16S rRNA gene identification of airborne pathogenic bacteria isolated from bioaerosols of wastewater treatment plant

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Background and objective
Wastewater treatment plants (WWTPs) represent a source of airborne bacteria. The presence of airborne bacteria in the environment of WWTPs could be considered as a potential health hazard for the exposed workers. This study aimed to isolate and identify cultivable bacteria from bioaerosols of different sites in a WWTP using 16S rRNA gene identification, as a first step to identify the pathogenic health hazards among the exposed workers.
Materials and methods
Air samples were collected from various locations in a selected WWTP. Airborne microorganism samples were collected on the nutrient agar plates by the settle-plate technique and were identified by the 16S rRNA gene sequencing technique.
Results
A total of 32 bacterial isolates were collected and sequenced. The study identified 25 different bacterial species. Of the 25 different strains, 10 (40%) belonged to pathogenic bacteria. Overall, 40% of the isolated pathogenic species were from the secretary room locations. The isolated bacterial species were *Staphylococcus sp.*, *Bacillus sp.*, *Rhodococcus sp.*, *Cellulosimicrobium funkei*, *Kytococcus sedentarius*, and *Kocuria rosea*. The highest percentage occurrence was *Bacillus sp.* (37.5%), followed by *Staphylococcus sp.* (18.75%).
Conclusions
Disseminated infection can be associated with isolated pathogen, and this result gives a warning of the danger of the spread of pathogenic aerobic bacteria in WWTPs and their existence in indoor environments.

Keywords:
16S rRNA gene, *Bacillus sp*, bacterial health hazard, gene sequencing, *Staphylococcus sp*, wastewater treatment plant

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Introduction

Most microorganisms are ubiquitous, and they are found in both air and on the surfaces. The sources of airborne bacteria that exist from wastewater treatment plants (WWTPs) are mostly from components of wastewater [1–3]. Some opportunistic pathogenic species are present among these airborne bacteria. These pathogenic species include different strains such as *Acinetobacter sp.*, *Bacillus sp.*, *Pseudomonas sp.*, *Enterococcus sp.*, and *Escherichia coli* [4,5]. The pathogenicity of such microorganisms is affected by temperature, humidity, and oxygen concentration [6].

WWTP employees are at risk of health problems as a result of exposure to airborne microorganisms, such as bacteria and fungi [7–10]. In addition to the health hazards of microorganism inhalation from air, contamination with microorganisms through hand, mouth, or eye contacts poses a great danger to the health of plant workers [11]. Airborne bacteria can be transferred to host through respirable fine particulates, where the bacteria are attached to surfaces. Moreover, they can be easily transferred by wind to considerable distances [12]. Besides, crowdedness and increased number of air conditioners inside building and poor ventilation can facilitate the spread of airborne particles and increase the number of people at risk of airborne infections [8,13].

During wastewater treatment, bioaerosols are released into the air as nuclei droplets, where small particles of water carry microorganisms. A small amount of bioaerosol causes infections to humans [14,15]. Bioaerosols have also been associated with non-infectious diseases, such as hypersensitivity pneumonitis, allergies, and asthma [16–18]. At

WWTPs, bacterial concentrations in the air usually range from 10^1 to 10^4 CFU/m^3 [19]. The inhalation of those high concentration and the complexity of bioaerosols during occupational activities are known to cause a variety of respiratory disorders like chronic pulmonary disease, decline in lung function, increased airway responsiveness, or hypersensitive pneumonitis in the exposed workers [20,21].

Previous studies proved that several species of potentially pathogenic microorganisms have been detected in the aerosols generated from sewage plants [22,23]. Korzeniewska *et al.* [19] reported the numbers (CFU – colony forming units – in 1 cm^3) of bacteria and fungi in untreated wastewater ranged from up to 1.9×10^5–6.4×10^7 and from 8.5×10^3 to 5.0×10^4, respectively. Therefore, measurement of airborne microorganisms are of public hygienic benefit. In our previous study, workers in the same WWTP were found to be at risk of elevation of AFP, a tumor biomarker of hepatocellular carcinoma, due to their exposure to high concentration of aflatoxin-producing *Aspergillus sp.* detected in the air of their work place [24].

