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Microbial quality evaluation of two pharmaceutical companies in Kano State, Nigeria

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Abstract
Environmental monitoring is one of the systems that helps determine the quality of product in the manufacture of pharmaceuticals in a pharmaceutical industry. This research was therefore carried out to evaluate the microbial quality of air, equipment and personnel in two pharmaceutical plants coded as Plant A and Plant B in Kano state, Nigeria. Parameters such as aerobic mesophilic bacterial and fungal count, and identification, were carried out. Data obtained from the study showed that some of the sampled air exceeds the limit of European Union (EU) and World Health Organization Good Manufacturing Practice (WHO GMP) with <100cfu/4hrs especially for Plant B. Meanwhile, the most prominent organism isolated from air was *Micrococcus luteus*. Similarly, some swab samples from personnel and equipment did exceed the permissible microbial limit with 198 CFU/25 cm^2 and 156 CFU/ cm^2 being the highest respectively. Organism isolated from these samples includes *M. luteus, Klebsiella* sp, *Citobacter* sp, *Providencia* sp and *Erwinea* (*Pectobacterium*). The *Citobacter* sp was isolated from the hands of personnel who manually fill methylated spirit and from the jug used in the course. Isolated fungi from this research were *Aspergillus niger, A. amstelodami, Penicillium spinulosum* and *Saccharomyces cerevisiae.* The results show that while the microbial limit for equipment are still within permissible levels, there is a need to improve on personnel hygiene and air quality in the plant.

Keywords: Environmental monitoring, Pharmaceuticals, Microbial quality, EU, WHO GMP, Swab sample.

INTRODUCTION

The microbial quality of pharmaceutical products primarily depends on the quality of raw materials, production process, production environment, hygiene of the personnel involved in manufacture and the storage conditions [1]. Thus, environmental monitoring is one of the systems that decide about the quality of product in the manufacture of pharmaceuticals in a pharmaceutical industry [2]. Microorganisms can arise in a manufacturing area from the atmosphere, equipment and work surfaces, personnel, water and from raw materials and their packaging. It is important to recognize that whilst each of these represents a potential source of contamination for a manufactured medicine, two of them, air and water, assume particular importance because they are also vectors, which facilitate movement of organisms from one place to another [3].

Microbiological monitoring of air quality is a key tool to determine the appropriateness of manufacturing area for microbiologically safe medicinal products production. Since aerial microbial

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distributions are highly dynamic and show significant variability from time to time depending on several influential variables, a monitoring system was adopted in order to establish control on trends, major sources of risks and defects [4]. In many human activities microorganisms in the environment represent a hidden but dangerous risk factor. Concern has increased with the introduction of advanced technologies in hospitals, industry and agriculture. In recent years, many studies have been carried out on related topic, and nowadays the evaluation of the level of air microbial contamination in places at risk is considered to be a basic step toward prevention [5]. Major ecological sources of microbial pollution that originates from aqueous, surface and air can act as reservoirs for viable particles and assumed to have crucial role as vehicles for transmission of infection [6]. Viable microorganisms are measured by methods including active and passive air sampling [7].

Microorganisms in the air are usually attached to dust particles, which, in a pharmaceutical factory, usually consist largely of skin flakes shed from the manufacturing personnel [3]. Microorganisms may be expected on most solid surfaces in a manufacturing unit, so it is common to monitor contamination on walls, floors, bench tops and equipment [3]. In addition to shedding skin scales into the air, operators in a manufacturing unit can introduce contamination into the product directly from their skin or from their nose in exhaled air. Skin contaminants are commonly *Micrococci*, *Staphylococci*, *Corynebacteria* (also called *Diphtheroids*) and (including the organisms associated with acne) [3]. As a result, non-sterile pharmaceuticals are not produced by aseptic processes and for this reason, not expected to be totally free from microbial contaminations, which can lead to significant economic loss to the industry as well as morbidity and mortality of the consumers [8,9]. Hence, the aim of the present research is to assess the microbial quality of the working environment: air, personnel and equipment in two pharmaceutical plants in Kano State.

EXPERIMENTAL

Sampling site. Samples of personnel, equipment and air were randomly sourced from two different pharmaceutical plants located in Kano State, Nigeria. The two plants were coded as Plants A and B.

Sample collection and size. Samples were collected in three (3) batches with two weeks interval for each plant. Samples obtained from the same plant on different days or batches were considered different samples. Therefore, a total number of 18 samples were collected from the two industries.

Study site and processing. All samples collected were transported to Microbiology Laboratory, Bayero University, Kano and analyzed within hours of collection. The samples were obtained between September and October 2016. Swab method for personnel and equipment, and settle plant method for air quality were used for enumeration; and isolation and identification was done for detection of pathogenic organisms.

