

Original Research Paper

VIRULENCE OF *FUSARIUM OXYSPORUM* ON KIDNEY ORGAN USING *IN VIVO* MICE MODEL

ABSTRACT

Aim: To isolate, identify, and determine the virulence of *Fusarium oxysporum* species using *in vivo* (mice model).

Study Design: Study was carried out using experimental design.

Place and Duration: The study was carried out in University of Nigeria, Enugu Campus. From May 2011 –August 2013

Methodology: Isolates from human infections were obtained from 2000 patients with apparent signs of fungal infections (mycotic keratitis). Plant isolates were obtained from symptomatic plants (decaying or rotting plant substrate) such as *Lycopersicum esculentus*, *Elaeis guineensis*, *Ipomoea batatas*, *Solanum tuberosum*, *Musa sapientum*, *Daucus carota*, *Musa paradisiaca*, *Carica papaya*, *Persea americana*, *Citrullus lanatus* and *Capsicum chinense* from five different markets and farm lands in Enugu state, Nigeria. A total of 3000 plants were used. Cultures were carried out on Sabouraud Dextrose Agar slants according to modified methods and identifications made. *In vivo* virulence studies in mice were carried out with each individual strain. Histopathology of the kidney organ was performed. Mean survival time (MST) was estimated by the Kaplan-Meier method.

Result: *In vivo* virulence studies of *Fusarium oxysporum* on mice kidney organ resulted in disseminated infection and death of the animals. Histopathological analysis revealed infected lungs with evidence of intense inflammation involving the glomeruli and severe, extensive as well as severe diffuse inflammatory cell infiltration in the interstitium of the cortex. Kidney of control mice showed no pathological features.

Conclusion: The various pathological manifestations and physical signs of infection in the injected mice were indications that *Fusarium oxysporum* isolated were virulence proving the hypothesis that a single strain of *Fusarium oxysporum* can produce disease both in plant and human hosts.

Keywords: *Fusarium oxysporum*; Kidney; Virulence; *In vivo*

1. INTRODUCTION

Fusarium infection is life threatening, causing serious morbidity and mortality, and is frequently fatal in immunocompromised host. Invasive mycoses are major cause of death in rapidly increasing population of patients undergoing immunosuppressive therapy in association with cancer and organ/tissue transplantation. The ability of animal and plant isolates to cause infection in mice model requires studies for improving the understanding of evolutionary distant hosts.

In recent years, there has been a marked increase in the incidence of fungal infections due to four factors, namely: more aggressive treatment for other conditions (use of immunosuppressants, transplantations and the use and abuse of antibiotics); the increased incidence of leukemia, lymphoma and AIDS; greater knowledge of clinical mycology; and the greater accuracy of diagnostic techniques [1-3].

Fungal keratitis is a leading cause of ocular morbidity throughout the world, particularly in warm climates and developing countries. It is estimated that corneal ulceration and ocular trauma result in 1.5 to 2 million new cases of corneal blindness annually. In certain parts of the world, such as south India, nearly half of these corneal ulcers may be secondary to fungal infection. Epidemiological studies in India have identified filamentous fungi, *Fusarium* and *Aspergillus*, as the two most common causes of fungal keratitis. While the incidence of fungal ulcers in the USA and Europe has historically been much lower, the 2006 outbreak of *Fusarium* keratitis related to contact lens wear and the use of specific contact lens solutions has highlighted the challenges of effectively treating fungal keratitis [4]. Some fungi cause disease on a single host species, while others have extremely broad host ranges. The molecular mechanisms that determine fungal host range specificity are not fully understood. Hence, a number of virulence determinants are clearly host specific [5]. There is also evidence for the existence of universal virulence mechanisms shared by fungal pathogens with highly diverse host ranges [6, 7]. Fortunately, common patterns of host defense also are found in evolutionary diverse groups such as plants, insects and mammals [8]. Hence evolutionary ancient mechanisms of fungal virulence and host defense might coexist with high virulence and resistance traits that have arisen during later stages of pathogen. The availability of the complete genome sequences of fungal plant and human pathogens and their model hosts, as well as the development of high-throughput protocol for gene function analysis, hold a huge potential for advancing our understanding on fungal pathogenesis and host defense [9]. However, pathogen host range imposes an important limitation on the comparative analysis of virulence mechanisms in evolutionary distant hosts because it requires gene identification and knockout in different pathogenic organisms.

The clinical manifestations of invasive fusariosis are often nonspecific, and the host status plays a crucial role in determining the severity, type and chronicity of the infection [10, 11]. Several patterns of skin lesions can be seen: subcutaneous nodules, palpable and non-palpable purpura, red or gray macules, macules or papules with progressive central necrosis, flaccid pustules, vesicles, and hemorrhagic bullae [12, 13]. In contrast with *Fusarium* infection in normal hosts, which are typically localized (example skin or eye infections and osteomyelitis) and frequently do not require systemic therapy, fusariosis in profoundly immunocompromised patients manifests in four major patterns namely: refractory fever of unknown origin, sinopulmonary infection or pneumonia, disseminated infection, and a variety of focal single organ infection [14, 15].

Many Fusarial species, notably *F. oxysporum*, *F. solani* and *F. verticilloides* (formerly called *F. moniliforme*) are recognized as being pathogenic to man and animals causing mycotic keratitis, infecting eyes, finger nails and hyalohyphomycosis, especially in burn victims and bone marrow transplant patients [16, 17, 18].

Fusarium species are therefore increasingly implicated as the causative agents of human mycoses particularly in the immunocompromised patient populations, as well as important plant pathogens [19]. Hence certain groups of *Fusarium* also exhibit a predilection toward human and plant diseases, a unique characteristic among the fungi [20].

The epidemiological pattern of corneal ulceration varies significantly from country to country and even from region to region. *Fusarium* species (47.1 %) and *Aspergillus* species (16.1%) are the most common aetiological agents of corneal ulceration in India [21]. *Fusarium* species are probably the most toxin producing fungi. The mycotoxins produced include trichothecenes, fumonisins, fusaric acid and moniliformin [22]. Human *Fusarium* infections are thus likely to be truly opportunistic, caused by a diverse set of competent pathogens that are commonly encountered by humans (20). Multilocus phylogenetic analyses indicate that diverse isolates within each complex are associated with human infections, with certain specific clones and lineages showing the greatest association.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY)

2.1 Collection of samples

The mycological specimens used in this study were collected from both humans and plants. Clinical isolates of *Fusarium oxysporum* from humans were isolated from different mycological clinical specimens of 2000 patients seen at different hospitals within Enugu State, while the *Fusarium oxysporums* from plants were isolated from 3000 various plants within Enugu State. Ethical approval for the study was obtained from the Ethical Committee of the Department of

Pharmaceutics, University of Nigeria, Nsukka. Isolates from human infections were obtained from patients with apparent signs of fungal infections (mycotic keratitis) such as ocular infections (corneal ulcer), skin and nail infections. Plant isolates were obtained from symptomatic plants (decaying or rotting plant substrate) such *Lycopersicum esculentus* (tomatoes), *Elaeis guineensis* (palm fruit), *Ipomoea batatas* (sweet potato), *Solanum tuberosum* (Irish potato), *Musa sapientum* (banana), *Daucus carota* (carrot), *Musa paradisiaca* (plantain), *Carica papaya* (pawpaw), *Persea americana* (Avocado pear), *Citrullus lanatus* (water-melon) and *Capsicum chinense* (fresh red pepper)

