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Pathogenicity Profiles of *Aspergillus* Species Isolated from *Phaseolus vulgaris* (Bean) Seeds

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ABSTRACT

The role of *Aspergillus* Species in cases of food borne infection have been established and reported to have substantial degree of morbidity and mortality among immunoincompetent individuals. This study was carried out to determine the pathogenicity of *Aspergillus* species isolated from bean seed samples. A total of 90 representative bean seed samples of different varieties were randomly collected from different shops and open markets in Ihiala Local Government Area, Anambra State, and screened for the presence of *Aspergillus* species. The isolates obtained were characterized and identified using macroscopic, microscopic and molecular characteristics. The pathogenic potentials of the isolates on immunocompetent and immunoincompetent mice were investigated by oral administration of 0.5 ml of the inoculum (10^8cells/ml) into the mice. The mice were kept under complete observation for 4 weeks for obvious pathological features and mortalities. It was observed that 32 (35.56 %) and 14 (14.44 %) of the studied bean seed samples recorded Aspergillus species for surface and internal contaminants. *Oloka* bean seeds (70 %/30 %) were most contaminated. *Aspergillus flavus* strain HUS6 (AFHUS6), *Aspergillus niger* strain HG48 (ANHG48), *Aspergillus niger* strain HUS1 (ANHUS1), *Aspergillus tubingiensis* strains EM-CN1 (ATEM-CN1), *Aspergillus aculeatus* strain AN5 (AAAN5) and *Aspergillus awamori* strain DN-SN2 (AW DN-SN2) were isolated from the bean seed samples, of which AN HUS1 was mostly seen in the studied samples There were significant (P<0.05) obvious pathological features and lesion on the internal organs, majorly the lungs of the infected immunoincompetent mice, and the pathological features of those mice infected with AFHUS6 and ANHUS1 were most pronounced. There were significant (P<0.05) mean plate counts of the test isolates from the organs of immunoincompetent mice, and decreased in organ weight to body weight ratio. Thus, this study has shown that the fungi isolates showed obvious pathological features majorly on infected immunoincompetent mice, of which AFHUS6 was most pathogenic.

INTRODUCTION

Studies have shown that beans are one of the most important domestic legumes in the world, because of its high concentration of protein, fiber and complex carbohydrates. Globally it has been estimated that 18.7 million tons are grown in nearly 150 countries on 27.7 million hectares. Beans comes in a wide varieties of shapes, sizes and colours, from pinto to pink, black and white, interesting enough, despite this diversity in colour and size, the wild and domestic beans belong to the same species, as do all of the colourful varieties of beans, which are believed to be the result of a mixture of population bottlenecks and purposeful selection. The main difference between wild and cultivated beans is domestic beans are less exciting. There is of course a significant increase in seed weight, and the seed pods are less likely to shatter than wild forms. But there is a primary change in decrease variability of grain size, seed coat thickness and water intake during cooking (Lerner, 2009). Common beans plants are annual plants and last only one growing season and range greatly in size. The common beans is used as a pulse and green vegetable eaten fresh or cooked. The beans can be dried, cooked in sauce and canned (Lerner, 2009).

Fungi can be spread by equipments used in contaminated fields, and by people or animals walking through the fields. Contaminated seeds carry fungi from one region or farm to another. Bean seeds can also be contaminated with other seeds in the field during harvesting. Susceptible varieties have more prevalent seed contamination than the resistant varieties. *Aspergillus* is one of the most common seed born fungi on dry beans (Domijan *et al.,* 2015). *Aspergillus* is a widely distributed genus of more than 250 species of largely saprophytic filamentous fungi belonging to the phylum Ascomycota. It was originally described in 1729 by the botanist and priest Antonio Micheli. Morphologically *Aspergillus* species are very similar and hard to distinguish. *Aspergillius* causes a wide spectrum of infections which includes cutaneous manifestations, otomycosis and invasive infections such as pulmonary aspergillosis and endocarditis. Pulmonary aspergillosis may range from invasive pulmonary aspergillosis (IPA) in severely immunocompromised patients to chronic necrotizing aspergillosis in mildy immunocompromised populations (Pagano *et al.,* 2007). *Aspergillus fumigatus* remains the predominant agents of IPA (Balajee *et al.,* 2007)

