Sequential pathological studies in Asian water buffaloes infected intra-tracheally with *Absidia corymbifera*

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Summary

Zygomycosis was produced experimentally in Asian water buffalo calves (*Bubalus bubalis*) by intratracheal inoculation of sporangiospores of *Absidia corymbifera*. Infected animals exhibited dullness, depression, partial anorexia, initial pyrexia, mucopurulent nasal and ocular discharge and coughing during the first week. There was no mortality at any stage of the experiment, which continued for 30 days. The gross and microscopic lesions were restricted to the lungs and there was no dissemination of the fungus to other organs. Gross and microscopic changes in the lungs were observed up to the 20th day post-infection. Gross lesions consisted of pneumonic consolidation of the antero-ventral lobes of the lungs. Microscopic changes consisted of granulomatous reactions with well developed pulmonary granulomas. Distorted hyphae of *A. corymbifera* were demonstrated in tissue sections up to 15 days post inoculation. Re-isolation of the fungus was achieved consistently for up to 15 days. It is concluded that intratracheal inoculation of *A. corymbifera* in buffalo calves leads to significant pathological changes in the lungs, but the disease appears to be self limiting 20 days following inoculation.

Key words Zygomycosis, Water buffaloes, *Absidia corymbifera*, Pathology

Estudios patológicos secuenciales en búfalos acuáticos asiáticos infectados intra-traquealmente con *Absidia corymbifera*

Resumen

La zigomicosis se produjo experimentalmente en crías de búfalos (*Bubalus bubalis*) mediante inoculación intratraqueal de esporangiosporas de *Absidia corymbifera*. Los animales infectados manifestaron torpeza, depresión, anorexia parcial, pirexia inicial, descarga mucopurulenta nasal y ocular y tos durante la primera semana. No se produjo mortalidad en ninguna etapa del experimento que duró 30 días. Las lesiones se limitaron a los pulmones sin observarse diseminación del hongo a otros órganos. Se observaron cambios macroscópicos y microscópicos hasta el día 20 post-infección. Las lesiones macroscópicas consistieron en consolidación neumónica de los lóbulos pulmonares antero-ventrales. Las lesiones microscópicas consistieron en reacciones granulomatosas con granulomas pulmonares bien desarrollados. Se demostró la presencia en hifas distorsionadas de *A. corymbifera* en secciones de tejidos hasta el día 15 post-infección. Era posible aislar el hongo hasta 15 días después de la inoculación. La inoculación intratraqueal de *A. corymbifera* en crías de búfalo produce alteraciones patológicas significativas en los pulmones, pero parece que la enfermedad se autolimita 20 días después de la inoculación.

Zigomicosis, Búfalos acuáticos, *Absidia corymbifera*, Patología

Zygomycosis is a rapidly fatal fungal infection affecting both humans and other animals [1]. The disease is caused by species of *Absidia, Apophysomyces, Cunninghamella, Mortierella, Mucor, Rhizopus, Rhizomucor* and *Saksenaea* of which *Absidia corymbifera* is particularly significant because it produces local and systemic zygomycosis in domestic animals [2] and humans [3].

Most reports of infection in non-humans deal with cases of zygomycosis that were diagnosed at necropsy. Although cases of zygomycosis have been reported by several workers [4-9] in water buffaloes, there have been no systematic studies regarding the pathogenesis and pathology of this disease in the water buffalo in contrast to mycotic abortion reported in cattle [21] *A. corymbifera* has been identified as the causative agent of naturally occurring pulmonary zygomycosis in Indian buffaloes [7]. The present study describes the clinical signs and sequential pathological changes in buffalo calves following intratracheal inoculation with *A. corymbifera*.

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MATERIAL AND METHODS

Experimental animals. Twenty four male asian water buffalo calves (*Bubalus bubalis*) 4-6 months old, weighing 60-70 Kg, were kept under observation in thoroughly cleaned rooms for 15 days prior to starting the experiments. Food and water were given ad libitum The animals were immunologically normal and were not immunosuppressed.

