Middle-East Journal of Scientific Research 27 (1): 55-63, 2019
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2019.55.63

Seasonal Variations of Fungi Isolated from Swimming Pools in Jeddah, Saudi Arabia

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Abstract: Swimming pools may act as a vehicle for the spread of fungal diseases. The study aimed at evaluating the seasonal variations of fungal populations in swimming pools in Jeddah, Saudi Arabia. Seventy-two samples were collected from 17 swimming pools during the summer and winter seasons in 2017. The Temperature, pH and chlorine levels of the swimming pools were measured. Also, the samples were cultured on potato dextrose agar medium (PDA) and incubated at 28 °C for 7 days. Fungal isolates were initially identified based on their morphology. Suspected isolates were analyzed further using internal transcribed spacer (ITS) rDNA analysis. The findings of this study revealed that there was a significant difference in temperatures between summer and winter seasons, but similar values were recorded for pH and chlorine levels. The results confirmed the presence of 10 species belonging to 7 different genera in swimming pools water. The prevalence of fungal species during summer were as follows: *Penicillium chrysogenum* (22.4%), *Talaromyces pinophilus* (15.1%), *Aspergillus fumigatus* (13.3 %), *Byssochlamys spectabilis* (10.3%), *Aspergillus niger* (9.8%), *Aspergillus sojae* (9.8%), *Alternaria alternate* (8.6%), *Galactomyces geotrichum* (4.1%), *Aspergillus terreus* (4.5%) and *Fusarium oxysporum* 2.6%. In contrast, a different distributions pattern was noted during the winter season with less species diversity including: *P. chrysogenum* (25.3 %), *A. Alternate* (15.1%), *A. niger* (12.6 %), *A. fumigatus* (11.3%), *A. sojae* (10.9%), *B. spectabilis* (10.9%), *G. geotrichum* (8.3%) and *T. pinophilus* (5.7%). In conclusion, swimming pools in Jeddah may be hazardous to swimmers because of the physiochemical parameters of swimming pools were not standard and having a high prevalence of saprophytic fungi. There is an urgent need for monitoring the physiochemical parameters that affect the proper efficacy of the disinfection system employed and may reduce fungal population in swimming pools water.

Key words: Fungal Isolates · Swimming Pools · Disinfection · Seasonal Variations

INTRODUCTION

Swimming is a healthy activity that is favored by many people to spend their leisure time, especially during the summer season in Jeddah city, where temperatures often exceed 40 ºC. It is important to note that many physiochemical factors such as temperature, pH and free residual chlorine residual are extremely significant in determining the microbial quality of swimming pools. In the summer season, high temperatures between 27 - 29 °C are reported in swimming pools in many areas in the world which provide a conducive environment for the growth of many disease-causing microbes [1]. The ideal pH level for swimming pool waters varies between 7.2 to 8 with a continuous level for free residual chlorine presence ranging between 1 to 2 mg/L. When the level of free

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residual chlorine goes below 0.7 mg/L, the microbes will multiply faster resulting in an increase in the contamination levels of swimming pools and will compromise the quality of the water [2]. Furthermore, lower pH contributes to many swimmers related problems including sore eyes, skin spots ando ther related problems [3]. Concerning the fungal quantity and quality of swimming pool waters, it is extremely important to comply with certain hygienic standards to reduce infections and ensures the safety of swimmers. Hence, continuous monitoring of swimming pool water's quality and associated physiochemical parameters are extremely essential in reducing associated infections [3]. The hygienic quality of the swimming pool is dependent on the disinfection system used. Physical and chemical variations in the quality of the water as well as the swimmer's behaviors could greatly affect the water quality. This is an addition to many environmental factors that affect the quality of the water including temperature, pH and number and behavior of swimmers and the time of the day [4].