Toxigenic group of Aspergillus fungi are known to produce one or more toxic secondary metabolites in bean seeds, it is well established that not all molds are toxigenic and not all secondary metabolites from molds are toxic. The presence of these toxins in foods and food products is a serious health hazard to consumers (Betina, 2012). Seed borne fungi pathogens are the principal producers of mycotoxins associated with fungal growth on crops in the field and in storage. It is widely acknowledged that *Aspergillus* species are the most important mycotoxin-producing fungi in tropical countries, seen mostly among adults in rural populations with a poor level of nutrition for whom common beans is the staple food. (Tulpule and Bhat, 2012).

The dramatic increase in fungal diseases in recent years can be attributed to the consumption of contaminated food products and other human activities. Jim *et al. (*2007) stated that most of *Aspergillus* species that cause diseases in humans and animals have contributed significantly to increase in mortality rate and economic losses in animal husbandry.

Anambra State is one of the few States in Nigeria with large consumption of bean seeds (*Phaseolas vulgaris*). Previous studies focused on Physiochemical properties and microorganisms associated with bean seeds (Ama *et al.,* 2015). Hence infections associated with *Aspergillus* remain one of the causes of reported periodic cases among the immuno compromised individuals within the developing countries (Gouge and Pizzomo, 2014). This shows that there is still paucity of information on the actual species of *Aspergillus* associated with bean seeds, and their pathogenic potentials that led to the reported menacing diseases among the consumers. Therefore, this study was designed to evaluate the pathogenic potentials of *Aspergillus* species isolated from bean seeds sold in Ihiala in Anambra State.

MATERIALS AND METHODS

Sample Collection: A total of 90 samples of bean seeds were collected randomly, from different shops and open markets in Ihiala Local Government Area (L.G.A.), Anambra State. Sampling was performed manually from different bags and basins, such that the bean seeds were collected from different parts of the bags and basins. The samples were aseptically pooled and mixed properly and formed one cup of the bean seeds in sterile nylon bag, then the bean seeds were taken for analysis. The samples were carefully labeled and then kept in a disinfected cooler, to maintain its temperature and stability of the number of the isolates. The samples were transported to the laboratory for analysis.

Isolation of the Fungi Isolates: This was carried out using the method of Suleiman and Omafe (2013). Each sample was shared into two groups. First group was aseptically soaked into distilled water for 30 minutes, and the second group was disinfected by soaking for 1 minute in 1% Sodium hypochloride and washed three times with distilled water, and then soaked in the distilled

water for 30 minutes. A 0.1 ml aliquot from the first group was plated on Sabouraud Dextrose Agar (SDA) containing chloramphenicol antibiotics (0.05%). Seeds from the second group was placed at the rate of 25 seeds Per Petri dish containing 20 ml of SDA supplemented with chloramphenicol antibiotics (0.05%).These were incubated at room temperature (30±2ºC) for 5 days. The fungi obtained were aseptically sub cultured on SDA containing chloramphenicol antibiotics (0.05%) and incubated at room temperature (30±2°C) for 5 days.

Identification of Fungal Isolates: The fungal isolates were identified to the genus/species level based on macroscopic, microscopic and molecular characteristics of the isolates obtained from pure cultures (Watanabe, 2002).

Pathogenicity Test

Inoculum preparation: The isolates were first sub-cultured on SDA and incubated 30±2ºC for 5 days. The inoculum was prepared by flooding the surface of the agar plate with sterile normal saline (0.85% NaCl) and scrapping the sporulating mycelium with sterile spatula and drawing up to the resultant suspensions with a sterile pasture pipette. The suspension was filtered using a sterile filter paper. The turbidity of the suspended cells was adjusted to match the turbidity standard of 0.5 Macfarland's standard which was prepared by mixing 0.6ml of 1% barium chloride dehydrate ($BaCl.2H_2O$) and 99.4ml of 1% concentrated tetraoxosulphate (VI) acid (Conc. H_2SO_4). The turbidity was standardized using spectrophotometer at 660 nm which was equivalent to approximately 10^8 cells per millilitre (Umedum and Iheukwumere, 2013).

