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Research paper

Evaluation of pathogenicity and antifungal resistance of frequently occurred *Aspergillus* and *Fusarium* species in swimming pools in selected hotels in Ekiti state

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Abstract

Background: Swimming pools have been reported as critical fungal reservoirs. This study was designed to investigate fungal populations in swimming pools as well as detect the presence of virulence factors in the recovered isolates.
Methods: Estimation of the mycobiota in the swimming pools sampled was done and isolates recovered were identified using colonial, morphology and molecular methods. Also, virulence factors: lipase, keratinase, laccase, hydrophobicity and biofilm formation were also determined using standard methods while antifungal susceptibility was done using clinically approved methods. Also, the presence of metalloprotease and protease gene was detected using PCR.
Results: Out of the 75 samples, 47 showed fungal growth, with an isolation rate of 25.5%, 34.0%, and 40.4% from the surface, bottom, and edges of the pools respectively. Six fungi species were isolated viz: *Candida tropicalis, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum,* and *Fusarium verticilloides. C. tropicalis* had the highest isolation rate at 41.7%. The fungi possessed virulence factors, with keratinase and lipase being the most common. All isolates were resistant to itraconazole, while none were resistant to fluconazole. The metalloprotease gene was detected in *A. flavus, F. verticilliodes,* and *F. oxysporum*.
Conclusion: Our study revealed that the fungi recovered from the swimming pool possessed virulence factors that can enhance their infectivity.

Keywords: *Antibiotics; Fungi; Pools; Virulence Factors; Virulence Genes.*

1. Introduction

A swimming pool is an artificial structure designed to hold water and visited by a variety of people for sport, recreational, educational, exercise or rehabilitation purposes [1-3]. Public pools are usually extra amenities available in recreational complexes [4]. Water supply to pools is usually sourced from municipal public supply or private underground water [5], [6]. Public swimming pools are among the water leisure centers that attracts many admirers. The use of water for recreational purposes can deliver important benefits to health and well-being; however, there may also be potential health hazard associated if the water is polluted or unsafe [7]. Pools are frequently associated with water-borne and water related infections. The pathogens accumulated in the pools often comprise of diversities of microorganisms [8], [9]. Some fungal diseases such as otitis media, eczema and other dermatitis have been traced to contaminated pools [10], [11]. Bathers, who bath in contaminated pools, are susceptible to fungal infections especially at the external ear, inter-digital spaces and on the skin generally. Pools with high temperature encourage fungal contamination.
Different species of fungi cause wide range of human infections that can be transmitted from person-to-person or through other means of transmission [12]. Swimming pools have been reported to serve as reservoir of different agents of human diseases especially in areas where users do not take their bath before and after using the swimming pools [13], [14]. In most cases users are not educated and those with fungal infection on their skins go swimming with other people. Swimming pools allow the survival of pathogenic fungi propagules [15]. Though members of saprophytic fungi, *Aspergillus* and *Fusarium* species have been reported to be responsible for the opportunistic infections of human and the risk of their infections are very high among immunodeficient and immunocompromised subjects. Evidence-based reports have shown that these fungi can be recovered from the surfaces, bottoms and edges of swimming pools [13], [14], [16].
In this study, the occurrence of *Aspegillus* and *Fusarium* species in swimming pools was investigated, considering the public health importance of swimming pools and possibility of habouring potential fungal pathogens as a result of contamination. We focused on these species because of their clinical importance and previous records that have implicated them in different human diseases. In addition, the presence of virulence factors and antifungal resistance in *Aspergillus* and *Fusarium* species isolated from the pools in the study location have been reported. Information on the safety level of the swimming pools in hotels in Ado-Ekiti, the capital of Ekiti State, Nigeria, is also lacking.

2. Experimental (materials and methods)

2.1. Selection of sample sites and sample collection

Hotels in Ekiti State were surveyed and twenty-five (25) hotels having swimming pools were selected based on the accessibility and consent of the hotel management. Water samples from various swimming pools were collected between the hours of 7 to 10 am. Each water sample was collected into sterile sampling bottles. Samples were collected from three different points viz; 20 cm from the water surface, the edges and the floor of the pools. The sample were kept in the ice pack and transferred to the within two hours of collection for examination.

