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COMPARATIVE ASSESSMENT OF MACROSCOPIC AND MICROSCOPIC CHARACTERISTICS OF PATHOGENIC AND POTENTIALLY PATHOGENIC STRAINS OF ASPERGILLI

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Abstract

Macroscopic features of colonies and microscopic characteristics of certain strains of the aspergilli– *A. flavus* and *A. niger*- isolated from soil, air and patients suffering from aspergillosis were examined. The samples obtained from patients yielded *A. flavus* and *A. niger*, not *A. fumigatus* which is the leading cause of aspergillosis in certain geographical areas of the world. Different media supported the growth of the strains to different extent. The strains of *A. niger* exhibited exudation on PDA and green velvety growth on RSA medium. The pathogenic strains of *A. flavus* exhibited white and fluffy margins on CSNAM and SA upto 15 days.

Keywords: *Aspergillus*, Aspergillosis, *A. flavus*, *A. niger*, Cultural characteristics of aspergilli

INTRODUCTION

Aspergillus spp. are ubiquitous, saprobic fungi that play a noteworthy role in global carbon and nitrogen recycling. Although their primary ecological niche is soil or decaying vegetation, about 40 species of the genus have been reported as human pathogens (Klich, 2006), the respiratory system being the normal portal of entry. Human aspergillosis is gaining importance in modern medical care (Latge, 1999; Latge and Steinbach, 2008). Different forms of aspergillosis *i.e.* aspergilloma and invasive aspergillosis involve direct growth of the fungus inside the host tissues; the former is often found associated with tuberculosis and is 'semi-invasive' while the latter, also called systemic aspergillosis, is the most life threatening form of *Aspergillus* infection. As the numbers of immuno- suppressed individuals increase within the human population, the cases of invasive aspergillosis are bound to increase.

Aspergillus flavus Link (Link, 1809) is the name now used to describe a species as well as a group of closely related species. It is a major cause of human invasive aspergillosis after *A. fumigatus* (Hedayati *et al*., 2007). *A. flavus* has been found to be associated with a wide variety of diseases *viz*. (i) granulomatous sinusitis; (ii) keratitis; (iii) cutaneous aspergillosis; (iv) wound infections; (v) osteomyelitis; (vi) acute and chronic invasive and granulomatous *Aspergillus sinusitis*; (vii) otitis; (viii) pulmonary and systemic infections in immunocompromised patients. On the other hand, *Aspergillus niger* is not believed to be so important cause of human diseases, though it may lead to aspergillosis, if large amount of spores are inhaled. Aspergillosis is, in particular, frequent among horticultural workers that inhale peat, dust, which can be rich in *Aspergillus niger* spores.

Accurate diagnosis of pathogen is an important pre-requisite for rational treatment of any disease. Identification of the species of *Aspergillus* is based on both the morphological features of the colony and microscopic characters (McClenny, 2005). Although molecular methods continue to improve and become more rapidly available, microscopy and culture remain commonly used and essential tools for identification of *Aspergillus* spp. The major cultural features used in identification of *Aspergillus* spp. are the (i) colour of the colony; (ii) the growth rate, and (iii) thermotolerance. Aspergilli exhibit varying morphological and growth response to different nutrients and species identification is facilitated by the study of pure cultures grown on known media.

In the present study, macroscopic features of colonies and microscopic characterstics for different strains of two *Aspergillus* spp. *i.e. A. flavus* and *A. niger* isolated from environmental (soil and air) and from clinical specimens were investigated on five different culture media. The object was to evaluate the possibilities of utilising the information for distinguishing potentially more pathogenic strains from non-pathogenic ones.

MATERIALS AND METHODS

Three clinical specimens, expected to be fungal infested, were collected from three different patients (two males and one female) suffering from aspergillosis. These specimens were collected aseptically during the surgery and were preserved in saline solution (9 gm NaCl/lit D.W.) and brought to the laboratory. All the three specimens were processed in Microbiology Laboratory, C.C.S. University, Meerut, for isolation of infesting fungus on Sabourauds Agar Medium followed by incubation in BOD incubator at 25±2°C for 5–6 days. Two of these samples yielded *Aspergillus flavus* (FS1 and FS2) and one sample yielded *Aspergillus niger* (NS1).