Animal Inoculation

Immunocompetent mice: A total of twenty eight (28) albino mice that were bought from Nnobi market Anambra state were used (4 for each isolate) for this study. A 0.5 ml saline suspension (viable count at 10^8 cells per ml) was administered orally into each of the albino mouse and observed for obvious pathological signs and symptoms for four weeks. The mice were sacrificed at the end of four weeks. Each animal was autopsied for the gross morphological lesions of the internal organs. The liver, lungs and kidney tissues were removed, portions were homogenized and macerated in peptone water, and 0.1 ml aliquot was aseptically cultured on Sabouraud Dextrose Agar supplemented with chloramphenicol (0.05 %) (Umedum and Iheukwumere, 2013).

Immunocompromised mice: A total of twenty eight (28) albino mice that were bought from Nnobi market Anambra state were used (4 for each isolate) for this study. Twenty-four hours before inoculation, 0.25% of hydrocortisone acetate was intramuscularly administered to the albino mice in other to suppress their immunity. The mice were starved for twelve hours in order to increase their appetite. A 0.5 ml saline suspension (viable count at 10^8 cells per ml) was administered orally into each of the albino mouse and observed for obvious pathological signs and symptoms for four weeks. The mice were sacrificed at the end of four weeks. Each animal was autopsied for the gross morphological lesions of the internal organs. The liver, lungs and kidney tissues were removed, portions were homogenized and macerated in peptone water, and 0.1 ml aliquot was aseptically cultured on Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (0.05 %). The remaining tissue materials were fixed and examined histological (Umedum and Iheukwumere, 2013).

Statistical Analysis: The data generated from this study were represented as mean ±Standard deviation and then charts. The test for significance at 95% confidence interval was carried out using Student's T test and Chi- square (Iheukwumere *et al.,* 2017).

RESULTS

The identities of the fungal isolates is shown in table 1.The obvious pathological signs of the test isolates were significantly ($P<0.05$) seen among the immunocompromised infected mice (Table 2). It was observed that cough and weight loss were commonly seen among the infected immunocompromised mice. The pathological signs were significantly ($P<0.05$) most on those immunocompromised mice infected by AFHUS6 whereas AAAN5 showed the least pathological manifestations. Death was recorded among the immunocompromised mice infected by AFHUS6, ANHG48 and ANHUS1, and AFHUS6 recorded the highest mortality rate. No significant ($P>0.05$) obvious pathological signs were seen among immunocompetent infected mice.

The gross lesions were significantly ($P<0.05$) seen among the immuno incompetent infected mice as shown in Table 3. Air sacculitis, an inflammation of the air sac was common seen the lungs of immunocompromised infected mice. There was liver inflammation (perihepatitis) among the immunocompromised infected mice. Lungs hemorrhage and lung hypertrophy was seen among those immuno incompetent mice infected by AFHUS6, ANHG48, AN HUS1, ATEM-CN1 and AWDA-SN2, and these were significantly ($P<0.05$) most in those mice infected by AFHUS6. Twenty five percent (25%) of immuno incompetent mice infected by AFHUS6 and ANHU1 recorded inflammation of kidney (perinephritis). No significant ($P>0.05$) gross pathological lesions were seen among immuno competent infected mice.

There was significant ($P<0.05$) decreased in the mean organ weight to body weight ratio (majorly the

lungs) among the immunocompromised infected mice, of which those mice infected by AFHUS6 were mostly affected (Table 4). The liver of the immunocompromised infected mice also showed decreased in organ/body weight. There was no significant (P>0.05) decreased in the kidney of immunocompromised infected mice except those infected by ATEM-CN1 which showed a slight decreased. There was no significant (P>0.05) decreased in organ weight to body weight ration observed among the immunocompetent infected mice.

