Proceedings of the 7th Congress of Federation of Asian Veterinary Associations

4 - 7 November 1990

Pattaya, Thailand

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the 7th Congress of the Federation of Asian Veterinary Associations

(FAVA)

4-7 November 1990, Pattaya, Thailand

Edited by Prachak Poomvises

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CONTENT

THE ORIGIN AND PATHOGENICITY OF *BASIDIOBULUS* SPECIES IN NORTHERN AUSTRALIA

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ABSTRACT

Basidiobolus haptosporus Drechsler causes human and animal disease in the tropics. This paper reports the isolation of the organism from natural substrates and describes their pathogenicity. *Basiodibolus spp* were recovered from faecal samples of amphibians, reptiles and macropods, from woodlice and from granulomatous skin lesions of horses. Some isolates were heat-tolerant. Almost all of the heat-tolerant isolates were pathogenic to suckling mice and had smooth or undulate or smooth plus undulate zygospore walls. When inoculated intracerebrally they caused encephalomalacia, necrosis, meningoencephalitis, congestion, haemorrhage and a granulomatous reaction in the central nervous tissue, hydrocephalus and nervous signs. Pathogenic isolates were recovered from certain amphibians, reptiles, macropods, woodlice, and a granulomatous skin lesion of a horse. There was a variety of animal sources of pathogenic strains of the organism widely distributed in the sampling area, thus the potential hazard of pathogenic infection could be more serious than previously considered.

INTRODUCTION

Basidiobolus spp have been isolated from tropical and subtropical environments throughout the world (3). The organism is widely distributed in nature in decaying vegetation, insects and the gastrointestinal tract of many reptiles, amphibians, insectivarous bats, and macropods (1;3;5;8;12;13;14).

Basidiobolus haptosporus Drechsler causes human and animal disease in the tropics. In horses, it has been reported as a granulomatous disease of the skin. Miller and Campbell (10) in their survey of granulomatous and neoplastic disease of equine skin in North Queensland, reported that 75% of fungal granulomas were caused by Pythium sp, 20% by Basidiobolus sp and 5% by Conidiobolus sp. Several pathogenicity studies have been reported, where viable Basidiobolus has been inoculated via the intradermal, intravenous, intraperitoneal or subcutaneus routes. These routes failed to initiate progressive disease, however following intracerebral inoculation of adult mice, progressive granulomatous lesions of the central nervous system frequently developed (4;6;8;9).

In the present work we report the isolation of Basidiobolus sp from natural substrates in Northern Australia and their pathogenicity in suckling mice.

MATERIALS AND METHODS

One hundred and eighteen natural substrate samples were collected from the Townsville district, North Queensland, Australia. These included the faeces of amphibians, reptiles, macropods, avian and

domestic animals as well as whole insects and litter and seven tissues (kunkers) from horses diagnosed clinically as suffering from equine phycomycosis. The technique of Coremans-Pelseneer (3) was used to isolate Basidiobolus sp from natural substrates, while the method used by Miller & Campbell (10) was applied to isolate the agent from clinical material.

Strain identification and characterisation

Fifteen isolates from the survey were examined microscopically and their identification was based on the zygospore wall characteristics. For the heat tolerance studies, all the isolates were cultured on Sabouraud's dextrose agar (SDA) and incubated at 25°C, 37°C, and 40°C. Colony diameter was measured after 48 hours inoculation. All treatments were conducted in triplicate. The data obtained from this study were tested by student "t" test.

Preparation of material for animal inoculation

The test cultures were grown in Sabouraud's dextrose broth (SDB) inoculated with hyphal fragments which were prepared by scraping the mycelium from the surface of a three day SDA culture and statically incubated at 27°C for three days. Mycelium was then harvested and washed three times over one hour with 50 ml sterile PBS at each washing. The washing was resuspended in 10 ml sterile PBS and sonicated for one to two minutes. Following sonication, the large mycelial fragments were allowed to sediment under gravity and the supernatant decanted. This suspension was also examined microscopically to determined the length of the viable hyphae. The concentration of viable units was estimated by plating serial dilutions onto SDA. The suspension was stored at

4°C during preparation and used for animal inoculation within one hour. Most of the suspensions contained viable units between 100 to 500 microns in length. A few zygospores were usually present.

