ORIGINAL RESEARCH

MODELS OF DAMAGES CAUSED BY MICROSPORIDIA INFECTION AMONG HIV-POSITIVE PATIENTS IN WHITE ALBINO MICE (*mus musculus domesticus*)

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Received: 16/12/2024; accepted for publication 1/3/2025

ABSTRACT

Background: Microsporidiasis has been reported mostly in patients with Human-Immuno Deficiency Virus (HIV)/AIDS with diarrhea and in more than 50% of these patients, no enteric pathogen are identifiable.

Aim: This study attempted to establish the natural route of human infection of microsporidiasis and study the pathologically induced manifestations in experimentally infected mice.

Methods: We isolated microsporidia spores from stool specimens of HIV-infected patients aged 2-61years and experimentally infect Albino mice with these isolates in an attempt to establish the natural route of human infection and study the pathologically induced manifestations in these experimentally infected mice. Seven hundred and fifty stool samples were

collected from HIV-infected patients and 375 samples from their non-infected counterparts to determine the prevalence of Microsporidia infection. Chromoptrope 2R and Ficoll-Hipaque techniques were used to isolate Microsporidia spores. Purification of these isolates was conducted using sucrose gradient centrifugation technique. Prednisolone was used to suppress the immune system in the experimental albino mice. Immuno-compromised and immuno-competent albino mice were orally, intranasally and intravenously inoculated with 55 spores/10µl in Phosphate Buffer Saline. At intervals of 2, 5, 10, 17 and 28 days post-infection, the infected mice were sacrificed and their internal organs processed for histopathological studies, using heamatoxylin and eosin stain.

Results: The prevalence of microsporidia isolates in the stool samples of 750 HIV-infected patients (42.4%) was significantly higher than in the HIV-non-infected subjects (19.2%) ($P<0.05$). In the immuno-suppressed Albino Mice, pathological damages were induced in the lungs, small intestines, kidneys and livers, unlike in the non-immuno-suppressed group. Oral ingestion and/or intranasal inhalation are considered to be the natural routes of infection because viable microsporidia spores were recovered only from the stools of treated mice which received microsporidia spores inoculums orally and intranasally.

Conclusion: Microsporidiasis is prevalent among immuno-compromised (HIV/AIDS) patients and the associations of microsporidiasis with overt pathology in vital internal organs have been demonstrated in the immuno-suppressed mice.

Keywords: Models, Damages, Microsporidia, HIV-Positive, White Albino Mice

INTRODUCTION

Microsporidiasis has been found mostly in patients with Human Immuno Deficiency Virus (HIV)/AIDS and chronic diarrhea is common in these patients[1]. However, in more than 50% of these patients, no enteric pathogen are identifiable[2]. Two possible reasons for these cases of unexplained Diarrhoea are; intestinal infection with HIV that has been associated with enteropathy in these patients and infection with yet to be identified enteric organisms[3]. The HIV/AIDS pandemic has revealed the propensity of microsporidia species to infect man, where they have being implicated in causing intestinal, ocular, pulmonary and renal diseases[3]. However, latent cases of the infection in healthy individuals becoming exacerbated when such individuals become immunosuppressed have been reported[4]. The high prevalence of microsporidia infection in immuno-compromised patients has posed serious public health and socio-economic challenges among these patients[5].

At present, microsporidiasis is being recognized as an important medical and public health problem both by the World Health Organisation and the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland[6]. A major complication and cause of death among the

HIV-infected patients is Diarrhoea and *E. bieneusi* has been strongly implicated as a major aetiological agent of the chronic Diarrhoea and excessive wasting, high morbidity and high mortality rates among the HIV-infected patients[7]. Elsewhere, these microsporidia have also been reported to have infected even patients with competent immune system[8] and in symptomatic immuno-competent individuals, a self-limited diarrhoea is the most common clinical manifestation too[9].

In spite of its medical, public health and socio-economic implications, there is paucity of information on microsporidia species, particularly in developing countries where no concerted effort has been made to ascertain the prevalence, intensity, mode of transmission and tissue pathology caused by microsporidiasis, especially in the HIV-infected patients. Therefore, it should be a concern that while HIV prevalence, morbidity and mortality rates are rising, the prevalence of microsporidiasis remains unknown and unstudied among HIV/AIDS patients in developing countries. In Nigeria, studies on human microsporidia and microsporidiasis, either among HIV-infected patients or among any other human groups is limited to many. We determined the prevalence of microsporidiasis among HIV-infected patients, isolated the microsporidia spores in human stool specimens of these patients and then study the histopathology of microsporidiasis in experimentally infected laboratory mice.

