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Bacterial Isolates from Pharmaceutical Industry Environment and Water System

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ABSTRACT

Bacterial populations inhabiting pharmaceutical environment and water systems were investigated over a 30 days of sampling period. The systems analyzed were different production area grade and different water types including, raw water, treated water, drinking water, purified and Water-for-Injection (WFI). Samples of water were tested by membrane filtration and the samples cultured using R2A agar. Culture based methods and phenotypic identification methods were used to characterize the isolates. The research was undertaken to produce an in-depth study of the microbial load of pharmaceutical grade water systems as well as the environment. The results presented act as a benchmark for industrial and pharmaceutical microbiologists to review comparable systems against, to present a review of the typical cultivable microorganisms recoverable from pharmaceutical water systems and environment. Further susceptibility patterns of these isolates were studied towards clinically significant antibiotics such as meropenem, cloxacillin, amoxicillin, ampicillin, methicillin and cephalosporin. The mean value of antibiotic sensitivity pattern shows that ampicillin was found to be most inert antibiotic as it was ineffective against all isolates, whereas meropenem was found to be most promising antibiotic followed by cephalosporin, methicillin and cloxacillin.

Keywords: Water, Water Systems, Water-For-Injection, Purified Water, Pharmaceutical Manufacturing grade, Bacteria, Sampling types, Beta lactam antibiotics.

INTRODUCTION

Pharma industry provides a lot of job opportunity to the people who reside in rural area as well as in urban area. Besides the consequence of imminent production of chemicals due to pharma industry through air, water and ground lifelines become questionable. The frequent monitoring of microbial life around the industry is necessary one and it will reveal the condition of environment.

They act as early warning sensors to detect pollution level. **The pharmaceutical industry is now facing new challenges in controlling and preventing environmental pollution as it is expanding.** In various parts of the world, the relationship between the pharma industry and the destiny of environment has been a controversial one. Environmental monitoring describes the microbiological testing undertaken in order to detect changing trends of microbial counts and microflora growth within clean room or controlled environments [1].

Microorganisms regarded as an important bioresource of our environment because they can be obtained in large quantities using cultural techniques within a shortest possible time by established fermentation methods, and they produce a regular and abundant supply of the desired product. Because of the presence of microbes in all walks of human life, there is constant interaction between microbes and humans. The vast majority of the bacteria in the body are rendered harmless by the protective effects of the immune system, and a few are beneficial. In fact, the relationship between microbes and humans is delicate and complex.

Microorganisms found in the manufacturing environment, water for pharmaceutical use, raw materials and ingredients, intermediates, and finished products are frequently identified to assist in product investigations [2]. The value of the data from an environmental monitoring program is greatly reduced if the microorganisms isolated are not characterized to some degree. Identification of isolates is an essential part of understanding the microbial ecology of a manufacturing facility, monitoring the effectiveness of microbiological control in aseptic environments and investigating of normal microbial populations or sterility failures.

Routine investigation might include characterization by colony and cellular morphology, gram reaction, and key enzyme activities. This information may be sufficient to confirm that the bacteria found in the sample are typical for that material or manufacturing area or to indicate the effectiveness of environmental control in an aseptic process [2].

The consumption of antibiotics gives red alarming in the human therapy has been reported [3]. The extensive usage of antibiotics in both human and animal medicine has resulted in the development of antibiotic-resistant bacteria which affect the treatment of infections [4]. Antibiotic resistance has therefore become a major public health issue [5] and its presence in waste water, surface water, and drinking water is well documented. The hazard associated with the pathogenicity of microbes is aggravated by its ability to resist destruction by antibiotics [6]. This study shows the importance of properly designed sampling technique, identification tools, careful interpretation and analysis of the results obtained in order to discover sources of contaminations in controlled environment to make immediate actions to prevent spreading of contaminant which may finally influence the microbiological quality of the final products which may lead to sever financial losses for pharmaceutical company. Besides, it also provides a new approach for detecting source of fault in controlled environment.