Fungus strain and preparation of inoculum. A strain of *A. corymbifera* (MTCC no. 379) obtained from the Institute of Microbial Technology, Chandigarh, India, was used. The isolate was grown on Sabouraud glucose agar (SGA) containing 0.3% chloramphenicol. After incubation for 3-4 days at 37°C, the growth was flushed with sterile phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween-80. The sporangiospore suspension was shaken for 2 h on a mechanical shaker and its sporangiospore concentration was determined and adjusted to $4x10^8$ sporangiospores/ml using a haemocytometer .

Design of experiment. After two weeks of rearing the animals in an animal house, the animals were randomly divided into two groups consisting of 16 animals to be infected and eight controls. Each animal in the infected group was inoculated intratracheally with 10 ml of a 3-4 day-old culture of *A. corymbifera* containing $4x10^8$ sporangiospores/ml. The animals in the control group were similarly inoculated with 10 ml of sterile PBS containing 0.05% Tween-80.

The animals in the two groups were kept in separate rooms. The control animals were always attended, fed and watered before handling the infected animals or contaminated material. The animals in both the groups were closely observed daily for clinical signs of disease.

The experiments were continued for 30 days and two randomly selected animals from the infected group and one animal from the control group were killed at 1, 3, 5, 7, 10, 15, 20, and 30 days post infection (DPI). They were subjected to a comprehensive post-mortem examination. Gross changes, if any, were recorded and portions of different organs were obtained and fixed in 10% buffered formal saline for histopathological study. Paraffin sections, 5 µm thick, were cut and stained with haematoxylin and eosin (H&E). For demonstration of fungi in tissue, sections were stained with the periodic acid Schiff (PAS), Grocott's methenamine silver nitrate (GMS) and combined GMS-HE stains [10].

Re-isolation of fungus. Re-isolation of *A. corymbifera* was attempted from lung tissue cultured on SGA slants incubated at 37°C for 48 h to 7 days.

RESULTS

Clinical signs. Infected buffalo calves developed dullness, depression, partial anorexia and initial pyrexia along with mucopurulent nasal and ocular discharge, and coughing during the first week. However, there was no mortality. The control animals did not show clinical signs.

Gross lesions. The gross lesions were mainly confined to the lungs. The animals sacrificed on DPI-1 and 3 showed patchy areas of moderate to severe red hepatization in all the lobes of the right lung. Cut surfaces of the lungs revealed pin point white foci. On DPI-5, lesions in the lungs were more extensive and severe. The entire apical, cardiac and intermediate lobes and anterior parts of the diaphragmatic lobes of the right lung were hepatized and showed pneumonic consolidation (Figure 1) and by 7DPI, firm nodules about 5 mm in size were palpated in

Figure 1. Lungs (DPI-5). Pneumonic consolidation of antero-ventral lobes.

the pneumonic portion of the lungs. The cut surfaces of the pneumonic lungs showed pin head sized white foci and mucopurulent exudate in the bronchi and bronchioles. On DPI-10,15 and 20, the extent and severity of the lesions in the lungs reduced and there were only patchy areas of consolidation. On DPI-30, the lungs, were almost normal grossly except for fibrinous adhesions between the pulmonary and costal pleura and pericardium. The tracheal, bronchial, and mediastinal lymph nodes were enlarged from DPI-5 to 30, in all the infected animals. There were no substantial variation between the two animals studied at each time interval. No significant gross lesions in lungs, associated lymph nodes and other visceral organs were seen in the control animals.

Histopathology. Histopathological changes were primarily seen in the lungs. The sequence of microscopic changes in buffalo calves were: On DPI-1, lungs showed acute interstitial pneumonia with marked congestion, infiltration of mononuclear cells in interalveolar septa leading to thickening along with perivascular lymphocytic cuffing. The lumina of the bronchioles were filled with purulent exudate containing sporangiospores and hyphae of *A. corymbifera* (Figure 2). In places, hyphae were seen penetrating through the wall of the bronchioles into the parenchyma, forming early pyogranulomas consisting of neutrophils surrounded by a few macrophages (Figures 3 and 4). In many places, the hyphae were seen entering the wall of blood vessels from the parenchyma, leading to mycotic thrombosis and perivasculitis (Figure 5). On DPI-3, the interstitial pneumonia became more severe with increased numbers of mono-nuclear cells in the interalveolar septae, presence of perivascular and peribronchiolar lymphocytic cuffing and formation of lymphoid aggregates in the parenchyma. Pyogranulomas in the pulmonary parenchyma were also bigger and contained an increased number of longer hyphae than seen at DPI-1 (Figure 6). In