Detection and identification of airborne microorganisms, such as bacteria, are of interest in different research fields, including occupational health. Various methodological tools are available to identify airborne microorganisms. PCR represents a more sensitive and specific detection method. These methods have been used to detect microorganisms in specific samples including bioaerosol [25,26]. In addition, quantitative real-time PCR may provide a potential technique for quantification of microorganisms in different working locations [27,28].

This application of PCR technique advances environmental and health sciences, because it identifies pathogens in aerosols that could not be diagnosed by way of culture methods [29]. It also leads to an increased understanding of the background populations of microorganism in air [30]. At present, the comparative sequence analysis of 16 S rDNA genes is the most commonly used method of 'phylogenetic' division of prokaryotes.

As aforementioned, workers in WWTPs may be at a high risk of diseases owing to exposure to bioaerosols. This study aimed to isolate and identify cultivable bacteria from bioaerosols of different sites in WWTP, as a first step to identify the pathogenic health hazards among the exposed workers.

Materials and methods

Sampling sites

In the present study, six different locations in a sewage treatment station were chosen for the collection of air samples to study the presence of airborne bacteria. These locations were maintenance building area (S1), sludge building area (S2), primary sludge raising station (S3), coarse strainer area (S4), secretary rooms (S5), and laboratories (S6). Various types of human activities characterize these selected sites.

Isolation of bacteria from air

Airborne microorganisms were collected on nutrient agar plates by the settle-plate technique using a sampler at 1.2–1.5-m height above the surface (the breathing zone of the workers). The air sampler device used was set at an air sampling rate of 100 l/min. Prepared plates of nutrient agar (HiMedia Laboratories Pvt, Mumbai, India) were exposed to the air sampler for 5 min at the six selected sites in the sewage treatment station. The air sampler was sterilized by 70% alcohol between each sample collections. After sampling, all plates were immediately taken to the microbiology laboratory and incubated at 37°C for 24 h for bacterial isolation. The isolated colonies were counted and expressed as CFU/m^3 of the air. The colonies were subcultured into a new fresh medium to obtain pure culture. Stock cultures were prepared from each isolate in 30% glycerol and stored at −20°C [13].

Bacterial identification

The bacterial isolates were first identified based on Gram's staining according to Cheesbrough [31]. A smear was prepared and heat fixed. The crystal violet stain (primary stain) was flooded over the fixed culture for 60 s; the stain was washed with water. Iodine solution was added onto the smear for 60 s, poured off, and rinsed with water. A few drops of decolorizer (ethyl alcohol) was added and washed with water immediately after 5 s, and finally, safranin (secondary stain) was added for 60 s and washed. The smear was allowed to air dry. After drying, the slide was mounted under a microscope to identify the stained bacteria.

DNA extraction and sequence analyses: complete identification of bacterial isolates was performed using the 16S rRNA gene sequencing method. Genomic DNA was extracted and purified from pure bacterial culture from each isolate using the EZ-10 Spin Column Genomic DNA (Biobasic, Ontario, Canada) following the instructions of the

manufacturer. For amplification of 16S rRNA genes of each bacterial isolate, PCR reaction mixtures (25 µl) were used, which contained 5 µl of the extracted DNA, Dream Tag Green PCR master mix(2X) 12.5 µl, 2 µl of forward primer (0.4 µM), 5′-GTTTGATCCT
GGCTCA-3′, 2 µl of reverse primer (0.4 µM), 5′-TACCAGGGTATCTAATCC-3′, and water nuclease completed to 25 µl. The PCR conditions were (a) one cycle of 2 min at 96°C, (b) 30 cycles of 25 s at 95°C, 1 min at 50°C, and 50 s at 72°C, and (c) one cycle of 1 min at 72°C. The PCR was performed in a Mastercycler (Eppendorf), and the amplified products were analyzed on a 1% agarose gel in TAE buffer. These sequences amplify an approximately 800 bp product from the 3′ end of the 16 S target [32].

