean state of the cleanrooms. The cleanrooms are classified acoording to ISO-14644. HVAC units are functioning. Low microbial counts are expected from the samples taken after cleaning.

The test results after cleaning conformed to expectation. Low microbial counts were obtained in the surface and air samples.

5.1 Microbial Count Comparison

Microbiological test result comparisons of the floor, wall and air samples before and after cleaning are given in figures 5, 6 and 7.

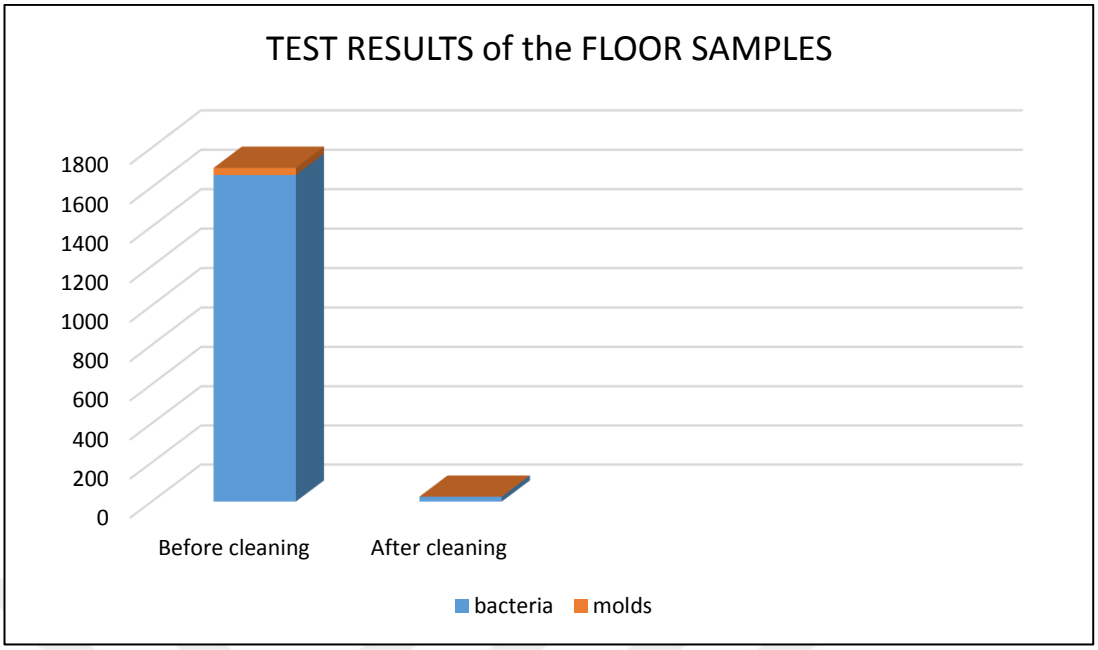

Figure 5. Test results of the floor samples

Total microbial count decreased by 98.5% after cleaning. Molds were completely eliminated.