Published studies have indicated that swimming pools may act as a vehicle for the transmission of pathogenic microorganisms including fungi [3]. Pathogenic fungi could be transmitted from one swimmer to another by physical contact resulting in a variety of skin infections like dermatophytosis which accounts for 10 to 20% of worldwide fungal infections [2]. Agababova and Hon [5] reported that swimming pools may be recognized for their potential ability to spread respiratory tract infections and other diseases. Many fungal genera have been identified in contaminated waters that are responsible for hypersensitivity pneumonitis including *Aspergillus*, *Penicillium*, *Cryptostroma*, *Rhodotorula*, *Trichosporon* and *Pullularia* [6, 7]. Several reports have indicated that increasing fungal presence in outdoor pools is mainly attributed to the waste discharge from swimmers or animals like feces, mucus, vomit, skin and saliva [8, 9]. Such secretions are hazardous and considered to be as a possible source for the spread of pathogenic fungal infections. Based on that, proper monitoring of those factors will result in improving the quality of swimming pools leading to a reduction in the frequency of the spread of pathogenic fungi and will result in less public health associated hazards [10].

Research Problem: Most ofthe swimming pools in Jeddah are not subject to oversight, as well as the general physiochemical requirements for the safety of swimming pools are not applied. This could result to spread of microbes specifically fungi which cause many diseases especially when temperature exceeds 45°C in Jeddah during summer. Therefore the aim of the current work was to assess cleaning level of swimming pools in Jeddah through sampling from swimming pools in two different seasons (summer and winter), studying the physical and chemical factors (temperature pH and chlorine) affecting in infection levels, isolation of fungi found in swimming pools and identification of the main fungi found in public swimming pools in Jeddah.

MATERIALS AND METHODS

Measurement of Physiochemical Parameters: Digital thermometer device (The Hanna CAL Check™, Mauritius) was weekly calibrated and used to measure the parameters required according to manual manufacturer's instructions. Both ice-point and boiling point were checked for thermometer sensitivity. Under antiseptic conditions, the thermometer probe was immersed at 30 cm depth inside the swimming pool water and temperature reading was recorded repeatedly. Also, pH and chlorine levels were measured using one set of pH and Chlorine was assayed using a chlorine test kit (Yuen Hoi pool products, China). This kit was provided with comparator test tubes with covers, reagents and standard manufacturer's chart for pH and chlorine concentrations, separately. Before using, comparator tubes, they were rinsed several times in the pool and filled up to the 9 ml mark. Five drops of reagents 1 and 2 were added to the water in each one of the test tubes and mixed thoroughly by inverting them several times. The pH result of the water was compared to the right color column standard scale, while the chlorine result was measured using the leftcolor column on the same set as described by Pool Water Treatment Advisory Group [1].

Sample Collection: Seventy-two samples were collected from 17 selected public swimming pools frequented by many swimmers in the Jeddah region, Saudi Arabia during summer and winter seasons in 2017. Water samples were taken from pools 3 times monthly. Manual collection procedure was followed as described by Naghipour *et al*. [11]. Briefly, water samples were scooped at a 30 cm depth in 100 ml a sterilized glass bottle. All samples were sealed, placed in ice cooler on ice (2 to 8 °C) till delivered to the laboratory. The time needed to reach the laboratory was around 30 min. Upon arrival at the laboratory, the water bottles were immediately cultured as shown below.

Isolation of Fungi: Each bottle was gently mixed before culturing 50 µl of its content was inoculated onto potato dextrose agar medium plates (PDA) [8]. Those plates were sealed using parafilm and incubated at 28 °C for 7 to 14 days in order not to miss any organism. We noted that all the fungi appeared at 7 days of incubation and no new ones were found. For this reason, we opted to use 7 days only for incubation in all our future cultures throughout the study. At day 7, fungal colonies were counted and pure cultures were prepared by inoculating the selected colonies in universal glass tubes with caps containing PDA slants. The slants were incubated at 28 °C for 7days and stored at 4 °C for further analysis.

Fungal Morphological Structures Analysis: Fungal macro-morphological structures were evaluated and the colonies were visualized by naked eyes. An inoculum taken from pure colony PDA slants was transferred to PDA plates and incubated at 28 °C for 7 days. One plug disk (6 mm diameter) of each pure colony was transferred to PDA plate and incubated for 7 days to measure their growth. The criteria used for such an evaluation included the following observations: exudate formations, color productions and colony diameters (Ref.??). For microscopic structure examination, a needle was used to transfer an inoculum onto PDA plug on a glass slide. This was immediately followed by staining it with lactophenol cotton blue stain (LPCB) and incubated at 28 °C for 7 days. At 7 days, the slide cultures were observed using light microscopy for micro-morphological structures. The conidia and phialides structures were recorded using 20 µm scale bar and macro and micromorphological structures were identified up to the genus level using morphological reference keys as reported by others [12].