MATERIALS AND METHODS

The Study Area. This work was carried out at the University of Ilorin Teaching Hospital (U.I.T.H), a tertiary and referral hospital located in Ilorin, Kwara State, Nigeria. UITH is involved in teaching, research and training of medical students, resident doctors and other paramedical studies. Kwara State is the gate way between the south-west and the north and is located south-east of the river Niger. It covers above 75000 km^2 on land mass and shares common boundaries with Benin Republic in the West, Oyo in the Southwest, Niger in the North, Ekiti, and Osun in the South, and Kogi in the East. Ilorin the State capital is located at longitude 8^0 30N and latitude 4^0 30E.

Study Design: A cross sectional study was designed to determine the prevalence of microsporidia species from human stool specimens of immuno-compromised (HIV-infected) patients and immuno-compromised (non-infected) individuals as control.

Preliminary Sampling: All the subjects (HIV-Positive and HIV-negative) in this study were screened for HIV-sero-status as a prerequisite for stool collection. This prerequisite distinguishes the HIV-infected patients from the HIV-non-infected control. Three screening methods, Antec HIV test, Determine test and Uni-Gold test were concurrently used to test and confirm HIV-sero-status.

Copies of the questionnaire were administered on each subject whose stool samples would be collected. They provided information such as; name, sex, age,

address, tribe State local government area, profession/occupation etc. A tag number was given on the questionnaire to correspond with the specimen bottle so as to ensure that each specimen corresponds with the given information.

Determination of Sample Size: This sample size was predicted on the prevalence (39-97%) rate of chronic Diarrhoea among the HIV-infected patients as was determined using Fisher's formula[10]. An overall sample size of one thousand one hundred and twenty five (1125) humans were examined for this study. This composed of seven hundred and fifty (750) HIV-infected patients and three hundred and seventy five (375) HIV-non-infected control, matched for age, sex, and other socio-economic variables.

Ethical Approval: Ethical approval was sought for and obtained from the Ethical Review Committee of the University of Ilorin Teaching Hospital with Reference number: UITH/021/ERC2023. Relevant cooperation and assistance of various heads of clinical departments were mobilized for and granted.

Informed consent sheet: The purpose of what the study is all about was given to all subjects and participation was voluntary, the confidentiality of whatever information given for the purpose of this study was guaranteed.

Data collection procedure

Stool Specimen Collection: Stool specimens were obtained from both the HIV/AIDS patients aged between 2 and 61years and the HIV-non-infected control in the same age group in sterile universal bottles. Sterile universal bottles were used for this study for collection of stool samples for two main reasons; to avoid contamination which would have been acquired environmentally that could affect isolating microsporidia spores in its pure form and to conform with the internationally accepted standard on stool collection in the contemporary world.

The Modified Trichrome Stain (Chromotrope2R) Staining Method: The Trichrome staining method was modified by the CDC Atlanta Georgia, USA, for the identification of microsporidia spores from human stool samples. The main dye, Chromotrope2R, was acquired from Sigma-Aldrich^{Tm} (Sigma-aldrich Co.3050 Sprule St. Louis, mo, C 3143-25G; Cust PO No. ZSA/NGO/ISA/AMASE/001. procedures were done according to manufacturers' instruction.

Ficoll-hypaque technique: This technique was used in this study to isolate pure microsporidia spores from the stool specimen. It is a sterile, ready to use method of density gradient mechanism for purification from human fluids, substances according to their densities using simple centrifugation procedures. Ficoll-hypaque is an aqueous solution of density 1.077±0.0019/ml, containing 5.7g Ficoll 400 and 9g sodium diatrizoate with 0.0231g

calcium disodium ethylenediamintetra-a-cetic acid in every 100ml. It was acquired from Sigma chemicals co. (ca + No SL – 2).