2.2 Fungal Isolation

All the samples collected, were immediately cultured in triplicates on Sabouraud Dextrose Agar slant containing 50 mg/l of chloramphenicol and 5 mg/l of gentamicin. This was according to the modified method of Ran *et al.* (19). The wire loop was sterilized by flaming till red hot and was allowed to cool, it was then inserted into the sterile medium to moisten it and was used to pick up portions of the human specimen to inoculate three agar slants by pressing the material at 2 or 3 points at intervals of about 1cm into the agar to be partly submerged. The infected portions of the various plant samples were excised and cut into 2 mm² pieces, surface sterilized with 1% NaOCl and rinsed in 4 successive changes of sterile distilled water. The excised infected portions were then plated on to Sabouraud Dextrose Agar (SDA) slants. The cultures were incubated at 28°C for 1 week in the dark. Slide cultures, from the pure cultures, were carried out to determine further characteristics for identification using standard methods of Gupta *et al.* (23). Conidial morphology and ontogeny were examined microscopically after 3-10 days of incubation at 28°C. Identification was made according to the overall micro and macroscopic appearance, including the typical appearance and morphology of colonies.

2.3 Direct Microscopy using Potassium Hydroxide (KOH) Mount

Preliminary microscopic examinations of the fungal specimens were also carried out, using 10 % potassium hydroxide except for nail clippings, where 40 % was used. A drop of 10 % potassium hydroxide solution was placed on a slide. A small portion of the specimen was transferred to the drop with the aid of sterilized cooled moistened wire loop. The preparation was covered with a cover slip and the slide placed in a Petri dish together with a damp piece of filter paper to prevent the preparation from drying out. The preparation was allowed for 20-30 minutes for digestion to take place. The specimen was examined microscopically using x10 and x40 objective lens for fungal elements (hyphae and spores). This was according to the modified method of Prajna Lalitha *et al.* (24).

2.4 Macroscopic Cultural Characteristics (Colonial Morphology)

Pure cultures obtained were examined for the following: colour seen on the surface of the medium, texture, topography (colony appearance) and pigment production on the reverse of the medium.

2.5 Microscopic Examination (Lactophenol Cotton Blue Mount)

Two or three drops of lactophenol cotton blue were placed on a clean slide. A small portion of the fungal growth was collected by digging into the agar with the aid of a sterilized straight needle. The collected material was transferred to the drop of the lactophenol cotton blue on the slide. A second sterilized straight needle was used to hold the material in position and the other straight wire was used to tease out the material into smaller fragments. The preparation was covered with a cover slip and was examined using x 10 and x 40 objective lenses (25)

2.6 Animal Studies

Virulence studies were determined according to the modified method of Ortoneda *et al.* (16). The turbidity standards (McFarland Nephelometry) were prepared. This was used for standardizing the inocula of the fungal isolates. Verification was by plating dilutions of the suspension on SDA plates.

One hundred and sixty test mice and 8 controls (94 males and 74 females, including six pregnant mice); with a weight range of 30-32 g obtained from the laboratory animal centre of the College of Medicine, University of Nigeria, Enugu Campus, Enugu, upon certifying their health conditions and approval by the committee for animal experiments of our institute were housed (eight per cage) in aluminum cages, with corncob bedding and free access to food and water, under standard conditions. Six pregnant mice were included so as to study the effect of the *Fusarium oxysporum*

on them. Rules for the protection and care of animals were strictly adhered to following the guiding principles for the Care and Use of Animals in the field of Physiological Sciences (26).

2.7 In vivo virulence studies in mice

Suspensions (0.2 ml of 10^6 conidia / ml) of each of the 20 randomly selected fusarial strains were injected intravenously via the lateral tail vein into groups of eight mice per cage (16). The different strains used for the inoculation are shown in table 1. Mortality was recorded daily for 30 days. Virulence study with each individual strain was performed in 3-6 replicates.

2.8 Tissue Burden and Histopathology

Randomly chosen surviving mice (one from each cage) were sacrificed 30 days after inoculation with overdose of halothane using International Standard Procedure. Kidneys of the experimental mice were aseptically removed, and one-half of each was weighed and homogenized in 1 ml of sterile saline. Tenfold serial dilutions of this homogenate were made with sterile saline and spread onto SDA plates which were then incubated at 28°C. Colonies were counted after 3 days, and the numbers of colony forming units (CFU) per gram of organs were calculated. The remaining half of each organ studied was fixed in 10 % neutral buffered formaldehyde for 10 days embedded in paraffin wax and automatically processed. Sections (3 µm in thickness) of the embedded tissues were stained with hematoxylin-eosin, periodic acid-Schiff, and methenamine silver (Grocott) for light microscopy observations.

2.9 Staining Procedures for Histopathological Studies

2.9.1 Mayer's Acid –Alum Haematoxylin and Eosin stain

Sections and control slide were de-waxed, hydrated and stained in solution 1 for 10- 30 minutes and were washed thoroughly in running tap water. They were differentiated in solution 2 until only the cell nuclei retain the stain, blued in solution 3 for 1-2 minutes, followed by running tap water for 5-10 minutes. Sections and control slide were counter-stained in solution 4 for 1- 2 minutes and was washed with running tap water until the excess eosin was removed. They were finally dehydrated, cleared and mounted in neutral balsam (28)

2.9.2 Grocott's Methenamine- Silver nitrates

Sections and control slide were brought to water after dewaxing and hydration. They were oxidized in solution 1 for 60 minutes and were washed in running tap water. Sections and control slide were transferred to solution 2 for 1 minute to remove residual chromic acid and were washed in running tap water for 5-10 minutes. They were then rinsed in several changes of distilled water and impregnated with solution 3 for 30- 60 minutes at 58°C. The control slide was examined under the microscope to check for adequate impregnation. They were rinsed in several changes of distilled water, toned in solution 4 for 2-5 minutes and rinsed in distilled water. Sections and control slide were fixed in solution 5 for 2-5 minutes and were washed in tap water. They were stained with solution 6 for 30-45 seconds, dehydrated, cleared and mounted in neutral balsam (29).

2.9.3 Periodic Acid-schiff Stain

Sections and control slide were de-waxed and hydrated. Stained in solution 1 for 5 minutes and were rinsed thoroughly in tap water and in distilled water. The slides were placed in Schiff's reagent (solution 2) for 5 minutes and were placed in 3 baths of solution 3 for 2 minutes in each bath. The slides were rinsed in tap water and were stained in solution 4 for 30 seconds, blued in tap water, dehydrated, cleared and mounted in neutral balsam (30)

2.10 Statistical Analysis

Data were collected, computed and analyzed statistically using SPSS, version 15.0. Analysis of variance (ANOVA) formulas by Post Hoc Test using Tukey HSD and Games - Howell comparisons were used for multiple value comparisons, while Chi-square tests by cross tabulations were used for Correlations. The correlation tests were also carried out to determine the Pearson coefficient (r) value. Students T-test was used for frequency calculations. Fungal colony counts were converted to $\log_{10}$ values and compared by using the analysis of variance test. Mean survival time (MST) was estimated by the Kaplan-Meier method. Colony count (colony forming unit) in different organs per cages was compared by the log-rank test.