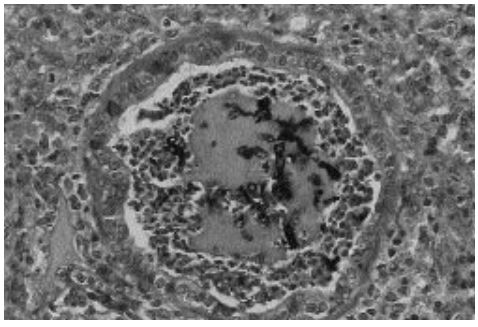

Figure 2. Lung (DPI-1). Hyphae and spores of *Absidia corymbifera* along with purulent exudate in the lumen of a small bronchiole. GMS-H&E, X 300.

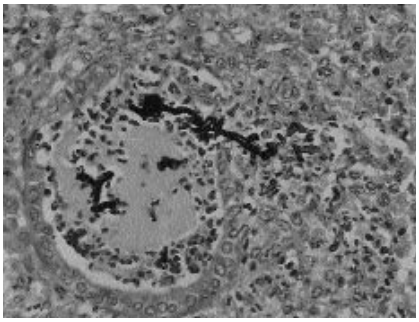

Figure 3. Lung (DPI-1). Fungal hyphae extending from the lumen of the bronchiole into the adjoining parenchyma. GMS-H&E, X 300.

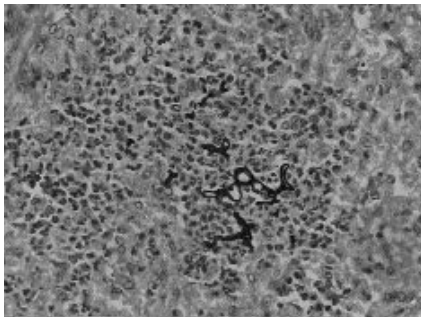

Figure 4. Lung (DPI-1). Fungal hyphae surrounded by early pyogranuloma. G.M.S.-H&E, X 300.

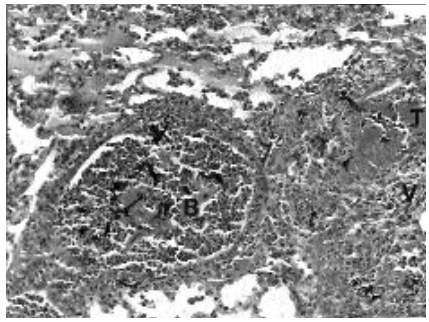

Figure 5. Lung (DPI-I). Mycotic thrombosis (T), vasculitis (V) and purulent brochiolitis (B) along with fungal hyphae in the lumen of a bronchiole. GMS-H&E, X 150.

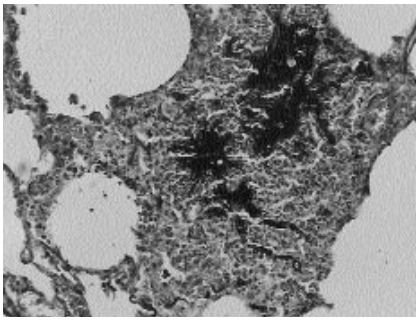

Figure 6. Lung (DPI-3). Large pyogranuluma in the parenchyma with increased number of long hyphae. GMS-H&E, X 150.

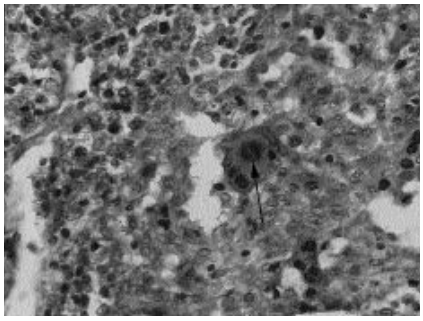

Figure 7. Lung (DPI-3). Giant cell granulomas, showing phagocytized digested fungal particles in the giant cells (arrow). GMS-H&E, X 300.