Enumeration of bacteria and fungi. Aerobic mesophilic bacterial and fungal count was done using settle plate method for air, while swab technique was employed for personnel and equipment. Open Petri dishes containing Nutrient agar and Potato dextrose agar of about 20 ml were distributed in different point of the industry and exposed for 15-30 minutes. The Petri dishes were closed, transported to laboratory and incubated appropriately. This was done in accordance to [5,10,11] with modification. Meanwhile the swab samples which were sourced from equipment and personnel palm in working section of the plant were moistened in 0.9%

normal saline (NaCl solution) and transported to the laboratory for enumeration and further analyses [10].

Identification of bacterial isolates. Various selective media such as MacConkey agar, Mannitol salt agar, *Salmonella-Shigella* agar, and Eosine methylene blue agar were used. Swab samples were directly placed on them and Open Petri dishes containing this media were exposed for 15-30 minutes, closed, transported and incubated as employed in enumeration. The biochemical tests conducted include catalase, coagulase, methyl red (MR), Voges-Proskauer (VP), indole, triple sugar iron (TSI), citrate utilization [12].

Identification of fungi. Where possible, fungi were identified to species level directly from colonies on PDA media using well established techniques of macroscopic and microscopic examination and standard reference works for the identification of moulds using lactophenol blue stain. A portion of the obtained culture was placed and teased out into a clean glass slide upon a drop of lactophenol cotton blue using sterile inoculating needles and covered with clean cover slip [13]. The light microscope depended on studying the morphological characteristic and microscopic characteristic, which was compared to the mycological atlas for Confirmatory identification such as [14, 15].

Statistical analysis. The statistical analysis was done with PC using GraphPad InStat – [DATASET1.ISD], Version 3.05 (2000). Student's t-test statistics was used for comparing the geometric means of the bacterial and fungal counts in the two plants.

RESULTS

Table 1 shows the Aerobic Mesophilic Bacterial and Fungal count present in examined personnel, air and equipment. The highest microbial air count was 164 CFU/4hrs for plant B and the lowest 16 CFU/4hrs for Plant A. Some swab sample from personnel and equipment exceed the permissible limit with 198 CFU/25cm^2 and 156 CFU/ cm^2 being the highest respectively. A comparison of the two plants using data obtained showed no significant difference ($p>0.05$), however, Aerobic Mesophilic Fungal count of personnel swab sample differed significantly ($p<0.05$).

Table 2 reveals the characteristics of microbial growth on agar plate for all the samples. Table 3 reveals the biochemical characteristics of the isolated microorganisms. Four organisms were isolated from Plant A, *Micrococcus luteus* from air and personnel sample*; Klebsiella* sp and *Providencia* sp from personnel; *Salmonella* sp from air; and *Citrobacter* sp from hand of personnel and equipment during manual filling of methylated spirit. Three identified organisms were isolated from Plant B, which include: *Providencia* sp isolated from raw water*; Erwinea (Pectobacterium)* and *Klebsiella* sp from personnel*; Micrococcus luteus* from air in all the three batches; and one unknown (unidentified) bacteria from air was isolated as well from Plant B. Bacteria such as *Staphylococcus aureus*, *P. aeruginosa*, *Shigella* sp., and *Candida albicans* were not be detected in any of the tested samples. Table 4 shows the isolated fungi from PDA which include: *Aspergillus amstelodami, A. niger, Penicillium spinulosum* and *Saccharomyces cerevisiae.*

DISCUSSION

The current research indicate that all sampled air quality of Plant A (16 CFU/ 4hrs, 66 CFU/4hrs for lowest and highest count respectively) fell within the range of [16] and WHO GMP which is 50 – 100 CFU/4hrs for grade c and d. But only the 3rd batch of Plant B is within the range and the first two batch samples exceed the limit. This is due to the fact that the airborne microbial concentration is correlated with suspended particulate

matter sized 5-7 μm, [17], human activity, number of people in a space, and apparel worn by personnel in working area [18]. The frequencies with which people enter and exit specific area also increase the number of microorganisms in indoor environments [19]. Airborne droplets usually harbour microorganisms as Gram-positive cocci and gram-negative rods, whose presence is considered objectionable in pharmaceutical products [4]. Indoor air contamination is linked with inappropriate environmental control measures of the buildings, including materials-of-construction, heating, ventilation and air conditioning (HVAC), and the other sources are related to human-being , such as inappropriate behaviour and numbers of people in constrained spaces[4, 17, 20].