MATERIALS AND METHODS

Microorganisms are present in a variety of milieux in the pharmaceutical manufacturing environment. The first step to identification is to isolate a pure colony for analysis. This purification is normally accomplished by sub culturing one or more times on solid media to ensure purity, each time streaking for single colony. This technique also allows full phenotypic expression and growth of sufficient inoculums for the identification.

Isolation of bacteria from adverse environment

The sample for the present study was collected from a pharmaceutical industry, Orchid Chemicals and Pharmaceuticals Pvt Ltd. is a leading Pharma industry located in SIPCOT, Alathur, Chennai, Tamilnadu, India.

They manufacture almost all pharmaceutical preparation like Injection, Ointments, Syrups and Tablets etc. The Company has a well-established Microbiology Department and they perform different tests pertaining to GMP of medicine before moving to the market.

Sampling locations

Samples were taken from representative different classes and locations in pharmaceutical facility including:

1-Water system:

2- Class 100 (The highest grade of cleanliness)

3-Class 10000 (High grade of cleanliness)

4-Class 100000 (Intermediate measure of cleanliness)

Specimen collection

Water sampling [7]

The whole process of water sampling was performed under strict aseptic conditions. Water samples were collected in sterile polypropylene sample containers with leak proof lids. When collecting the sample, enough air space was left in the bottle to allow for proper mixing before examination. Sample ports were flushed and disinfected with 70% IPA (Iso Prophyl Alchocol) to avoid sample contamination. Before collecting the sample 5 to 10 liters of water drained out as pere USP. The sample bottle was kept closed until it had to be filled. Cap was removed carefully and was replaced immediately. The volume of sample was collected to be sufficient to carry out all tests required (not less than 100 ml).

Active air sampling [8]

Air sampler was placed in the center of each room at a height of approximately 1meter above the floor. Before sampling the instrument was disinfected with 70% IPA. One thousand liters of air sampling were obtained by Merck air sampler. The TCA plates were incubated for 48 hours at 30ºC - 35ºC and then for 72 hours at 20ºC - 25ºC. After incubation, the colonies were counted and recorded.

- Active air sampling locations in class 100 area-garment changing airlocks, near reactors, near sampling table and sterilized material unloading room.
- Active air sampling locations in class 10000 area-media preparation and storage room, sterilization room, Water testing room, and incubator room.
- Active air sampling locations in class 10000 area- used media discarding room, corridor, and garment washing room.

Surface monitoring

I-Contact plate method [9]

1-Surface sampling was performed with raised RODAC plates. The tryptone soya agar in contact plates was mixed with neutralizers (Tween 80 and lecithin), which inactivate many residual disinfectants.

2-During sampling, a contact plate was pressed onto the area to be tested. Any microorganisms on the surface of the area tested (which should ideally be flat) were transferred onto the contact plate.

3-After the sample had been obtained; the area tested had been wiped down with isopropyl alcohol 70 % to remove any residue left by the contact plate. The plates were incubated under upside down condition with two different temperature condition such as for 48 hours at 30ºC - 35ºC and then for 72 hours at 20ºC - 25ºC.

- Surface monitoring locations at class 100 area-sampling scoop, aluminium containers, surface of reactors and air curtains
- Surface monitoring locations at class 10000 area-garment cabinet, air locks cross over bench, corridor wall
- Surface monitoring locations at class 100000 area- meduia discarding room wall, autoclave jacket wall, waste material storage room

II-Swabbing method [10]

1-Swab samples were collected by a sterile swab, moistening it by inserting it into a second tube which contained a sponge soaked with sterile 1.5 ml of phosphate buffered saline (PBS) at pH 7.2. The sampling area was wiped with swab with a radiated fashion. After sampling, the swab was streaked on SCDA plates and the plates were incubated for 48 hours at 30ºC - 35ºC and then for 72 hours at 20ºC - 25ºC.

- Swabbing locations at class 100 area- cross over bench, electrical panels, view glass, valves and knobs and unloading port of the reactors.
- Swabbing locations at class 100000 area- inner surface of the discarding autoclave, waste material discarding drum and wash basin of the discarding room.