places, some giant cells also contained phagocytized digested remnants of hyphae (Figure 7) and distorted hyphae. On DPI-5, the lungs showed a severe granulomatous pneumonia with a marked increase in macrophages and foreign body giant cells. On DPI-7, the changes in the lungs were, by and large, similar to those seen on DPI-5 but the granulomatous inflammation was also more severe. There was chronic pleuritis with proliferation of fibrous tissue and infiltration of lymphomononuclear cells. The fungus was observed less frequently than that at DPI-5 and distorted fungal hyphae were seen phagocytized by foreign body giant cells, particularly in the bronchioles. On DPI-10, the lung parenchyma showed resolution. Giant cell granulomas and fungus were not seen in the lung parenchyma. Bronchioles showed severe chronic granulomatous bronchiolitis characterized by giant cell granulomas containing distorted phagocytized hyphae occluding their lumina. The bronchiolar wall had fibrosis and marked peribronchiolar lymphocytic cuffing. On DPI-15, changes in the lungs were similar to those seen on DPI-10, but occasional granulomas consisting of caseous necrosis in the center surrounded by neutrophils, macrophages, foreign body giant cells and lymphocytes and fibrous tissue capsule encircling such granulomas were seen (Figures 8 and 9). Cross sections of distorted hyphae were also detected occasionally in the centers (Figure 9). Some bronchioles showed marked hyperplasia of epithelium leading to the obliteration of their lumina. On DPI-20, besides, occasional pyogranulomas in the lung (Figure 10), lung parenchyma showed resolution with only occasional lymphoid nodule around a few bronchioles. On DPI-30, the lungs revealed nearly complete resolution. Granulomas and fungus were not seen. The mediastinal lymph nodes showed reactive lymphadenitis characterized by infiltration of neutrophils and lymphoid cell hyperplasia and the presence of digested fungal mate-

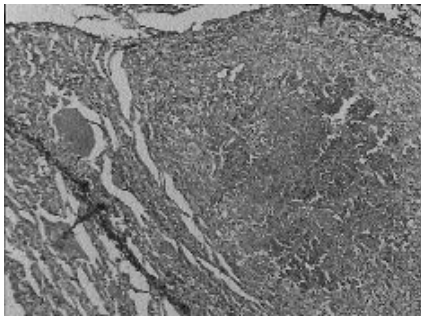

Figure 8. Lung (DPI-15) Large chronic pyogranuloma. GMS-H&E, X 75.

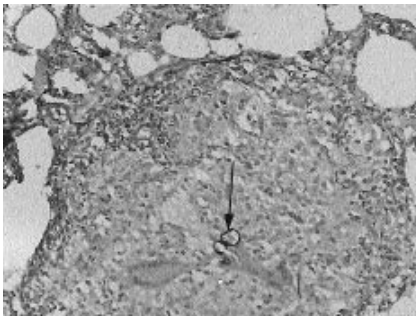

Figure 9. Higher magnification of Figure 8 showing cross sections of distorted fungal hyphae (arrow) phagocytized by giant cells. GMS-H&E, X 150.

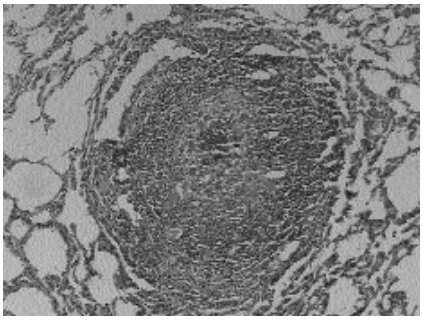

Figure 10. Lung (DPI-20). Chronic pyogranuloma encircled by severe infiltration of lymphocytes and fibrous tissue capsule. GMS-H&E, X 75.

rial in the macrophages.

The brain revealed mild lymphocytic meningitis besides there was also congestion, perivascular edema and hemorrhages, neuronal degeneration and necrosis of neurons. In many places, mild spongiosis, gliosis resulting in glia nodules formation (Figure 11) and marked

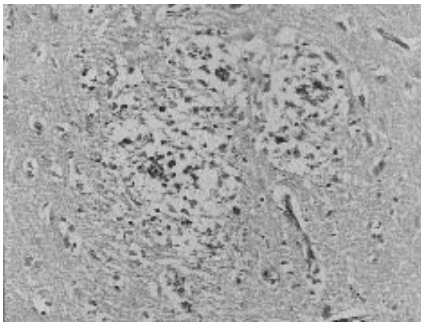

Figure 11. Brain (DPI-5). Cerebrum revealing glia nodule formation in the neuropil. GMS-H&E, X 150.

