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ISOLATION, CHARACTERIZATION AND PATHOGENIC POTENTIAL OF FUNGI FROM WASHING MACHINES

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ABSTRACT

This study aimed to profile the antifungal resistance pattern of fungi isolated from washing machines sampled from hospitals, hotels, student hostels, homes and laundry services shops in Ogbomoso. Isolation procedures for collected samples were carried out using standard microbiological techniques. Fungal isolates were subjected to antifungal drugs to determine their susceptibility pattern. Isolates with multiple resistance ≥ 60 % were selected for molecular characterization. The ability of the selected isolates to produce biofilm was assessed and quantified at 492nm and toxin production was also determined. The potential of the fungi to produce enzymes such as protease, amylase, cellulase, pectinase and collagenase were screened. Questionnaires were given to the staff of hotels and laundry service shops on the treatment of washing machines. A total of 24 fungal isolates belonging to 5 genera of *Aspergillus*, *Trichoderma*, *Candida*, *Cladosporium* and *Alternaria*. *Aspergillus* species were found to be the predominant (70.8%). The highest resistance to anti-fungal drugs employed was from *Aspergillus niger* with 80% resistant. *Aspergillus niger* was the only biofilm producer, belonging to the weak biofilm former. Two isolates out of the selected four fungi were able to produce aflatoxins while they all have the potential to produce more than five enzymes. Both the hotels and laundry services are frequently (82%) treated and their source of water is treated once in a month as reported by 63% of the respondents. Washing machines sampled in this study were reservoirs for multi-anti-fugal drug- resistant isolates, and this could pose serious threat to public health.

Keywords: Washing machine, antifungal, Resistance, Biofilm, Enzymes

INTRODUCTION

Washing machine is regarded as electronic equipment popular nowadays in almost every home, hotels and hospitals for washing of cloth to target clean. In this present time, this appliance is well known due to its great use in washing clothes quickly, thus saving a lot of time and energy as compared to manual washing (Rainer *et al.*, 2020). Nevertheless, as effective as cleaning of clothes and textiles soiled with dirt is, the appliance is not capable of sterilizing these fabrics (Rainer *et al.*, 2020). They are essential household appliances that can provide an ideal breeding ground for microbial growth (Adeoyo and Omolola, 2022). Their warm, moist environment encourages the proliferation of bacteria and fungi. Additionally, detergent and fabric softener residues often accumulate on various parts of the machine, such as the drum, seals, and detergent drawers, contributing to microbial growth. Although, washing process by this appliance is expected to deliver hygienically and visually clean laundry but microorganisms can gain entry into the washing machine through water from effluent discharge, household linen and worn clothing (Teufel *et al.*, 2010). Various researches have shown that some microorganism can attach themselves to clothes from washing machine during laundry process. Dirk *et al.* (2019) noted that some microbial infections of the skin such as rashes, nosocomial infections are attributed to microorganisms associated with washing machines. Washing machines present suitable conditions for fungal colonization, which could pose health risks. Washing machines have become a focus of research due to their potential as a habitat for fungal growth, the spread of fungi and diseases caused by these fungi (Elsheikh *et al.*, 2023). Fungi have the potential to survive in both visible and hidden parts of washing machines when the environmental conditions, that include low washing temperatures, moisture, and the use of biodegradable detergents, enhances their growth, thereby leading to the formation of biofilm and fungal proliferation (Whitehead *et al.*, 2022).

Biofilm refers to the complex communities of microbes that may be found attached to a surface or may form aggregates without adhering to a surface and buried firmly in an extracellular matrix (ECM) (Haaber *et al.*, 2012). The biofilm lifestyle allows microorganisms to withstand hostile environmental conditions like starvation, desiccation and makes them capable of causing a broad range of chronic diseases (Haaber *et al.*, 2012). Biofilm formation may have detrimental effects in healthcare, drinking water distribution systems, food industries (Ali *et al.*, 2023). They are known to cause nosocomial infections and chronic illness, food spoilage, industrial pipe fouling, contamination of food products (Ali *et al.*, 2023). Biofilms contain either the homogenous or heterogeneous populations of different microorganisms especially bacteria which remain in the matrix made up of extracellular polymeric substances secreted by component population of the biofilm (Zhang *et al.*, 2022). Some populations of biofilm-associated bacteria exhibit antibiotic resistance (Vasudevan, 2014). The formation of microbial biofilms can be described in three main stages - attachment, growth (maturation), and detachment (Zhang *et al.*, 2022). Biofilms harbour microorganisms which may act as reservoirs, contaminating laundry and posing a threat to individuals, especially those with immune compromised systems. A study by Nix *et al.* (2015) identified the Basidiomycota and Ascomycota fungal groups as the most common fungal colonizers in washing

machines. Species of *Aspergillus, Cladosporium, Penicillium, Fusarium, Rhizopus, Alternaria and Cephalosporium* are commonly isolated from washing machines.