The Probability values were determined by, .05 and .01 significance level, at 95 % and 99 % confidence limits respectively.

3. RESULTS AND DISCUSSION

Culturally, colonies of the various *Fusarium oxysporum* grew easily and rapidly within three to five days on Sabouraud Dextrose Agar, beginning as a white patch which quickly developed into pale or brightly coloured patches, with some having cottony aerial mycelium. The colour of the *Fusarium oxysporum* isolated was lilac (purple) shade and showed no pigment production. The macroscopic appearance showing the usual loose cottony texture is seen in Plates 1- 4. The total positive cases recorded from various human samples was 1265 (63.3 %) where *F. oxysporum* was 544 (43.0%) while a total of 2,140 (71.3 %) plant samples were positive and 1177 (55.0%) was *F. oxysporum*.

The result of the slide cultural technique further confirmed the loose cottony texture of the *Fusarium oxysporum* macroscopically and pattern of arrangement and shape of the macroconidia and microconidia microscopically. Direct microscopy based on potassium hydroxide mount revealed fungal elements (hyaline septated hyphae and spores) from which *F. oxysporum* were subcultured.

Microscopic features and arrangement of macroconidia were usually hyaline, multiseptate, and fusiform to sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell. The microconidia on the other hand were 1-2 celled and curved. The typical microscopic appearance of the *F. oxysporum* from the human sample which was recovered from the mice is shown in plate 5, while that from the plant source is shown in plate 6.

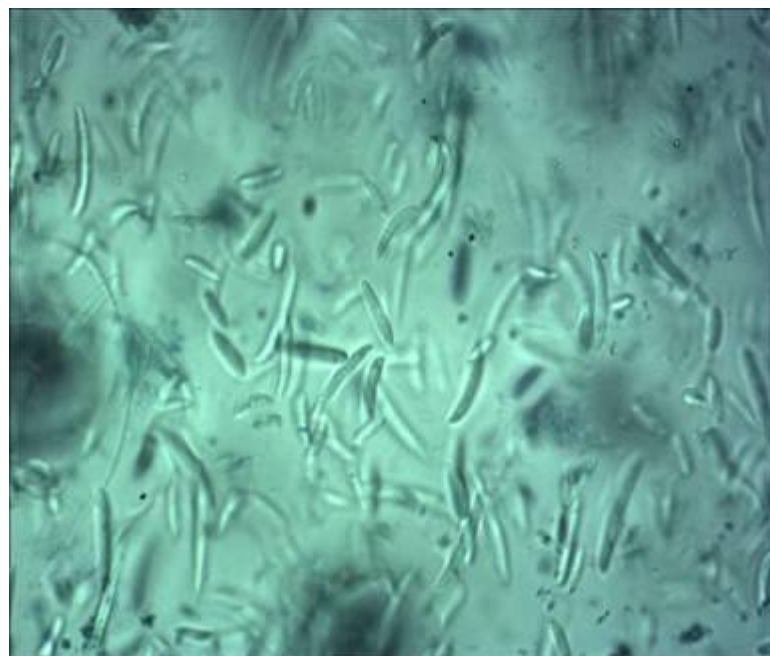


Plate 5: Microscopic features of *Fusarium oxysporum* from human sources, showing macroconidia (multiseptate and sickle-shaped) and microconidia (ovoid)

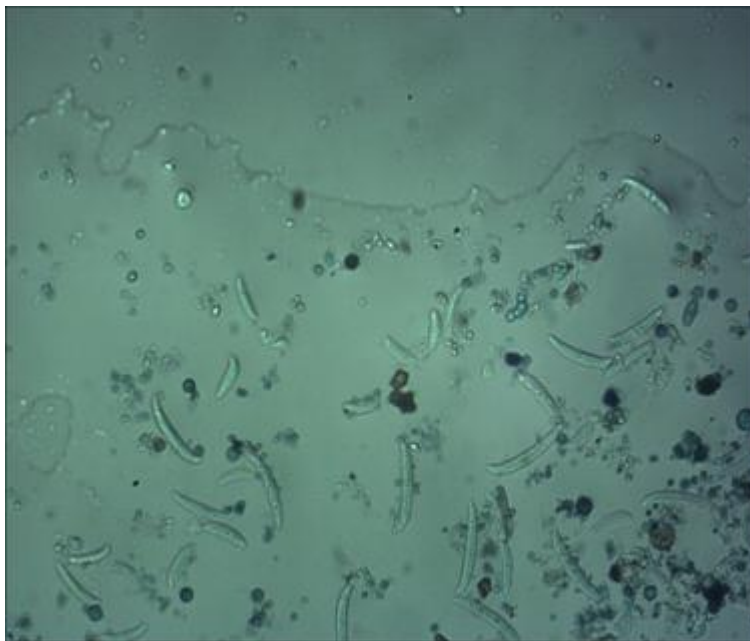


Plate 6: Microscopic features of *Fusarium oxysporum* from plant sources, showing the macroconidia (multiseptate and sickle-shaped) and microconidia (ovoid)

Generally, the mice inoculated with *Fusarium oxysporum* conidia from humans and plants were very dull, malnourished, and could not feed well, for three days, after which, they picked up again. The mice developed various signs of infections such as purplish ring-like lesions on the upper/undersurfaces of their bodies, towards the waist in some of the mice and on the tails in some others. Some of the mice developed lesion on the foot spreading to the toes along with hair loss (Plate 7, 8 and 9).



Plate 7: Mouse injected with *Fusarium oxysporum* (P6) showing signs of infection