Fungal genomic DNA extraction and PCR amplification: The fungal genomic DNA extraction method was used [13]. An inoculum was taken from PDA pure fungal colony slant and transferred into 100 ml Erlenmeyer flask containing 20 ml of potatoes dextrose broth (PDB) and then incubated at 28 °C for 7 days. After that, the broth was filtered through a 0.22µm nitrocellulose sterilized filter paper and mycelia were collected onto the filter, separately. Then, they were washed several times with deionized sterilized water and stored at -20 °C. Frozen mycelia were mixed with liquid nitrogen and ground using a sterilized pestle and mortar until they turned into fine powder. The powder was distributed into 1.5 ml Eppendorf centrifuge tubes and stored at -20°C. For DNA extraction, 30 mg of frozen grounded mycelia were lysed in lysis buffer provided in the Gene JET Genomic DNA extraction kit (Thermo Scientific, USA, Put catalog No.??) and suspended in 500 µl lysis buffer. The tubes were, then, incubated in a water bath at 37 °C for 60 mins. Polymerase chain reaction (PCR) was performed by amplifying the ITS region of ribosomal DNA (rDNA) ITS1 (CTTGGTCATTTAGGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) using thermal cycler (Esco health care, Swift max pro, Malaysia). The 50 µl reaction mixture contained 3 µl of template DNA, 5 µl of each primer, 25 µl of green PCR mix (Promega, Go Taq ® Green Master Mix, USA) and 50 µl of nuclease-free water. The PCR program was as follows: the initial cycle of 96 °C for 1 min and 35 repeated cycles of 94 for 1 min, 56°C for 45 s, 72°C for 1 min and final cycle of 72 °C for 6 min [14]. The negative control was prepared with DNA extract with no additional solution.

The DNA loading dye was mixed with each PCR product and 20 µl was transferred into 1.5% agarose gel. Electrophoretic gel (Horizontal gel electrophoresis, Cleaver scientific, UK) was stained using ethidium bromide and run for 45 mins at 130 volts. The 20 µl of DNA marker (100 bp, Invitrogen, USA) was used to quantify and identify the PCR products. All sample bands were visualized under UV light (gel doc imager, BioRad, USA). Then the sharp band samples were cut and sent to Macrogen Company, South Korea for purification and sequencing.

The sequence results for each PCR product from both strands were assembled using big dye terminator cycle sequencing kit (Applied Biosystems, U.K). Sequence identities were characterized using Basic Local Alignment search tool (BLAST general GenBank databases from the National Center for Biotechnology Information (NCBI) database [13]. A sequence alignment of constructions of neighbor analysis was done by MEGA -X. The result reports were received from Macrogen molecular analysis laboratory.

Statistical Analyses: The statistical analyses were performed using T-test and SPSS 22 software to determine the significant difference among the data. The criterion for statistical significance was set at $p \leq 0.05$.

RESULTS

Table 1 shows the seasonal physiochemical parameters of the water tested during the summer and winter. The results indicated that there was a significant

