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CLINICAL OBSERVATION AND ISOLATION OF EQUINE *ASPERGILLUS* SPECIES IN ABIA, ENUGU AND RIVERS STATES, NIGERIA

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ABSTRACT

This study attempted to observe and isolate equine Aspergillus species in Abia, Enugu and Rivers State, Nigeria. Observation of animals with disseminated aspergillosis showed that it is difficult to diagnose this condition early enough for successful treatment but adequate prognosis could be possible based on presence of lipid band in spun sera of sick horses. Affected animals had episodes of drastic weight loss prior to first appearance of creamy white crusty lesions on the lower parts of the limbs which progress to all parts of the body. Physiological parameters and liver enzymes remained non indicative of disease until close to recumbency. In fatal disease there was increase respiratory rate and subnormal rectal temperature. Ninety six sera and nasal swab samples each obtained from (34) ObolloAforin horse market, Udenu, Enugu State, 7 research and domestic horses from Umuahia Abia State, 55 horses from Polo Club Port-Harcourt, Rivers State were screened for aspergillus infection by culture and isolation. There was no growth from serum and nasal swab samples of market and domestic horses managed on extensive and semi-extensive system respectively. Aspergilli growths were identified from nasal swab (four) of horses from polo club, managed intensively. *Aspergillus fumigatus*, A. *flavus*, *Citrobacter diversus*,*Citrobacter freundi* were isolated from blood, skin and nasal swab of animals with fatal infection. There was no sex, breed or age predisposition to infection using χ^2 test (χ=0.79, 0.81, 0.52) respectively at 5% confidence interval, but a positive susceptibility related to management and uses of animals was observed (χ= 0.027and 0.028) respectively. Though a fatal disease, aspergillosis was found uncommon in horses screened using basic laboratory procedures.

Key words: Clinical Observation, Isolation, Equine *Aspergillus species*

INTRODUCTION

This study was carried out to detect subclinical aspergillosis in apparently healthy horses and relate to observed clinical disease. Aspergillosis in horses has been primarily caused by *A. fumigatus, A. flavus* and *A. niger* which are the most common pathogenic species worldwide (Verweji and Brandt., 2007; Segal, 2009). Ante mortem diagnosis of aspergillosis can be difficult because Aspergilli are ubiquitous and can be isolated from the nasal cavities of healthy animals (Kahn, 2005). The clinical signs of upper respiratory involvement, chronic weakness, weight loss, and change in voice or exercise intolerance, skin lesions are non specific to Aspergillus infection it is thus difficult to make the right laboratory request.

Most advanced diagnostic techniques such as thoracocentesis, examination of tracheal washings, radiographic changes which may not be visible in early cases except in advance stage with organ involvement, Cross-sectional imaging via computer tomography (Ramirez *et al*., 2002; Kahn, 20052) are expensive and not commonly performed in this environment. In chronic infection, there is non-regenerative