2.2. Isolation of fungi from swimming pools samples

The sample was primarily inoculated onto Potato Dextrose Agar (PDA) supplemented with chloramphenicol and incubated for 48 h at 37℃. Subcultures of distinct fungi colonies were made on PDA supplemented with chloramphenicol to get pure cultures. The Pure cultures were stored in PDA slants at 4 ℃.

2.3. Cultural characterization of fungal isolates

The isolated fungi were characterized based on cultural characteristics and morphological characteristics plus spore type, mycelia and other fruiting bodies in a lactophenol cotton blue wet mount by compound microscope at X100. Detected characteristics were noted and interpreted according to Faiza et al. [17].

2.4. Molecular identification of fungal isolates

The DNA extraction of genomic DNA from the fungi was steered from a week-old PDA culture using DNeasy Plant Mini Kit (supplied by QIAGEN). Primers (ITS 1 and ITS 4) were used to intensify ribosomal interior transliterated spacer (ITS). PCR products were purified using the QIA quick PCR purification kit [18]. The amplicons sequencing was then analysed using MEGA 7 to compare the sequenced amplicons to the GenBank database using BLAST (Basic alignment search tool) to find regions of local similarity between sequences in order to identify the fungal species.

2.5. Detection and estimation of lipase activity among the isolates

As described by Falony et al. [19], lipase activity of the fungal strain isolated from the swimming pools was determined by incubating the test fungi on basal medium contained in ($g{\cdot}L^{-1}$): NaH_2PO_4 (12), $MgSO_4{\cdot}7H_2O$ (0.3), KH_2PO_4 (2), $CaCl_2$ (0.25) and agar agar (15). Also, ammonium sulphate [$(NH_4)_2SO_4$] at 1% and olive oil at 2% were used as nitrogen and carbon sources respectively. Clear zone observed around the fungal growth was noted for lipase production.

2.6. Determination of keratinase production

As described by Shabaan et al. [20], feather meal agar (FMA) was prepared by supplementing Minimal Growth Agar with 10% agar meal. In g/mL, the Minimum Growth Agar contain NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4.7H_2O$, 0.1; agar agar 15. The pH was adjusted to 7 in phosphate basal solution. The fungal isolates were inoculated on the sterile plates of FMA and incubated at 25°C after 72 h. Isolates with degradation halo surrounding it was taken to be positive for keratinase production.

2.7. Detection of laccase in the fungal isolates

Selection for laccase producing organisms was prepared on plates comprising following conformation (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH-6) accompanied with 0.02% guaiacol. The fungi isolates were inoculated into the plates and the plates were incubated at 25°C for 7 days. Laccase activity was visualized on plates. Plates with reddish brown zones in the medium was taking to be positive [21].

2.8. Molecular detection of virulent genes in the fungal isolates

2.8.1. Detection of metalloprotease and protease genes

Molecular detection of metalloprotease and protease genes in the fungi isolated were done from swimming pools by simple PCR on the extracted DNA using metalloprotease and Protease regions specific primers (Primer sequence 5'-3'). Reaction cocktail used for all PCR per primer set included (Reagent Volume µl) - 5X PCR SYBR green buffer (2.5), $MgCl_2$ (0.75), 10 pM DNTP (0.25), 10 pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06) and made up to 10.5 with sterile distilled water to which 2 µL template was added. Buffer control was also added to eliminate any probability of false amplification. PCR was carried out in a GeneAmp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair. Table 1 below shows the primer sequence for the amplification of virulence genes in the fungal isolates isolated from swimming pools.