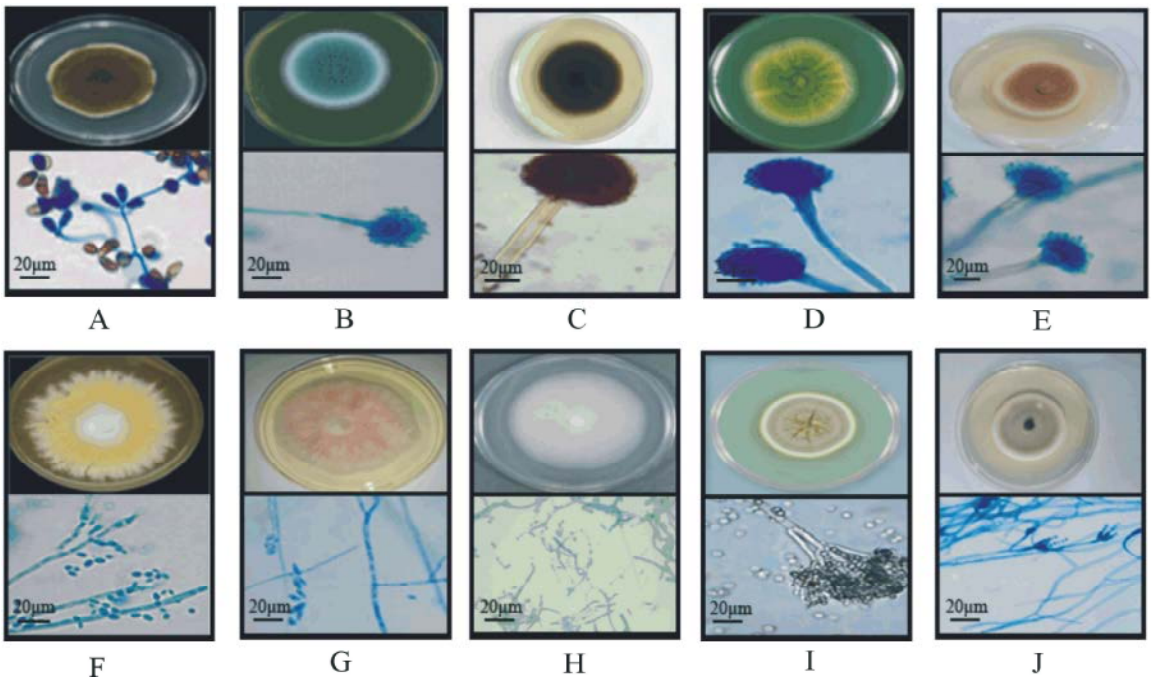

Fig. 1: Filamentous fungi isolated from water samples collected from 17 swimming pools on PDA and incubated at 28°C for 7 days. Seven fungal genera were identified based on microscopic structures including *Alternaria* (A) *Aspergillus* (B-E) *Byssochlamys* (F) *Fusarium* (G) *Galactomyces* (H) *Penicillium* (I) and *Talaromyces* (J).

Table 1: Seasonal measurements of physiochemical parameters of tested swimming pools waters

	Seasons	
Factors	Summer	Winter
Temperature (ºC)	35.31*±0.14	24.21*±0.13
pH	5.92±0.15	6.04±0.15
Chlorine(mg/L)	0.57±0.03	0.58±0.02
Mean± Stander error	*Significant difference at ($p \leq 0.05$)	

difference between the summer and winter water temperature as expected the water was warmer in the summer season. However, this was not the case in terms of pH and chlorine level.

Ten opportunistic saprophytic isolates belonging to 7 genera of fungi were detected in 72 water samples isolated from 17 swimming pools during both summer and winter seasons in Jeddah at 2017. Macromorphological structures were observed as cotton, filamentous and colorful aspects between light to dark colonies on dishes included white, withpink, white-gray, yellow-green, brown, green and grey-green with some dark colors such as olivaceous and black. Fungal colony diameters ranged from 3.5-7.5 cm (Fig. 1). Microscopically, different genera were observed based on basic micromorphological. For example, the genus *Aspergillus* has conidial head and another genus known as *Galactomyces* poses only septated hyphae. However, in the case of the genera of *Penicillium, Byssochlamys* and *Talaromyces*, they all demonstrated chains of conidia. Besides, curved and pyriform conidia shaped were seen in the case of *Fusarium* and *Alternaria* respectively (Fig. 1). The combination of fungal observations based on both macro and microscopic structures represented 7 genera are included in the following: *Alternaria*, *Aspergillus*, *Byssochlamys*, *Fusarium*, *Galactomyces*, *Penicillium* and *Talaromyces*.

Modern molecular techniques were used in order to identify the fungal associated species for the different isolated genera. The PCR profiles of pair ITS1/4 regions amplified from different isolates showed intense sharp bands using agarose gel with an expanded ranging between 400 to 569 bp (Fig. 2). This was followed by PCR genetic sequences alignment of all isolates were identified as species based on their 96 – 100% similarity in their sequences based on what has been published by NCBI databases. All of the 10 isolates belonging to 7 genera were confirmed to the appropriate species as follows: *A. alternate*, *A. fumigatus*, *A. niger*, *A.sojae*, *A.terreus*, *B. spectabilis*, *F. oxysporum*, *G. geotrichum*, *P. chrysogenum* and *T. pinophilus.*Those fungal isolates were deposited in the GenBank database as presented in Table 2.