For obtaining soil samples, four different hospitals of Meerut namely Subharati Medical College (Meerut), Military Hospital (Meerut), Pyarelal Sharma Hospital (Meerut) and Cantonment Hospital (Meerut) were selected. Soil samples were collected from ENT waste dumping section of these hospitals aseptically in sterile polythene bags and were brought to laboratory. Serial dilution plate method (Waksman, 1927) was followed for isolating fungi from the samples. For collection of samples from air, already prepared plates of Sabourauds Agar Medium were exposed for 15–20 minutes near the ENT waste dumping sections of the aforementioned hospitals and were brought to the laboratory followed by incubation at 25±2°C for 5–6 days.

Pure cultures of *A. flavus* and *A. niger* were prepared. In all, ten different strains of *A. flavus* (four from soils; four from air and 2 from patients) were purified, and were designated as FSS1, FSS2, FSS3, FSS4 (strains of *A. flavus* obtained from soil samples); FAS1, FAS2, FAS3 and FAS4 (strains of *A. flavus* obtained from air samples) and FS1 and FS2 (strains of *A. flavus* isolated from clinical specimens). In the same manner, pure cultures of 9 different strains of *A. niger* (four from soil; four from air and one strain from patient) were obtained; and were designated as NSS1, NSS2, NSS3, NSS4 (strains of *A. niger* obtained from soil samples); NAS1, NAS2, NAS3 and NAS4 (strains of *A. niger* obtained from air samples) and NS1 (single strain

of *A. niger* isolated from clinical specimen). Altogether, nineteen (10 strains of *A. flavus* and 9 strains of *A. niger*) were obtained. In order to study strain variability, each of the nineteen strains was cultivated on five different culture media *viz.* Potato Dextrose Agar medium (PDA), Czapek's Sucrose Nitrate Agar medium (CSNAM), Sabourauds Dextrose Agar medium (SA), Oat Meal Agar medium (OMA) and Richard's Synthetic Agar medium (RSA).

A set of fifteen Petri plates (a set of 3 Petri plates for each medium) was used for culturing a particular strain. 15–20 ml of each of the given medium was poured in each of a set of 3 Petri plates and allowed to cool and solidify. A 0.5 mm disc of each strain was cut from pure culture of that particular strain, with the help of a cork borer, and was placed in the centre of each of the freshly prepared culture plates. Thus, a total of 150 Petri plates (15 for each strain and total 10 strains of *A. flavus*) were prepared and incubated at 25±2°C. Various macroscopic and microscopic features were observed after 2, 4 and 6 days of incubation. Similar procedure was followed for studying the strains of *A. niger*. In this case, a total of 135 Petri plates (15 for each of the 9 strains of *A. niger*) were prepared and studied.

Macroscopic characteristics, studied after two, four and six days of incubation, include (i) size of colony (radial growth in cms); (ii) chromogenesis, and (iii) growth rate/day. The microscopic characteristics studied were (i) size of the vesicle; (ii) size of the head; (iii) length, width and the type of wall and septation of conidiophores; (iv) size and wall texture of conidia.

RESULTS AND DISCUSSION

The present study was conducted with an aim to obtain diversity spectrum of human pathogenic and non-pathogenic strains of *A. niger* and *A. flavus* with respect to their growth patterns on five different culture media. The results are presented in the tables 1 to 4.

A. fumigatus is believed to be the most common species of *Aspergillus* causing allergic and invasive diseases, though *A. flavus* has also been recognised as an important pathogen (Denning *et al*., 1990). After *A. fumigatus*, *A. flavus* is believed to be the second leading cause of invasive and non-invasive aspergillosis (Denning, 1998; Morgan *et al*., 2005). *A. flavus* has been reported to be particularly frequent in some areas like Middle-East and India (Thakar *et al*., 2004; Saravanan *et al*., 2006). Other less common pathogenic aspergilli include *A. glaucus*, *A. niger* and *A. terreus* (Barnes and Denning, 1993). The results of the present study confirm that not *A. fumigatus* but *A. flavus* is the more common cause of aspergillosis in the patients under study followed by *A. niger*.

