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Invasive Factors Recognition in *Aspergillus* Section Nigri Isolates from Patient and Environmental Samples in the Centre Region, Cameroon

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TC and DJP conceived and designed the experiments. Author EAI performed the experiments. Authors DJP and EAI analyzed the data. Author EAI drafted the manuscript. Author DJP finalized the paper. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Aspergillus* section *Nigri* species are invasive opportunistic pathogens, seen in individuals with various immune disorders. The invasive capacity involves the production of varieties of enzymes such as lipases, phospholipase, proteases and hyaluronidase. The determination of proteinase, phospholipase, esterase and biofilm production in patient and environmental isolates approve the pathogenic strength of the species.
Aims: To evaluate the invasive capacity of *Aspergillus* section *Nigri* isolates from patients and environmental samples.
Methods: Our study is cross sectional and experimental, performed at the outpatient clinic of the otorhinolaryngology department of Central and University teaching hospital during a period of 12

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months from March 2018 to February 2019. To determine the invasive capacity of *Aspergillus* section *Nigri* species, 400 samples were evaluated in the study (that is; 250 from patients and 150 samples from environment). Patient samples and hospital environment samples were evaluated by standard phenotypic methods for detecting of Proteinase, phospolipase, esterase and biofilm. The variables were statistically analyzed using Chi-square test of independent and SPSS (Version 16.0).

Results: The isolates recovered from the patient sample shows maximum invasive capacity as compared to the environmental isolates, that is for 44 isolates; 42 isolates showed proteinase activity and biofilm production, followed by phospholipase activity 36, and then esterase 32.The isolates recovered from the hospital environment also showed the production of the various invasive factors, that is for 16 isolates; 15 isolates showed biofilm production, followed by proteinase activity 6, phospholipase 5 and esterase 4.The disparities of the invasive capacity in patient and environment isolates virulence were statistically significant for proteinase, phospholipase and esterase (that is; p-value <0.05). Majority the isolates recovered from patients and the environment were potential producers of biofilm.

Conclusion: The isolates recovered from patients sample showed high invasive capacity as compare to the isolates recovered from the environment. This highlights the implications of phospholipase enzyme, proteinase enzyme, esterase enzyme and biofilm used by *Aspergillus* section *Nigri* isolates as means of survival in the host system.

Keywords: Aspergillus section Nigri; proteinase; biofilm; phospholipase; esterase.

1. INTRODUCTION

Aspergillosis is a fungi infection caused by *Aspergillus* species, which posed severe health disorder, with high unwholesome and dead which can be attained via inbreathing especially in immune depressed patients (patients with acute leukemia, transplant recipients, autoimmune diseases, and Acquired Immune Deficient Syndrome (AIDS)) [1,2,3]. *Aspergillus* species are widespread in the environment; in which their growth and reproduction occurs in the soil, dust and decomposing organic matter under favorable conditions such as; the presence of nutrients and appropriate temperature, pH and humidity [1]. The incidence of *Aspergillus* species as opportunistic fungal pathogens has emerged due the increase in predisposing factors such as; diabetes, use of hearing aids, HIV-AID, use of antibiotic drugs [3,4].

Several studies have demonstrated that 80% of *Aspergillus* infections are caused by *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus tereus*, and *Aspergillus nidulans* [4,5]; where *Aspergillus niger* is ranked as third *Aspergillus* pathogen involved in causing fungi infections to humans [3,6,7]. *Aspergillus niger* is also classified as the third prevalent species which causes pulmonary aspergillosis (especially Broncho pulmonary, aspergilloma and invasive aspergillosis) and otomycosis [3,6,8,9,10].

Moreover, the clinical involvements of other species in *Aspergillus* section *Nigri* are not generally reported and they are generally classified as *Aspergillus niger* [3,11]. Recent studies have approven the implication of other species in *Aspergillus* section *Nigri* causing infection to humans [12,13], these include; *Aspergillus tubingensis*, *Aspergillus brasiliensis* and *Aspergillus foetidus* forming a species complex called *Aspergillus* section *Nigri* [14]. Some studies have demonstrated that for *Aspergillus* species to invade the host barrier, they must be capable of producing invasive factors which enhance their pathogenicity [3,15]. The following factors has been identified as invasive factors for *Aspergillus* species such as adhesins, hydrolytic enzymes such as proteases, phospholipases, ribonucleases, restrictocin; catalases, superoxide-dismutases, mycotoxins and low-molecular-weight non-protein metabolites [3,15,16,17].