Procedure for isolation of pure microsporidia spore from stool samples: Using Ficoll-hypaque: The refrigerated portion of the microsporidia spore-positive stool-samples was saturated in a 10-fold concentrated phosphate buffer saline (PBS), pH 7.2 to 7.4 and filtered through series of nylon sieves (pore size, 210, 100, 70, 50 and 20µm; small parts, Inc Miami Lakes Florida, USA). The filtrate was then separated, 2ml into a 10ml test-tube (plastic). With the help of a syringe with needle attached, the aqueous Ficoll-hypaque was withdrawn 3ml and put in another 10ml test-tube. The 2ml sieved stool sample was then gently added on top of the 3ml aqueous Ficoll-hypaque. This was then centrifuged, using a cold centrifuge at 400g for 35 minutes at 20^{o}C. The middle band which contained microsporidia spores was carefully collected through a syringe attached with needle. Pure microsporidia spore became heavily concentrated when re-stained with Chromotrope2R. They (the pure microsporidia spores) were stored in a PBS solution and refrigerated under seal for further studies.

Quality Control: As a quality control measure, a control slide of microsporidia spores in a 10% formalin preserved specimen from the CDC Atlanta, USA (No. UN 3373) [25] was viewed alongside with the prepared slides. Spore walls of microsporidia stain a pinkish-red colour and measured about 3 µm were confirmed from both self-prepared and the control slides. All solutions subsequent to chromotrope stain were changed after every 10 slides to obtain proper rinsing and dehydration.

Animal Study design

A total of 43 Albino mice (*Mus musculus domesticus*) were used in this study. These animals were aged between 7-12 weeks, with an average weight of 148g, and their normal CD_4+ cell count ranges between 18-22 cell/mm^3. They (the animals) were divided into three major groups (the immuno-suppressed group– 18, the immuno-competent group– 18, and the Prednisolone administered free line group-6). One of the mice was sacrificed for normal histopathology of the targeted organs (the heart, lungs, livers, kidney, small intestine and the spleen). There were three routes of parasite inoculation (orally/perors, intranasally and intravenously) in both the immuno-compromised and the immuno-competent groups.

Determination of immuno-suppression in the animals

The Determination of immuno-suppression in the animals was done using prednisolone as the immuno-suppressant. The first group of animals whose immune system was suppressed, modeled the HIV-seropositive patients. A 8.6mg/kg body weight of prednisolone serves as the LD50 that reduced the CD_4 cell count of the animals by 50%. Half of this dosage was given to the animals after every 24hours in line with the 1/2 life of the drug. The same process was

applicable for the 6 animals left to move freely within the two groups (immuno-suppressed and immuno-competent groups) serving as control.

Grouping and parasite Inoculation.

In each of the immuno-suppressed and immuno-competent groups (18 each=36) there were three routes of inoculation.

Immuno-suppressed Group -18
- 6 for oral inoculation.
- 6 for intranasal inoculation.
- 6 for intravenous inoculation.

immuno-competent Group– 18
- 6 for oral inoculation.
- 6 for intranasal inoculation.
- 6 for intravenous inoculation.

These animals were marked according to the route of infection in each case and kept in the same feeding and environmental conditions, but in different cages.

Free living/immuno-suppressed Group -6
– 2 mixed with those infected orally
- 2 mixed and with those infected intranasally
- 2 mixed with these infected intravenously

This group of animals (free living) have their immune system suppressed just as the 18 animals in the immuno-suppressed group, but, they were not infected with the parasite and were allowed to move freely with the infected animals in each group, serving as control for the prednisolone administered animals.

Determination of sacrifice days.

The prime number interval selection by Silveira and Canning[10] was used in this section of the study. It is based on the duration of the developmental biology of the parasite in the target organs. Five days of sacrifice were used as shown below:

Day 2 = 1st prime number = 2nd day after inoculation = 1st day of sacrifice

Day 2+ (3, 2nd prime number) = 5th day after inoculation = 2nd day of sacrifice

Day 5 + (5, 3rd prime number) = 10th day after inoculation = 3rd day of sacrifice

Day 10 + (7, 4th prime number) = 17th day after inoculation = 4th day of sacrifice

Day 17 + (11, 5th Prime number) = 28th day after inoculation = 5th day of sacrifice

From the 1st to the 4th day of sacrifice, six animals were sacrifice each day. One for each, in the oral, intranasal and intravenous routes of inoculation.

On the 5th day of sacrifice, 9 animals were sacrificed, including the free living immuno-suppressed mice who were infected with the parasite.