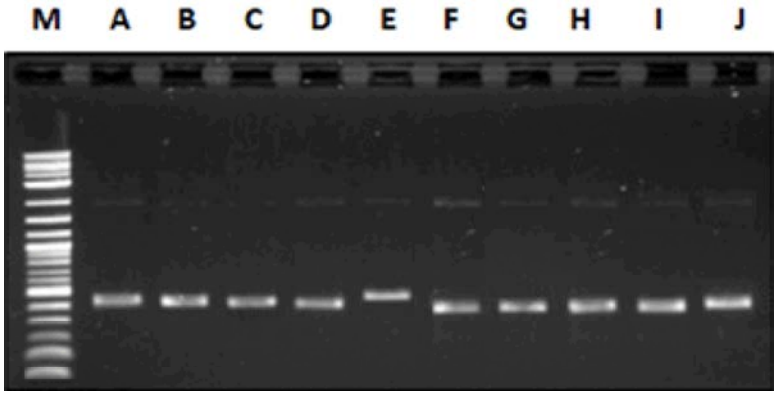

Fig. 2: PCR analysis of 10 fungal isolates with ITS1/4 regions indicated the range of DNA isolates samples (A-J) molecular size of bands were from 400 to 569 bp compared to the standard M (100 bp marker). Put Mol. Wt. on the DNA marker??

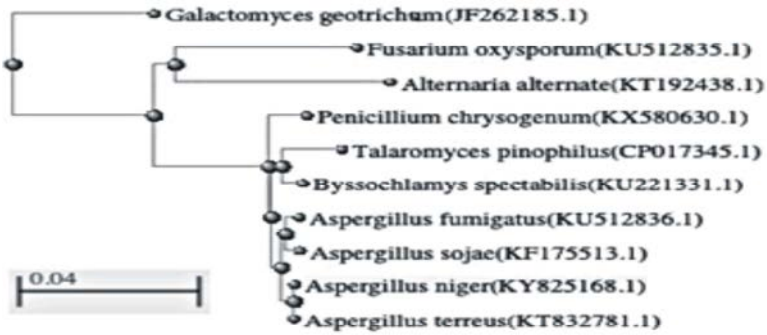

Fig. 3: Dendrogram showing phylogenetic analysis based on the ITS4/1 region and NCBI GenBank database for 10 fungal species.

Table 2: List of fungal species GenBank accession number isolated from swimming pools and most closely related fungal species with their similarity percentage found in the NCBI website

Fungal species accession numbers	Closest related species	Similarity (%)
MK500615	*A.alternate* KT192438.1	100
MK500616	*A. fumigatus*KU512836.1	100
MK500614	*A. niger* KY825168.1	100
MK500620	*A.sojae* KF175513.1	100
MK500619	*A.terreus* KT832781.1	100
MK500623	*B. spectabilis* KU221331.1	99
MK500621	*F. oxysporum* KU512835.1	99
MK500618	*G. geotrichum*JF262185.1	99
MK500617	*P. chrysogenum* KX580630.1	96
MK500622	*T. pinophilus* CP017345.1	100

The dendrogram of phylogenetic analysis indicted the taxonomic relationship for all of the 10 fungal species isolates based on ITS4/1 region and NCBI GenBank database. Bootstrap percentages from 100 replicates are shown in Fig. 3 which reveals the neighbor-joining tree by using the interactive tree. The following fungi *Aspergillus*, *Byssochlamys*, *Penicillium* and *Talaromyces* belong to the same order as Eurotiales. While *Alternaria* belongs to the Pleosporales order. Concerning *Fusarium*, it belongs to the Hypocreales order, finally, the *Galactomyces* belongs to Saccharomycetales. All data obtained based on the rDNA-ITS sequences of the 10 isolates could be grouped under Ascomycota phylum.

A total number of 942 fungal colony forming units (CFU) were isolated in the summer, while the number decreased to 288 CFU in the winter seasons. The most frequently present genera were the following: *Alternaria*, *Aspergillus*, *Byssochlamys*, *Fusarium*, *Galactomyces*, *Penicillium* and *Talaromyces*. In both seasons, *Aspergillus* genus has always shown the highest percentage distribution colonizing frequency (% CF) of 36.7 and 35.4% in summer and winter seasons respectively. This was followed by *Penicillium* with a % CF of 21 and 26.7%, in summer and winter seasons respectively. Furthermore, the most two dominant species that were found in the summer included *P. chrysogenum* (21%) and *A. fumigatus* (17.6%). In contrast to those present in summer, *P. chrysogenum* (26.7%) and *A. niger*