The amplified products were purified using the QIA quick PCR purification kit, according to the manufacturer's directions. The resulting 16 S DNA purified sequences were sequenced using an automated DNA sequencer (ABI model 377; Waltham, Massachusetts, USA Applied Biosystems).

DNA sequence analyses

The sequences of bacterial isolates of this study were then compared with those in GenBank (National Centre for Biotechnology Information; *http://www.ncbi.nih.gov/*) using the basic local alignment search tool for nucleotide sequences (*blastn*). Multiple sequence alignment was carried out by CLUSTAL W, and later phylogenetic analysis was performed using software MEGA X [33]. Phylogenetic tree construction was performed using the Unweighted Pair Group Method with the Arithmetic Mean method [34].

Results

An airborne bacteriological investigation was carried out at six different sites of a sewage station to isolate and identify airborne bacteria. Under the microscope, 32 (100% of the collected samples) were gram-positive bacteria (Fig. 1).

Identification of bacteria by 16S rRNA sequence analysis

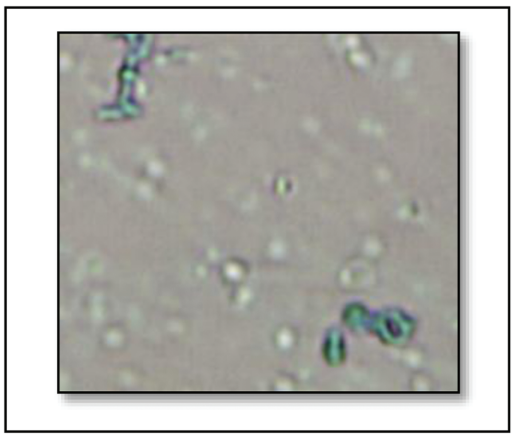
The 16S rRNA amplicons of obtained isolates were showed on electropherograms. All the isolates were shown to have a PCR product of ~800 bp (Fig. 2).

From the 32 bacterial isolates, the sequencing revealed 25 different bacterial types in the different sewage locations by the phylogeny of the 16S region of RNA sequences (Table 1). Sequence analysis showed a significant alignments of 80–100% with the isolated bacterial species.

A phylogenetic tree made from sequenced 16S rRNA region of 10 pathogenic bacterial isolates identified is shown in Table 3, and evolutionary analyses were conducted in MEGA X program. The phylogenetic grouping indicated that strains with similar sequences were typed in the same group and probably were considered as close relatives. Most of the isolates were phylogenetically related to *Staphylococcus* and *Bacillus* strains (Fig. 3).

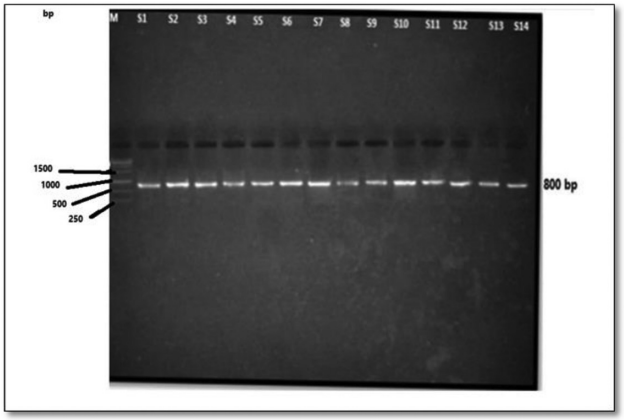
The results of the calculation of airborne bacterial concentration at different workplaces are presented in Table 2. The concentration of bacterial aerosol

Figure 1



Gram-positive bacteria.