anaemia; the differential count in haematology usually reveals neutrophilia with a left shift (Aguilar and Redig, 1995; Heidenreich, 1997). In serum chemistry, an increase in total serum protein especially in the globulin portion, an increase in Aspartate Transaminase and bile acids relating to hepatic involvement (Lee *et al*, 2004). Indirect ELISA for detection of antibodies is useful for the diagnosis and monitoring of the progress and response to treatment of aspergillosis. This test can detect antibodies against *Aspergillus fumigatus* as early as one week post infection. This is much earlier than the time clinical sign of the disease are obvious and the treatment can be started having better result (Heidenreich, 1997; Harris, 2000; Redig, 2000). The choice of ELISA may not be justified in an early infection. Laboratory diagnosis of aspergillosis based on isolation and identification has to be complemented, by history of poor sanitation in the environment (Agarwal *et al*., 2013), weak and immunocompromise horses not responsive to antibiotic treatment (Redig *et al*., 2000), and by other techniques on the basis that, microscopically fungus mycelia generally are intimately associated with tissues making them rarely visible in exudates or body fluids (Oglesbee, 1997; Ivey, 2000). *Aspergillus* species grow at temperatures from 25 to 37^0C, on sabouraud dextrose agar or blood agar with antibiotics like chloramphenicol, they are sensitive to cyclohexamides (Biberstein and Zee, 1990; Redig *et al*., 2000; Akan *et al*., 2002). *Aspergillus fumigatus* colonies have a diameter of approximately 3 to 4cm in 7 days. The flat colonies are white at first, then bluish green as conidia begin to mature, especially near to the centre of the colony. As the colony matures, conidial masses become gray-green, while the colony edge remains white. A distinctive feature of *A. fumigatus* is the development of columnar masses of chains of conidia arising from the vesicle (Richard, 1997). This organism is resistant to high temperature and can grow well at 45^0C (Quinn *et al*., 1994; Abram *et al*., 2001). The above are the most typical characteristic but variations occur in colony colour, surface and reverse and colony morphology (Lopez *et al*., 1995). *Aspergillus flavus* grows very rapidly, obtaining a colony diameter of approximately 6 to 7 cm in 10 days at 25^0C. The colony begins with a white colour, turning yellowish to yellow-green with a white edge as conidia develop. Massive colonies may become somewhat olive-green (Richard, 1997). *Aspergillus niger* begins with a white colour but rapidly develops a black colour as conidia mature. The colony reverse is yellowish (Quinn *et al*., 1994). Samples of *Aspergillus* species can be seen microscopically using wet preparations with 20% kolt, lacto phenol blue, calcofluor white or new methylene blue, potassium hydroxide, Hematoxlin-Eosin, periodic acid Schiff or Grocotts' stains (Cork *et al*., 1999). Some drops of the stain are put on the slide, the sample added and a cover slip placed. When kolt is used, the slide may be heated over a flame (Bauck, 1994; Dylestra *et al*., 1997; Cork *et al*., 1999). *Aspergillus fumigatus* presents smooth conidiophores and single series of phialides arranged upward paralleling the axis of the conidiophores. Conidia are echinulated, spherical to semispherical (Richard, 1997). Aspergillus flavus Conidia are elliptical or spherical and echinulated. Vesicles are spherical, phialides are in 2 series over the vesicle surface, but can be in one serie (Carter *et al*., 1994). *Aspergillus niger* is observed with big fruiting bodies. Vesicles are spherical; conidia are black, rounded and smooth with phialides in one or two series (Lopez *et al*., 1995).

MATERIALS AND METHODS

Study Area: Samples were collected from three different geographical locations in the South Eastern and South- South regions of Nigeria. The locations include ObolloAforinin Enugu State, located at Latitude 6^o 91" N and longitude7^o55"E 6^o91N, Umuahia located at Abia State and lies within latitudes 5^o 32' 0''North and 7^o 29' 0'' East and longitudes 5^o.417North and 7^o50"East (Hoiberg and Dale, 2010) while Port-Harcourt is located in Rivers state in Latitude 4^o 45" E and longitude 6^o50" E in the South-south region of Nigeria. Fifty-five samples were collected from the polo club

at Port-Harcourt, Rivers State and 34 from horse market at Obollo Afor in Enugu State. Three samples were collected from research horsesat the Veterinary Teaching Hospital, Michael Okpara University of Agriculture,Umudike, Abia State and four private horse owners.

Observation of horses: Sick horses with natural infection were observed over a period of one and half year and screened for the presence of Aspergillus. Sera, nasal swab and skin specimen were collected from these sick horses for serum chemistry and isolation of Aspergillus organism.

Sample collection: A total of 96 sera samples and nasal swabs were collected using 30 to 50% random sampling method from apparently healthy horse of the polo club, market and research. Samples were screened for subclinical or asymptomatic aspergillosis. Information on breed, sex, age, uses and type of management practice in husbandry were obtained from owners during sample collection and recorded in a form sample below. Vital parameters of the animal were recorded.