Identification using BBL CRYSTAL identification systems

The tests used in the BBL CRYSTAL E/NF identification system are based on microbial utilization and degradation of specific substrates detected by various indicator systems. **Gram-positive ID kit** includes tests for fermentation, oxidation, degradation and hydrolysis of various substrates. Chromogenic substrates upon hydrolysis produce color changes that can be detected visually. Identification is derived from a comparative analysis of the reaction pattern of the test isolate to those held in the data base [11]. Prior to BBL CRYSTAL

E/NF panel set-up, Oxidase and Indole tests should be performed from a nonselective isolation plate no more than 24 hours old.

Identification: Identification can be obtained by using the analytical profile index. The pattern of the reactions obtained must be coded into a numerical profile.

Characterization of bacterial isolates

The first step is to determine the G**ram reaction** and cellular morphology of the bacteria isolates. This is a critical step for phenotypic identification schemes.

The biochemical studies for the identification of isolate were performed by different tests such as Catalase test, Oxidase test, Methyl red (MR) test, Voges-Proskauer test, Citrate utilization test, Fermentation of carbohydrate test according to the methods described in manual of methods for general bacteriology [12].

Inoculums Preparation for antibiotic sensitivity

Eight bacterial isolates were sub cultured on non-selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted using normal saline (0.85% NaCl) using Spectrophotometer to bacterial population of 1.0 x 108 cfu/ml.

Antibiotic Susceptibility Test

An antibiotic susceptibility test was performed using the Kirby-Bauer disk diffusion method [13]. The following antibiotic discs at the final concentrations that are indicated were used: ampicillin (AP) 10 μg, cephalosporin (KF) 5 μg, chloramphenicol (C) 30 μg, amoxycillin (A) 10 μg, and meropenem (MP) 10 μg. These antibiotics were chosen because they are either used in both human medicine and animal veterinary practice. Besides most of the gram positive bacteria are sensitive to these beta lactom antibiotics [14]. All these antibiotics were obtained from local pharmacy store and working solution having 10mg/ml concentration of each antibiotic was used for the study.

Three colonies were picked from each sample and each colony was transferred in to 3mL of sterile distilled water to prepare bacterial suspension. Aliquots of 100 μL from each suspension were spread-plated on Mueller-Hinton agar plates. Antibiotic discs were applied on to the plates using sterile needles and the plates were incubated at 37^{o}C for 24 hours. After incubation, the antibiotic inhibition zone diameters were measured. Results were

observed and the sensitive isolates were selected using standard reference values of National Committee for Clinical Laboratory Standards [15].

RESULTS

Result of samples taken from different area in pharmaceutical industry:

1-**Water system**: All the water samples were collected by daily basis for 30 days and the results were recorded and shown in table 2. But the number sampling points are varied with the type of water. It is ranging from 2 to 98. Raw water samples were taken from the water station while the treated water from the corresponding water treated plant. Drinking water samples were taken from the canteen and the pantries. Both the purified water and the WFI water sample were taken from the sterile and non sterile area of our facility. The total numbers of raw water samples were 60 samples among which all samples (100%) showed positive growth none of the samples showed no growth whereas the WFI water samples were showed very minimum (<1%) positive growth. However, TW, DW, and PW were observed positive growth as 40.3%, 26.35% and 21.2% respectively. The number of CFU in PW and WFI samples were recorded as per ml of samples but the rest of the samples were recorded as 100 ml of samples.

Table 2: Isolates from different type water samples for 30 days

S No.	Types of water samples	No. of sampling points	Total No. of samples	Inhouse Declared limit of CFU	Positive samples		Negative samples	
					No.	%	No.	%
1.	Raw water	02	02×30	<500/100ml	60	100	-	-
2.	Treated water	10	10×30	<500/100ml	121	40.3	179	59.7
3.	Drinking water	09	09×30	<500/100ml	71	26.3	209	73.7
4.	Purified water	98	98×30	80-100/ml	623	21.2	2317	78.8
5.	WFI	57	57×30	5-10/ml	1710	<1	-	-

2- **Class 100** (The highest grade of cleanliness) **Active air sampling, Surface monitoring and Swabbing method** Samples were taken from both microbiology laboratory and production area in facility by following appropriate techniques**.** Results were shown in table 3. All samples of class 100 showed no growth.