Though some species belonging to the genus *Aspergillus* section *Nigri* are used as producers of industrial enzymes and metabolites in food processing, some species are known as potential producers of mycotoxin and toxin which has effect on the nephron, immune system and capable of causing cancer in animals [3,7].

This study was carried out to evaluate the pathogenic strength between the isolates of *Aspergillus* section *Nigri* isolated from patient and environment.

2. MATERIALS AND METHODS

2.1 Study Group

We carried out a cross sectional study on 250 patients clinically diagnosed to have otomycosis infection. This was achieved by visiting the outpatient clinic of the otorhinolaryngology department weekly during a period of 12 months from March 2018 to February 2019 at the Central Hospital and University Teaching Hospital, Yaoundé, Cameroon. General information like age, sex, occupation, diabetic status, trauma, history of ear surgery or any fungal infection in other parts of body and laterality of symptoms were recorded. Any history of habits like use of oils/ear drops; wooden sticks or metal wax picks for removal of wax were also recorded.

2.2 Inclusion Criteria

All patients clinically diagnosed of otomycosis of age above one year presenting symptoms like itching, pain, feeling of blocked ear, tinnitus, deafness, discharge and in which otoscopic examinations reveals wet or dry masses of hyphae/spores were included in the study.

2.3 Exclusion Criteria

All patients presenting with symptoms of severe otitis media, tympanic membrane perforations, prior ear surgery were excluded in our study.

2.4 Collection of Samples and Processing

The clinical sample was collected from the external auditory canal from patients that were clinically diagnosed to have otomycosis under aseptic conditions with the help of sterile cotton swab containing a preservation medium (Sigma transwab-liquid amies-Germany). Samples were collected from either the right or left ear presenting signs or symptoms of otomycosis infection. The environmental samples were air borne sample and the surface borne in the hospital milieu. The air borne sample was recovered by the exposure of petri dishes containing the prepared culture medium at the hospital toilet and the open air environment. The surface borne sample was recovered from patients ward by the used of cotton swab. To do this, the cotton swabs were rubbed on the surface of patient's bed, on the walls of patient's room and consultation.

For mycological identification, direct microscopic examination was carried out by 10 % KOH examination and inoculation of material was done on two prepared Sabouraud Dextrose Agar (SDA) plates supplemented with chloramphenicol. One plate was incubated at room temperature 25 ^{0}C for 3-5 days and another at 37^{o}C for 48 hours. Both plates were observed for fungal growth daily. Fungal growth was identified by standard procedures [18]. Identification was done on the basis of colony morphology and Lactophenol Cotton Blue (LPCB) mount microscopy. *Aspergillus* isolates were characterized by varying length of conidiophores and extent of coverage of vesicles by phialides.

Various virulence factors were studied in *Aspergillus* section *Nigri* isolates from patient's and environment by standard phenotypic methods such as biofilm formation (by crystal violet) [19] adherent biofilm layer was scored visually as either non adherent (NA=OD≤ODC) for negative, or weak adherent (WA = ODC < OD ≤ (2 × ODC)), moderate adherent (2 × ODC) < OD ≤ (4 × ODC)), or strong adherent (SA = (4 × ODC) < OD) for positive [20], esterase production (using Tween 80 agar plates) [21], proteinase activity (using gel of bovine serum) [22], phospholipase activity (using egg yolk agar) [23]. The dense white zone of precipitation around the colonies of esterase, proteinase and phospholipase were positive isolates. The esterase, proteinase, and phospholipase activities were calculated using the formula described by Charu et al. [24]. Pz = diameter of colony/total diameter of colony+ precipitation zone (Pz ≥ 0.64 to <1 high activity, Pz <0.64 very high activity present, Pz = 1 null or no activity).