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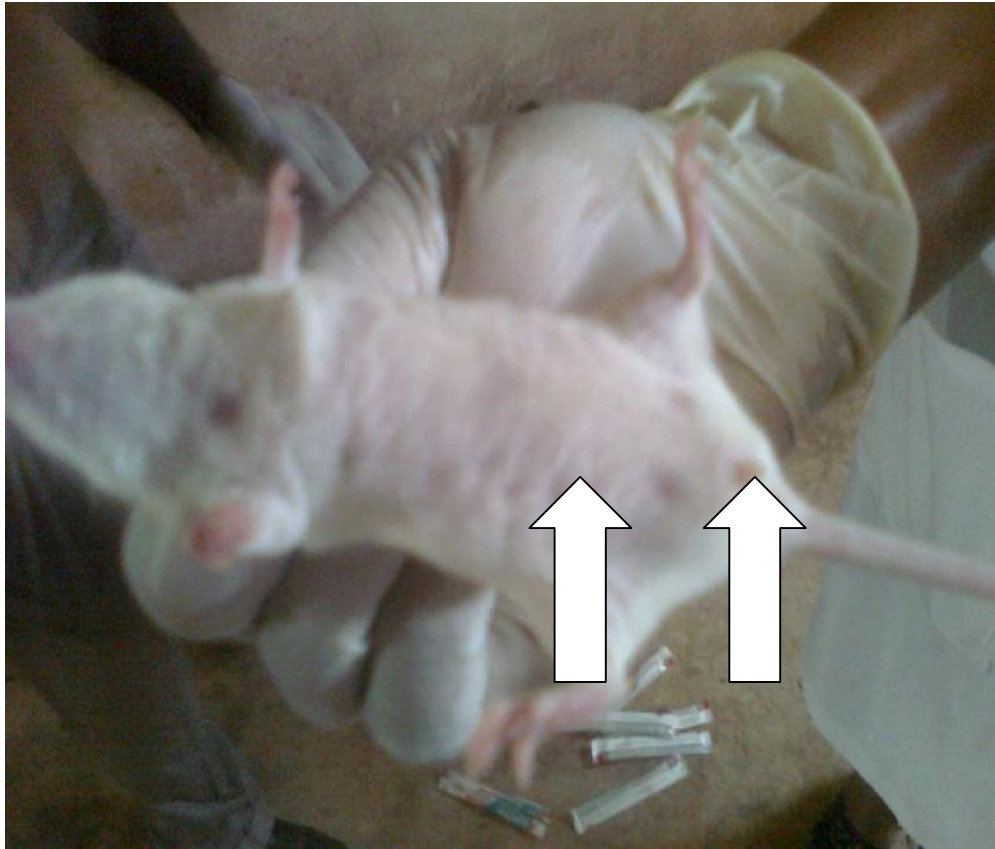


Plate 8: Mouse injected with *Fusarium oxysporum* (H 7) showing signs of Infection



Plate 9: Mouse injected with *Fusarium conidia* (H131) showing signs of infection

Development of granuloma was also seen in the mice (Plate 10a & 10b). The six pregnant mice also infected with the *Fusarium oxysporum* delivered safely after the third inoculation. Two weeks later, the pinkies (litters) also developed similar signs of infection leading to the death.



Plate 10a: A female mouse from Cage 5 with a very large solid granuloma after injection with *Fusarium oxysporum* conidia



10b: A male mouse in Cage 8 with similar granuloma after injection with *Fusarium oxysporum* conidia (H43)

The kidney of most of the sacrificed mice showed intense inflammations (Plate 12 and 13), compared to the control where there was no inflammation seen (Plate 13). The *F. oxysporum* inoculated into the different mice were recovered again from the kidney organ of the sacrificed mice (plate 14).

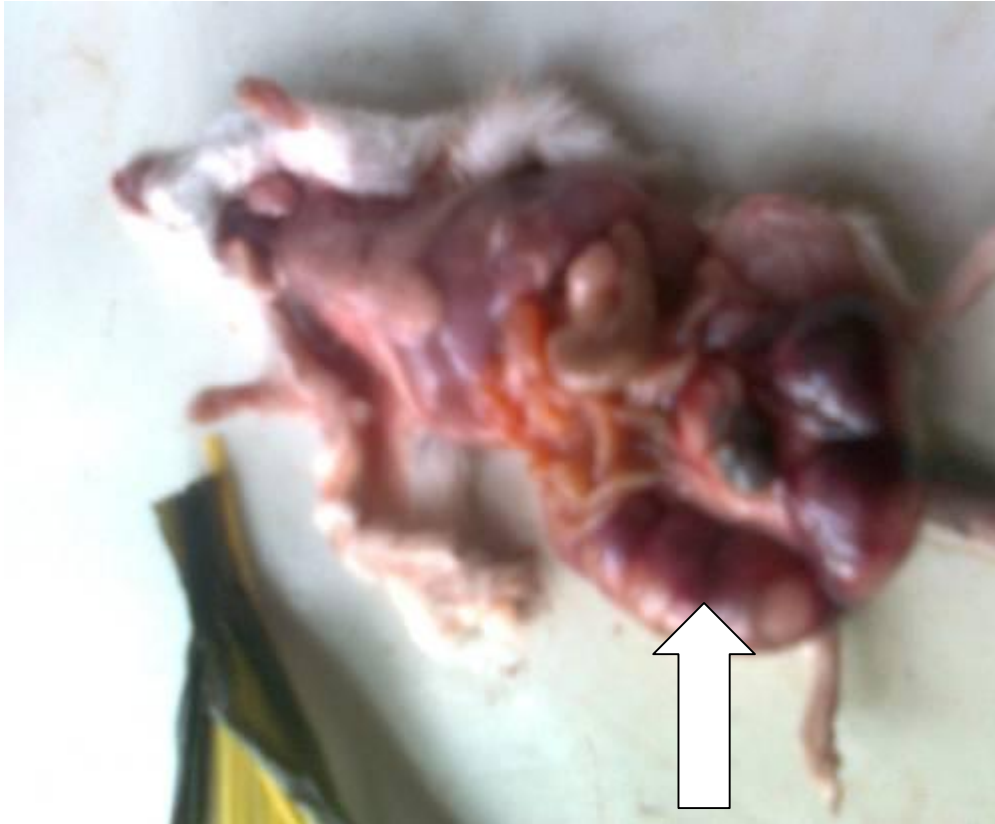


Plate 12: Inflamed kidney of a typical mouse infected with *Fusarium oxysporum*

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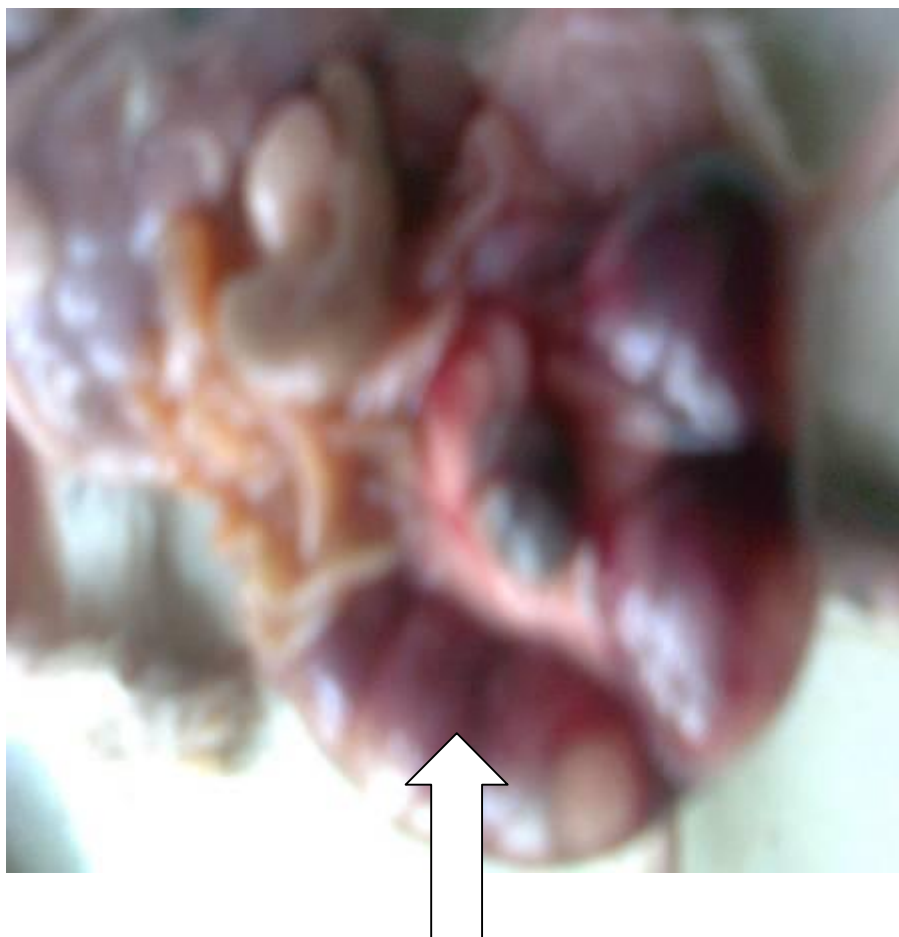