There was significant (P<0.05) total mean viable plate counts of the test isolates in the internal organs of the infected immunocompromised mice (Table 5). Significant (P<0.05) growth was record in the lungs, of which AFHUS6 recorded the highest counts whereas ATEM-CN1 recorded the least counts. There was also significant (P<0.05) growth in the liver but no growth was seen in the kidney except ATEM-CN1 which recorded a single colony. It was also observed that the mean viable plate counts of the test isolates recorded in the lungs of the immunocompromised mice were slightly higher than that recorded in the liver. No significant (P>0.05) counts were recorded from the internal organs of the immunocompetent infected mice.

Significant damages were observed in the infected organs of immunocompromised mice, majority the lungs (Table 6). The kidney showed little or no alteration but cystic congestion within the cystic and weakening of sinusoid walls was on the kidney from infected by ATEM- CN1. There were slight alteration mainly congestion and slight or complete enlargement of sinusoids. AFHUS6 showed dilation of sinusoids and multifocal necrosis. Major destructions were observed majority on the lungs of infected mice. There were enlargement or airways, degeneration of connective tissues, degeneration of alveoli, congestion, vascular thrombosis, peribronchial degeneration and reduction in alveolar size, and these were most sever in those mice infected by AFHUS6.

Table 1: Molecular identities of the isolates

Isolate	Max score	Total score	Query cover	GCP (E-value)	Identity	Accession Number	Description
X	719	719	100%	0%	100%	MF 163443.1	*Aspergillus flavus* strain HUS 6
Y1	701	701	100%	0%	100%	KX 099668.1	*Aspergillus niger* strain HG48
Y2	832	832	100%	0%	100%	MF 163441.1	*Aspergillus niger* strain HUS 1
M	832	832	100%	0%	100%	KY 509548.1	*Aspergillus tubingiensis* strain EM-CN1
Q	793	793	100%	0%	100%	KU 527791.2	*Aspergillus aculeatus* strain AN 5
R	785	785	100%	0%	100%	KY 509551	*Aspergillus awamori* strain DA-SN2

Table 2: Obvious pathological signs of the test isolate on the experiment mice

N= 4

Parameter	Immuno competent						Immuno compromised mice						Control
	AF HUS 6	AN HG48	AN HUS 1	AT EM-CN 1	AA AN 5	AW DA-SN2	AF HUS 6	AN HG48	AN HUS 1	AT EM-CN 1	AA AN 5	AW DA-SN2	
Weakness	2	1	1	0	0	0	4	3	4	3	2	3	0
Anorexia	2	0	0	0	0	0	4	3	4	3	2	3	0
Diarrhea	0	0	0	0	0	0	1	0	0	1	0	0	0
Cough	2	1	1	0	0	0	4	4	4	4	4	4	0
Isolation	2	0	0	0	0	0	4	3	4	3	2	3	0
Frequent Urination	0	0	0	0	0	0	1	0	0	0	0	0	0
Weight Loss	2	1	1	0	0	0	4	4	4	4	4	4	0
Death	0	0	0	0	0	0	2	2	2	0	0	1	0

AF HUS6 – *Aspergillus flavus* strain, Hus 6; ANHG48 – *Aspergillus niger* strain HG48;
ANHUS1 – *Aspergillus niger* strain Hus 1; AT EM-CN1 – *Aspergillus tubingiensis* strain EM-CN1
AA ANS – *Aspergillus aculeatus* strain AN5; AW DA-SN2 - *Aspergillus awamori* strain DA-SN2

Table 3: Gross pathological lesion on the internal organs of the infected mice

N = 4

Parameter	Immuno competent						Immuno incompetent mice						Control
	AF HUS 6	AN HG48	AN HUS 1	AT EM-CN 1	AA AN 5	AW DA-SN2	AF HUS 6	AN HG48	AN HUS 1	AT EM-CN 1	AA AN 5	AW DA-SN2	
Perihepatitis	1	1	2	1	1		3	3	4	3	2	3	0
Liver hypertrophy	0	0	0	0	0	0	0	0	0	0	0	1	0
Liver hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0
Air sacculitis	2	1	2	0	0	0	4	4	4	4	4	4	0
Lungs hypertrophy	0	0	0	0	0	0	4	2	3	2	0	2	0
Lungs hemorrhage	0	0	0	0	0	0	3	1	2	1	0	1	0
perinephritis	0	0	0	0	0	0	1	0	0	1	0	0	0
Kidney hypertrophy	0	0	0	0	0	0	0	0	0	0	0	0	0
kidney hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0