In the case of *A. niger*, Sabourauds Dextrose Agar medium (SA) supported best growth of both pathogenic and non-pathogenic strains. A patchy appearance of colonies with concentric rings was observed on OMA medium in case of all the strains of the fungus. After 6 days of incubation, strains isolated from patients exhibited (i) exudation when cultured on PDA medium and (ii) a cream velvety outgrowth on RSA medium; such a growth was not observed in case of non-pathogenic strains. In the pathogenic strain, head: vesicle ratio was 1:1.74 which was very different from all other strains except NSS3 (soil sample 3). NS1 could be distinguished from NSS3 because of much shorter conidiophores (89.10 µm) as compared to 1108.23 µm in NSS3.

Table 1: Macroscopic observations on the colonies of pathogenic and non-pathogenic strains of *Aspergillus flavus* after 6 days of incubation

Strain	Characterstics	Media				
		PDA	CSNAM	SA	OMA	RSA
FSS1	Colony diameter	5.9±0.43	4.4±0.81	5.2±0.26	6.1±0.7	5.1±0.65
	Chromogenesis	Dark Green	Yellow Green	Yellow Green	Grey Green	Grey Green
	Surface	Rough	Rough	Rough	Dull	Dull
	Growth/day	0.98	0.73	0.86	1.01	0.85
FSS2	Colony diameter	7.2±0.85	7.2±0.34	6.7±1.60	5.7±1.5	5.3±0.7
	Chromogenesis	Dull Green	Deep Yellow Green	Dull Green	Dark Green	Golden Green
	Surface	Dull	Rough	Rough	Rough	Rough
	Growth/day	1.2	1.20	1.11	0.95	0.88
FSS3	Colony diameter	7.6±1.15	6.5±0.79	7.9±0.8	6.7±0.81	6.8±0.6
	Chromogenesis	Green	Deep Yellow Green	Dark Green	Grey Green	Dark Green
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth/day	1.26	1.08	1.31	1.11	1.13
FSS4	Colony diameter	6.7±0.78	4.4±0.3	6.9±1.6	5.9±1.34	5.5±1.04
	Chromogenesis	Dark Green	Deep Yellow Green	Dull Green	Dark Green	Golden Green
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth/day	1.11	0.73	1.15	0.98	0.91
FAS1	Colony diameter	8.1±0.43	6.9±0.45	8.3±1.04	7.2±1.34	6.1±0.7
	Chromogenesis	Dark Green	Deep Yellow Green	Yellow Green	Grey Green	Green
	Surface	Rough	Rough	Rough	Dull	Rough
	Growth/day	1.35	1.15	1.38	1.2	1.01
FAS2	Colony diameter	8.2±1.68	5.7±1.66	7.6±0.62	7.1±1.2	5.6±1.93
	Chromogenesis	Yellow Green	Yellow Green	Dark Green	Grey Green	Green
	Surface	Rough	Rough	Rough	Dull	Rough
	Growth/day	1.36	0.95	1.26	1.18	0.93
FAS3	Colony diameter	8.2±0.69	6.3±1.27	7.2±0.75	6.6±0.52	5.4±0.4
	Chromogenesis	Yellow Green	Deep Yellow Green	Deep Green	Grey Green	Green
	Surface	Rough	Rough	Rough	Dull	Rough
	Growth/day	1.36	1.05	1.2	1.1	0.9
FAS4	Colony diameter	7.4±0.62	5.1±0.32	6.8±1.30	6.8±0.4	5.2±0.36
	Chromogenesis	Yello Green	Deep Yellow Green	Yellow Green	Dull Green	Dull Green
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth/day	1.29	0.85	1.13	1.13	0.86
FS1	Colony diameter	7.7±1.15	6.8±0.52	8.6±0.26	6.6±0.43	7.4±0.78
	Chromogenesis	Yellow Green	Deep Yellow Green	Yellow Green	Dull Green	Green
	Surface	Rough	Rough	Rough	Dull	Rough
	Growth/day	1.28	1.13	1.13	1.1	1.23
FS2	Colony diameter	7.8±0.79	7.1±0.88	7.9±1.41	5.8±0.75	6.6±1.05
	Chromogenesis	Green	Yellow Green	Green	Grey Green	Green
	Surface	Rough	Rough	Rough	Dull	Rough
	Growth/day	1.3	1.18	1.31	0.96	1.1