Figure 2

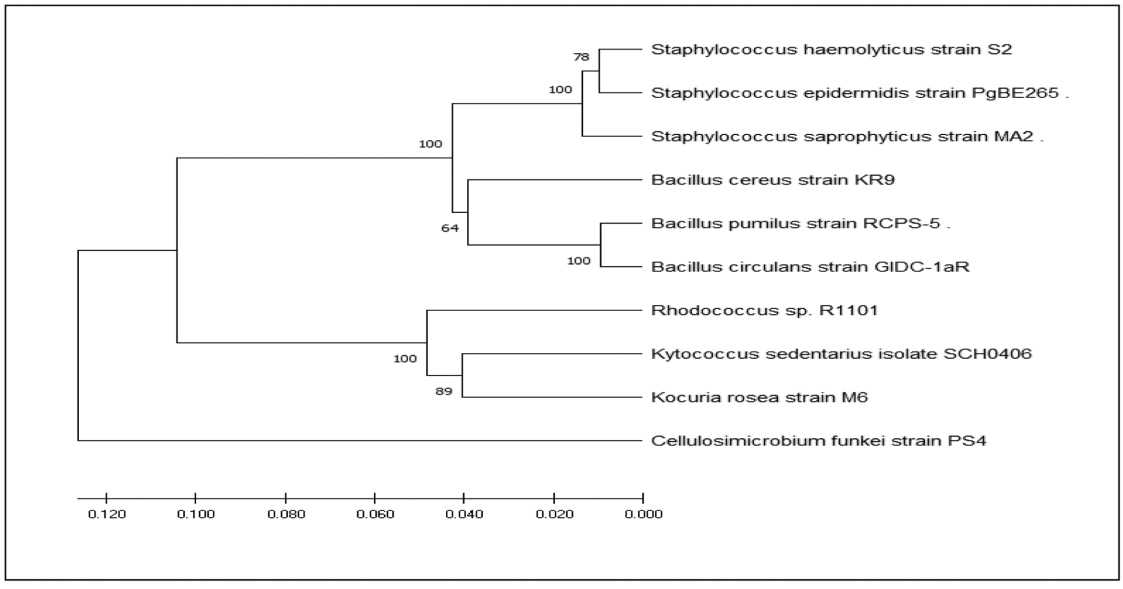


PCR amplification of the 16S rRNA from bacterial isolates.

Table 1 Phylogeny of the isolated bacteria

Numbers	Isolated organisms	Accession number	Length (nt) bp	Identity %
1	*Carnobacterium divergens*	LC279607	1494	99
2	*Bacillus circulans*	MH031304	912	94
3	*Rhodococcus sp.*	JQ775394	1512	96
4	*Micrococcus sp.*	MH671522	1451	99
5	*Kocuria sp.*	LC416394	1488	86
6	*Enterococcus hirae*	LC279607	1494	90
7	*Bacillus sp.*	KX374900	785	83
8	*Cellulosimicrobium funkei*	MF828441	718	80
9	*Bacillus sp.*	FJ379319	1462	98
10	*Bacillus pichinotyi*	MG705701	1403	98
11	*Bacillus pumilus*	HM172502	1500	98
12	*Bacillus thuringiensis*	KX057531	1034	97
13	*Planomicrobium glaciei*	HF545326	1510	98
14	*Bacillus aerophilus*	HF545320	1509	99
15	*Micrococcus yunnanensis*	KF217126	1449	99
16	*Kytococcus sedentarius*	AY881239	1516	97
17	*Kocuria rosea*	MH196919	809	99
18	*Staphylococcus hominis*	MG815842	981	97
19	*Bacillus firmus*	KJ691874	1454	84
20	*Bacillus cereus*	KX082775	1467	98
21	*Staphylococcus warneri*	KC787352	1511	86
22	*Staphylococcus haemolyticus*	MN093881	1507	98
23	*Staphylococcus epidermidis*	MN689679	1519	98
24	*Staphylococcus saprophyticus*	MN294684	888	99
25	*Staphylococcus hominis*	MK874940	1487	99

Figure 3



Phylogenetic tree of pathogenic strains with the 16S rRNA gene detected in the studied air samples using the MEGA X program by the UPGMA method. UPGMA, Unweighted Pair Group Method with the Arithmetic Mean.

was noted to be in the range from 80 to 340 CFU/m^3, at outdoor opened areas (S1, S2, S3, and S4), with the highest concentration at sludge building area (340 CFU/m^3). On the contrary, the indoor closed areas (S5 and S6) had aerosol bacterial concentration ranging from 600 to 1600 CFU/m^3, with the highest concentration at secretary rooms (1600 CFU/m^3).