AF HUS6 – *Aspergillus flavus* strain, HUS 6; ANHG48 – *Aspergillus niger* strain HG 48;
ANHus1 – *Aspergillus niger* strain HUS 1; AT EM-CN1 – *Aspergillus tubingiensis* strain EM-CN1
AA ANS – *Aspergillus aculeatus* strain AN5; AW DA-SN2 - *Aspergillus awamori* strain DA-SN2

Table 4: Mean organ weight to body weight ratio of the experimented mice

Isolate	Immuno competent mice			Immunocompromised mice		
	Liver	Lungs	Kidney	Liver	Lungs	Kidney
AF-HUS6	0.021 ± 0.002	0.013 ± 0.001	0.008 ± 0.000	0.017 ± 0.002	0.008 ± 0.001	0.007 ± 0.001
AN HG48	0.024 ± 0.003	0.014 ± 0.001	0.008 ± 0.000	0.019 ± 0.001	0.010 ± 0.001	0.007 ± 0.001
AN HUS1	0.022 ± 0.001	0.014 ± 0.001	0.008 ± 0.000	0.018 ± 0.002	0.009 ± 0.001	0.007 ± 0.001
AT EM-CN1	0.024 ± 0.003	0.015 ± 0.001	0.008 ± 0.000	0.019 ± 0.001	0.012 ± 0.001	0.006 ± 0.001
AA AN5	0.025 ± 0.001	0.016 ± 0.001	0.008 ± 0.000	0.020 ± 0.002	0.012 ± 0.001	0.007 ± 0.001
AW DN-SN2	0.023 ± 0.002	0.014 ± 0.001	0.008 ± 0.000	0.018 ± 0.001	0.011 ± 0.001	0.007 ± 0.001
Control	0.026 ± 0.001	0.018 ± 0.001	0.008 ± 0.000	0.026 ± 0.001	0.018 ± 0.001	0.008 ± 0.001

AF HUS6 – *Aspergillus flavus* strain, HUS6; ANHG48 – *Aspergillus niger* strain HG 48;
ANHus1 – *Aspergillus niger* strain HUS1; AT EM-CN1 – *Aspergillus tubingiensis* strain EM-CN1
AA ANS – *Aspergillus aculeatus* strain AN5; AW DA-SN2 - *Aspergillus awamori* strain DA-SN2

Table 5: Total mean viable plate counts of the isolates from the internal organs of the infected mice

Isolate	Immuno competent mice			Immunocompromised mice		
	Liver	Lungs	Kidney	Liver	Lungs	Kidney
AF-HUS6	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	6.00 ± 0.82	8.00 ± 0.00	0.00 ± 0.00
AN HG48	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00
AN HUS1	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.00	6.00 ± 0.48	0.00 ± 0.00
AT EM-CN1	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	1.00 ± 0.00
AA AN5	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	0.00 ± 0.00
AW DN-SN2	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

AF HUS6 – *Aspergillus flavus* strain, HUS 6; ANHG48 – *Aspergillus niger* strain HG 48;
ANHus1 – *Aspergillus niger* strain HUS 1; AT EM-CN1 – *Aspergillus tubingiensis* strain EM-CN1
AA ANS – *Aspergillus aculeatus* strain AN5; AW DA-SN2 - *Aspergillus awamori* strain DA-SN2

Table 6: Histological features of the internal organs of infected immunocompromised mice