Multiple studies have highlighted the role of fabrics and textiles in the transmission of infections, suggesting that microbial respiratory infections could spread via washing machine use during laundering (Rawson *et al.,* 2022). Fungi in washing machines can spread into other areas, such as kitchens or surrounding environments, through aerosols, wastewater, and direct contact between contaminated machines and users' hands (Tischner *et al.,* 2019). The conditions inside washing machines, with moisture, humidity, and nutrient availability, create an environment conducive to fungal growth, deterioration of materials, and discoloration. These machines are often exposed to fluctuating temperature and humidity levels, with residual moisture that may persist. Additionally, organic matter such as lint and detergent residues in WMs can serve as nutrient sources for fungal growth (Adeoyo and Omolola, 2022). Thus, this present study aimed to isolate, characterize and evaluate the pathogenic potentials of fungi associated with washing machines.

MATERIALS AND METHODS

Collection of Samples

Samples were collected from 35 different washing machines by using sterilized swab stick to swab the inner tub of these machines and then introduced into 10 ml of sterile peptone water and then incubated overnight at 37^0C. For isolation, Potato Dextrose Agar (PDA) was used for the isolation of fungi. Standard microbiological procedures using pour plate method for the isolation of fungi was used.

Identification of Fungal Isolates

The isolated fungi were identified according to their macroscopic and microscopic structures. Slides of isolated pure fungal cultures were prepared in lactophenol – cotton blue stain for identification purpose. The slides were observed under oil immersion microscope at x100.

Assay for Antifungal Activity

Antibacterial Susceptibility Testing

Antibiotic susceptibility testing of fungi was carried out on freshly prepared Mueller Hinton agar (MHA) (NEOGEN, UK). The isolates' spores were serially diluted to obtain the McFarland Standard of 0.5; these were introduced on the MHA with sterile swab sticks and allowed to set for 30 minutes before anti-fungal disc was placed on it (Ayandele *et al.,* 2019; Agboola *et al.,* 2021). Antifungal disc used include Miconazole (50 µg), Econazole (50 µg), Ketoconazole (50µg), Clotrimazole (50µg) and Amphotericin B (100µg), all were products of Bio-Rad (France). The zones of inhibition were measured according to the method described in Clinical and Laboratory Standard Institutes (CLSI, 2010) to determine sensitive, intermediate or resistant patterns

Determination of Biofilm Production by Fungal Isolates

The abilities of fungal isolates to produce biofilm were determined with the methods of Shuwa and Rao, (2017) as described by Amao *et al*. (2019).

OD_{cut}=OD_{avg} of negatives control + 3xs S.D of ODs of negatives control.

OD= OD_{cut} (Non biofilm formers)

OD $< OD \leq 2 \times ODcut$(weak biofilm formers)

$2\times OD_{cut} < OD \leq 4 \times OD_{cut}$ (moderate biofilm formers)

OD $> 4 \times OD_{cut}$ (strong biofilm formers).

Molecular Characterization of Selected Organisms

Molecular characterization of isolates with high anti-fungal activities was carried out using 18SDNA gene; using the primer pair ITS1 (F): TCCGTAGGTGAACCTGCGGG and ITS4 (R): TCCTCCGCTTATTGATATGC. Phylogenetic relationships of the isolates were defined by the obtained 18SDNA gene sequence compared with those of other fungi in the NCBI Gene Bank, using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A neighbour-joining phylogenetic tree was constructed for the isolates using MEGA X software.

Aflatoxin Test of Fungi Isolates

Ammonia Vapor Test

The fungal isolates were inoculated on desiccated coconut agar medium as single colonies using cork borer of diameter 5 mm at the center of plate and was incubated in the dark at 28 °C for 7 days. The petri dishes were inverted and 2 drops of concentrated ammonium hydroxide solution was placed on the lid of the dish. Then the Petri dish was then inverted over the lid containing the ammonium hydroxide. Aflatoxigenic isolates change pink to red colour colonies in inverted petri dish (Raed *et al*., 2016).

Fungal Enzymatic Screening

Lipase Assay

The culture medium for screening lipase contains peptone (10 g/L), NaCl (5 g/L), $CaCl_2{\cdot}2H_2O$ (0.1 g/L), and agar (16 g/L) and autoclaved at 121 °C for 20 min. Ten milliliters of Tween-20 was also autoclaved separately and was added into the medium and inoculated with 5 mm of mycelia of isolated fungi. After incubating for 5 days at 25 °C, the lipolytic activity was indicated by the appearance of a visible precipitate. All the assays were done in triplicate.