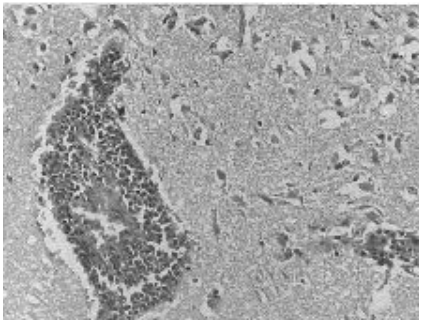

Figure 12. Brain (DPI-20). Severe perivascular lymphocytic cuffing in the cerebrum. GMS-H&E, X 150.

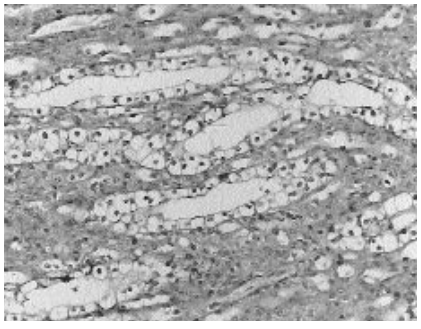

Figure 13. Kidney (DPI-5). Vacuolar degeneration in the tubular epithelium of the kidney. GMS-H&E, X 150.

perivascular lymphocytic cuffing was also seen by 20-DPI (Figure 12).

The kidneys revealed nephrotic changes characterized by vacuolar (Figure 13) and granular degeneration of the tubular epithelial cells. In places, chronic periglomerolitis characterized by atrophy of glomeruli, marked thickening of Bowman's capsule, mild fibrosis and infiltration of chronic inflammatory cells around the glomeruli were also recorded.

The liver showed congestion, hepatosis with mild centrolobular fatty change, bile duct hyperplasia and foci of necrosis infiltrated with neutrophils.

The myocardium showed vacuolar degeneration of myocardial cells and mild lymphocytic myocarditis. There was also degeneration of Purkinje fibers and fibroelastic thickening of the endocardium.

There was no substantial variation in the histopathological changes between the two animals studied at each time interval. No histopathological lesions were seen in the lungs, associated lymph nodes and other visceral organs of the control animals.

Re-isolation of fungus. *A. corymbifera* was consistently re-isolated from lung lesions from days 1 to 15 post-infection. It was not isolated from the lungs of the control animals.

DISCUSSION

The clinical signs observed after intratracheal inoculation of buffalo calves with the sporangiospores of *A. corymbifera* were similar to those recorded in naturally occurring zygomycosis in sheep and goats [11,12].

The gross mycotic lesions were observed only in the lungs and not in other organs. The reason for the other organs not being significantly affected might be due to the considerable inhibitory effect of germinating inhibitors and the concentration of the basic proteins present in them [13]. In experimental intratracheal Aspergillus infection in goats also, gross lesions were observed in the lungs only [14,15]. As already mentioned, in our study, the lesions were mainly confined to the anteroventral lobes of the right lung. Similar findings were recorded in experimental aspergillosis in goats [14,15].

Microscopic changes also were seen mainly in the lungs. The congestion and edema, observed initially, are the earliest responses of tissues to infection [16] In addition, the physical irritation by fungal propagates might play a role in invoking the early inflammatory response [17]. Almost similar lesions were recorded by Mandal and Gupta [15] and Sood [14] in early stages of experimental intratracheal aspergillosis in goats. However, these workers did not record the migration of fungal hyphae from the bronchioles into the adjoining parenchyma and pulmonary blood vessels. Blood vessel invasion in natural zygomycosis has been reported in the literature [8,11,12,18-21].

The pyogranulomas as observed by us on DPI-3 may be regarded as the hall mark of *A. corymbifera* infection as both the macrophages and neutrophils play their roles in their defense against fungi [22]. Pyogranulomatous inflammation of the lungs have been documented in experimental intratracheal aspergillosis in goats [14,15].

On DPI 5, 7 and 10, there was a severe granulomatous pneumonia with marked increase in the macrophages and foreign body giant cells. Neutrophils were comparatively less in number. The fungal hyphae were distorted. These hyphae and digested fungal material were phagocytized by large foreign body giant cells and alveolar macrophages. The presence of granulomas, composed of macrophages, epitheliod cells, lymphocytes, plasma cells and giant cells have been reported to occur as a part of the allergic response to fungal infections in humans [23-25].