Isolate	Liver	Lungs	Kidney
AF-HUS6	Dilation of sinosoids and multifocal necrosis	Enlargement of airways and degeneration of connective tissues	Minor passive congestion
AN HG48	Intact parenchymal cells and some necrotic cells	Peribronchial degeneration of connective tissues	Minor passive congestion and glomerular atrophy
AN HUS1	Congestion of central vein	Degenerated alveoli and multiple infiltration fluids	Minor vascular congestions extended vacuolations
AT EM-CN1	Severe enlargement and congestion of sinusoids	Congestions and widening of air sacs with degeneration of alveoli	Congestion cystic cavity and weakening of sinusoidal walls
AA AN5	Minor enlargement of sinusoids and inflammatory cells mainly PMNs	Minor degeneration of alveolar ovals with broken septa	Minor vacuolations with intact renal corpuscles
AW DA-SN2	Enlargement of sinusoids with deposit of granules	Reduction in alveolar size and vascular thrombosis	Minor passive congestion and intact renal corpuscles
Control	Normal liver morphology with intact hepatocytes	Normal being architecture with intact alveolar cells	Normal kidney morphology with intact medulla with prominent distal and proximal tubules

DISCUSSION

The obvious pathological signs such as weakness, anorexia, cough and weight loss significantly seen among the infected immunocompromised mice corroborated with the findings of McCree (2012) and Omar (2013). Cough was commonly observed among the infected immunocompromised mice due to the tendency of *Aspergillus* species spores to cause infection of the lung which could present cough as one of the clinical manifestations (Omar, 2013). The cases of mortality associated with *Aspergillus flavus* strain HUS6, *Aspergillus niger* strain HG-48 and *Aspergillus niger* strain HUS1, could be attributed to the toxigenic potentials of these fungi. *Aspergillus niger* is one of the fungal species that produces ochratoxins (OTA), which is known to be growth inhibitor, carcinogen and has lethal effect (Banford and Adebanjo, 2011). *Aspergillus flavus* is known for aflatoxins production, majorly aflatoxins B_1 and B_2 (AFB_1 and AFB_2), and these aflatoxins are associated with malabsorption of nutrients, uptake of vitamins A and D leading to nutrient deficiencies, carcinogenicity of visceral organs, interference in normal protein synthesis, inhibition of several metabolic systems and lethal to human (Bbosa *et al.,* 2013; Milani, 2013; Omar, 2013;; Mohammed and Metwally, 2014).

The occurrences of air sacculitis, perihepatitis lungs hypertrophy, lungs hemorrhage and other histopathological changes in the infected immunocompromised mice could be attributed to the capability of the organisms to invade the visceral organs of the mice (Dashe *et al.,* 2013). The infections of the lungs were significantly observed in this study. This could be traced from the fact that the studied isolates have the ability to disrupt the unfold protein response (UPR) through deletion of the hac A gene which make them to thrive and grow on mammalian lung tissue (Taylor and Nancy, 2009). Also, several studies have shown that pathogenic *Aspergillus* species possess adhesions on the surface of their conidia for attachment into host lung cells, and esterase for invasive mechanism and pathogenicity (Tobias and Marta, 2007; Taylor and Nancy, 2009; Ghazaei, 2017).

The significant decreased in the mean organ weight to body weight ratio majorly the lungs weight to body weights ratio among the infected immunoincompetent mice supported the findings of many researchers (Bbosa *et al.,* 2013; Damijam, 2015; Damijam *et al*., 2015). Nirogi *et al.* (2004) reported that analysis of organ weight of body weight ratio is optimum for most of the organs for prediction of toxicity. Similar conclusion was drawn by other researchers (Dashe *et al*., 2013; Iheukwumere *et al*., 2017).

The significant mean viable plate counts of the test isolates recorded in the internal organs of the infected immunoincompetent mice, majorly the lungs supported the findings of Damijam (2015). The presence of these organisms in the lungs suggests that the organ contains sufficient nutrients and favourable environment for the growth of *Aspergillus* species (Dashe *et al*., 2013; Iheukwumere *et al*., 2017). The activities of the invading fungi in the lungs might cause degradation of the nutrients, obstruction of lumen of the lungs, deterioration and deformation of the lungs, thereby manifested on the infected mice (Dashe *et al*., 2013; Iheukwumere *et al*., 2017).

CONCLUSION

The isolates showed obvious pathological features which were pronounced in immune compromised mice. This is an indication that the organisms are pathogenic & capable of causing disease in immune compromised individuals.

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