2.5 Statistical Analysis

The variables were statistically analyzed using Chi-square test of independent and SPSS (Version 16.0). Where, p-value <0.05 was considered statistically significant.

3. RESULTS

3.1 Samples Distribution, Culture Morphology of the *Aspergillus* Section *Nigri* Isolates on the Various Culture Media Showing the Virulence Activity of the Isolates

From the 250 clinical samples collected, 44 *Aspergillus* section *Nigri* were identified and used in the study of invasive factors recognition while 16 *Aspergillus* section *Nigri* were recovered from

150 hospital environment samples. A total of 60 *Aspergillus* section *Nigri* were evaluated for their capacities of producing the various invasive factors; Phospholipase C, proteinase, esterase and biofilm formation. The rate of production of invasive factors in clinical and environmental samples has been shown in (Table 1).

36 out of 44 from patients sample were found to produce phospholipase C, i.e., 81.82% as compared to 5 out 16 from environmental samples, i.e., 31.25% (Fig. 1A). 42 out of 44 from patients sample were found to produce proteinase (Fig. 1B) i.e., 95.45% as compared to 6 out of 16 from environmental samples i.e., 37.5%. 32 out of 44 from patients sample were found to produce esterase was seen in patients samples (Fig. 2) i.e., 72.73% as compared to 4 out of 16 environmental samples i.e., 25%. 42 out of 44 from patients sample were found to produce biofilm i.e., 95.45% (Fig. 3) as compared to 15 out of 39 environmental samples i.e., 38.46%.

3.2 Comparison of Virulence Factors in the Clinical and Environmental Isolates

We observed a statistically significant difference from the various invasive factors evaluated in both the patients and the environmental samples (Table 1). That is; phospholipase C, proteinase and esterase activities were statistically different from the clinical sample, as well as the environment (P-values<0.05). Higher productions of the invasive factors were seen in the patient samples than the environmental samples (Table 2 and Table 3).

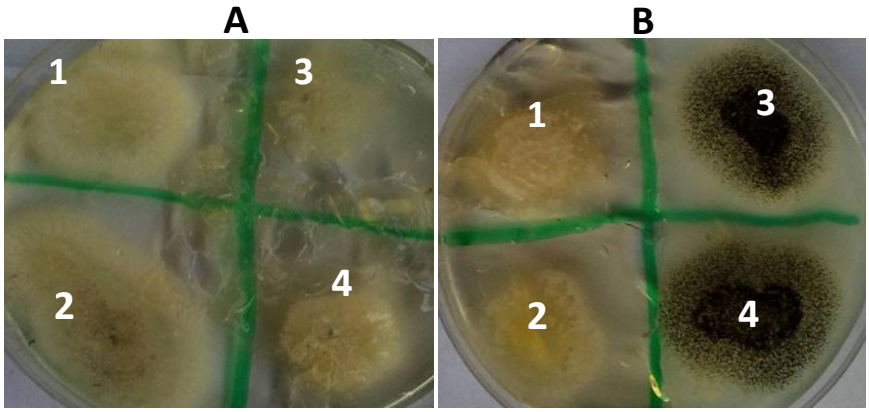

Fig. 1. (A) *Aspergillus* section *Nigri* showing phospholipase activity; 1 and 2: positive; 3 and 4: negative. (B) *Aspergillus* section *nigri* showing proteinase activity; 1 and 2: negative; 3 and 4: positive

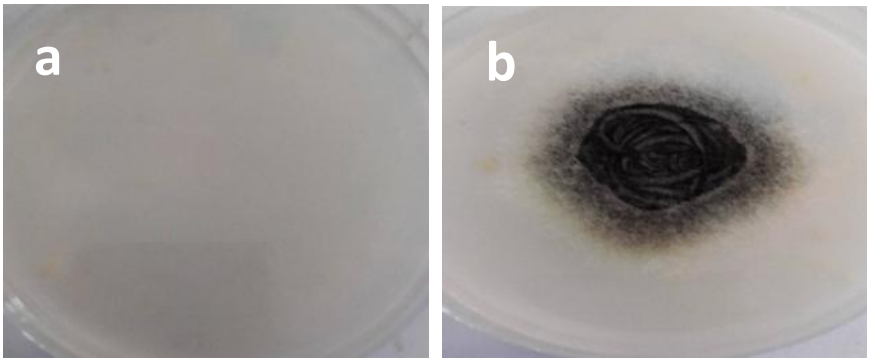

Fig. 2. Showing esterase activity on tween 80 agar medium; a) showed negative for esterase activity; b) showed positive for esterase activity