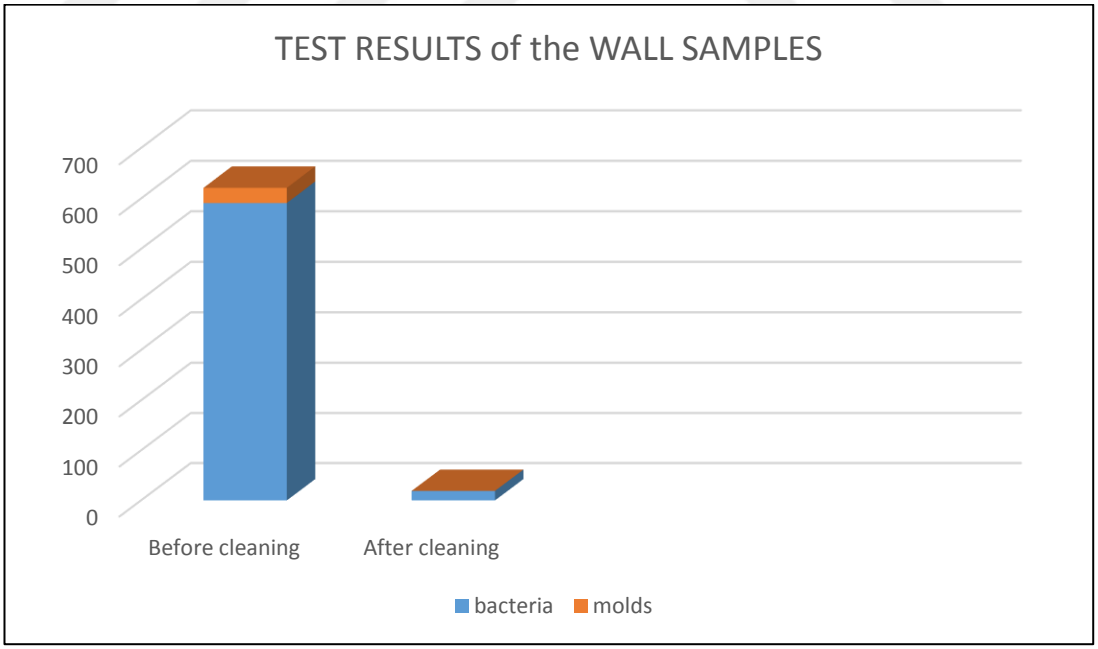

Figure 6. Test results of the wall samples

Total microbial count decreased by 96.8% after cleaning. Molds were completely eliminated.

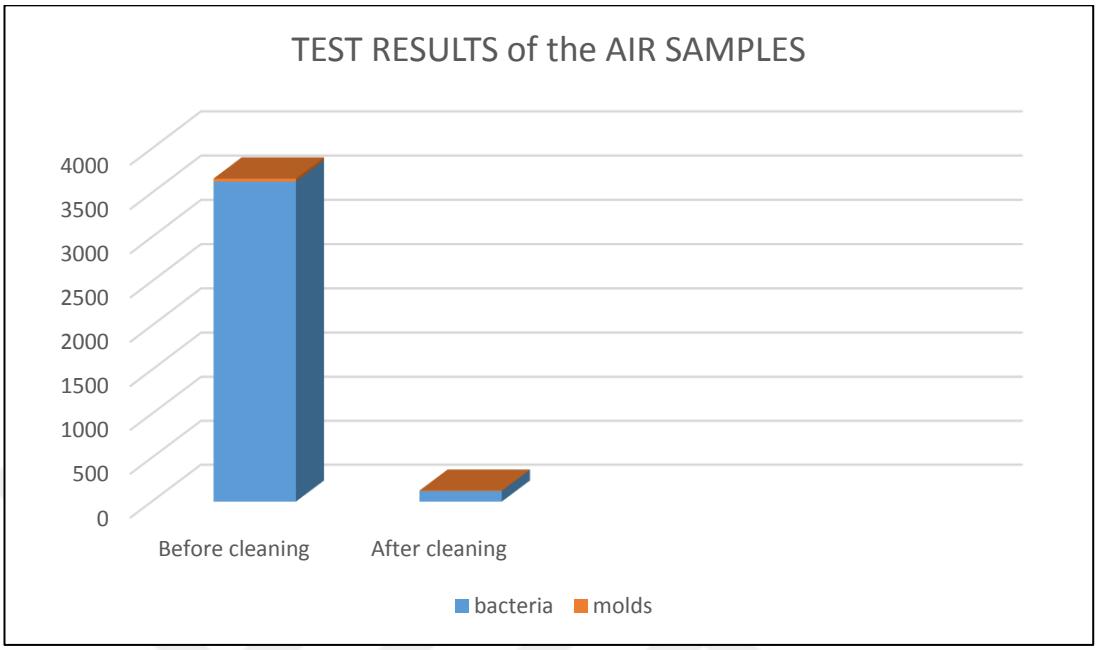

Figure 7. Test results of the air samples

Total microbial count decreased by 96.7% after cleaning. Molds were completely eliminated.

The test results after cleaning indicate that the use of chosen disinfectant (KlercideTM Sporicidal Active Chlorine Unit Dose Concentrate (5000ppm)) provided sufficient and effective disinfection. Both surface and air test results conform to specifications of EU GMP Annex 1: Manufacture of Sterile Medicinal Products (2).

5.2 Comparison of Types of Microbial Colonies

The colonies isolated in the tests pre-commissioning cleaning were identified to genus and species level. The genus and species information of the microorganisms identified are given in table 10.