On 15 DPI lesions were markedly reduced and only occasional granulomas were visible containing distorted fungal hyphae in their centers. Some bronchioles showed marked hyperplasia of their epithelium leading to obliteration of their lumina. Bronchiolar hyperplasia, as observed by us on DPI 1-15, has been recorded in naturally occurring zygomycosis in sheep [12] and in experi-

mental aspergillosis of goats [14]. It appeared to be an outcome of increased fibrinous exudation and retention of exudate in the bronchioles [26]. On DPI-20, only a few pyogranulomas surrounded by fibrous tissue capsule were seen and the rest of the lung parenchyma showed resolution. These granulomas did not have fungal elements. The lungs appeared nearly normal at DPI-30 indicating that in normal non-immunosuppressed animals the disease is self limiting. Similar observations have been reported in experimental intratracheal aspergillosis in goats [14,15] after 30 days of infection. Lymphoid hyperplasia in the mediastinal lymph nodes might indicate triggering of local humoral mechanism [27,28]. The presence of hyphae in pulmonary lymph nodes has been documented in zygomycosis in pigs [29].

It is speculated that changes in the brain were indicative of the damage by the mycotoxins or toxic metabolites excreted by *A. corymbifera* as similar alterations in the brain have been reported in experimental intratracheal aspergillosis in goats [14,15]. Fungal hyphae were demonstrated in meningeal blood vessels in naturally occurring zygomycosis in sheep [11,30]. Microscopic changes in the heart, liver, and kidneys, observed in buffalo calves in this study appeared to be due to the anoxic and toxigenic influences exerted by *A. corymbifera*. Similar changes have been reported in experimental intratracheal aspergillosis [14,15]. Metabolites like 7-hydroxydodecanoic acid, ergost-7-en-3β-01, fungisterol, (24S) 24-methyl-5α-cholest-7-en-3β-01, ergosta-7,22-dien-3β-01, 5, 6-dihydroergosterol and ergoline alkaloids, are known to be produced by zygomycetes group of fungi [31]. *A. corymbifera* was re-isolated from the lungs up to DPI-15 only.

On the basis of these studies, it is inferred that the intratracheal inoculation initiates infection by *A. corymbifera* in buffalo calves resulting in characteristic clinical symptoms and pathological alterations. The lesions were mainly restricted to the lungs and were self limiting after 20 days of infection.

Our findings in buffalo calves are different from those of Corbel *et al.* [32] and Sodhi [33] in rabbits, who observed mycotic gross and microscopic lesions only in the kidneys and not in the other organs including the lungs, after intravenous inoculation of sporangiospores of *A. corymbifera*. This difference in the site of predilection may be either due to different routes of infection or due to species differences and needs further investigation.