Table 1: Aerobic mesophilic bacterial and fungal count for the three batches of samples

Para meter	Sample	Plant	Mean of 1st batch	Mean of 2nd batch	Mean of 3rd batch	Limit for Viable Particles
AMBC	Personnel (CFU/hand)	A	16	14	198	25 – 50 CFU/25cm^2
		B	14	56	68	
	Air (CFU/4hrs)	A	20	16	66	50 – 100 (EU & WHO GMP)
		B	154	164	42	
	Equipment (CFU/plate)	A	12	5	156	25 – 50 CFU/25cm^2
		B	26	56	72	
AMFC	Personnel (CFU/hand)	A	5	7	7	25 – 50 CFU/25cm^2
		B	12	16	24	
	Air (CFU/4hrs)	A	7	6	22	50 – 100 (EU & WHO GMP)
		B	20	22	15	
	Equipment (CFU/plate)	A	4	11	9	25 – 50 CFU/25cm^2
		B	10	8	13	

Key: AMBC = Aerobic Mesophilic Bacterial Count; AMFC = Aerobic Mesophilic Fungi Count

Table 2: Characteristics of microbial growth on agar plate

Sample	Plant	MSA	SSA	EMB	MAC
$Personnel_1$	A	Yellow colony	NG	NG	Colourless colony
$Personnel_1$	B	NG	Pink to red colony	Pink colony	Light pink colony
Air_1	A	Yellow colony	NG	NG	Colourless colony
Air_1	B	Yellow colony	NG	Colourless colony with purple centre	Colourless colony
$Equipment_1$	A	NG	NG	NG	NG
$Equipment_1$	B	NG	NG	NG	NG
$Personnel_2$	A	NG	Colourless colony	Pink colony, Colourless colony	Pink colony
$Personnel_2$	B	NG	NG	NG	NG
Air_2	A	NG	Colourless colony	Colourless colony	Colourless colony
Air_2	B	Yellow colony	NG	NG	Colourless colony
$Equipment_2$	A	NG	NG	NG	NG
$Equipment_2$	B	NG	NG	NG	NG
$Personnel_3$	A	NG	NG	Greenish metallic sheen	Red colony
$Personnel_3$	B	NG	NG	NG	NG
Air_3	A	NG	NG	NG	NG
Air_3	B	Yellow colony	NG	NG	Colourless colony
$Equipment_3$	A	NG	NG	Greenish metallic sheen	Red colony
$Equipment_3$	B	NG	NG	NG	NG

MSA = Mannitol Salt Agar; SSA = *Salmonella – Shigella* Agar; EMB = Eosine Methylene Blue Agar; MAC = MacConkey Agar; NG = No Growth, the subscript indicate the batch of sample

Table 3: Biochemical Characteristics for all the three batches of samples

Sample	Plant	GS	I	M	VP	C	TSI S	TSI B	TSI G	H_2S	Cat	Coag	Inference
P_1	A	+	–	NA	–	+	R	R	–	–	+	–	*Micrococcus luteus*
	B	–	+	+	+	+	Y	Y	+	–	+	NA	*Erwinea (Pectobacterium*)
		–	+	+	–	+	Y	Y	+	–	+	NA	*Klebsiella* sp
A_1	A	+	–	NA	–	+	R	R	–	–	+	–	*M. luteus*
	B	+	–	NA	–	+	R	R	–	–	+	–	*M. luteus*
		–	+	+	–	–	R	R	–	–	+	NA	Unknown
P_2	A	–	+	+	–	+	Y	Y	+	–	+	NA	*Klebsiella* sp
		–	+	+	–	+	R	Y	+	+	+	NA	*Providencia* sp
A_2	A	–	–	+	–	+	R	Y	–	–	+	NA	*Salmonella* sp
	B	+	–	NA	–	+	R	R	–	–	+	–	*M. luteus*
P_3	A	–	+	+	–	+	R	Y	+	–	+	NA	*Citobacter* sp
A_3	B	+	–	NA	–	+	R	R	–	–	+	–	*M. luteus*
E_3	A	–	+	+	–	+	R	Y	+	–	+	NA	*Citobacter* sp

P = Personnel; A = Air; E = Equipment (the subscript indicate the batch of sample); I = Indole; M = Methyl red, VP = Voges – Proskauer; C = Simmon citrate; S = Slant; B = Butt; G = Gas, H_2S = Hydrogen Sulphide; Cat = Catalase; Coag = Coagulase; NA = Not Applicable; TSI = Triple Sugar Iron; R = Red (Alkaline); Y = Yellow (Acid); GS = Gram stain; + = Positive; – = Negative; Unknown = Unidentified bacteria.