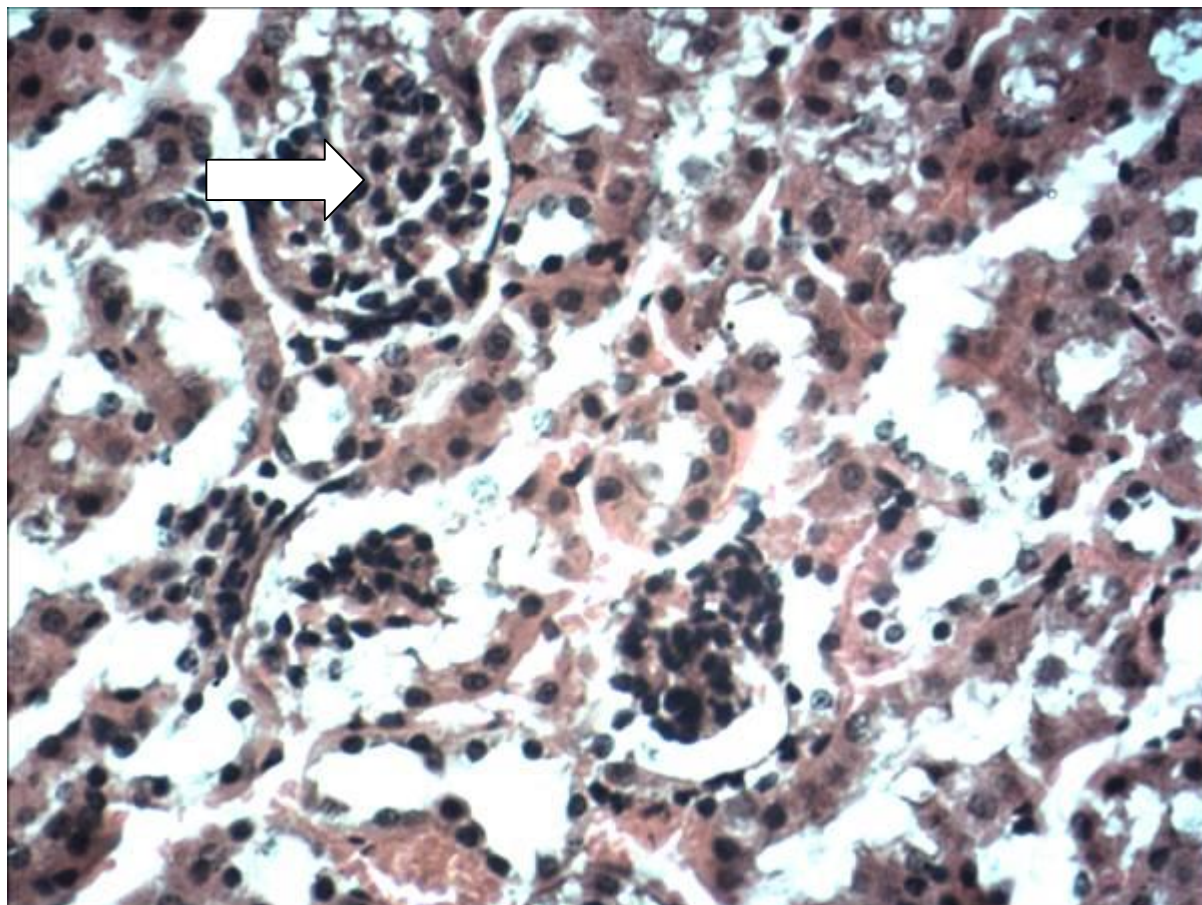
Plate 13: Typical mouse infected with *Fusarium oxysporum* showing intense inflammation of the kidney

Plate 15 shows the control kidney while plate 15 to 21 revealed the damage caused by various strains to the kidney tissue. Plate 16 shows the kidney infected with conidia of *Fusarium oxysporum*. Photomicrograph of kidneys infected with conidia of *Fusarium oxysporum* (H1) showing background haemorrhage focally, prominent mononuclear cell infiltrates in the cortical interstitial areas with oedema of the tubules below. Although the micrograph shows no glomerulus, they were preserved.

H127 showed kidney infected with conidia of *Fusarium oxysporum* with much more intense inflammation now involving the glomerulus. The straight arrow, points to the glomerulus and the other curved arrow to the interstitium. This strain is more toxic than the immediate preceding strain (Plate 17). There was severe extensive and severe diffuse inflammatory cell infiltration in the interstitium of the cortex in between the tubules (Interstitial nephritis) with P189 (Plate 18).

The kidney infected with conidia of *Fusarium oxysporum* (P191) showed interstitial nephritis with swelling and destruction of the tubules. The tubular lumen was widened (Plate 19). Photomicrograph of kidney infected with conidia of *Fusarium oxysporum* (H131) showed diffuse toxic tubular damage (Plate 20). The kidney organ infected with conidia of *F.oxysporum* (P6) moderate pyelonephritis (Plate 21).

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Plate 15: Photomicrograph of normal kidney (control). Arrow shows a normal glomerulus. The rest background shows tubules cut in both transverse and longitudinal sections. There is clearly no background inflammation.