Table 2: Macroscopic observations on the colonies of pathogenic and non-pathogenic strains of *Aspergillus niger* after 6 days of incubation

Strain	Characterstics	Media				
		PDA	CSNAM	SA	OMA	RSA
NSS1	Colony diameter	6.1±1.37	1.2±0.2	7.1±1.04	3.9±0.81	6.2±0.81
	Chromogenesis	Black	Dull Black	Black	Black	Yellow Black
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	1.01	0.2	1.18	0.65	1.03
NSS2	Colony diameter	6.1±0.96	2.1±0.75	7.0±0.5	4.1±1.3	5.5±0.98
	Chromogenesis	Black	Yellowish Black	Black	Black	Yellow White
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	1.01	0.35	1.16	0.68	0.91
NSS3	Colony diameter	3.3±0.72	1.6±0.69	7.5±1.05	3.8±1.01	6.2±0.75
	Chromogenesis	Black	White Black	Black	Black	Golden Brown
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	0.55	0.26	1.25	0.63	1.03
NSS4	Colony diameter	4.5±0.79	1.0±0.26	6.5±1.83	4.1±0.43	5.4±0.60
	Chromogenesis	Black	Dull Black	Black	Black	Golden Brown
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	0.75	0.16	1.08	0.68	0.9
NAS1	Colony diameter	4.0±0.60	0.8±00	6.8±0.85	4.7±1.77	6.6±0.81
	Chromogenesis	Black	Dull Black	Black	Black	Golden Brown
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	0.66	0.13	1.13	0.78	1.1
NAS2	Colony diameter	4.2±0.51	1.0±0.26	7.3±1.37	3.8±1.21	7.2±1.15
	Chromogenesis	Black	Dull Black	Black	Dull Black	Golden Brown
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	0.7	0.16	1.21	0.63	1.2
NAS3	Colony diameter	5.0±1	1.1±0.3	8.0±0.1	3.6±0.69	6.2±0.69
	Chromogenesis	Black	Dull Black	Black	Black	Golden Brown
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	0.83	0.18	1.33	0.6	1.03
NAS4	Colony diameter	4.5±0.60	0.9±0.1	7.7±1.2	3.9±0.8	5.4±0.3
	Chromogenesis	Black	Dull Black	Black	Black	Golden Brown
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	1.5	0.15	1.28	0.65	0.9
NS1	Colony diameter	3.9±0.65	0.8±0.05	6.5±0.55	3.9±0.65	7.0±0.55
	Chromogenesis	Black	Dull Black	Black	Dull Black	Cream
	Surface	Rough	Rough	Rough	Rough	Rough
	Growth / day	0.65	0.13	1.08	0.65	1.16

Table 3: Microscopic characteristics of pathogenic and non-pathogenic strains of *Aspergillus flavus* after 6 days of incubation

Strain	Characteristics						
			Conidiophores			Conidia	
	Vesicle Size (µm)	Head Size (µm)	Length (µm)	Width (µm)	Wall and septation	Size (µm)	Wall
FSS1	48.5±2.28	102±6.06	601±23.2	10.4±0.58	SW, NS	4-6	Smooth
FSS2	15.08±1.08	30.5±1.08	509.12±18.7	6.2±0.57	RW, S	4-5	Spinulose
FSS3	16.00±0.74	32.38±32.38	530±20.9	7.1±0.68	SW, NS	4-6	Smooth
FSS4	18.95±1.03	57.96±1.48	826.92±17.8	7.72±0.640	SW, NS	4-6	Smooth
FAS1	30.17±1.43	63.66±2.51	749.98±38.3	10.48±0.58	SW, NS	3-5	Smooth
FAS2	27.41±1.05	63.48±2.97	748.01±33.9	11.22±0.76	SW, NS	3-5	Smooth
FAS3	27.04±1.78	60.53±3.13	717.78±47.2	10.67±0.78	SW, NS	3-5	Smooth
FAS4	35.14±1.199	73.04±1.66	817.79±28.0	11.04±0.56	SW, NS	4-5	Smooth
FS1	18.76±0.91	33.8±0.89	593±28.8	6.07±6.07	SW, NS	4-6	Smooth
FS2	15.64±0.85	33.48±2.24	564.1±19.7	7.17±0.86	SW, NS	4-6	Smooth