Tissue Staining Procedure: The conventional Hematoxylin and Eosin stains were used in this study for Histopathology as contained in Manual of Histologic and Special Staining Techniques and Atlas of Protozoan Parasites in animal tissues, 2nd ed. and used by Gardiner *et al*.[12].

RESULTS

This study established that the prevalence, of microsporidia species at the University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria, in a total of 1125 patients examined is significantly high (34.7%) P<0.05. Seven hundred and fifty (66.7%) were HIV-infected patients while 375 (33.3%) were HIV-non-infected patients. The prevalence of microsporidia infection among the 750 HIV-infected patients was 42.4%. The corresponding prevalence among the HIV-non-infected patients was 19.2%. This difference in the prevalence of microsporidia infection among HIV-infected patients and their HIV-non-infected counterparts was also statistically significant (p<0.05) (Table 1).

Table 1: Prevalence of Microsporidiasis among HIV-infected and HIV-non-infected Patients (N=1125).

Infection Status	Total No. Examined	No. (%) +ve*
HIV-infected patients	750	318 (42.4)
HIV-non-infected patients	375	72 (19.2)
Total	**1125**	**390 (34.7)**

P<0.05, * = Microsporidiasis.

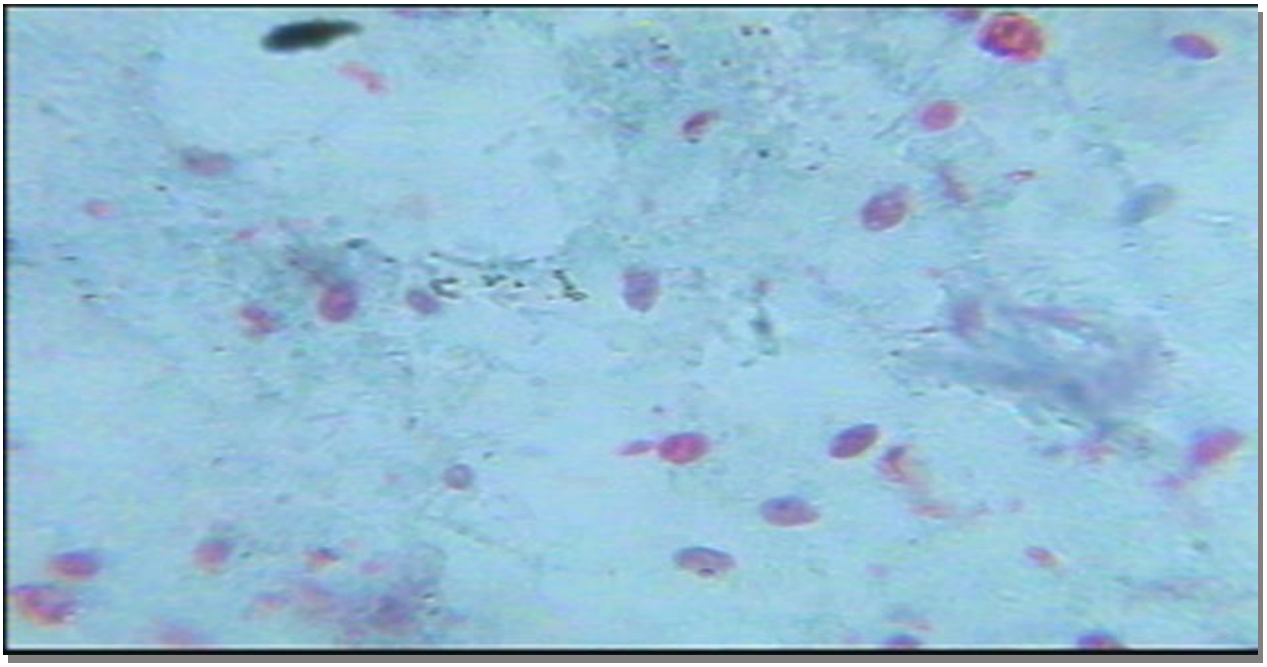
Table 2: Prevalence of Microsporidiasis among HIV-infected and HIV-non-infected Patients by Gender (N = 1125)