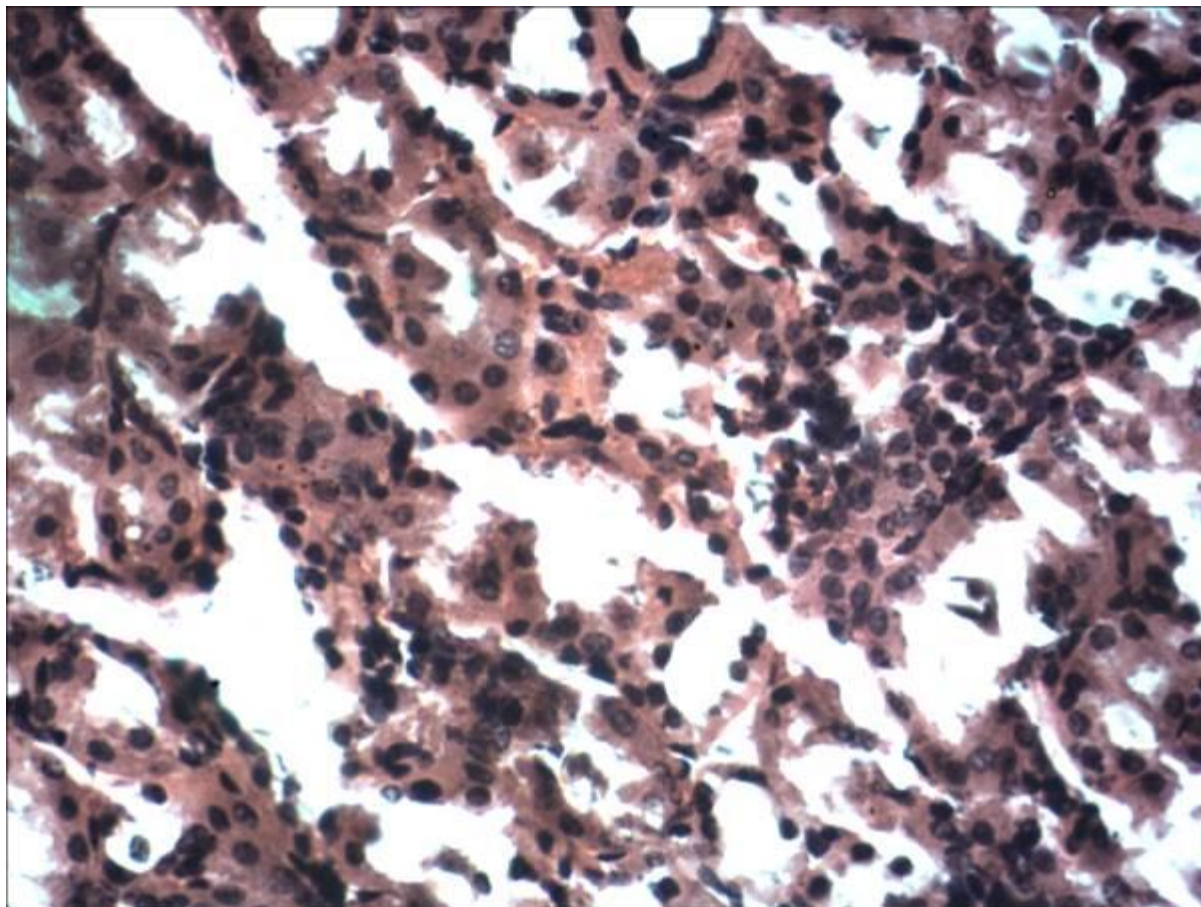


Plate 16: Photomicrograph of kidneys infected with conidia of *Fusarium* species (H1) showing background haemorrhage focally, prominent mononuclear cell infiltrates in the cortical interstitial areas with oedema of the tubules below. Although the micrograph shows no glomerulus, they were preserved.

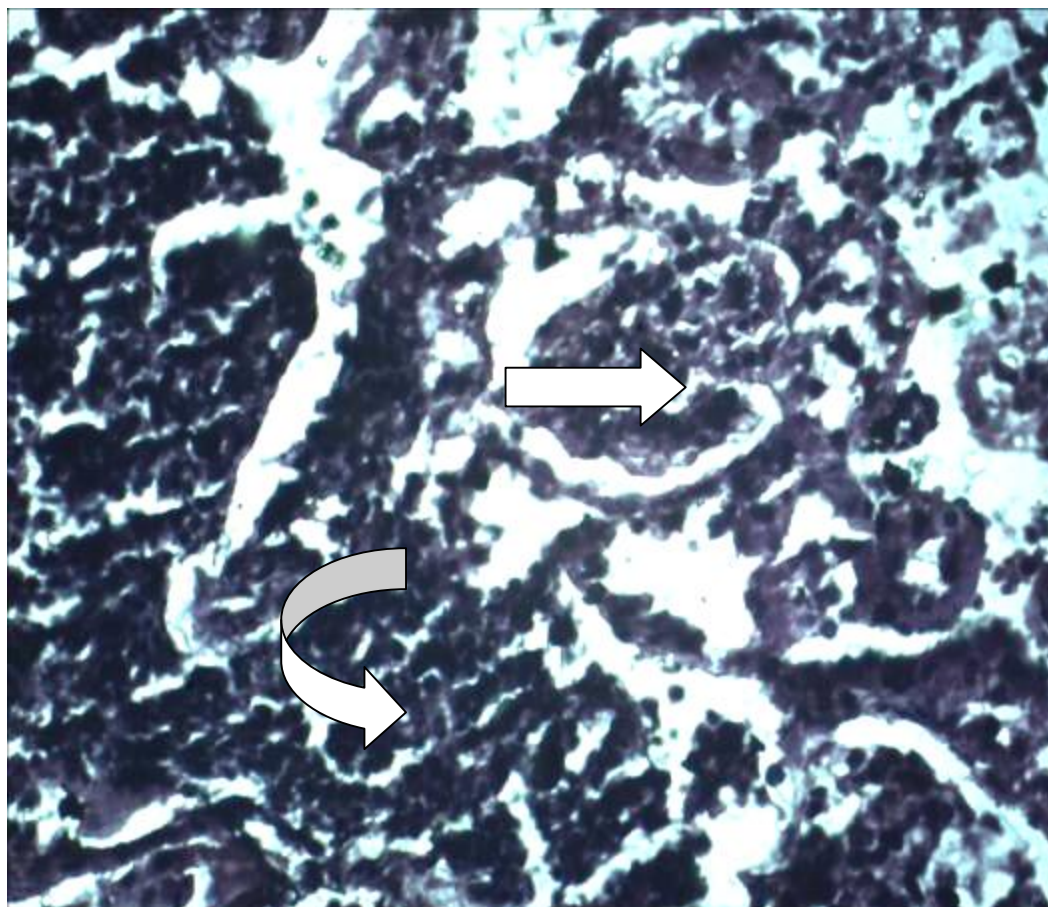


Plate 17: Photomicrograph of kidney infected with conidia of *Fusarium oxysporum* (H127) showing much more intense inflammation now involving the glomerulus. The straight arrow, points to the glomerulus and the other curved arrow to the interstitium. This strain is more toxic than the immediate preceding strain.