SW= Smooth walled; RW= Rough walled; S= Septate and NS= Non-septate

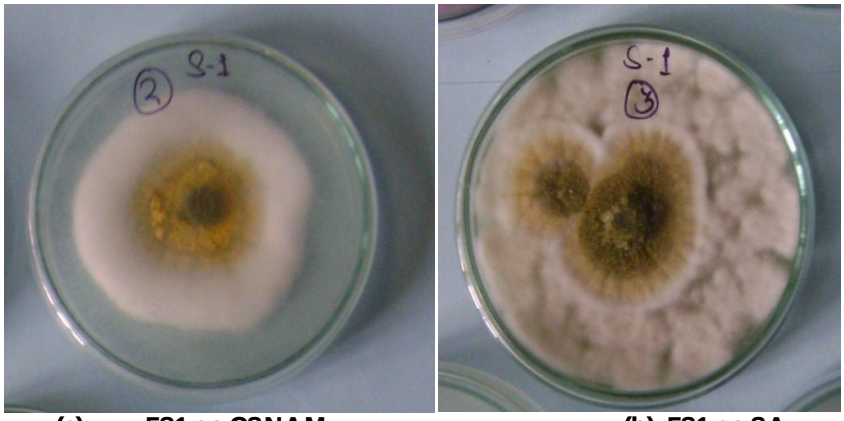
Table 4: Microscopic characteristics of pathogenic and non-pathogenic strains of *Aspergillus niger* after 6 days of incubation

Strain	Characteristics						
			Conidiophores			Conidia	
	Vesicle Size (µm)	Head Size (µm)	Length (µm)	Width (µm)	Wall and septation	Size (µm)	Wall
NSS1	36.24±1.60	104.32±4.34	741.88±23.3	14.16±0.58	SW, NS	3-4	Smooth
NSS2	44.52±2.40	97.704±5.24	1163.8±62.1	15.08±0.64	SW, NS	3-4	Smooth
NSS3	66.42±3.21	115±6.26	1108.23±34.6	15.45±0.61	SW, NS	3-4	Smooth
NSS4	43.60±3.21	112.42±5.56	1290.20±71.9	13.8±0.63	SW, NS	3-4	Smooth
NAS1	31.09±0.68	71.2±3.96	396.15±10.9	14.9±0.73	SW, NS	3-4	Smooth
NAS2	33.30±1.19	89.97±3.01	409±10.1	13.24±0.6	SW, NS	3-4	Smooth
NAS3	31.28±1.76	74.15±3.88	533.41±13.27	12.69±0.60	SW, NS	3-4	Smooth
NAS4	55.3±2.13	143.33±4.5	1118.72±29.6	17.29±0.65	SW, NS	3-4	Smooth
NS1	57.22±4.17	99.91±5.03	829.10±51.5	15.82±0.57	SW, NS	3-4	Smooth