3-**Class 10000** (High grade of cleanliness) **Active air sampling and Surface monitoring**

All samples were taken using contact plates from production area and microbiology laboratory in facility. In class 10000 area the swabbing method was not practiced. In classes 10000 area by air sampling (178 samples) and by surface monitoring (227 samples) showed positive growth. Out of 390 active air samples of class 10000 area 212 samples (54.4%) and 450 surface samples of class10000 area (49.6%) showed no growth. Results were shown in Table (4).

4-**Class 100000** (Intermediate measure of cleanliness) **Active air sampling, Surface monitoring and swabbing method**

Table 3: Isolates from class 100 area (Days of sampling 30)

S No.	Types of sampling	Total No. of samples	Inhouse Declared limit of CFU	Positive samples		Negative samples	
				No.	%	No.	%
1.	Active air sampling	59x30=1780	<l	00	00	1780	100
2.	Surface monitoring	08x30=240	<1	00	00	240	100
3.	Swabbing	02x30=60	<1	00	00	60	100

Table 4: Isolates from class 10000

S No.	Types of sampling	Total No. of samples	Inhouse Declared limit of CFU	Positive samples		Negative samples	
				No.	%	No.	%
1.	Active air sampling	13x30=390	10-60	178	45.6	212	54.4
2.	Surface monitoring	15x30=450	10-40	227	50.4	223	49.6

Table 5: Isolates from class 100000

S No.	Types of sampling	Total number of samples	Inhouse Declared limit of CFU	Positive samples		Negative samples	
				No.	%	No.	%
1.	Active air sampling	18x30=540	<500	540	100	00	00
2.	Surface monitoring	22x30=660	<100	660	100	00	00
3.	Swabbing	41x30=1230	<200	1230	100	00	00

All the samples from classes 100000 area showed positive growth (Table 5). None of the samples showed negative growth.

Distribution of identified microorganisms from the positive samples of environmental monitoring specimens

There are 16 bacterial species (Table1) were isolated from various facilities of pharmaceutical industry. Among which the most predominantly isolated species is M*icrococcus luteus* and the rare or less commonly isolated species were *Kocuria rosea, Staphylococcus epidermidis, Staphylococcus auricularis, Staphylococcus warneri, Helococcus kunzii* and *Streptococcus vestibularis.*

There were two gram positive bacilli isolates and the rest were gram positive cocci but all the isolates showed positive Catalase and Oxidase test. However, none of the gram negative organism was isolated.

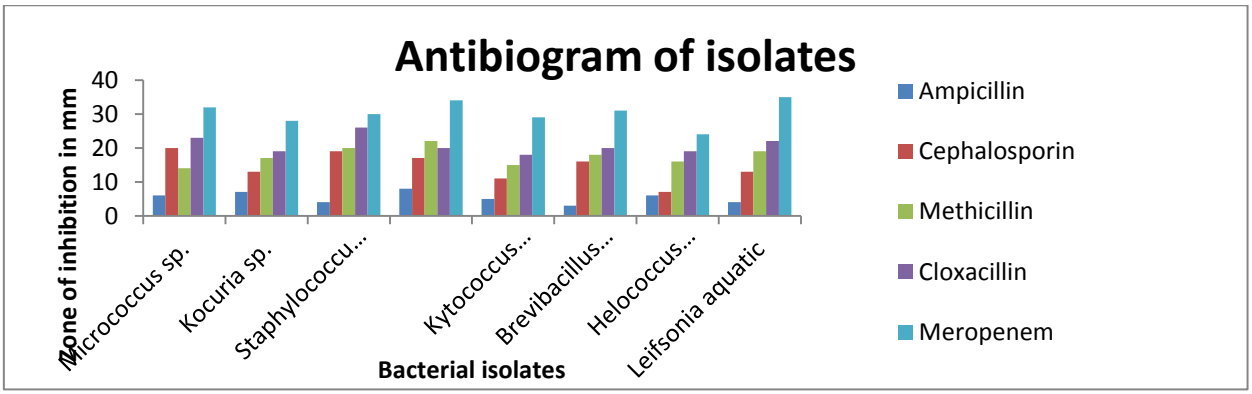
Table1: Identification of bacterial isolates by BBL CRYSTAL system