Cellulase Assay

Cellulase activities of the fungal isolates were determined by plate screening medium (PSM) that contained Mendel's Mineral Salt (g/L): Urea -0.3, $(NH_4)_2SO_4$ -1.4, KH_2PO_4- 2.0, $CaCl_2$- 0.3, $MgSO_4$ - 0.3, yeast extract- 0.25 and protease peptone -0.75 with 10 g L-1 of carboxymethyl cellulose (CMC) and 17.5 g L-1 agar (Mandels, 1974). Agar blocks (8 mm in diameter) from one-week old fungal colony grown on PDA plates were cut and inoculated into the centre of the basal media plates. The plates were incubated at 25±2 °C for seven days. Cellulolytic fungal species was selected based on the diameter of the zone of hydrolysis surrounding the colonies. For observations, plates were stained with 1% Congo red dye (30 min), followed by destaining with 1 M NaCl solution for 20 min. clear zones could be observed only around colonies of the active fungal strains.

Protease Assay

Protease activity of isolated fungal was evaluated on Yeast Peptone Dextrose (YPD) agar medium supplemented with 0.4% (w/v) of gelatin (Sigma-Aldrich, USA). The plates were inoculated with 5 mm of mycelia from fungal isolate. After incubating for 5 days at 25 °C, the plates were then flooded with saturated aqueous ammonium sulfate. The clear zone around the fungal colony indicates the hydrolysis of gelatin. This assay was done in triplicate for all experiment.

Questionnaire Administration

The analysis of responses of users of washing machine in the laundry services and in hotels and suites were also investigated. Questionnaire which was a part of the research procedure was given to all selected and suites and

laundromats. This helps to gather adequate information and ensures more credibility and authenticity to this work. Information related to factors that could contribute to cross contamination were assessed in the sampling sites.

Statistical Analysis

The data were analyzed on the average of three replicates obtained from independent determinations. Statistical analyses of these averages were analyzed using one-way analysis of variance (ANOVA) and were carried out with IBM SPSS version 20 software at 95% significance level and Microsoft Excel 2007 version. Charts were drawn for all values of analysis using the Microsoft Office Excel 2007 version.

RESULTS AND DISCUSSION

Isolation of Microorganisms from Washing Machine

A total of twenty-four fungi were isolated from 35 different washing machines under study. These belong to 5 different genera namely; *Aspergillus, Candida, Trichoderma, Alternaria* and *Cladosporium* .Table 1 shows the morphology and microscopic identification of fungi isolates from these samples.

Prevalence of Bacteria and Fungi Isolates

Aspergillus spp was found to be most dominated genus representing 70.83% while *Alternaria* spp and *Cladosporium* spp were the least dominant genus in the fungi group (Figure 1).

Antimicrobial Susceptibility Testing of Isolated Microorganisms

The antifungal susceptibility pattern to antifungal agents was represented in Table 2. Multiple antifungal resistance was observed for all isolates. Miconazole was least sensitive to all fungi isolates (91.67%) while Econazole and Ketoconazole were most sensitive to all fungi isolates (4.17 %).

Molecular Characterization and Phylogenetic Relatedness of Selected Fungi

The molecular characterization and phylogeny with closest isolates by maximum-likeliwood from NCBI for four (4) selected fungi in this study were represented below. These were identified belong to the genera of *Aspergillus* and *Trichoderma.* Figure 2 show the phylogenetic relatedness of selected fungi with closest isolates from NCBI.

Potentials of Isolated Microorganisms to Produce Biofilm

Table 3 represents the abilities of fungi isolates to produce biofilm. Only *Aspergillus niger* produces biofilm with forming potentials 0.042 ± 0.0047 among the selected fungi and it is a weak biofilm producer.

Aflatoxin Screening

The result of fungi toxin screening by ammonia vapour test by the four selected fungi isolates are represented in Table 4. *Trichoderma harizianum* and *Aspergillus aculeatus* were found not to produce aflatoxin by ammonia vapour test.

Fungi Enzymes Activity

The Table 5 shows the result of enzymes activity of selected fungi conducted in this study. All the selected fungal isolates were able to produce 3 to 5 of the enzymes tested for.