References

1. Smith JMB, Jones RH. Localization and fate of *Absidia ramosa* spores after intravenous inoculation of mice. J Comp Pathol 1973, 83: 49-55.
2. Davis CL, Anderson WA, McCroy BR. Mucormycosis in food producing animals; a report of twelve cases. J Am Vet Med Assoc 1955, 126:261-267.
3. Darja M, Dary MT. Pulmonary mucormycosis with cultural identification. Can Med Assoc J 1963, 89: 1235-1238.
4. Gill BS, Singh B. Phycomycotic pneumonia in buffaloes- A note. Zbl Vet Med B 1976, 23: 782-784.
5. Gir AK, Sharma NC, Sikdar A. Fungal infection in repeat breeder cattle and buffaloes. Indian J Anim Sci 1994, 64:1158-1160.
6. Kharole MU, Gupta PP, Singh B, Mandal PC, Hothi DS. Phycomycotic gastritis in buffalo calves. Vet Pathol 1976, 13:409-413.
7. Mandal PC, Gill BS, Grewal GS. Spontaneous pneumonia in She-buffalo caused by *Absidia corymbifera.* J Res. Punjab Agr Univ, Ludhiana, India 1981; 18: 452-456.
8. Sood N, Gupta PP. Pulmonary mycosis, tuberculosis and echinococcosis in Indian water buffaloes. Indian J Anim Sci. 1989, 59:648-651.
9. Vanamayya PR, Mouli AC, Parihar NS. A note on phycomycotic pneumonia in buffalo (*Bubalus bubalis*). Indian Vet J 1983, 60:490.
10. Luna G. Manual of histological staining methods of the Armed Forces Institute of Pathology, 3rd ed. New York, McGraw Hill Book Company, 1968.
11. Chattopadhyay SK, Iyer PKR, Rao M. Systemic phycomycosis in sheep: a case report. Indian J Vet Pathol 1985, 9:66-69.
12. Chattopadhyay SK, Parihar NS. Zygomycotic pneumonia in sheep and goats. Indian J Vet Pathol 1986; 12: 82-85.
13. Corbel MJ, Eades SM. Observations on the localization of *Absidia corymbifera in vivo.* Sabouraudia 1978; 16: 125-132.
14. Sood N. Pathology and pathogenesis of *Aspergillus flavus* in the respiratory tract of goats. PH. D thesis. C.S. Azad University of Agriculture and Technology, Kanpur, UP, India, 1996.
15. Mandal PC, Gupta PP. Sequential pathological studies in goats infected intratracheally with *Aspergillus fumigatus.* Mycopathologia 1993; 121: 77-81.
16. Slauson DO, Cooper BJ. Mechanism of Disease- A Textbook of Comparative Pathology, 2nd ed. Baltimore, Williams and Wilkins, 1990.
17. Srivastava AK. Mould allergy- an overview. In Advances in Medical Mycology. Mukerji KG, Srivastava AK, Singh KP, Garg KL (Eds.) New Delhi, Aditya, Books Pvt. Ltd., 1992: 64-80.
18. Rippon JW. Medical Mycology. 3rd ed. Philadelphia, W.B. Saunders Company, 1988. p:681-713.
19. Arora DK, Ajello L, Mukerji KG. Handbook of Applied Mycology-Humans, Animals and Insects. Vol II. New York, Marcel Dekker, 1991.
20. Konig H, Nicolet J, Lindt S, Raaflaub W. Some phycomycoses in cattle, pig, cat, deer, and flamingo. Schweiz Arch Tierheilk 1967, 109:260-268.
21. Kharole MU, Chand P, Monga DP, Sadana JR. Pulmonary zygomycosis in a bovine foetus. Vet Rec 1988; 122:236.
22. Fromtling RA, Sahdomy HJ. An overview of macrophage- fungal infections. Mycopathologia. l 986; 93: 77-93.
23. Waldorf AR, Diamond RD. Aspergillosis and mucormycosis. In Cox RA (Ed.) Immunology of Fungal Diseases by Bra Ratou, C.R.C. Press Inc, 1989.
24. Bardana EJ Jr. The clinical spectrum of aspergillosis Part I: Epidemiology, pathogenicity, infection in animals and immunology of *Aspergillus.* CRC Crit Rev Clin Lab Sci. 1980; 13:21-83.
25. Howard DH. Fungi pathogenic for humans and animals. Vol. III part B: pathogenicity and detection. Marcel Dekker, Inc., New York, USA. 1985.
26. Spencer H. Pathology of Lung. Vol II 4th ed. New York, Pergamon Press, 1985.
27. Tizard I. Veterinary immunology - An Introduction. 3rd ed. Philadelphia, W. B. Saunders Company, 1987.
28. Outteridge PM. Veterinary Immunology. New York, Academic Press, 1985.
29. Sadana JR, Kalra DS. A note on pulmonary phycomycosis in a pig. Indian J Anim Sci 1973; 43: 961-964.
30. Chihaya Y, Matsukawa K, Okada H. A case of bovine cerebral mucormycosis. Jpn J Vet Sci. 1986; 48: 1271-1274.
31. Turner WB, Alridge DC. Fungal metabolites. Vol II. London, Academic Press, 1983; p 47, 312, 313, 402.
32. Corbel MJ, Hambleton P, Baskerville A, Bailey NE. Biochemical and pathological changes in experimental phycomycosis. J Comp Pathol 1983; 93:219-234.
33. Sodhi MPS. Studies on the pathology of *Absidia corymbifera* infection in rabbits. M.V.Sc Thesis . Chaudhary Charan Singh Haryana Agriculture University, Hisar, India, 1994.