Table 1: Sequence and Conditions for the Amplification of Virulence Genes in the Fungal Isolates Isolated from Swimming Pools

Virulence Gene	Primer sequence 5'-3'	Conditions
Metaloprotease	Forward - GAG ATG ATG AAG CAG CCC Reverse - CTC GTC CAT GTT TTC AAC	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30 secs, 55ºC for 30 secs. 72ºC for 60 secs and terminate at 72ºC for 10 min.
Protease	Forward - AATCCTTCCGACAGTTCC	

	Reverse ACCCCAAATTCCCTATCTTAC

2.9. Antifungal susceptibility testing of the fungal isolates

Different antifungals containing different concentrations in µg were tested against the isolates. The antifungals include Griseofulvin (25), fluconazole (10), itraconazole (10), ketoconazole (15) and nystatin (100). The method of European Committee on Antimicrobial Susceptibility Testing (EUCAST) was used for the sensitivity test. The Antifungal susceptibility testing was carried out using the disk diffusion method. Disc preparation (5 mm) and was sterilized in oven at 160 ºC for 2 hours. The sterilized disks were impregnated by different concentrations of the antifungals the discs were ascetically placed on plates seeded with spores of the isolates and 0.5 MacFarland standard culture of candidal isolates. The plates were incubated at 25 ºC for 72 h for the molds and 37 ºC for 24 h. Zone of inhibition were measure and results interpreted according to EUCAST standard [22].

3. Results

A total of twenty-five swimming pools were investigated and samples were collected from three different points: surface, base and edge in the pools. The highest number of fungal isolates were isolated from the edge of the pool (n=50), while a total of 30 and 42 fungal isolated from the surfaces and the base of the pools respectively. Seven of the isolates recovered showed greenish to black mycelium with a brown colour on the reverse. The microscopic examination of the seven isolates revealed that they had septate hyphae with colourless hyaline conidiophores and tentatively identified as *A. niger*. Also, sixteen of the recovered fungi isolates were woolly green with a cream reverse and the microscopic examination showed that they had smooth conidia, septate hyphae and were suspected to be *A. flavus*. The identity of the recovered isolates is shown in Table 2.

A number of the isolates showed a flat velvety woolly filament with microscopic showing septate hyphae and was tentatively identified as *F. verticilloides* while two of the isolates were pinkish white with cottony mycelia and red pigmentation on the reserve, and the microscopic examination of the 2 isolates showed the presence of hyaline hyphae septate and branched short conidiophores and were presumptively identified as *F. oxysporum*. Fungal isolates that showed creamy colonies that produces budding and were identified to the genus level and tentatively identified as *Candida* species. Molecular characterization of the isolates further confirmed the identities of the fungi from the swimming pools in hotels in Ekiti State.

Table 2: Identification of the Fungi Isolated from Different Parts of the Swimming Pools

Fungi	Surface	Base	Edge	Total (%)	*Similarity
Aspergillus niger	13 (28.89)	6 (13.33)	26 (57.78)	45 (100.00)	90.2
Aspergillus flavus	13 (36.11)	5 (13.89)	18 (50.00)	36 (100.00)	98.3
Fusarium verticilloides	9 (33.33)	4 (14.81)	14 (51.85)	27 (100.00)	98.0
Fusarium oxysporum	4 (36.36)	2 (18.18)	6 (54.55)	11 (100.00)	97.4
Total	39 (32.77)	17 (14.29)	64 (53.78)	119 (100)	

*Percentage similarity of the representative isolates after molecular characterization

Table 3 shows the results of detection of virulence factor in the fungal strains isolated from the swimming pools. It was observed that the *A. flavus* isolates were able to produce laccase [n=32 (71.11%)], lipase [n=26 (57.78%)] and keratinase [n=32 (71.11%)]. Out of 36 *A. flavus* isolated from the pools only 55.56%, 22.22%, 83.33% were able to produce laccase, lipase and keratinase respectively. Out of the three factors screened for keratinase was the least produced factor. Plates 1 and 2 showed the agarose gel electrophoresis of metalloprotease and protease (met and pro) genes. It is showed in the plate that only four out of the ten selected isolates showed the positive amplification of met gene with band size of approximately 400 bp. None of the isolates was positive for protease gene.