Table 1: Morphological and Microscopic Identification of Isolated Fungi

Isolates	Pigmentation	Form	Elevation	Margin	Surface	Spore	Probable Identity
DTL 10^{-3b}	Yellow	Irregular	Raised	Entire	Rough	Conidiospore	*Trichoderma harzianum*
AAL-3	Inner Margin Green, Outer Margin White	Circular	Raised	Entire	Rough	Zygospore	*Aspergillus aculeatus*
WLS 10^{-3}	Whitish Brown	Circular	Raised	Lobate	Rough	Acospore	*Aspergillus brasiliensis*
BLS 10^{-3}	Cream	Irregular	Umbonate	Lobate	Dull	Zygospore	*Candida parapsilosis*
KLS-3	Black	Circular	Raised	Lobate	Rough	Zygospore	*Aspergillus niger*
DTL 3a	Pale Green	Irregular	Raised	Entire	Dull	Conidiospore	*Apergillus flavus*
HCL 3	Brown	Circular	Flat	Lobate	Dull	Acospore	*Aspergillus fumigatus*
NHL 3	Black	Filamentous	Umbonate	Entire	Rough	Conidiospore	*Cladosporium* spp
HCR 3b	Black	Rhizoid	Convex	Undulate	Rough	Conidiospore	*Alternaria* spp
SUN 10^{-3b}	Black	Irregular	Raised	Curled	Smooth	Zygospore	*Aspergillus niger*
DTL-3c	Whitish Brown	Circular	Raised	Lobate	Rough	Acospore	*Aspergillus brasiliensis*
NHL-3c	Yellow	Irregular	Raised	Entire	Rough	Conidiospore	*Trichoderma harzianum*
SUN 10^{-3a}	Inner Margin Green, Outer Margin White	Circular	Raised	Entire	Rough	Zygospore	*Aspergillus aculeatus*
NHL 3a	Black	Irregular	Raised	Curled	Smooth	Conidiospore	*Aspergillus niger*
HCR 3a	Black	Irregular	Raised	Curled	Smooth	Conidiospore	*Aspergillus niger*
HGH 10^{-3}	Black	Irregular	Raised	Curled	Smooth	Conidiospore	*Aspergillus niger*
MSH 10^{-3}	Black	Irregular	Raised	Curled	Smooth	Conidiospore	*Aspergillus niger*
HCR 10^{-3}	Whitish Brown	Circular	Raised	Lobate	Rough	Acospore	*Aspergillus brasiliensis*

Isolates	Pigmentation	Form	Elevation	Margin	Surface	Spore	Probable Identity
CCL 10^{-3}	Creamy	Irregular	Umbonate	Lobate	Dull	Zygospore	*Candida parapsilosis*
JFH 10^{-3b}	Black	Irregular	Raised	Curled	Smooth	Zygospore	*Aspergillus niger*
JFH-3a	Creamy	Irregular	Umbonate	Lobate	Dull	Zygospore	*Candida parapsilosis*
NHL 3b	Inner Margin Green, Outer Margin White	Circular	Raised	Entire	Rough	Zygospore	*Aspergillus aculeatus*
RES 10^{-3a}	Black	Irregular	Raised	Curled	Smooth	Zygospore	*Aspergillus niger*
TWM 10^{-3}	Black	Irregular	Raised	Curled	Smooth	Zygospore	*Aspergillus niger*

KEYS: AAA = Adeniji Ajoke Adenike Sample; AAL = A.A. Luxury Suite Sample; AAU = Adeshina Adeola Underg Sample; AGV = Amazing Grace Villa Underg Sample; BCH = Brachan Hostel Sample; BLS = Beth Laundry Service Sample; BTH = Bowen Teaching Hospital Sample; CCL = Crystal Clean Laundry Sample; DPH = De-Pillar Hotel Sample; DTL = Divine Touch Laundry Sample; GDC = Goshen Dry Cleaning Sample; GHS = General Hospital Sample; HCR = Hossana Cleaning Route Sample; HWL = Household Washing Machine Isale General Sample; HGH=Hecky Guest House Washing Machine; IHH =Imperal Heritage Hotel Sample; JAL = Joland Adenike Laundry Sample; JFH =Jesutofunmi Household Sample; JHL=Joke Hostel Sample; JPH=Joseph Peters Hostel Sample; KHW=Khossana Household Sample; KLS=Kleanit Laundry Sample; LAA= A.A Laundry Underg Sample; LTH= Lautech Teaching Hospital Sample; LHC=Lautech Health Centre; NHL=Nest Hotel Laundry; MHS=Mainspring Hospital Sample; MLS=Mountain Laundry Services Sample; RCH=Royal Crown Hotel; RES=Ragaray Executive Suite Sample; SUN=Sunsun Household Sample; TWM=TOSIN Household Washing Machine; VHH=Vicky Householld Sample; WLS=Wonderland Laundry Services