Infection status	Male		Female		Total
	No. Examined	No. (%) +ve	No. Examined	No. (%) +ve	No. (%) +ve
HIV-infected patients (N = 750)	356	141 (39.6)	394	177 (44.9)	218 (42.4)
HIV-non-infected patients (N=375)	176	35 (19.9)	199	37 (18.6)	72 (19.2)
Total (N=1125)	**532**	**176 (33.1)**	**593**	**214 (36.1)**	**390 (34.7)**

P>0.05

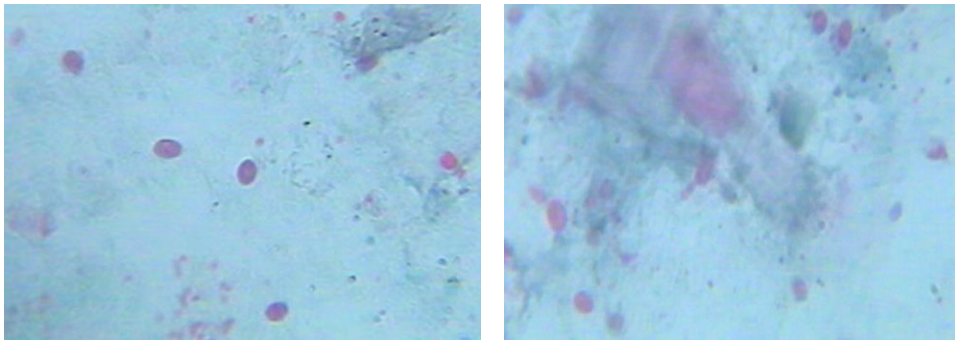
Prevalence of Microsporidiasis by Gender

Table 2 shows that, the prevalence of microsporidiasis among HIV-infected males was 39.6% and was 44.9% among their female counterparts. The difference in the prevalence of microsporidiasis among HIV-infected males and HIV-infected females was not statistically significant P>0.05. Microsporidiasis prevalence among HIV-non-infected males was 19.9% and among HIV-non-infected females was 18.6%. The difference was not statistically significant P>0.05. The total prevalence (both HIV-infected and non-infected patients) among males was 33.1% and among females was 36.1%. The difference however was not statistically significant P>0.05).



The CDC control slide, (No.UN3373)

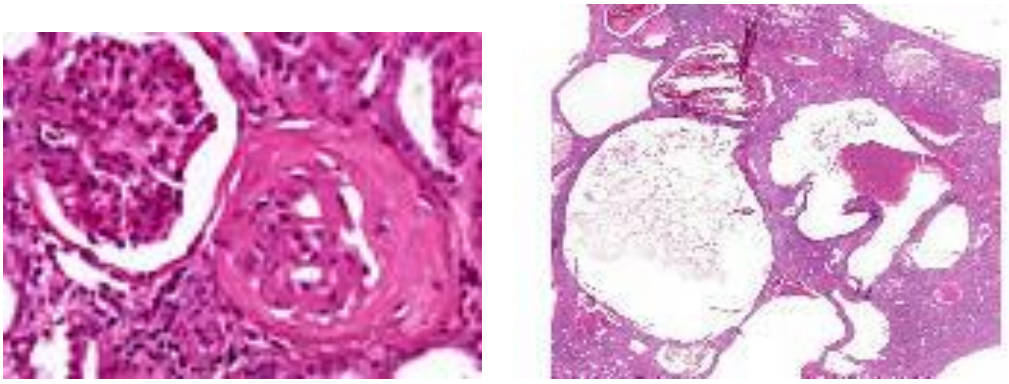
FIG. 1: Images (Photomicrographs) of Microsporidia Stained Samples from Stool Specimens in this Study in Comparison with the Control (the CDC prepared slide 100X)



A B

Figs. 1:A & B (100X); are the Chromotrope 2R stained slides of microsporidia spores from this study, measuring from 1.0-1.8µm x 0.6-1.0µm to 2.0 – 3.5µm x 1-2.0µm using PMIAS 3.0.