Table 3: Screening for Virulence Factor in Fungi Isolated from Swimming Pools in Ekiti State

Isolates	Virulence factors		
	Laccase	Lipase	Keratinase
A. niger (n=45)	32 (71.11)	26 (57.78)	32 (71.11)
A. flavus (n=36)	20 (55.56)	8 (22.22)	30 (83.33)
F. oxysporum (n=12)	10 (83.33)	8 (66.67)	5 (41.67)
F. verticillioides (n=27)	16 (59.26)	20 (74.07)	13 (48.15)

The results of the antifungal susceptibility tests of the fungal strains from the hotel swimming pool in Ekiti State is shown in Table 4. The results revealed that was 9 (25.00%) of *A. flavus* were resistant to itraconazole, while 7 (19.44%) and 6 (16.67%) were resistant to nystatin and griseofulvin respectively. More than the half of *A. niger* was resistance to griseofulvin, itraconazole and ketoconazole. None of the isolates belonging to the genera *Fusarium* was resistant to both fluconazole and itraconazole. All the *F. oxysporum* isolates were resistant to Ketoconazole.

Table 4: Antifungal Resistance [N (%)] of the Fungi Isolated from the Swimming Pool Cited in Hotels in Ekiti State

Isolates	Antifungals				
	Griseofulvin	Fluconazole	Itraconazole	Ketoconazole	Nystatin
A. niger (n=45)	23 (51.11)	21 (46.67)	26 (57.78)	30 (66.67)	12 (26.67)
A. flavus (n=36)	6 (16.67)	5(13.89)	9 (25.00)	5 (13.89)	7 (19.44)
F. oxysporum (n=12)	3 (25.00)	0	0	11 (100.00)	4 (36.36)
F. verticillioides (n=27)	20 (74.07)	0	0	26 (96.30)	25 (92.59)

4. Discussion

Both *Aspergillus* species and *Fusarium* species were recovered from the samples screened in this study. This presence of fungi in these swimming pools indicate contamination and this pose a major threat to the general populace given the regular use of the swimming pools and also because the pool water can be a reservoir for transmission of fungal diseases. This is in accordance to the investigation of Zayni

et al. [23] who reported that swimming pools may contribute to the spread of fungi that are responsible for different fungal infections. The presence of these saprophytic fungi in swimming pools seems to be an indicator of their resistance to several agents used in treatments of the pool. Itah and Ekpombok [24] and Hossein et al. [25] reported that numerous fungi recognized to be human pathogens have also been obtained from different pools.

Janardhan and Vani [26] in a study carried out in India reported that *Aspergillus* sp., and *Fusarium* sp. had high isolation rate in the swimming pools they sampled. Badiee et al. [27] reported that these cosmopolitan fungi could only cause infections almost exclusively in debilitated patients whose normal defense mechanisms are impaired. The detection of the virulence factors in the isolates showed that some of the isolates are potential pathogens because these factors have been linked with pathogenicity in fungi [27]. The molecular detection of the metalloprotease gene in the isolates further confirm the possible pathogenicity of these isolates.

In this study, the antifungal resistance among the isolates in this study showed a similar trend with the one reported by Davey and Blaxter [28]. The researcher reported that fungal isolates showed resistance to fluconazole, ketoconazole and griseofulvin antifungal with the percentage resistance of 100%. Barben et al. [29] reported that *Aspergillus* sp. possess various resistance mechanisms that enhance the efflux of triazoles outside the fungus cell, keeping their internal concentration low [30], [31]. All these might have contributed to their high resistant rate reported in this study.

5. Conclusion

In this study the association of antifungal resistant *Aspergillus* and *Fusarium* species that also possess virulence factors in swimming pools has been established. This poses a risk to the users especially immune suppressed or immunocompromised individuals. Swimming pools could be a medium of transmitting these fungi. Therefore, continuous surveillance and control should be carried out to reduce the incidence of pathogenic fungal growth in swimming pools.

6. Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

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