Of the 25 different strains, 10 (40%) belonged to pathogenic bacteria. Overall, these pathogenic bacteria in the isolates were *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Bacillus circulans*, *Bacillus pumilus*, *Bacillus cereus*, *Rhodococcus sp.*, *Cellulosimicrobium funkei*, *K. sedentarius*, and *K. rosea*. Four (40%) of the 10 pathogenic species were isolated from the secretary room locations, followed by 20% from maintenance building, 20% from laboratories, 10% from sludge building area, and 10% from primary sludge raising station (Table 3).

Pathogenic bacteria were present in 50% of the isolates (16 out of 32 isolate samples). *Bacillus sp.* was the most frequent, followed by the *Staphylococcus sp.*, then *C. funkei*, *K. sedentarius*, and *K. rosea* (Table 4).

Discussion

The sewage station environment is an important concern for public health, as some infectious agents could be suspended in the air during wastewater treatment processes and may cause environmental pollution. During the wastewater treatment processes, fine particles of water present in air of WWTPs serve as carriers of microorganisms. Pathogenic bacteria have been isolated from aerosols generated from sewage [20]. Bacteria in the air can affect the health of sewage workers and are responsible for many infections such as respiratory, digestive tract, and skin [35].

In the present study, it was seen that among the isolated bacterial species, 100% were gram-positive bacteria (Fig. 1). Prazmo *et al.* [36] also detected that the predominant isolated bacteria from the air of sewage treatment plant were gram-positive bacteria. The same results were also reported by Cyprowski *et al.* [37]. Gram-positive cocci and gram-positive rods were present in WWTPs more than other bacterial types [38].

A total of 25 different bacterial types from the 32 isolates were identified by the phylogeny of the 16 S region of RNA sequences in the six selected locations in the present study (Table 1). Amplification of the 16S

Table 2 Levels of cultivable airborne bacteria in the air of wastewater treatment plant workplaces

S/N	Sample site	Concentration of airborne bacteria (CFU/m^3)
S1	Maintenance building area	250
S2	Sludge building area	340
S3	Primary sludge raising station	300
S4	Coarse strainer area	80
S5	Secretary rooms	1600
S6	Laboratories	600

Table 3 The isolated pathogenic bacteria at various sites

S/N	Sample site	Isolated organisms	Number isolated/ total pathogenic isolates (%)
S1	Maintenance building area	*Bacillus circulans, Rhodococcus sp.*	2/10 (20)
S2	Sludge Building area	*Cellulosimicrobium funkei*	1/10 (10)
S3	Primary sludge raising station	*Bacillus pumilus*	1/10 (10)
S4	Coarse strainer area	–	0
S5	Secretary rooms	*Kytococcus sedentarius*, *Kocuria rosea*, *Bacillus cereus*, *Staphylococcus haemolyticus*	4/10 (40)
S6	Laboratories	*Staphylococcus epidermidis*, *Staphylococcus saprophyticus*	2/10 (20)

Table 4 Percentage of occurrences of pathogenic bacteria

Bacterial species	Number of isolates/sites (*n*) S	Frequency of occurrence	Percentage of occurrences	Total species (%)
Bacillus cereus	(3) S5	3	18.75	37.5
Bacillus pumilus	(2) S3	2	12.5	
Bacillus circulans	(1) S1	1	6.25	
Staphylococcus haemolyticus	(1) S5	1	6.25	18.75
Staphylococcus epidermidis	(1) S6	1	6.25	
Staphylococcus saprophyticus	(1) S6	1	6.25	
Cellulosimicrobium funkei	(2) S2	2	12.5	12.5
Kytococcus sedentarius	(2) S5	2	12.5	12.5
Kocuria rosea	(2) S5	2	12.5	12.5
Rhodococcus sp.	(1) S1	1	6.25	6.25

rRNA gene has been reported as a useful technique to amplify the 16S rRNA gene from cultivable and noncultivable bacteria, and the heterogeneous species in many research fields [39]. Of 25 identified bacterial species, 10 (40%) belonged to pathogenic bacteria. Most of these bacteria may lead to pathogenic infections among the exposed workers if their immune defenses were lost or weak, such as gastrointestinal tract, and urinary tract infections [35].