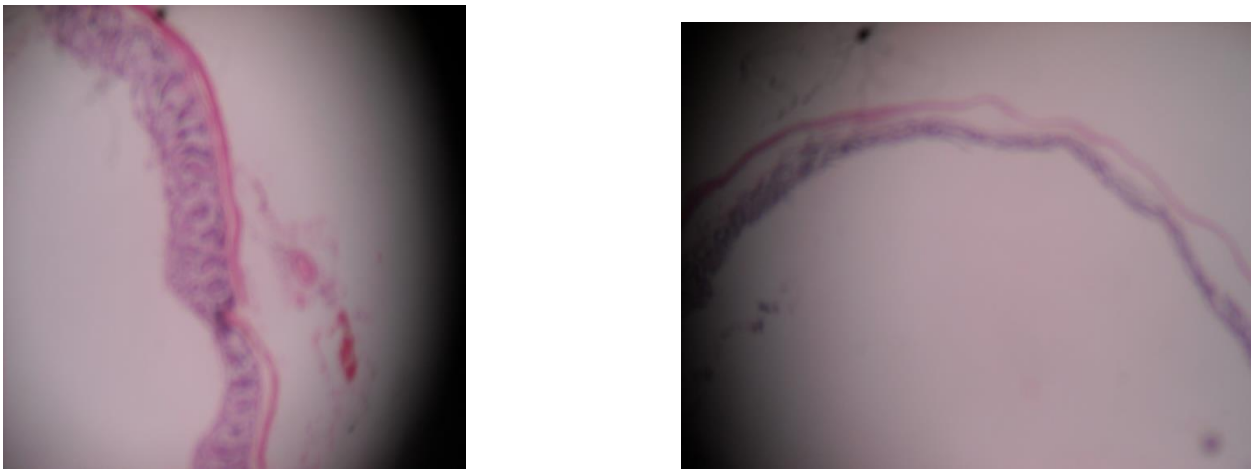
Experimental Study of vital organs of White Albino Mice (*Mus Musculus domesticus*) Infected with Microsporidia Spores.



A= Kidney at day 17 B= Kidney At day 28

Fig. 2: Histopathology of the kidney (x52).

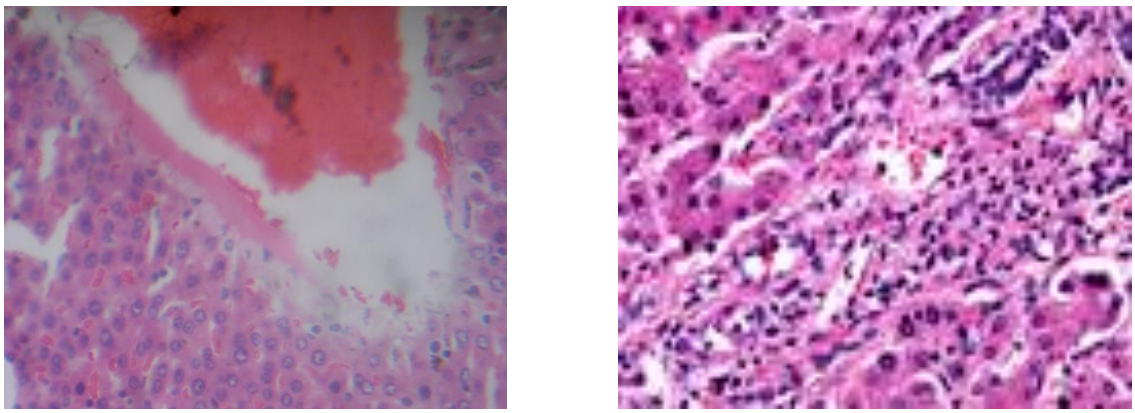
In figs.2A&B above, the glomeruli are obliterated by amorphous amyloid deposits. The vessels are also virtually occulated by the deposition within the walls. Extensive obliteration of the glomerular interstitial tissues as containing heavy infiltrates of white cells, causing widening of the intertubular spaces seen in day 28.



A= Intestine at day 17 B= Intestine at day 28

Fig.3: Histopathology of Small intestines (x52).