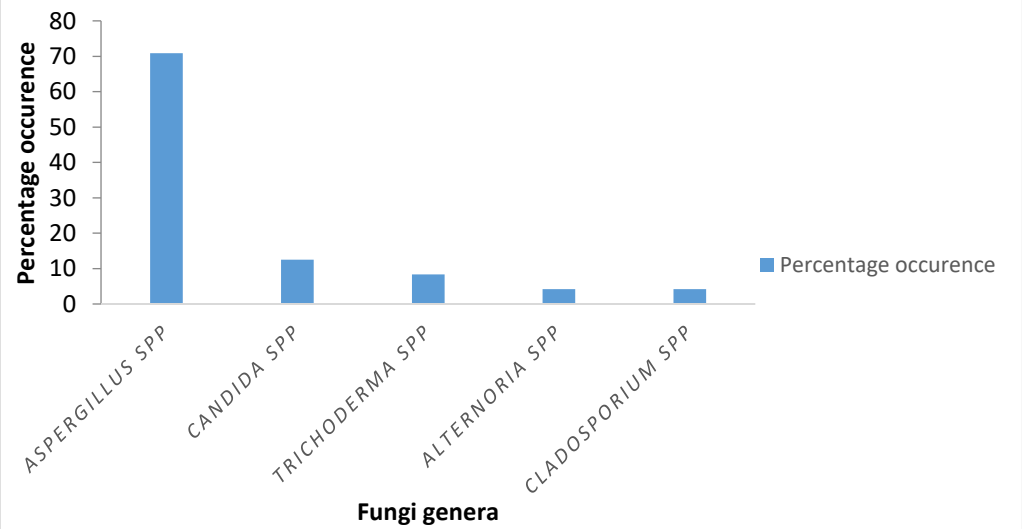

Figure 1: Prevalence of Different Genera of Fungi Isolated from Washing Machine

Table 2: Antifungal Susceptibility Test

ISOLATES		MCZ (50µg)	EC (50µg)	KET (50µg)	CTR (50µg)	AB (100µg)	Resistance (%)
Trichoderma harzianum	DTL 10^{-3b}	R	S	I	R	R	60.00
Aspergillus aculeatus	AAL-3	R	S	S	I	R	40.00
Aspergillus brasiliensis	WLS 10^{-3}	R	S	I	R	R	60.00
Candida parapsilosis	BLS 10^{-3}	R	I	I	R	I	40.00
Aspergillus niger	KLS-3	R	S	R	R	R	80.00
Apergillus flavus	DTL 3a	R	S	S	R	R	60.00
Aspergillus fumigatus	HCL 3	R	S	S	I	I	20.00
Cladosporium spp	NHL 3	R	S	S	S	I	20.00
Alternaria spp	HCR 3b	R	S	S	I	R	40.00
Aspergillus niger	SUN 10^{-3b}	R	S	I	R	R	60.00
Aspergillus brasiliensis	DTL-3c	R	S	S	I	R	40.00
Trichoderma harzianum	NHL-3c	R	S	S	I	R	40.00
Aspergillus aculeatus	SUN 10^{-3a}	R	S	S	R	R	60.00
Aspergillus niger	NHL 3a	R	I	S	R	R	60.00
Aspergillus niger	HCR 3a	R	S	I	R	R	60.00
Aspergillus niger	HGH 10^{-3}	R	S	S	R	R	60.00

Aspergillus niger	MSH 10^{-3}	R	S	I	R	R	60.00
Aspergillus brasiliensis	HCR 10^{-3}	I	S	S	I	R	20.00
Candida parapsilosis	CCL 10^{-3}	R	S	S	R	R	60.00
Aspergillus niger	JFH 10^{-3b}	S	S	S	R	R	40.00
Candida parapsilosis	JFH-3a	R	I	S	S	R	40.00
Aspergillus aculeatus	NHL 3b	R	R	S	I	R	60.00
Aspergillus niger	RES 10^{-3a}	R	S	S	R	R	60.00
Aspergillus niger	TWM 10^{-3}	R	S	S	R	I	40.00
Number of resistances		22	1	1	15	20	
Percentage resistance		91.67	4.17	4.17	62.50	83.33	

Ketoconazole; CTR: Clotrimazole;AB: Amphotericin B.
KEYS: R: Resistant; S: Sensitive; I: Intermediate; MCZ: Miconazole; EC: Econazole; KET:

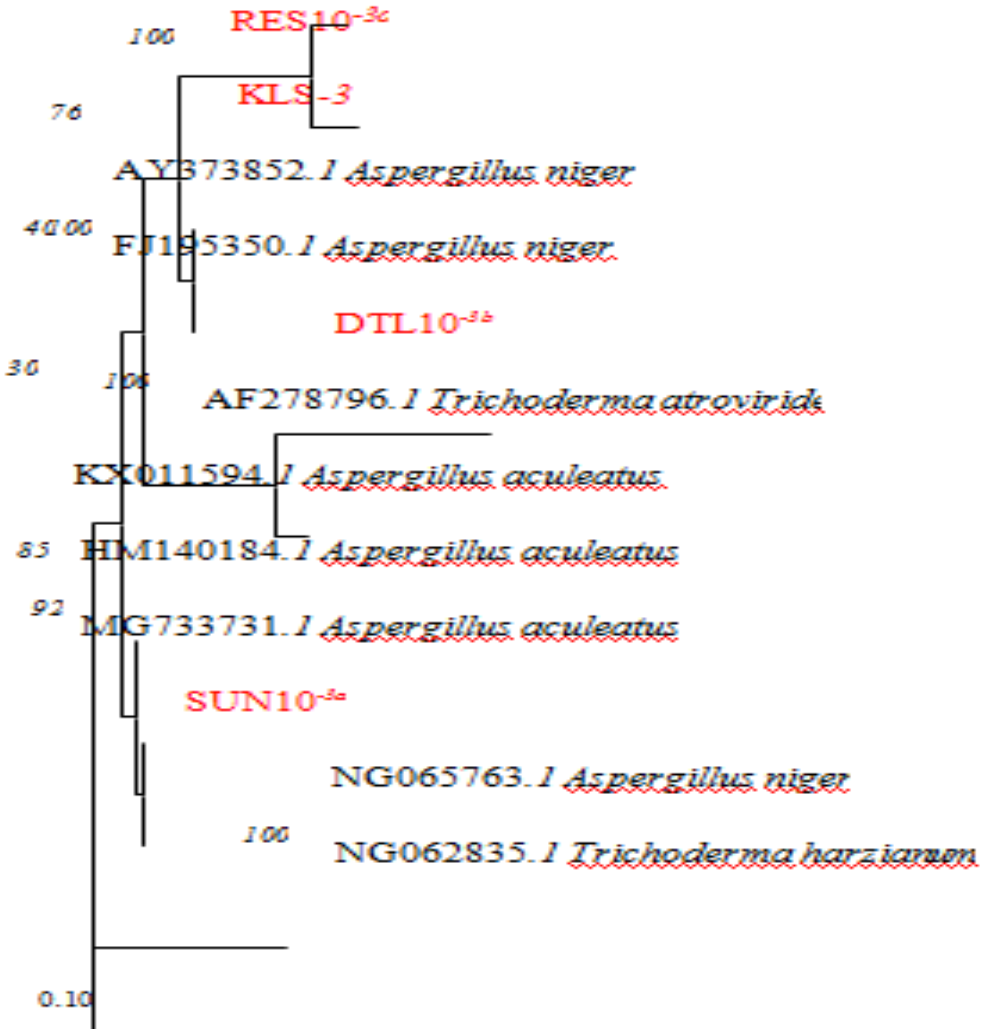

Figure 2: Phylogenetic Tree of Fungal Isolates by Maximum-likelihood with Closest Isolates from NCBI

Table 3: Biofilm Production Potentials of Selected Fungi

Probable Identity	**Mean ± SD**	**Biofilm forming group**
Trichoderma harizianum	0.007 ± 0.0035	None
Aspergillus niger	0.042 ± 0.0047	Weak
Aspergillus aculeatus	0.001 ± 0.0013	None
Aspergillus niger	0.006 ± 0.0031	None
Control	0.142 ± 0.0005	None

Table 4: Aflatoxin Screening by Ammonia Vapour Test

Identity	Screening result
Trichoderma harizianum	-
Aspergillus niger	+
Aspergillus aculeatus	-
Aspergillus niger	+

Keys: + = Positive screening; - = Negative screening

Table 5: Enzyme Assay for Selected Fungi Isolates

Identity	Cellulase	Protease	Lipase	Pectinase	Keratinase	Collagenase
Trichoderma harizianum	+	+	-	-	+	-
Aspergillus niger	+	+	+	+	+	-
Aspergillus aculeatus	+	-	+	-	-	+
Aspergillus niger	-	+	+	+	-	-

Keys: + = Positive reaction; - = Negative reaction

Table 6: Responses Analysis of Users of Washing Machine in the Laundry Services

S/N	Items	Description of variables	Frequency (n = 11)	Percentage (%)	% Total
1.	**Size of factory**	Large	2	18%	
		Medium	6	54%	
		Small	3	28%	
					100%
2.	**Type of washing machine**	Top loader	8	72%	
		Front loader	3	28%	
		Both	-	-	
					100%
3.	**No of cloth washed daily**	< 50	10	91%	
		51 – 100	1	9%	
		101 – 150	-	-	
					100%
4.	**Use of washing machine**	Once a day	2	18%	
		Twice a day	5	45%	
		Thrice a day	4	37%	
		Twice a week	-	-	
					100%
5.	**How dirty is cloth**	Very dirty	1	9%	
		Moderately dirty	4	37%	
		Mild dirty	6	54%	
					100%
6.	**Temperature choice of washing machine**	High temperature	2	18%	
		Low temperature	9	82%	
					100%
7.	**Expertise of use**	Very expert	7	63%	
		Expert	4	37%	
		Non expert	-		
					100%
8.	**How often is cleaning and sterilizing machine**	Everyday	2	18%	
		Frequently	9	82%	
					100%
9.	**Treatment of water**	Once a month	4	63%	
		Once in three months	7	37%	
		Once in six months	-	-	
		Once a year	-	-	
					100%