Toze [40] showed that a wide spectrum of pathogenic bacteria are detected in WWTPs, many of which are enteric bacteria. Wastewater can carry many opportunistic pathogens, which can cause infections among people with immune system defect. Bioaerosols exist in stages that involve aeration and mechanical agitation, which may contain potentially pathogenic microorganisms [41]. Numerous publications suggested that sewage treatment plants may contain pathogenic microorganisms that cause health hazard for workers. Subsequently, there is an importance for regular monitoring of air quality in these plants.

There are no official standards for permissible level of airborne microorganisms. The results of the present study indicated that there were differences in the concentration of bacteria among different sites in the WWTP. The airborne bacterial level of most of the studied sites exceeded the suggested occupational exposure limit value of 100 CFU/m^3 recommended by the Polish Standards [42]. Such comparison indicates a high air microbial pollution at wastewater facilities. The authors attributed the elevated microbial level in air to the high degree of emissions from sludge and wastewater. Several investigations have documented a high degree of microbial emissions in the air of workplaces in the WWTPs [3,7,43,44]. Wastewater and sludge were the main sources of pathogenic bacteria, as was determined from the inlet and outlet of WWTPs [45].

The authors also observed that the levels of bacteria were found to be in the range from 80 to 3.4×10^2 CFU/m^3 at opened area worksites and from 6×10^2 to 1.6×10^3 CFU/m^3 in closed work site areas. Similarly, certain studies demonstrated that the total bacterial content of sewage atmosphere was 10^3–10^4 CFU/m^3, as reported by Lavoie and Dunkerley [46], 10^1–10^4 CFU/m^3 by Korzeniewska *et al.* [19], 2.4–70.7×10^2 CFU/m^3 by Prazmo *et al.* [36], and in Egypt, the concentrations ranged from 1.06×10^2–7.36×10^3 CFU/m^3 [44].

Subsequently, we found a wide presence of airborne bacteria in the closed and opened areas of WWTP workplaces. The highest level of bacteria was detected in the atmosphere of the closed spaces with concentration reaching up to 1600 CFU/m^3. Different human activities such as talking, coughing, and sneezing can contribute in generating or increasing the droplets. The presence of such droplets in air adds protection to bacterial cells and results in enhanced survival of airborne microorganisms [47]. Additionally, the elevation of bacterial load in indoor environments may be owing to inadequate ventilation system and crowdedness in restricted areas.

Detailed analysis of the pathogenic bacteria detected in the isolates in the current work showed similarity in the gene sequences of the 10 isolates to those within the Gen Bank. The frequency of occurrence of pathogenic isolate was 16 (50%) of 32 total isolates. Most of the isolates showed similarities that approached 100% (Fig. 3). Among of them, the most frequent bacteria types occurred were *Bacillus* (three *Bacillus.* species), followed by *Staphylococcus* (three *Staphylococcus* species). Similarly, Kowalsk *et al.* [38] reported that *Staphylococcus* (six *Staphylococcus.* species) and *Bacillus* (three *Bacillus* species) were the most common airborne bacteria in WWTPs. The high percentage of the presence of *Bacillus sp.* in the tested isolated samples compared with the other isolated bacteria might be due to their nature as spore former that could tolerate adverse condition. Ashgar and El-Said [48] mentioned the same explanation.