Blood sample collection: Horses from 2 to 15 years of age were sampled. Aging was done from information given by owners and confirmed by aging using dentition. The blood samples were collected by jugular puncture using 10ml syringes and 18 gauge needles under adequate restrain aided by groomsmen. After collection some of the samples were allowed to settle by placing the syringes in a slant position after which they were immediately preserved in a flask containing ice packs then later they were transferred to a refrigerator and kept at a temperature of -4^0C to -8^0C. Where the laboratory was close to the location, samples were collected in plane vacutainer tubes and spun in centrifuge at 1500g for 5minutes.

Serum extraction: The serum was carefully decanted into Eppendorf tube from the blood samples that had been allowed to clot in the syringes for about 12 hours in a slanting position. On clotting of the blood, the serum was transferred into a sample bottle and immediately taken to a freezer and stored at a temperature of -20^0C to – 30^0C till when needed.

Nasal swab collection: Nasal swabs were collected by inserting the sterile swab sticks into the nostrils, rolling the sterile swab sticks anticlockwise or clockwise manner and thereafter preserved in a flask containing ice blocks after which they were transferred in the refrigerator and kept at a temperature of -4^0C to -8^0C.

Culture and Isolation of Aspergillus species from sampled horses

Media was prepared using the appropriate amount of agar and water at the given concentration of 65g of agar to 1000ml of water from the manufacturer. The mixture was dissolved using Bunsen burner after it was well stirred. Then covered using a foil and sterilized in an autoclave at 121^0C, 15mmHg in 15mins. After sterilization, the media was cooled to 50^0C and then dispensed into the Petri dishes within a hood chamber, and was allowed to set. It was turned upside down to prevent moisture and kept in the refrigerator till the next day. Plates were labeled and inoculated with sera and nasal swabs samples of screened horses then incubated at 37^0C for 48 to 72hours. Plates on which growth were not seen after 48 hrs were allowed for up to 72 hours or more. Those plates that had colonies were observed for colony morphology and other descriptions related to the fungus of interest. Organism was harvested from colonies on positive plates, placed on a clean slide, fixed with methanol, air dried then stained with lactophenol cotton blue. Stained slides were observed under low power microscope for spores and hyphae of *Aspergillus* species.

RESULTS

Clinical observation of horses affected with aspergillosis

The two affected horses were found to be concurrently infected with *Aspergillus fumigatus* and *Aspergillus flavus. Citrobacter*

diversus and *C. freundi* were isolated alongside with *Aspergillus* species from the blood of infected horses. The horses presented overt clinical disease that had a fatal course. Disseminated systemic disease seen in infected horses had an initial phase of a periodic loss of weight with no history of alterations on feeding or other management conditions. The animal regains fair body condition temporarily and looses it in a cyclical manner with repeated episodes two to three months after. Within this period other signs especially skin lesion intensify and become more obvious. At this stage of aspergillosis more common ailments such as helminthosis and ectoparasites infestation are most likely to be diagnosed. In this particular case there was tick infestation and a misdiagnosis of tick bite dermatitis and dermatophilosis tentatively was made. Animal were screened and treated for helminthosis and ectoparasites. Three to four of such observations were made over a period of about eighteen months. The primary skin lesions in affected animals were infested by ticks and were confused with tick bite dermatitis (Plate 1 A). These lesions appeared first below the fetlock of the hind limbs the lighter skin animal such lesions may not be easily noticed. This stage of equine aspergillosis can easily be confused with bacteria or fungi dermatitis such as dermatophilosis, equine blastomycosis or sporotrichosis. The vital parameters which have remained within normal physiological ranges gradually became subnormal (Table 3). Centrifuged sera of affected horses presented a large lipid band suggesting advanced liver damage (Plate 2). Serum chemistry (Table 1) showed high level of alkaline phosphatase which could not be assigned specifically to liver damage.