Table 3: The total number of seasonal CFU isolated from 17 swimming pools during both summer and winter seasons (2017) in Jeddah with their percentage CF

	Seasons			
	Summer		Winter	
Isolates	CFU	%CF	CFU	%CF
1. Alternaria	83	8.8	38	13.1
a) A. alternate	83	8.8	38	13.1
2. Aspergillus	346	36.7	102	35.4
a) A. fumigatus	166	17.6	39	13.5
b) A. niger	92	9.8	46	15.9
c) A.sojae	92	9.8	28	9.7
d) A. terreus	31	3.3	0	0
3. Byssochlamys	91	9.7	25	8.7
a) B. spectabilis	91	9.7	25	8.7
4. Fusarium	26	2.8	0	0
a) F. oxysporum	26	2.8	0	0
5. Galactomyces	32	3.4	23	7.9
a) G .geotrichum	32	3.4	23	7.9
6. Penicillium	198	21	77	26.7
a) P. chrysogenum	198	21	77	26.7
7. Talaromyces	146	15.5	23	7.9
a) T. pinophilus	146	15.5	23	7.9
Total	942*	76.60%	288*	23.40%

* Significant difference at ($p \leq 0.05$)

(15.9%) were the most two dominant species during the winter. *A. terreus* and *F. oxysporum* were least frequently present fungi (3.3 and 2.8 %, respectively) during the summer and were absent during the winter (Table 3)

DISCUSSION

Swimming pools are very attractive places for recreation especially during the summer season for humans. However, these pools could be a major public health hazard to swimmers especially when the fungal levels exceed those recommended by the WHO [3]. Our study investigated the effect of seasonal variations of fungal levels in swimming pool waters in Jeddah taking into consideration the physiochemical characteristics of those pools.

The results of the current study revealed that the physiochemical parameters did not comply with the recommended standards [11]. The temperature of swimming pool waters ranged between 24-35 °C, with significantly higher summer temperatures. This finding is not in agreement with those published by Rasti *et al*. [2] in Iran who reported that the standard swimming pool temperature should be controlled between 27 to 29°C. A wide variation in temperature may create diverse fungal communities which may pose health hazards to human [3]. In our study, variations in temperature have possibly resulted in an increase in the total number of fungi. Such an increase may lead to higher rates of human infections. A study by Agababova and Hon [5] carried out in Canada investigated the effect of a physical factor in relation to fungal counts. The data obtained from this study revealed that the recorded temperature in 6 swimming pools did not lie within the nationally recommended standards and was significantly higher than 29 °C providing a more suitable and conducive environment for fungal growth. Regarding chemical parameters, the pH and chlorine levels were not affected by seasonal changes. In this study, the effect of pH on fungal swimming pool water contamination was indicated that fungal levels go higher as a result of low pH because fungi grow better at low pH. The pH values were not in compliance with the levels recommended in swimming pools in both summer and winter seasons and ranged between 5.92 and 6.04. Pool Water Treatment Advisory Group [1] stated that the standard pH for swimming pools should be within the range of 7.2 to 8.0. The low levels of pH in pools may lead to many health problems among swimmers and may cause: eye infection, allergies, skin pigmentation and itch [3]. Also, the chlorine level of 0.58 mg/L that was detected in our swimming pools was much lower than the recommended standard levels of 1-2 mg/L. These chlorine levels will not be sufficient to efficiently control the growth of microbes resulting in higher microbial contamination and more associated health care hazards. These findings are consistent with what has been published by Antia and Umoh [15]. Based on the data obtained in the current study, the characteristics of swimming pools in Jeddah did not comply with the international set standards and as such may lead to more fungal contaminations and associated health hazards. Such problems were noted more in the summer season because more people swim and affect the overall physiochemical characteristics. Swimmers can be a major source of the problem because of their behavior which may lead to an increase in the chlorine demand and result in less free chlorine residual which is the main disinfectant species which is recommended to be kept between 1-2 mg/L to ensure proper disinfection (Ref.??). Such behaviors include not showering before using the pool, accidental fecal release, mucus, urination, vomiting and other unwarranted behaviors.