Pathogenicity studies

Seven day old Quackenbush suckling mice were used. Each litter was housed individually with the mother in a cage with a minimum of six in each group. The mice were inoculated intracerebrally with 0.025 ml of a mycelial suspension which contained approximately 10^3 viable units per dose.

The animals were observed daily for six weeks. Those dying within 24 hours after inoculation were not included, since death was considered to be the result of injection trauma. The remaining animals were necropsied as soon after death as possible. The survivors were killed at the end of the observation period.

At autopsy, the cranial cavity was opened and examined for gross changes. Samples of brain were removed asseptically for culture and one part was fixed in 10% buffered formalin for microscopical examination. Cultures were made on SDA as described by Miller and Campbell (10) Microscopical examination was made of paraffin-embedded brain, sectioned longitudinally and stained with haematoxylin eosin (H&E) or with the special fungal stains Priodic-Acid-Schiff (PAS) or Gomori-Methamine Silver (GMS).

RESULTS

From various substrates, 14 isolates were made from the faeces of bearded dragon (Amphibolurus barbatus Cuvier), cane toad (Bufo marinus Linn), gecko (Hemidactylus prenatus, Dumeril & Bibron),

two lined dragon (Diporiphora bilineata Gray), agile Wallaby (Macropus agilis), olive python (Liasis childreni), water python (Liasis fuscus Peters), skink (Carlia mundivensis Broome), nothern dtella (Gehyra Australis) and amethystine python (Liasis amesthystinus). The fungus was also isolated from whole woodlice (Porcellio laevis Latereille), leaf litter and grass and cutaneous granuloma (kunkers) from a horse.

All of the 14 isolates grew well at 25°C. Of these 10 grew better at 37°C than at 25°C and grew also at 40°C (Table 1) and were considered "heat-tolerant". The others 4 isolates were regarded as non heat-tolerant strains. The mean colony diameter (cm) and standard error (SE) of the heat-tolerant isolates (3.72 ± 0.05) was significantly greater than the mean colony diameter (cm) and SE of non-heat-tolerant isolates (1.67 ± 0.03) when grown at 37°C ($p<0.01$).

Eight of the 10 heat -tolerant isolates with smooth or undulate or undulate plus smooth zygospore walls (Table 2) were pathogenic. They induced clinical signs such as ataxia, hydrocephalus and loss of weight as well as high mortality in the first week of the experiment. Basidiobolus which conformed with the strains inoculated were isolated from the brain tissues of all mice inoculated. The non-heat-tolerant isolates were associated with low mortality and produced few clinical signs, except poor condition. None of the 4 non-heat-tolerant isolates could be recovered from brain tissue (Table 3).

The clinical signs, appearing in the first week post inoculation were an external distension of the cranial vault (hydrocephalus), and a general loss of condition indicated by ruffling of the fur.

The majority of deaths occured at this time. In the second week, some of animals with hydrocephalus developed clinical symptoms such as lethargy, ataxia and paralysis. Animals in this phase rapidly died or were killed before death.

At post-mortem examination, there were no other specific lesions in extracranial sites. Animals which showed nervous signs had severe distortion of the cranial vault and clear fluid containing white flecks of suspended material was found in the cerebral cavity. The cerebral hemispheres were grossly distended. In addition the cerebral hemispheres had atrophied to a thin shell or ruptured. The nervous parenchyma surrounding the ventricle was necrotic.

Histological examination of sections of brain one week post-inoculation showed necrosis and encephalomalacia, severe congestion and haemorrhage, meningoencephalitis with gliosis and perivascular infiltration and thrombosis in the cerebral vessels. Encephalomalacia was found in the subcortical and deep cortical level of the cerebrum and elsewhere. The cellular reactions consisted of lymphocytes, neutrophils, eosinophils and glial cells. Perivascular cuffing was mainly by neutrophils and lymphocytes. The meninges were infiltrated by neutrophils and macrophages.