Table 7: Responses Analysis of Users of Washing Machine in Hotels and Suites in the Study Location

S/N	Items	Description of variables	Frequency (n = 7)	Percentage (%)	% total
1.	**Hotel rating**	5 star	7	100%	
		4 star	-		
		3 star	-		
		2 star	-		100%
		2 star	-		
		No star	-		

S/N	Items	Description of variables	Frequency (n = 7)	Percentage (%)	% total
2.	**Type of washing machine**	Top loader	2	28.6%	
		Front loader	2	28.6%	
		Both	3	42.9%	100%
3.	**No of rooms in the hotel**	< 50	7	100%	
		51 – 80	-	-	
		81 - 100	-	-	100%
4.	**No of customers using the hotel per day**	>500	-		
		400 - 499	-		
		<300	7	100%	100%
5.	**Type of people using the facilities such as beddings**	Learned	3	57.1%	
		Moderately learned	4	42.9%	
		Not learned	-		100%
6.	**Use of washing machine**	Once a day	5	71.4%	
		Twice a day	2	28.6%	
		Thrice a day	-		
		Twice a week	-		100%
7.	**Expertise of use**	Very expert	6	85.7%	
		Expert	1	14.3%	
		Non expert	-		100%
8.	**Cleaning of beddings**	Everyday	7	100%	
		Three times a week	-	-	
		weekly	-	-	100%
9.	**Change of beddings**	Once daily	3	42.9%	
		Twice a day	4	57.1%	
		Twice a week	-		100%
10.	**How often is cleaning and sterilizing machine**	Everyday	2	18%	
		frequently	5	82%	100%
11.	**Treatment of water**	Once a month	4	63%	
		Once in three months	3	37%	
		Once in six months	-	-	
		Once a year	-	-	
					100%

Discussion

The use of washing machines in homes, laundromats and hospitals continue to rise and can be a reservoir for many pathogenic microorganisms of public health concern. So, this study is carried out on the potentials of fungi isolated from washing machines to produce biofilm, susceptibility to antifungal agents and their enzymatic activities. In this study, twenty-four fungi were isolated from these washing machines belonging to 5 different genera namely; *Aspergillus, Candida, Trichoderma, Alternaria* and *Cladosporium.* Many parts of domestic washing machines offer ideal living conditions for microorganisms that include fungi (Babic *et al.,* 2020). Prevalent genera of fungi identified in this study have been implicated to cause varying degree of infection in human population. *Aspergillus* produces aflatoxin which is toxic and affects human systems such as respiratory, nervous and reproductive systems. *Aspergillus* species are the most prevalent fungal species isolated from this study. Also, in a study carried out by Jacksch *et al.* (2021), they isolated *Aspergillus* as the most abundant genus from the washing machines. *Aspergillus* sp. are saprophytic fungi and can recycle organic debris. *A. fumigatus* is a prevalent airborne fungal pathogen that can cause severe infections in immune-compromised people. They largely enter the machine via soiled clothing, tap water, and maybe also from air (Babic *et al.,* 2020).

In this study, five different genera of fungi were recorded in all samples collected. The reasons for this might be due to the level of hygiene practiced in the use of the washing machine, disinfection techniques employed and migration of the microorganisms through the airflow leading to microorganisms developing resistance and the transfer of resistant gene. The sources of such contamination could be cross-contamination from a patient's flora, health care workers' hands, contaminated storage carts, or due to contamination during the washing process especially that of bed linens (Shemse *et al.,* 2020). This could be linked to the fact that the most hospital environments, particularly sinks, are conducive for the persistence of fungi (Parcell *et al.,* 2018).

Fungal isolates showed very high resistance to miconazole (91.67 %), followed by amphotericin B (83.33 %). The genus *Aspergillus* was observed to have highest resistance to all antifungal agents used. Antibiotic resistance by isolated microorganisms is of greater danger to worldwide public health (WHO, 2014). The epidemiological and clinical significance of fungi genera in this study such as *Aspergillus* and *Trichoderma* are based on their capacity to overcome antimicrobial actions. These microorganisms are also shown to have higher resistance to antimicrobial agents with wide distribution and pathogenicity (de Souza *et al.,* 2012). Isolated microorganisms from washing machine are common pathogens causing infection in human population and they have become relevant in hospitals (nosocomial infections) such as rash, skin infections and other related infections.