S. NO.	ORGANISMS	COLONY MORPHOLOGY	GRAM REACTIONS	BIOCHEMICAL TEST RESULT	ORIGION OF ORGANISM ISOALTION	AREA
1.	*Micrococcus luteus* 	Circular, convex, yellow, entire, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Surface monitoring	Class 10000
2.	*Micrococcus lylae* 	Circular, convex, yellow, entire, mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Surface monitoring	Class 10000
3.	*Kocuria rosea*	Circular, convex, yellow, opaque and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Surface monitoring	Class 10000
4.	*Kocuria kristinae*	Circular, convex, dull, yellow, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Swabbing	Class 100000
5.	*Staphylococcus capitis*	Circular, flat, white entire, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Swabbing	Class 10000
6.	*Staphylococcus hominis*	Circular, convex, white, entire smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Swabbing	Class 100000
7.	*Staphylococcus saprophyticus*	Circular, convex, yellow, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Swabbing	Class 100000

8.	*Staphylococcus epidermidis*	Circular, flat, white, opaque and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Swabbing	Class 10000
9.	*Staphylococcus auricularis*	Circular, convex, dull, white, opaque and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Swabbing	Class 10000
10.	*Staphylococcus cohnii ssp cohnii*	Circular, convex, white, opaque and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Water	Raw water
11.	*Kytococcus sedentarius*	Circular, convex, pale yellow, entire, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Water	Drinking water
12.	*Brevibacillus brevis*	Irregular, flat,creamy brown,butyrus, smooth and mucoid	G+ve bacilli	Catalase +ve Oxidase +ve	Air sampling	Class 10000
13.	*Staphylococcus warneri*	Circular, convex, grayish white, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Air sampling	Class 100000
14.	*Helococcus kunzii* 	Pinpointed, convex,grey, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Air sampling	Class 10000
15.	*Streptococcus vestibularis*	Circular, convex, whitish yellow, entire, smooth and mucoid	G+ve cocci	Catalase +ve Oxidase +ve	Surface monitoring	Class 100000
16.	*Leifsonia aquatica* 	Circular,convex,yellow, butyrous, opaque and mucoid	G+ve bacilli	Catalase +ve Oxidase +ve	Water	Raw water

Table 6. Antibiotic resistance patterns of different pathogens

Organisms	Zone of inhibition[a]				
	Ampicillin	Cephalosporin	Methicillin	Cloxacillin	Meropenem
Micrococcus sp.	06	20	14	23	32
Kocuria sp.	07	13	17	19	28
Staphylococcus sp.	04	19	20	26	30
Streptococcus vestibularis	08	17	22	20	34
Kytococcus sedentarius	05	11	15	18	29
Brevibacillus brevis	03	16	18	20	31
Helococcus kunzii	06	07	16	19	24
Leifsonia aquatic	04	13	19	22	35
Mean value	**5.4**	**14.5**	**17.6**	**20.9**	**30.4**



a-Zone of inhibition in mm; Values are average of replicates

Figure 1. Sensitivity pattern of bacterial isolates against antibiotics

The antibiotic resistance patterns in terms of average zones of diameter considering duplicate plates for bacterial isolates against each of five antibiotics of 10mg/ml concentration were calculated and shown in table 6. Eight genera of isolates were subjected to an antibiotic susceptibility test using 5 different antibiotics from which their antibiotic sensitive profiles and their phenotypes were compiled. The results obtained are depicted in figure 1. The results revealed that a large proportion of the environmental isolates were resistant to ampicillin, followed by cephalosporin, methicillin and cloxacillin. None of the isolates were resistant to meropenem.