The presence of *Citrobacter* organisms from blood samples of affected animals suggested possible enteritis and septicaemia; but there were no observable signs of colic or gastrointestinal upset. Skin lesions were temporarily cleared using common antiseptics such as topical iodine or gentian violet leaving a raw surface that heals to a smooth dark scar tissue. The white creamy powdery scabs which first appeared on the limbs soon become extensive covering virtually every other part of the body (Plate 1B and 1C). At this point the diagnosis was made but very late for a successful treatment. Organism was sensitive to Itraconazole at the dose of 10mg per kg daily. Duration of treatment was estimated to cover a minimum period of three to six months. The younger one of the affected horses had apparent good respond to therapy but because it had a fix economical value lesser than the cost of treatment euthanasia was advised to the owner. The older horse presented a lower resistance compared to the younger one. Owner was reluctant to euthanasia and both animals died from a normal course of illness.

Isolation of *Aspergillus* species from screened horses

The summary of samples collected (Table 2) shows that the dominating breed in the Polo Club was Thalon breed, followed by the Sudan, Local then the Thorough breed. From Market horses The Local breed was most common. Much older animals were encountered on the market and younger animals constituted the larger group in the Polo Club. A total of 63 males were screened and four of them had non clinical infection with Aspergillus organisms. Among thirty nine females, two had severe disseminated fatal disease described in this study (Table 2). There were more males than females in the total population sampled. Aspergillus organism was isolated from nasal swap of intensively managed horses (4) of the polo club Port-Harcourt and (4) of research horses. Aspergilli isolation from serum was only positive in clinical cases whereby isolation was possible from every other tissue (skin, blood, upper respiratory) owing to haematogenous dissemination of infection. Organisms were isolated from the nasal swab sample of both clinical and some apparently healthy; from skin lesions of sick animals. The growth from the sera was only from the clinical cases with invasive aspergillosis and none from apparently healthy animals using culture and identification.

Aspergillus flavus and *fumigatus* were isolated from the sera samples of the two research animals observed with clinical invasive aspergillosis. Out of the ninety six nasal swaps screened six swaps were positive from which *aspergillus* organisms were isolated based on colony description (Plate3), tubular septated hyphae (plate 4), conidophores and spores (Plate 5 and Plate 6) as observed under microscope.

A B C



Plate 1: Feet and body Lesions on animal with disseminated aspergillosis.

A - Lesions on the feet of affected horses

B and C - Disseminated creamy white powdery scabs on body of horse with aspergillosis

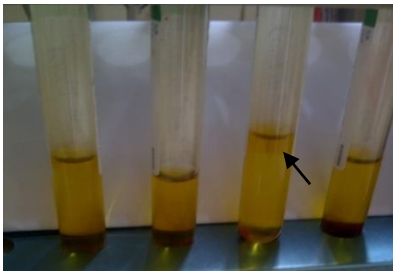


Plate 2: Lipid band on spun serum of horse with aspergillosis.

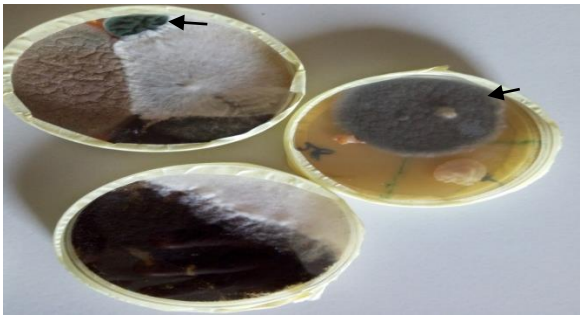


Plate3: Colonies of A.fumigatus on SDA

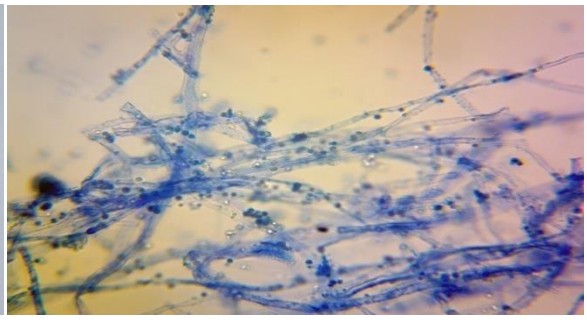


Plate4:Hyphae& Spores of Aspergillus (x400)