In the current study, 7 genera including 10 species were isolated from 17 swimming pool water during both summer and winter seasons. This data is in agreement with what has been published by others on the isolation

of fungal genera from swimming pools [3, 16]. In general, it is extremely difficult to isolate, characterize and confirm fungi at the species level because of the overlapping resemblance between macro and micro-morphological structures [14]. To ensure proper and indisputable differentiation and confirmation of the isolates to the species level, we used the powerful advanced molecular technique based on amplifying the ITS1 segment within fungal rDNA using PCR and sequencing the resulting PCR products. Many reported fungal species used this technique for confirmation of species identification [13]. For instance, consistent with our data which reported a molecular size ITS region of nearly 569 bp for *A. Alternate* was mentioned by Baranov *et al*. [17]. Similar findings to ours of a molecular size for ITS region of 565 to 613 bp in the case of *Aspergillus* species were found by Henry *et al*. [18]. In the case of *B. spectabilis* and *G. geotrichum*, similar findings to ours were reported by Imran and Jabbar [19] and Liu *et al*. [20] concerning the 400bp for both species. Similar to other published data, a550 bp molecular size for ITS region of *F. oxysporum*, *P. chrysogenum* and *T. pinophilus* were noted by others [21-23].

In our study, the total number of CFU was higher in the summer than in the winter seasons. Parallel results were reported by Lahouar *et al.* [17]. in Tunisia. The reason behind the increase in the fungal counts in the summer season may be due to humidity, higher temperature and the rise in the number of swimmers which can exert more demand on the disinfectant and as such provide a conducive environment for fungal growth. Our data have shown the dominance of both *Aspergillus* and *Penicillium* genera during both summer and winter seasons. This is consistent with the findings of others who reported based on studies conducted on swimming pools in many countries worldwide including Iran, Nigeria and Italy [25-27]. Based on published data, it was reported that *Aspergillus* and *Penicillium* genera were the most prevalent fungi isolated from swimming pool waters worldwide, which is agreeable to our findings [8, 25]. These fungi can be a major cause of health-related problems especially among immune-compromised individuals. For instance, *Aspergillus* can result in tracheobronchitis and pneumonia in those humans with immune-compression [28]. *Penicillium* are considered as air irritant and have been considered as a serious hazardous human pathogen [29].

During both seasons, *P. chrysogenum* was the most frequently isolated species in swimming pools. This is in agreement with what has been published by others [30]. *Penicillium chrysogenum* which has been isolated from the different indoor and outdoor environments including indoor air, dust and moist building materials [31]. This fungal species is an opportunistic pathogen resulting in hypersensitivity pneumonitis and asthmatoimmune-compromised individuals [32]. In another study by Ogórek *et al*. [33], it was reported that *P. chrysogenum* has the ability to cause allergies, superficial and invasive infections to humans. Several mycotoxicoses could be caused by species of *Penicillium*, the most important toxins are patulin and ochratoxin [29]. Concerning the second two fungal species that were most frequently present in both included *A. fumigatus* and *A. niger*. Study conducted by Gomes *et al*. [34] indicated that *A. fumigatus* a thermophilic species which can grow and survive at temperatures between 55 °C and survive up to 70°C. A. *fumigatus* has been mentioned as one of the most ubiquitous fungi capable of producing airborne spores and cause invasive aspergillosis in immune-compromised patients [35]. In the case of *A. niger* which has been frequently detected in air and could produce two groups of potentially carcinogenic mycotoxins including ochratoxins and fumonisins [36, 37]. In the case of the rarely isolates, *A. terreus* and *F. oxysporum,* they were the least detected of present fungal species in swimming pools during summer and winter seasons. Similarly, studies showed that these fungal species did not frequently detected and when present may cause allergy with hematological malignancy to humans [38, 39].

CONCLUSIONS

First, Give a Very Brief Summary of the Article??: In conclusion, it is recommended that much stricter measures needed to be exercised and enforced concerning the physiochemical parameters in swimming pools in Jeddah to reduce fungal populations and associated public health hazards. Educational training programs are needed to improve staff's knowledge about proper disinfection of swimming pools as well as increase the awareness and knowledge among swimmers to help keep cleaner recreational swimming pools waters.

ACKNOWLEDGMENT

Authors are appreciated by Dr. Mona G. Alharbi, Department Head of Biological Science at KAU for supporting and kind gaudiness.

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