Table 4: Fungi Isolated from the two pharmaceutical plants in Kano State

Sample	Plant	Fungi specie
Personnel	A	NG
Personnel	B	*Saccharomyces cerevisiae, Aspergillus niger, Aspergillus amstelodami*
Air	A	*Penicillium spinulosum*
Air	B	*P. spinulosum*
Equipment	A	*S. cerevisiae, P. spinulosum*
Equipment	B	*A. niger, A. amstelodami*

NG = No growth

This was evident in the trending where corridor and airlocks showed higher rate of excursions than observed in manufacturing rooms. So, by continuous monitoring of the buildings and its utilities and ensuring that all tests fall into its criteria, the remaining probable cause of the environmental microbiological excursions will be related to the operators factor with the quality and frequency of the associated activities and out-of-control states will increase as long as non-consistent GMP actions and attitudes are extended and growing. Thus, it is not surprising that progressive increase in microbial count trends could be attributed to the workers and adherence to GMP rules [4].

In this study, *Micrococcus luteus* was the predominant organisms isolated in the two plants. *Micrococci* can grow well in the environments with little water or high salt concentration. They occur in wide range environments, including water, dust and soil [21]. *Micrococcus* is generally thought of as harmless bacterium, though there have been rare cases of *Micrococcus* infection in people with compromised immune systems, as occur in HIV patients [22]. Similar work was reported by [11]. Aside from *M. luteus*, other organisms isolated were *Salmonella* in Plant A and one unidentified organisms in Plant B. The unidentified organism resembles *Shigella* in all biochemical respect but differ in the requirement for glucose utilization. *Shigella*

utilizes glucose present in Triple Sugar Iron [23] while the unknown isolate organism does not utilize glucose (refer to Table 3). *Penicillium* was the only isolated organism in the air of the two pharmaceutical plants. *Penicillium, which* are dominantly found in the atmospheric air, are known as fungal allergy sources [24]. Similar work was done by [13,25].

In the course of absence of general microbial limit for swab sample (of irregular surfaces) obtained from equipment and personnel palm, it will be hard to analyze or discuss this results. However, Guidelines on test methods for Environmental Monitoring for Aseptic Dispensing facilities produced by a working group [26] gives 25-50 CFU/25cm^2 for grade c and d operations. Going by this, only 3rd batch sample of personnel and equipment of Plant A deviated from this standard and 1st and 2nd sample fell within the range (16 and 14 for personnel; 12 and 5 for equipment). For Plant B, only the 1st sample of personnel and equipment are within the range stipulated and all other sample of both parameters exceed the limit.

Micrococcus, Pectobacterium (*Erwinea), Klebsiella, Providencia* and *Citrobacter* were isolated from personnel. Presence of *Micrococcus* might be due to the fact is normally present in the indigenous microflora of skin, also in the mucous membrane such as the nasal cavities, the upper respiratory tract and the lining of the mouth [27]. The genus *Pectobacterium*, which includes Gram-negative non-lactose fermentors, is closely related to the genus *Erwinia* [28]. The presence of *Pectobacterium* in the hand of personnel doing manual labeling with gum might be attributed to the fact that the bacterium is employed in bio – degumming technology [29]. This organism was reported in the work of Zeitoun *et al.* [28], which claimed to have isolated it from pharmaceuticals, which might be transferred from personnel to the product. *Klebsiella* are ubiquitous in nature. In human, they may colonize the skin, pharynx, or gastro-instestinal tract. The principal pathogen reservoirs of infection are the gastro – intestinal tract of patients, the hands of hospital personnel [30]. *Citobacter* was isolated from personnel during manual filling of methylated spirit. This is an indication that the isolated strain of *Citrobacter* is resistant to the methylated spirit produced in Plant A. Just like isolates of *Citrobacter* have been reported to be resistant to many other antibiotics as a result of plasmid-encoded resistance genes. More also, methylated spirit cannot be relied upon to completely eliminate all bacteria as it only helps to reduce the number of organisms. Moreover, the antimicrobial activity of this agent may be influenced by their formulations effects, level of organic matter (as methylated spirit lose its efficiency in the presence of contaminated organic matter), synergy, temperature, dilution rate and tests method as explained by [31].

It is recommended that more study should be done on overall assessment of microbiological quality of pharmaceutical industries as very little data of such studies are available. Stringent GMP have to be adopted in the manufacture of product. For instance (in Plant A) to have isolated pathogenic organism from a disinfectant! Further study should be done on resistivity of *Citrobacter* to methylated spirit, as this organism was isolated from the hands of personnel manually filling methylated spirit and also from the jug used in the filling.

Conclusion. The results of this study show that some of the analyzed personnel and air samples do not comply with Good Manufacturing Practice and therefore could lead to microbial contamination of the product.

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