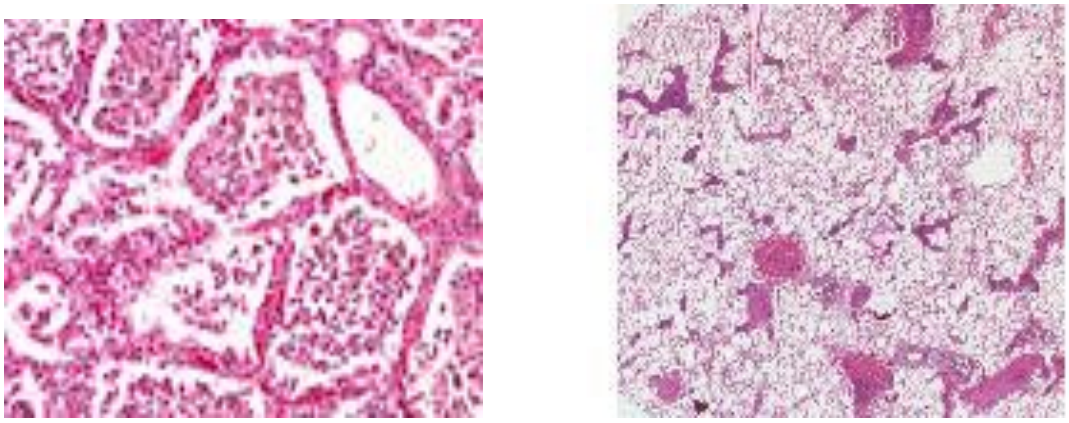
Figs.3 A&B above show partial to complete intestinal mucosa erosion due to the inversion of the microsporidia spores. The superficial mucosa is entirely obscured by the extensive hemorrhage, only the bases of the intestinal glands are visible. Sub-mucosa is also affected in day 28 above.



A= Liver at day 17 B= Liver at day 28

Fig.4: Histopathology of the Livers (x52).

In Figures 4 A&B above, an anaplastic carcinoma of the liver was observed. There are great versatility in size and shape of the cells. The nuclei are largely pleomorphic and several hyperchromatic trauma giant cells are readily evident, some with what appears to be multiple nuclei as observed in day 28.



A= Lungs at day 17 B= Lungs at day 28

Fig.5: Histopathology of the Lungs (x52).

In fig.**5** A&B above masses of acute inflammatory cells nearly obliterate lung structure. Panlobular emphysema, the distortion of the characteristically uniform air space was observed. The alveoli and alveolar ducts are dilated with focal areas of paenchymal destruction in day 28.

DISCUSSION

The prevalence of microsporidiasis among the HIV-Positive Patients recorded in this study (42.4%) using Chromotrope 2R is relatively high for an infection which is hardly known or considered significant. The high prevalence of the infection among the HIV-infected patients strongly suggests that microsporidiasis is relatively common among the HIV-infected patients at the University of Ilorin Teaching Hospital, Ilorin. The result also confirms the speculations that, if properly investigated, microsporidiasis prevalence among the immuno-compromised groups could be alarmingly high [12].

The prevalence of microsporidiasis found among the HIV-infected patients poses a concern particularly when it was discovered further from this study that the infection cuts across all ages, sex, educational status, religion and other socio-demographic variables. Presently, this study presents with a high prevalence and intensity of microsporidiasis in both HIV-infected patients and the HIV-non-infected control, creating an epidemiological challenge in both the immuno-compromised and the immuno-competent groups.

In an attempt to further study and morphologically characterize microsporidia species, the isolated spores from this study were measured and found to fall between 2-4µm and a comparison with the CDC control slide, strongly suggest that, the isolated spores are to be those of *Enterocytozoon bieneusi* and *Encephalitozoon cuniculi*.

While there are challenges of probes availability for molecular characterization of

microsporidia to species level, the morphological characterization used in this study and as earlier reported[13] hold promise for further studies on microsporidiasis as supported by their conclusion and that drawn from this study. The measured spores and as compared with the CDC control slide also conform with the report by Coyle *et al.* [(14)] and Yu *et al.*[15]. However, the conclusion they made that only *E. beneusi* was capable and responsible for the Diarrhoea in the HIV-infected patients differs from findings from this study and the conclusion by the CDC. Although this study does not support their conclusions in totality, morphological features presented by their findings agree with those found in the isolated species in this work particularly as concerns *E. beneusi.* The argument put forward by their conclusions and as contained in this study further stresses the need for the rapid development of molecular diagnostic tools that will help characterize microsporidia molecular and to species level.