DISCUSSION

Maintaining the integrity of a clean room is a constant battle [16]. There are 3 prime sources of contamination. The first is from human errors. To control this source of contamination, human hands must be washed with disinfectant. 70% IPA is the widely used skin disinfectant because of its mild nature. Contamination may also result from the room surface areas. To avoid such contamination, floors, walls and ceilings must be swept with disinfectants. The third contamination source is from the room air. UV irradiation is the most convenient way to sterilize room air although it is advised the fumigation with suitable disinfectant periodically reduce and limit the microbial load in the production area at pharmaceutical industry [17].

Reduction in the number of bacteria in the treated water could be due to the treatment process, when comparing drinking water to raw water. However, occurrence of bacteria in the water after treatment could also harbour potential pathogens and the health risk caused by these should be taken into consideration when water is distributed. This is of particular importance when the drinking water abstraction and purification facility are at a relatively short distance from the sewage treatment and effluent disposal facility. In Mafikeng, the latter is the case.

To date there are several techniques such as MALDI-TOF MS or ribotyping that seem to be the attractive technologies of rapid microbial identification. The absence of sample preparation, coupled with rapid analysis and high throughput make them indispensable for clinical investigations where precise identification affect diagnosis and treatment options. The ability of MALDI-TOF MS to identify bacteria to the species level in pure cultures and simple microbial mixtures has been established. Besides, this method is free from restrictions related to conditions of microbial cultivation [18].

These results are in agreement with the findings of other workers [19]. It was found that *Staphylococcus* and spore-forming *Bacillus* were more resistant to UV than the other vegetative bacteria [20, 21].

To ensure a clean room conforming to the designated classification, constant monitoring of contaminant sources and identification of the predominant contaminant bacteria is usually necessary [22]. This study found that the predominant contaminant bacteria were a group of Gram positive bacteria: either spore-forming *Bacillus*, or nonsporulating *Staphylococcus* and *Micrococcus*.

This study found that 8 bacterial genus identified isolates were Gram positive bacteria, either spore-forming *Brevibacillus*, which is known to confer resistance to extreme environmental conditions, or non-sporulating *Staphylococcus* and *Micrococcus*, which have a thick cell wall and the rest of the bacterial genus also. The thick wall of a cell or spore is a reasonable explanation for resistance to UV irradiation because this kind of non-ionizing radiation penetrates weakly. However, to 70% IPA and hydrogen peroxide solutions, the cell wall could not be a reasonable explanation for retarding disinfectant entrance.

A further objective of this study was to characterise the isolates using their antibiotic sensitive profiles. The results revealed that a large proportion of the environmental isolates were resistant to ampicillin followed by cephalosporin and methicilli. The trend was in accordance with earlier studies that showed resistance towards β-lactam antibiotics [23]. All these results could be attributed to the overuse of these antibiotics in the clinical and veterinary setting. All isolates were found to be susceptible to meropenem in line with an earlier observation [24].

CONCLUSION

This paper, in reviewing data from three inter-connected pharmaceutical water systems (raw water, treated water, drinking water, purified and Water-for-Injection) and different pharmaceutical environment grade has established a benchmark of the typical microorganisms that can be isolated and recovered on culture media. Whilst is recognized that each facility and geographical locale will differ; the types of organisms recovered bear some similarity to earlier reviews of industrial and laboratory water. Therefore, the results compiled provide empirical support for some of the more theoretical discussions about the microbial ecology of pharmaceutical grade environment and water. The study revealed that the most common isolates are M*icrococcus luteus* and other gram positive cocci. Tracking and trending of bacterial load is an important part of pharmaceutical microbiology, providing the basis for evaluating microbiological risks on products and environments, and this paper, through its long-term historical review, is designed to help with this review process. The fourth generation antibiotic i.e. Meropenem is found to have significant efficacy and can be considered appropriate for empirical treatment of above eight bacterial genera. Although the present study can lead to beneficially assistance in the identification and to control these bacterial strains in pharmaceutical production environment as well an in the water system.

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