From the results of this finding, it was only *Aspergillus niger* that is biofilm former and belongs to a weak biofilm former group. Biofilms are symbiotic communities of bacteria that reside on surfaces within different settings, including washing machines (Ali *et al.,* 2023). The microorganisms that comprise biofilms are notably more tolerant to antimicrobial agents, and are significant cause of recurrent human infections (Lebeaux *et al.,* 2014). Biofilms have also long been implicated in washing machine malodor caused by fungi (Stapleton *et al.,* 2013). Due to the potential for biofilm formation within a typical unsanitized public washing

machine, it is possible that microbial exchange between the washing machine and the textiles being laundered might occur during routine use. Biofilm formation within washing machines is a concern, not only due to the possible presence of malodor-associated microorganisms, but also the possible presence of pathogenic (Copeland and Purvis, 2017) and drug-resistant microorganisms in such biofilms (Johani *et al.,* 2017). In addition, the occurrence of many different species at a given site might increase interspecies communication and cross-feeding and positively affect biofilm biomass (Zupancic *et al.,* 2018). In addition, microbial biofilms might serve as reservoirs for potentially pathogenic microorganisms that might contaminate the laundry and thereby pose a health threat for susceptible persons (Jacksch *et al.,* 2021). The occurrence of many different species at a given site might increase interspecies communication and cross-feeding and positively affect biofilm biomass (Zupancˇic *et al.,* 2018).

There is a threat to human health due to the production of aflatoxin by microorganisms, because there is a correlation between aflatoxin exposure and death, the death rate for high dose exposure is around 25% (Marchese*et al.,* 2018). Chronic low-level exposure to aflatoxin, in particular aflatoxin B1, may lead to hepatocellular carcinoma, or liver cancer, malnutrition, and stunted growth in children (Ammann, 2003). The signs of aflatoxicosis are extremely diverse due to the chronic exposure aflatoxin (Marchese *et al.,* 2018).

Various enzymes are incorporated in the formation of detergent. Hence the study on potentials of fungi isolates to produce any of such enzymes such as protease, lipase, pectinase, cellulase, keratinase and collagenase were also investigated. All fungi isolates have the potential to produce one or more enzymes. This study is particularly important because enzymes have been reported to play an essential role and has the potential to increase virulence in pathogens and pathogenicity in cells that are directly related to clinical symptoms. Fungi colonization in parts of washing machines such as inner tub and rubber seal is a growing concern, thus creating an ideal environment for fungal growth, particularly species like *Aspergillus* and *Penicillium*, leading to health threat. Hence, maintaining proper hygiene and regular cleaning routines is important to prevent fungal buildup in washing machines.

The interconnection between mycotoxin and enzyme production and resistance to different antifungal agents by isolated fungal isolates is a complex relationship. *Aspergillus niger* isolated in this study represent one of the major mycotoxin-producing fungi especially the aflatoxin class (Marin *et al.,* 2013). The production of certain enzymes by *Aspergillus* spp. in this study can facilitate the breakdown of cell walls which can lead to the biosynthesis of aflatoxins. Fungi which showed higher resistance to antifungal agents may possibly produce toxins since those organisms can survive in the environment where susceptible fungi would possibly be inhibited. Also, certain enzymes such as cellulase, protease, lipase, pectinase and keratinase produced by these fungal isolates have the ability to break down or modify antifungal agents thereby contributing to resistance and transfer of resistance genes. A similar study was conducted by Schaller *et al. (*2018*),* also showed the ability of fungal enzymes to show resistance by breaking down or modifying antifungal agents. The ability of fungal isolates such as *Aspergillus niger* to form extracellular matrix on various surfaces can make them persist, difficult to treat and increase resistance, thus leading to increased virulence. Fox *et al.* (2014), also demonstrated the role of biofilm produced by fungi isolates in antifungal resistance.

Questionnaires distributed to users of washing machines in hotels and suites and laundromats featured questions related to the practice, expertise and hygiene level and water used in the laundry process. In this survey, it was observed that thorough cleanliness of machines was not performed by users and water used are mostly not treated. Eighty-two percent (82%) respondents agreed that they do not wash their washing machine every day (Table 6 and Table 7). The frequency of use and how dirty is the clothes, people using the hotel facilities among others could be contributing factors in the transfer of microorganisms. This could also culminate to persistence of microorganisms with potentials for pathogenicity.

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