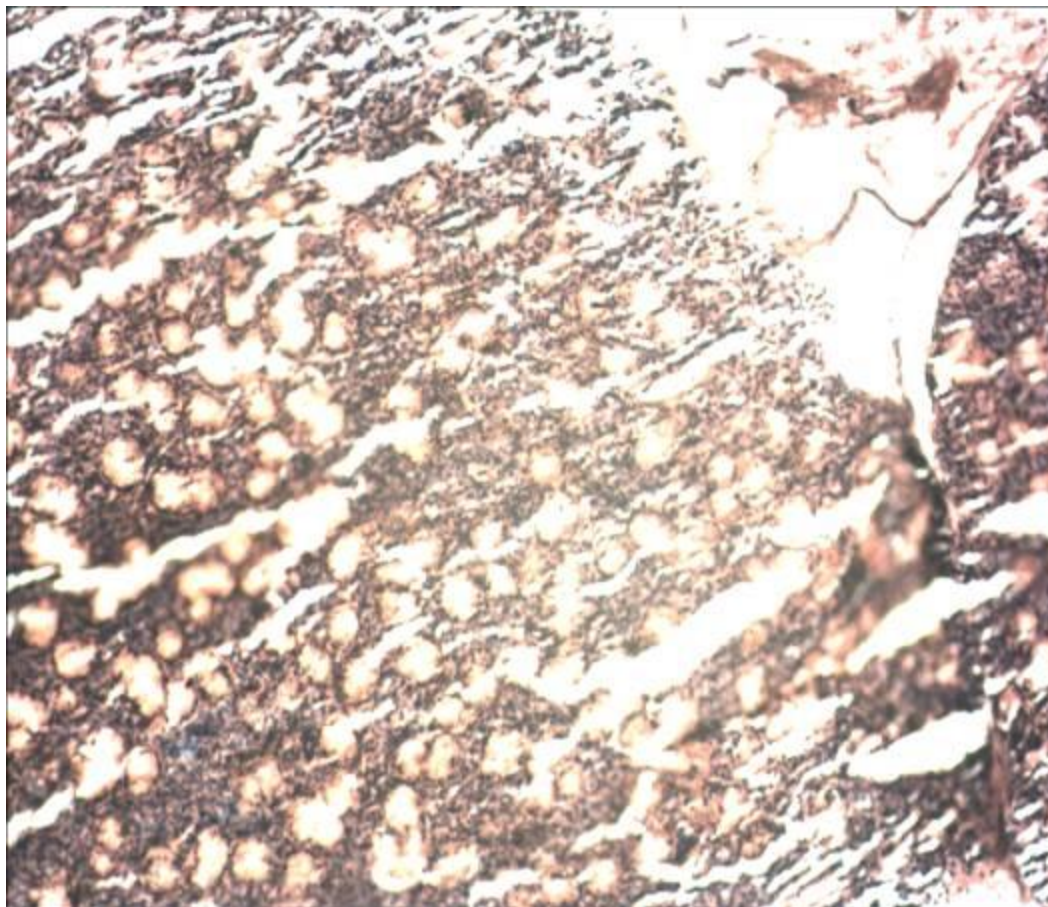


Plate 18: Photomicrograph of kidney infected with conidia of *Fusarium oxysporum* (P189) showing severe extensive and severe diffuse inflammatory cell infiltration in the interstitium of the cortex in between the tubules (Interstitial nephritis).

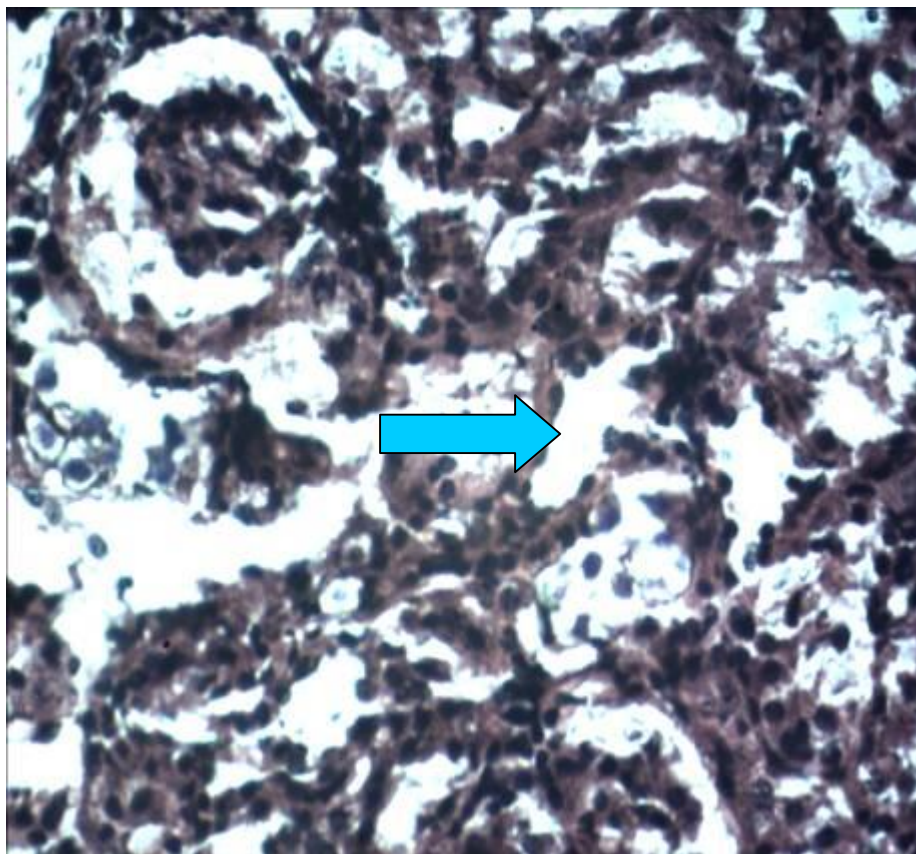


Plate 19: kidney infected with conidia of *Fusarium oxysporum* (P191) showing interstitial nephritis with swelling and destruction of the tubules. Note that the tubular lumen are widened