Bacillus sp. has a higher frequency of occurrence at 37.5%, as *B. cereus* (18.75%) was detected in secretory rooms followed by *B. pumilus* (12.5%) and *B. circulans* (6.25%). Airborne bacteria enter any building via the natural ventilation components such as windows and doors, whereas the nonairborne bacteria are transferred indoor from the shoes onto the floors and carpets [49]. According to the results of the present study, 80% of isolates containing pathogenic bacteria were detected in the indoor locations of the buildings of the included WWTP, as 40% of pathogenic species were isolated from the air samples of the secretary rooms, 20% from maintenance building, and 20% from laboratories. This could be attributed also to the high temperature that can lead to increase in the existence of pathogenic microorganisms [50]. Moreover, according to literature, *Bacillus sp.* are often detected in drinking-water supplies, although these water supplies were disinfected by acceptable procedures, as Małecka-Adamowicz *et al.* [51] reported that bacterial population in the air of WWTPs was predominated

by bacteria of genus *Bacillus* (64%). This is due to the resistance of spores to disinfectants [52].

B. cereus had the highest percentage of occurrence (18.75%) in secretory rooms in the present study. *B. cereus* is associated with food poisoning leading to several infections such as gastrointestinal infections and wound infections. In addition, wastewater treatment workers were at a high risk of infectious diseases [53].

This study reported that 12.5% of pathogenic bacteria isolated from sewage air are *B. pumilus.* Human infection by *B. pumilus* is rare; however, in 2007, researchers reported that *B. pumilus* can cause cutaneous infection in humans [54]. Clemente *et al.* [55] reported a case of severe sepsis caused by *B. pumilus* in a 7-year-old healthy child.

During the past decade, the numbers of infections caused by opportunistic pathogens has increased. Opportunistic pathogens are defined as pathogens that may cause disease in the immunocompromised population [56]. Wastewater is appearing as a vector that transfers opportunistic pathogens during irrigation [3]. In this study, we isolated *B. circulans* from sewage air in small concentration (6.25%) mainly from indoor maintenance building. The dangers of the presence of *B. circulans* in the maintenance building even in small concentration are attributed to its opportunistic pathogenic properties, as it was proved to be the cause of nosocomial infection responsible for sepsis [57].

In the present study, *Staphylococcus sp.* has a high frequency of occurrence (18.75%). It was dominated by the genus *S. haemolyticus*, *S. epidermidis*, and *S. saprophyticus*. This higher incidence of *Staphylococcus sp.* obtained in this study was similar to several findings of the studies conducted by several researchers. Niazi *et al.* [58] reported that *Staphylococcus* sp. was the most frequently observed bacteria types in the WWTPs. The results obtained by Małecka-Adamowicz *et al.* [51] indicated that *Staphylococci* constituted 27% of the bacterial community in WWTPs. Urinary tract infections caused by *Staphylococci* are due to *S. saprophyticus, S. epidermidis*, or *S. haemolyticus* [59].

The other types of bacteria detected in the air samples collected from the different sites in the WWTP (*C. funkei*, *K. sedentarius*, and *K. rosea*) were with low concentrations compared with the environmental concentrations of *Staphylococci* and *Bacillus* species.

Conclusion

Pathogenic bacteria were detected in the air of WWTP work place. The highest percentage of occurrence of pathogenic bacteria *Bacillus sp.* and *Staphylococcus sp.* were detected in closed areas. The results of this study alert to the occurrence of pathogenic airborne bacteria in the indoor WWTP working environment, especially the closed areas, which may cause adverse health problems in exposed workers.

Recommendations

Thus, it is important to check the sanitary conditions of air in the sewage work place, especially of the closed areas, and more attention should be given to safe indoor environments from the growth of pathogenic microorganisms. Further studies should be done to identify the pathogenic effects of the detected bacteria on the health of exposed workers.

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Author contributions: Gehan Moubarz collected the air samples from the working places, performed molecular and genetic analysis to identify the bacteria, and wrote the manuscript. Amal Saad-Hussein, the principal investigator of the project, designed the study, took the ethical approval for the study, interviewed the participants, and performed the statistical analysis of the data. Asmaa M. Elfiky conducted the phylogenetic analysis. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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