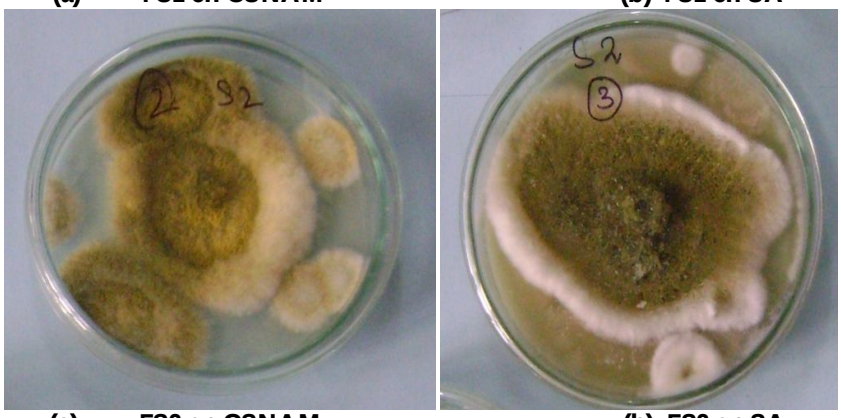
SW= Smooth walled; RW= Rough walled; S= Septate and NS= Non-septate

In the case of *A. flavus*, all the five culture media supported the growth of *A. flavus* to various extents as revealed by their colony diameter (radial growth in centimetres) after two, four and six days of incubation. No clear cut distinction between pathogenic and non-pathogenic strains could be observed as far as parameters mentioned in the table 1 are concerned. However, the margins of the colonies of both pathogenic strains of *A. flavus* on CSNAM and SA remained white and fluffy (Fig. 1) for upto 15 days.

The differences in growth patterns might be helpful for distinguishing pathogenic strains of A. *niger* and *A. flavus* from non-pathogenic ones thereby facilitating diagnosis and appropriate treatment of the patients.



(a) FS1 on CSNAM **(b) FS1 on SA**



(a) FS2 on CSNAM **(b) FS2 on SA**

Fig 1: Growth patterns of pathogenic strains of *Aspergillus flavus* (FS1 and FS2) on CSNAM and SA media.

REFERENCES

1. Barnes, A.J. and Denning, D.W. (1993). Aspergilli− significance as pathogens. *Rev. in Med. Microbiol.* 4; p. 176−180.
2. Denning, D.W.; Clemons, K.V.; Hanson, L.H. and Stevens, D.A. (1990). Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. *J. Infect. Dis.* 162; p. 1151−1158.
3. Denning, D.W. (1998). Invasive Aspergillosis. *Clinical Infectious Diseases* .26; p. 781–805.
4. Hedayati, M.T.; Pasqualotto, A.C.; Warn, P.A.; Bowyer, P. and Denning, D. W. (2007). *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. *Microbiology*. 153; p. 1677–1692
5. Klich, M.A. (2006). Identification of clinically relevant aspergilli. *Med. Mycol*. 44; p. S127–S131.
6. Latge, J.P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12(2); p. 310–350.
7. Latge, J.P. and Steinbach, W.J. (2008). *Aspergillus fumigatus and Aspergillosis.* DC: ASM Press, Washington.
8. Link, H.F. (1809). Observationes in Ordines plantarum naturales. Dissertatio prima, complectens Anandrarum ordines Epiphytas,Mucedines Gastomycos et Fungos Der Gesellschaft NaturforschenderFreunde zu Berlin. Magazin fu¨r die neuesten Entdeckungen in der gesamten Naturkunde 3; p. 1–42.
9. McClenny, N. (2005). Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach. *Med Mycol.* 43(1); p. S125–128.

10. Morgan, J.; Wannemuehler, K.A.; Marr, K.A.; Hadley, S.; Kontoyiannis, D.P.; Walsh, T.J.; Fridkin, S.K.; Pappas, P.G. and Warnock, D.W. (2005). Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. *Med. Mycol.* 43 (1); p. S49–S58.
11. Saravanan, K.; Panda, N. K.; Chakrabarti, A.; Das, A. and Bapuraj, R. J. (2006). Allergic fungal rhinosinusitis: an attempt to resolve the diagnostic dilemma. *Arch. Otolaryngol. Head. Neck Surg*. 132; p. 173–178.
12. Thakar, A.; Sarkar, C.; Dhiwakar, M.; Bahadur, S. and Dahiya, S. (2004). Allergic fungal sinusitis: expanding the clinicopathologic spectrum. *Otolaryngol. Head Neck Surg.* 130; p. 209–216.
13. Waksman, S.A. (1927). *Principles of Soil Microbiology*. Williams and Wilkins, Baltimore Md.