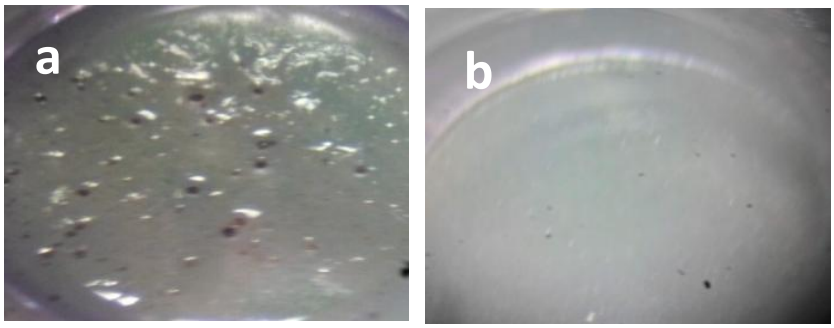

Fig. 3. Microscopic photograph of biofilm formation; a) showing no adhesion (negative); b) conidial adhesion (positive)

Table 1. Comparison of virulence factors in patient and environment samples

Isolates positive for virulence	Patient's sample (n=44)	Environmental samples (n=44)	P-value significance level(<0.05)
Phospolipase	36 (81.82%)	5 (31.25%)	0.0316
Proteinase	42 (95.45%)	6 (37.5%)	0.03225
Esterase	32 (72.73%)	4 (25%)	0.0407
Biofilm	42 (95.45%)	15 (93.75%)	0.2531

We can see that in the Phospholipase C, proteinase and esterase, we have a significant difference between Patient's sample and Environmental samples since our p-value is less than 0.05

Table 2. Variation of virulence factors in the patient's and environmental samples

Activity	Clinical isolates (n=44)			Environmental isolates (n=16)		
	Phospolipase	Proteinase	Esterase	Phospolipase	Proteinase	Esterase
High	16 (36.36%)	17 (38.64%)	14 (31.82%)	5 (31.25%)	6 (37.5%)	4 (25%)
Very high	20 (45.45%)	25 (56.82%)	20 (45.45%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
No activity	8 (18.18%)	2 (4.54%)	10 (22.73%)	11 (68.75%)	10 (62.5%)	12 (75%)

Table 3. Variation of biofilm production in the patient's and environmental samples

Adhesion	Clinical isolate (n=44)	Environmental isolate (n=16)
Non adherent	2(4.55%)	1(6.25%)
Weak adherent	2(4.55%)	1(6.25%)
Moderate adherent	24(54.54%)	10(62.5%)
Strong adherent	16(36.36%)	4(25%)

4. DISCUSSION

The various invasive factors were studied; phospholipase, proteinase, esterase and biofilm. The principal mode of action of these invasive factors is as follows; these invasive factors screened are capable of causing destruction of the composition of the cell membrane such as phospholipids degradation (phospholipase), protein degradation (proteinase), and lipids (esterase) [3,25]. Formation of biofilm by the *Aspergillus* species helps the mirobe to escape from the mechanism of phagocytosis employed by the body defence, enhancing the growth of the aspergilli [3,26]. The invasive factors associated with toxins initially cause perturbation of cellular function, which progresses gradually to necrosis/lysis of cells and tissues of larger organs, leading to organ failures [3]. Our study of invasive factors recognition revealed the present of these invasive factors in *Aspergillus* section *Nigri* isolates from patients as well as environment.

In our study, these invasive factors followed a descending order in patient isolates as follows; esterase, phospholipase, proteinase, biofilm. Moreover, this same descending order was observed in environment samples, showing a less number of positive results as compared to

patient sample. This is explained by the fact that, these invasive factors are intrinsically present in the environmental samples and less active for survival in the environment [3]. But on entry in human tissues, their activity is enhanced to oppose the adverse condition, protective mechanism of human bodies which tries to eliminate the microorganisms. Hence, for survival in human tissues, the virulence factors activity is geared up to oppose human protective mechanisms [3].