Animal models which hitherto remain the only veritable tool for the study on microsporidia was experimentally examined in this study, in an attempt to ascertain the route of microsporidial infection and study the pathological damages caused by microsporidiasis. And as revealed in the immuno-suppressed group of albinomice, where pathological damages were observed in the lungs, small intestines, kidneys and livers, Al-Brhami *et al*.[15] and Bojko *et al.* [17] had also observed similar symptom in rabbits and birds respectively. These observations suggest that such damages could possibly be seen in the HIV-infected patients who harbour microsporidia. As Didier *et al.*[18] reported granulomaotus lesions in similar organs in mice and consequent death in those mice, microsporidiasis as discovered from this study strongly suggest to be responsible for the mortality rate of its infected immuno-compromised patients.

In the mice, the onset of overt pathology which occurred on day 17 post-infection and attained the peak severity on day 28 in the affected organs were observed only in the group of mice whose immune systems were suppressed. This shows that microsporidia infection in the immuno-compromised (HIV-infected) patients could result in similar pathological damages capable of causing death in these patients. To affirm this, Ghoshal *et al* in 2021[19] had reported a fatal granulomatous lesion in the liver and lungs as a result of microsporidial infection that were suspected to be responsible for the death of the affected HIV/AIDS patient.

Observations from this study and other findings [20,21] had widened the scope of the morbidity and mortality in the HIV-infected patients as a result of microsporidiasis. Interestingly, these overt pathological damages were observed only from the immune-suppressed mice which received microsporidia inoculums through oral ingestion and intranasal inhalation. This point has confirmed the earlier assumptions by many[22] that oral route is the likely source of microsporidia infection. Furthermore, the result of this study evidenced by the overt pathology in the intranasally inoculated mice suggest that intranasal inhalation is another

route of microsporidia infection whose routes of infection were previously unknown by many [23,24]. By these pathological facts seen, and their absence in other routes of parasite inoculation, it has become established that oral ingestion and intranasal inhalation are the primary routes of microsporidia infection. Also, it is true that, these are the two basic routes where human existence is possible, through feeding and breathing.

Be that as it may, microsporidia can be viewed to be in existence since creation, causing unknown deaths in its host. Encouragingly as revealed from this study and just as the spectrum of microsporidia appears to be getting wider, more understanding about the parasite and the disease will keep unfolding. As reported by Ding *et al.*[25], it will not be out of place if these evidences about microsporidiasis provoke interest for aggressive research about microsporidia worldwide, aiming at making more findings concerning its mode of infection, damages caused, chemotherapy, prevention and control.

The recovery of viable microsporidia spores in the stool samples of the infected immuno-suppressed mice, reaffirms Diarrhoea condition caused by this parasite in the HIV-infected patients, more so with the fact that the immuno-competent mice do not present with Diarrhoea. As Ding *et al* [25] concluded in his remarks, that though parasitic disease have been highly neglected, the little talk about microsporidiasis is as a result of the high level of ignorance about the parasite and the disease, else the danger posed by microsporidiasis in future will not be ignored. As exposure about microsporidia begins to unwind, the danger, by this organism as evidenced by this study will soon remain far and above neglect. Furthermore, this study reveals that, microsporidia will remain a parasite of interest for further studies in the morbidity and mortality rates among the immuno-compromised patients worldwide. While much more is expected to be established about microsporidia, findings from these pioneering initiations are encouraging and enough to substantiate that, epidemiological significance of this parasite and knowledge about the parasite are relevant to man.

CONCLUSION

The prevalence of microsporidiasis (42.4%) (an opportunistic infection) is relatively common among the immuno-compromised (HIV/AIDS) patients, thus posing a serious threat to complicate morbidity and mortality in areas where HIV/AIDS is common. The association of microsporidiasis with overt pathology in vital internal organs of immuno-compromised (HIV/AIDS) patients has been demonstrated. We recommend that Routine laboratory search, screening in hospitals and identification of microsporidia spores should be made mandatory and/or reportable. More studies on the etiology of microsporidiasis should be undertaken including histopathology study of different organs at autopsy.

Funding: There was no external funding for this study.

Acknowledgements: We acknowledge the authorities of the University of Ilorin Teaching Hospital for allowing us access to their patients for this research work. All the technical staff in the lab who have contributed in one way or the other are dearly acknowledged. We also acknowledge

The CDC Atlanta Georgia who supplied the control slides for the identification of Microsporidia spores from stool samples.

Declaration of conflict of interest: The authors have declared that there is no conflict of interest concerning the research, authorship and/or publication of this article.

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