Table 8. The colonies isolated before cleaning

Gram stain	Genus and species	Source
Gram-negative bacillus	*Rhizobium radiobacter*	Opportunistic human pathogen (16)
Gram-positive cocci	*Micrococcus species*	Normal human flora (17)
Gram-positive spore forming bacillus	*Brevibacillus choshinensis*	Soil (18)
Gram-positive cocci	*Staphylococcus epidermidis*	Normal human flora (17)
Gram-negative bacillus	*Pseudomonas luteola*	Human pathogen (19)
Gram-positive cocci	*Staphylococcus lentus*	Normal human flora (17)
Gram-positive cocci	*Micrococcus luteus*	Normal human flora (17)
Gram-positive cocci	*Leuconostoc mesenteroides ssp. cremoris*	Normal human flora, human pathogen (20)
Gram-positive cocci	*Kocuria species*	Human skin and mucosa (21)
Gram-negative bacillus	*Brevundimonas diminuta / vesicularis*	Human, water, soil (22), (23)
Gram-positive cocci	*Kocuria kristinae*	Human skin and mucosa (21)
Gram-negative bacillus	*Sphingomonas paucimobilis*	Water, soil (24)
Gram-positive cocci	*Staphylococcus hom. hominis*	Normal human flora (17)
Gram-negative bacillus	*Pseudomonas fluorescens*	Moist environments, soil, water, human respiratory pathogen (25)
Gram-positive spore forming bacillus	*Alkalihalobacillus clausii*	Soil organism (26)
Gram-positive cocci	*Staphylococcus haemolyticus*	Normal human flora (17)

The colonies isolated in the tests post-commissioning cleaning were identified to genus and species level. The genus and species information of the microorganisms identified are given in table 11.

Table 9. The colonies isolated after cleaning

Gram stain	Genus and species	Source
Gram-positive cocci	*Staphylococcus epidermidis*	Normal human flora (17)
Gram-positive cocci	*Staphylococcus haemolyticus*	Normal human flora (17)
Gram-positive cocci	*Staphylococcus hominis ssp hominis*	Normal human flora (17)
Gram-positive cocci	*Staphylococcus xylosus*	Normal human flora (17)
Gram-positive cocci	*Staphylococcus gallinarum*	Normal human flora (17)
Gram-negative bacillus	*Pantoea spp*	Human, water, soil (27)
Gram-negative bacillus	*Sphingomonas paucimobilis*	Soil, water (24)
Gram-negative bacillus	*Serratia marcescens*	Human, plumbing system (17)

5.3 Evaluation of the Identified Microorganisms

The microorganisms isolated both before and after the cleaning are mostly human origin. This is consistent, because operators have worked without any cleanroom gown during construction. Water and soil microoganisms are also common, because the rooms were open to atmosphere and environment. The disinfection process reduced the total number of microorganisms greatly as shown in paragrah 5.1. The number of species also reduced to half, from 16 to 8.

Troclosene sodium is a chlorine containing disinfectant and has very strong bactericidal ability (28). Chlorine targets, cell wall, -SH groups, nucleic acids, thiol groups amino groups. It is also considered to be a general oxidant (29).

Human borne microorganisms were mostly identified after cleaning, mainly *Staphylococcus species.* This is consistent, because after the cleanroom classification HVAC units were functioning properly and the main microbial contamination source is the operators.

6 CONCLUSION

In this study, it is shown that the chlorine containing “KlercideTM Sporicidal Active Chlorine Unit Dose Concentrate (5000ppm)” disinfectant is effective on bacteria, bacterial and fungal spores. The number of microorganisms were reduced 98.5% on floor surface samples, 96.8% on wall surface samples and 96.7% on viable air samples. The number of species also reduced to half. Microbiological test results of ISO B, C and D Class cleanrooms after commissioning cleaning conforms to GMP microbiological specifications. Bacterial and fungal spore forming microorgansims were completely eliminated. Since bacterial and fungal spores are resistive to harsh conditions and disinfection, complete elimination of them is crucial for pharmaceutical cleanrooms. These test results indicate a successful commissioning cleaning.

After the commissioning cleaning a routine disinfection program will be implemented using two different types of disinfectants. One is quaternary ammonium compound, the other is a sporicidal low residue peroxide. These two disinfectants will be used rotationally. With the rotaional use of these disinfectants the total number and type of microorgansims will be still under control. Quaternary ammonium compound is a bactericidal and low residue peroxide is a sporicidal. When used rotationally, the number and types of microorganisms will be kept at a safer state.

The reason why “KlercideTM Sporicidal Active Chlorine Unit Dose Concentrate (5000ppm)” is not used in the routine disinfection is, it is corrosive on most surfaces. For this reason a low residue quaternary ammonium compound and a low residue peroxide will be used rotationally.

This study can further be expanded to capture seasonal deviations in the cleanroom flora. In this way a possible drift in plant flora may be detected. If objectionable microorgansims are detected, the use of disinfectants will be re-evaluated.

As identification data of this study indicate control of human borne contamination is essential in a pharmaceutical cleanroom environment. With the proper behaviour training in a cleanroom and proper gowning, most of the human borne microorganisms can be further controlled.

If a negative deviation occurs from normal plant flora, the concentration and use frequency of the disinfectants can be re-evaluated even a new disinfection program can be implemented with the use of new chemical compounds.

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