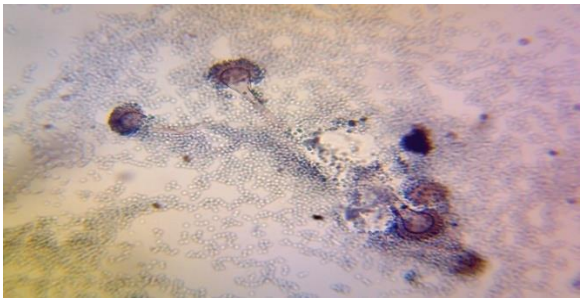


Plate5: Conidophores (x 400)



Plate 6: Aspergillus spores (x400)

Table 1: Serum chemistry of horse with disseminated aspergillosis

LIVER ENZYMES	
ANALYTE	**VALUES**
ALKALINE PHOSPHATASE (ALP)	326.5U/L
γ-GLUTAMYLTRANSFERASE (GGT)	20.8U/L
ALANINE TRANSAMINASE (ALT)	9.36U/L
ASPARTATE TRANSAMINASE(AST)	11.0U/L
TOTAL BILIRUBIN	0.4mg/dl
BILIRUBIN- DIRECT	0.1mg/dl
BILIRUBIN- INDIRECT	0.3mg/dl

Table 2: Summary details of 96 sampled animals from the three different locaions (Polo Club Falga Port-Hartcourt Rivers, Obollo Afor horse market Udenu Enugu, research horses Umudike Umuahia Abia).

	sex		**Breed**			**age**				**mgt**			**uses**		
	M	F	Thal	Sudan	Th/breed	Local	2-4	5-10	> 10	Int	Sem /Int	Ext	Pol	Rese arch	Mar ket
positive	4	2	3	1	0	2	1	5	0	2	4	0	4	2	0
negative	31	59	34	10	3	43	12	62	16	51	5	34	51	7	34
χ^2value	0.7990				0.8185			0.5229			0.027			0.0288	

Table 3: Mean values and standard deviation of vital parameters of sampled animals

Breed of horses	Temperature ($\bar{x} \mp$ SD)	Heart Rate ($\bar{x} \mp$ SD)	Respiratory rate ($\bar{x} \mp$ SD)
Thalon	37.32±.07	57.62±0.53	12.00±0.12
Sudan	37.65±0.15	58.30±1.00	11.80±0.20
Thorough breed	37.67±0.33	57.33±1.33	11.67±0.33
Local	37.62±0.08	59.68±0.44	12.58±0.21
Sick animals	37.7±0.9	39±1.5	20.5± 1

DISCUSSION

The fatal disease in this study was caused by concurrent infection with A*spergillus fumigatus* and *Aspergillus flavus*. There was severe loss of condition in this case and it occurred in different episodes before the primary cutaneous lesions were observed, similar observation has been reported by Krogh and Lundegaard (1986), Blomme *et al*, (2010) in guttural pouch and pulmonary disease. The nature of weight loss relatively differs from that described in literature in that it occurs in cycles of about three months interval. At the first instance of emaciation it could be difficult for a clinician to think of aspergillosis investigation may be focused on change in diet, malnutrition, gastrointestinal or blood parasites. In some cases the disease may be masked by other infections and can only be diagnosed when system and organ damage (with reference to the liver) has become irreversible. First manifestations of clinical signs such as drastic loss of weight could be confused with more common diseases of bacterial and parasitic origin such as helminthosis and haemoparasitic infections; possible skin involvement to condition as allergic dermatitis, ectoparasitic infections, dermatophilosis. A striking association between development of invasive pulmonary aspergillosis with previous acute enterocolitis has been described. It is said that enterocolitis in horses predisposes to pulmonary aspergillosis by facilitating mycotic invasion from the gut and subsequently development of embolic mycotic lung disease. In this study a similar finding could be described with the presence of *Citrobacter freundi* isolated in infected animals alongside with the pathogenic aspergilli. The low percentage (4.3%) of positive apparently healthy was obtained among intensively managed polo horses. Some of which were said to have shown signs of colic and this is similar to a finding reported by (Slocombe and Slauson, 1988). Age related immunocompromization could be reported on the basis that the older animals manifested signs of disease and died earlier than the younger ones even though statistically there was no age susceptibility to infection (P=0.52). It is important to make early diagnosis by means of simple laboratory techniques for effective and economical clinical decision. It could be noted that horses showing loss of condition should be screened for aspergillus infection given that early detection is paramount to successful management. Furthermore skin lesions on susceptible animals should be observed carefully so as to differentiate the powdery creamy white lesion in invasive aspergillosis from dry crusts and scabs in Dermatophilosis or moist erythematous lesions in other allergic bacterial or parasitic dermatitis. Liver enzymes test show relatively high levels of alkaline phosphatase value which is not