This our study corroborates with the study carried out by Raksha et al [3], in which out 78 isolates tested, 32 out of 39 from the patient samples were reported to produce biofilm as compared to 15 out of 39 environmental samples, 26 out of 39 from patients sample were reported to produce lipase as compared to 11 out of 39 environmental samples, 35 out of 39 from patients samples showed were reported to have α-amylase activity as compared to 14 out of 39 environmental samples, 24 out of 39 from patients sample were reported have pectinolytic activity as compared to 9 out of 39 environmental samples, 24 out of 39 from patients samples were reported to produce phospholipase as compared to 10 out of 39 from the environment and 31 out of 39 from patients samples were reported to produce hemolysin as compared to 13 out of 39 environmental samples. A similar study was carried out by Mezher MA et al., in which from the 62 patient's sputum samples, 19 out of 62 (30.6%) were reported to show positive growth of *Aspergillus* species [15]. In which majority of the isolates were *A. fumigatus* (12.9%) followed by *A. terreus* (6.5%), *A. niger* and *A. flavus* (1.6%). It was sited from their study that *A. fumigatus* showed production of haemolysin and phospholipase 62.5% each and protease production 87.5%, while *A. terreus* showed production of protease and phospholipase 50% each and haemolysin 25%, while *A. flavus*, showed no production of haemolysin, phospholipase protease. While all isolates of *A. niger* showed phospolipase production (100%) [3]. This our study also corroborate with a study carried out by Gharamah A et al., studied on 115 patients samples out of which 25 were *Aspergillus* species isolated, protease activity (80%), lipase (84%) and haemolysis (28%) [3,26]. Mythili A et al., studied on 108 clinical samples out of which 60 fungal isolates were obtained [3,27]. Highest lipase activity, protease activity, α-amylase activity and pectinase activity was also prominent for corneal isolate of *Aspergillus* species [3]. A study also carried by Birinci A et al., showed 30 *A. fumigatus*, nine *A. flavus* and four *A. niger* strains isolated from clinical specimens [3,28]. Where *A. fumigatus* showed proteinase 76.7% (23/30) and phospholipase activity 93.3% (28/30). No proteinase and phospholipase production was seen in *A. flavus* isolates. And *A. niger* was also reported to show phospholipase activity (25%) but did not express proteinase activity. Pathogenesis of aspergillosis is dependent on various factors of the host (immune status) and virulence factors of the pathogen [3]. Some putative virulence factors have been identified for different *Aspergillus* species. These include adhesions e.g., biofilm production and haemolysin, pigments hydrolytic enzymes such as proteases, proteinase, lipase, phospholipases, α-amylase, low-molecular-weight, non-protein metabolites [3,16,17]. These factors could play a major role in the pathogenesis of invasive *Aspergillus* infections [3,29,30]. These invasive factors of *Aspergillus* species confirm the pathogenicity and invasiveness nature of the fungi [3]. Hence, detecting of invasive factor can help to differentiate pathogenic from non-pathogenic aspergilla [3].

5. CONCLUSION

The isolates recovered from patients sample showed high invasive capacity as compare to the isolates recovered from the environment. This highlights the implications of phospholipase enzyme, proteinase enzyme, esterase enzyme and biofilm used by *Aspergillus* section *Nigri* isolates as means of survival in the host system.

CONSENT AND ETHICAL APPROVAL

An Ethical clearance was obtained from the Centre Regional Committee for Human Health Research bearing no: 00842/CRERSHC/2018 and an authorization from the Directors of Central Hospital and University Teaching Hospital. Verbal informed consent was sought from individual patients from whom the ear samples were collected. To do this, it was explained to the patients in languages they understood that, the isolates that would be obtained from their samples would be used in this study. Samples from patients who consented were processed for bacteria and fungi growth at the Zion laboratory of Microbiology, Yaoundé. The ear samples from patients who disagreed were not included in the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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