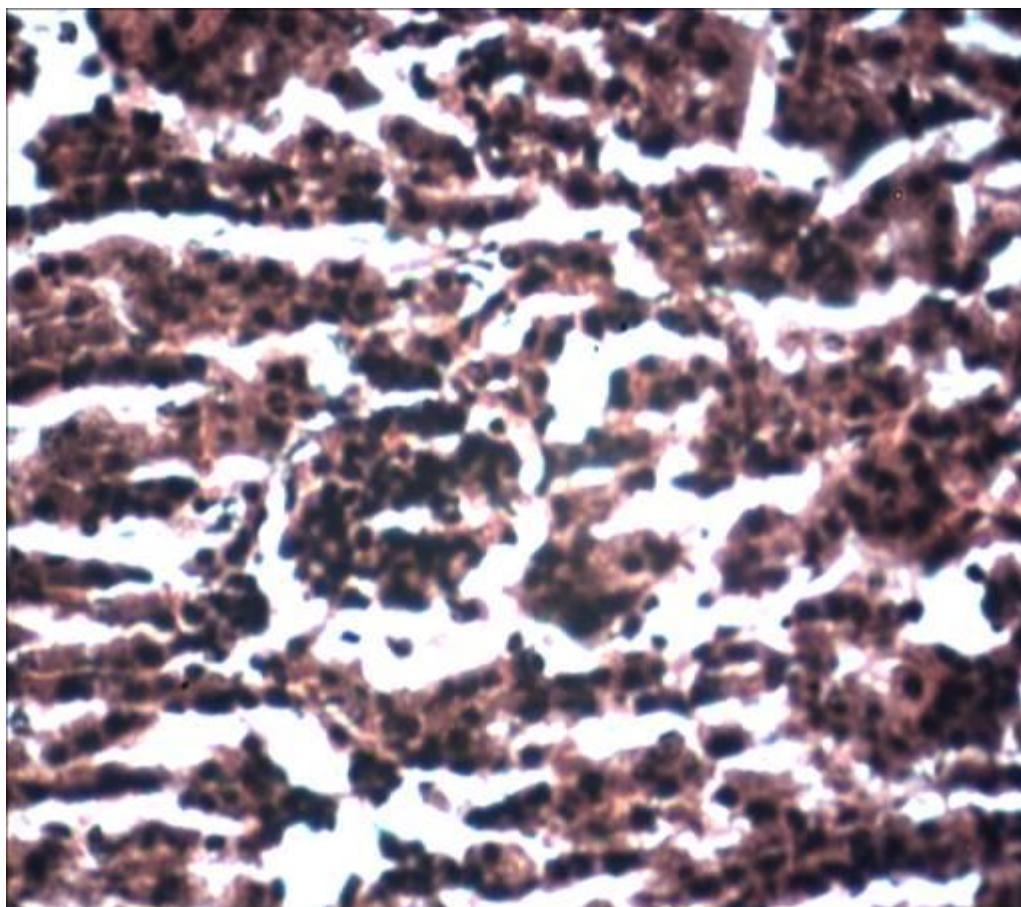


Plate 20: Photomicrograph of kidney infected with conidia of *Fusarium oxysporum* (H131) showing diffuse toxic tubular damage.

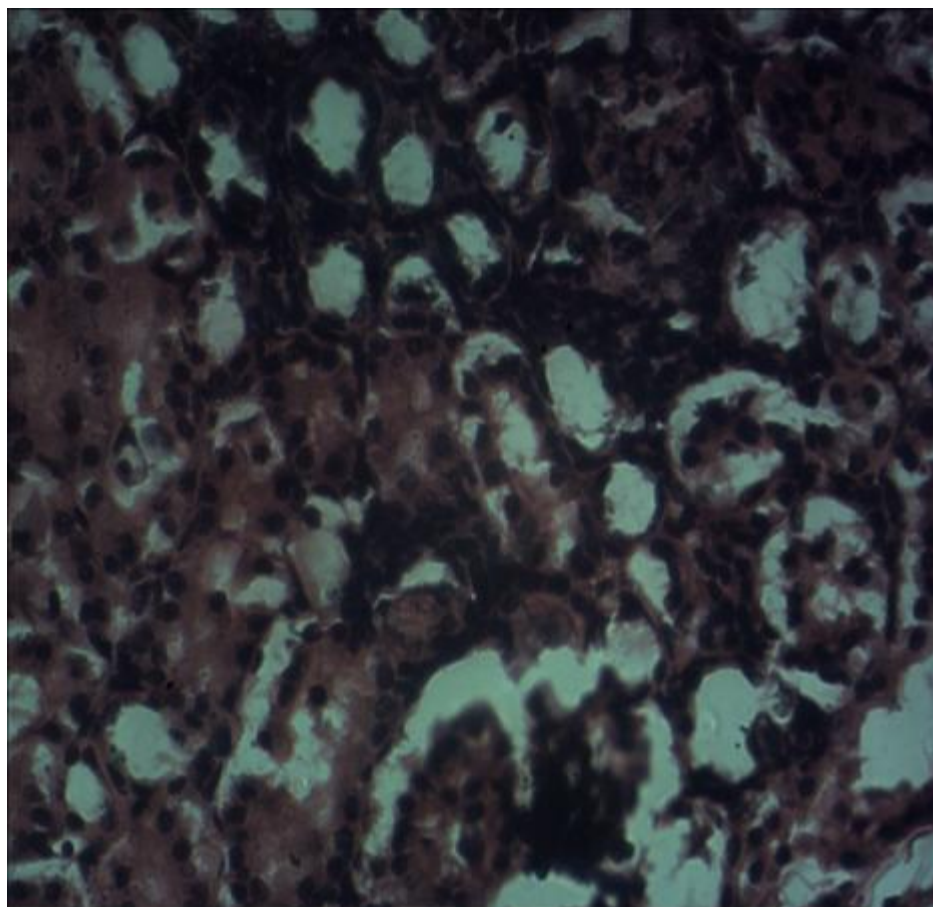


Plate 21: Photomicrograph of kidneys infected with conidia of *Fusarium oxysporum* (P6) showing moderate pyelonephritis

4. DISCUSSION

The high incidence of the *Fusarium oxysporum* from human and plant samples in this study showed that *fusarium* has a predilection towards both human and plants. This result is in conformity with the work of O' Donnell *et al* [20] who discovered that certain groups of *fusarium* did exhibit a predilection toward human and plant diseases, a unique characteristic among the fungi. Looking at the incidence of the *Fusarium oxysporum* among male and female subjects, this study recorded higher rate of fusarial infection in females than males. There was a statistically significant difference ($P = .001$) between males and females in the incidence of infections by *Fusarium* species. There was also a significant association between the variables, gender and site of isolation of samples (cornea, skin and nail), as shown by the p -value .001. The various pathological manifestations and physical signs of infection in the injected mice were indications that the various species of *F. oxysporum* isolated were virulence. This is in line with the work of Ortoneda *et al.* [16] who also had similar result when microconidia of tomato pathogenic isolate was injected into the lateral tail vein of immunodepressed mice in order to prove the hypothesis that a single strain of *Fusarium oxysporum* can produce disease both in plant and human hosts.

In this study the human and plants fusarial isolates caused various physical disease signs and pathological features. The ability of the human and plant fusarial isolates to survive and reproduce in mice, colonize multiple organs and finally lead to the death of the host suggests that some of the strains contain the basic pathogenicity determinants required to cause disease in mammalian hosts.

It has been speculated that the toxins produced by *Fusarium oxysporum* may enhance the breakdown of tissues and facilitate entry of *Fusaria* into the systemic circulation [31]. Histopathological analysis of the kidney tissue of these animals further supports this hypothesis; infected kidney showed much more intense inflammation involving the glomerulus and showing diffuse toxic tubular damage, indicating that the organism is very toxic. Severe extensive and severe diffuse inflammatory cell infiltration in the interstitium of the cortex in between the tubules (Interstitial nephritis) was also noticed. Kidney infected with conidia of *Fusarium oxysporum* showed interstitial nephritis with swelling and destruction of the tubules leading to widening of the tubular lumen.

After *Fusarium* species have been inoculated, the toxins produced by this pathogen may enhance the breakdown of tissues and consequently facilitate entry of fusaria into the vascular structures and then dissemination. *Fusarium* species have a propensity for vascular invasion, resulting in thrombosis and tissue necrosis [31].

5. CONCLUSION

This study expresses high occurrence of *Fusarium* species in both humans and plants. The ability of both isolates to cause infection in mice model represents a key step toward improving the understanding of how fungal pathogenesis on evolutionary distant hosts has evolved. These results further suggest that certain virulence factors essential in plant pathogenesis are dispensable in animal pathogenesis and vice versa. Several *Fusarium* species are toxigenic, and the mycotoxins and mycotoxicoses produced by these fungi present a potential health problem. Many of the toxins produced by these fungi are not yet characterized chemically.

ETHICAL APPROVAL

"All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee"

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