specific to liver damage as earlier reported by (Lee *et al*, 2004). The presence of lipid band in spun sera of affected horses is more suggestive of the liver disease as had been described by Agarwal *et al*. 2013 in systemic infection with *aspergillus* organism. The species of aspergillus isolated from these infections are known to actively produce aflatoxins which are most likely the cause of liver damage evidenced by presence of lipid band in the serum of affected animal (Plate 2). At this stage the disease is advanced and a rational clinical decision could be made based on prognosis after treatment. Adversely the cost of treatment in most cases could be higher than the fixed value of the affected animal, as such the economical decision remains euthanasia except when other values may be attached to the animal. Microscopically Aspergillus mycelia presented tubular septated hyphae (Plate 4) similar to the description in literature (Cork *et al*., 1999). *Aspergillus fumigatus* presents smooth conidiophores with a length up to 300μ and a diameter from 5 to 8μ. Vesicles are from 20 to 30μ in diameter, and are arranged upward paralleling the axis of the conidiophores (Plate 5). Conidia are echinulated, spherical to semispherical and from 2 to 3μ in diameter (Richard *et al*., 1997). *Aspergillus flavus* contains conidiophores of up to 100μ in length and 10 to 65μ in diameter. Vesicles are spherical (Plate 6), from 30 to 45μ in diameter and phialides in 2 series over the vesicle surface, but can be in one serie. Conidia are elliptical or spherical echinulated and from 3 to 6μ in diameter (Carter *et al*., 1994; Lopez *et al*., 1995).

The result of this study shows that disseminated aspergillosis however is not a common disease of horses as none of the apparently healthy animal screened had signs of ongoing infection. Aspergillosis has been considered over the years as a disease of poultry production and is predisposed by poorly managed and processed feed fed to poultry. Infected animals in this study were equally housed in a mixed farming system were poultry and other livestock feed was processed coupled with inadequate housing and nutrition. The disease appears to occur as a result of poor management since isolate were only obtained from animals in confine of intensive management system. From background they were hosted in a mixed farming system alongside with pigs, cattle sheep and goats; and some laboratory animals. Source of infection was thought to be from animal feed processed in the same environment. Chronic, highly fatal disseminated when lesions become obvious on the skin of infected horses. Aspergillosis cause a systemic disease and toxicosis leads to death if not diagnosed early and treated. This study shows that the primary source of aspergillus is from moldy feed which are given to the horses in confinement system of management, but it is not seen in free ranging horses fed on little or no processed feed.

Other source could be the unhygienic environment especially beddings as described by (Guillot *et al.*,1999). The polo horses are given feeds that are wet and have grown mould by handlers who may be inadequately informed on the possible disease risk. Aspergillosis has been considered over the years as a disease of poultry production and is predisposed by poorly managed and processed of feed fed to poultry. Infected animals in this study were equally housed in a mixed farming system were poultry and other livestock feed was processed coupled with inadequate housing and nutrition. The disease appears to occur as a result of poor management since isolate were only obtained from animals in confine of intensive management system. From background they were hosted in a mixed farming system alongside with pigs, cattle sheep and goats; and some laboratory animals.

Aspergillosis though a fatal disease of horses is not common in the horse population in the area of study, rather is predisposed by poor management and relative immune compromization. Diagnosis however could be made based on careful observation of clinical signs complemented with basic economical and reliable screening tests.

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