At two weeks post-inoculation, the lesions were less acute as shown not only by increasing numbers of neutrophils and glial cells in the necrotic areas but also by macrophages and a granulomatous reaction with some giant cells. Mineral deposits indicated early calcification. Neuronal degeneration

with chromatolysis was also found.

At three weeks, the brain tissue changes were similar but there was greater calcification.

Abundant structures identifiable as fungal elements were seen in H & E sections in the necrotic or encephalomalacic areas but more clearly by the PAS and Gomori-methamine silver stains. They consisted of short, rather irregular, broad septate hyphae.

DISCUSSION

Strains of Basidiobolus sp have been recovered as common saprophytes from the faeces of reptiles, amphibians and from debris or leaf-litter (4;5;11;12;14). Less commonly the fungus has been recovered from the intestinal tract of insectivorous bats and the faeces of macropod species in captivity (1;13). In this study, the organism was isolated from faecal samples of reptiles, amphibians and debris. It was also established that Basidiobolus sp is associated with a wild population of macropods and with woodlice (Porcellio laevis Latereille). This isolation of the fungus from woodlice represents a new record.

Pathogenic Basodiobolus sp isolates are reported to grow well at 37°C. Greer and Friedman (7; 8) in their studies using human and non-human isolates found that the former were adapted to grow at 37°C, while non-human isolates grew better at 25°C. In the heat tolerance studies reported here, all the isolates grew well at 25°C, but 11 grew better at 37°C and also grew at 40°C. These were considered heat tolerant.

Intracerebral inoculation of adult mice with viable fragments of

Basidiobolus has been shown to provoke the development of progressive granulomatous lesion of the central nervous system (4;6;8;9). In this study, eight of 10 heat-tolerant isolates induced clinical signs and high mortality in the first week of the experiment. Identical strains were recovered from dead or sacrificed animals. These pathogenic isolates had smooth, undulate or undulate plus smooth zygospore walls, thus the morphology of the zygospore wall could not be used as a reliable criterion to differentiate pathogenic from non pathogenic isolates (Table 2).

The four non-heat-tolerant isolates caused low mortality and produced few clinical signs. Furthermore, none of these four strains could be recovered from brain tissue, all of which confirms their comparatively low pathogenicity.

In this study, cranial enlargment developed in the first week and was followed by nervous signs in the second week post inoculation. Eades and Corbel (6) reported slower development of cerebral lesions and clinical signs. Similar observations were reported following intracerebral infection with Absidia ramosa (2). The more rapid and uniform rate of development of cerebral lesions and clinical signs in our study is probably related to the difference in the age of the experimental animals. Susceptibility to infection with zygomycetes has been reported to differ between 3 and 4 week old mice (2). The results reported here confirm the effectiveness of the intracerebral route of inoculation and provides evidence that suckling mice offer an improved experimental model for the study of the pathogenicity of

Basidiobolus sp. It is noteworthy that the pathogenicity of the single clinical isolate (Eq.7) from a horse with basidiobolomycosis was confirmed using this model.

The origins of the pathogenic isolates were the usual sources of Basidiobolus sp. and are distributed widely in Australia. They constitute the main source of the fungus for environmental contamination and the hazard is therefore more widespread than previously considered.

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Table 1 : The origin and mean colony diameter (cm) of Basidiobolus isolates after 2 days incubation at 25°, 37° and 40°

No	Isolate/ origin	Incubated at 25oC	37oC	40oC	Heat Tolerant
1	Eq7/kunker	3.10±0.00	4.57±0.1	1.57±0.06	+
2.	B51/nothern dtella	3.03±0.06	3.77±0.06	1.53±0.00	+
3.	B46/skink	3.00±0.00	4.43±0.06	1.23±0.06	+
4.	B16/woodlice	3.07±0.06	1.43±0.06	NG*	-
5.	B7/toad	2.40±0.00	3.90±0.17	1.46±0.06	+
6.	B25/litter	2.23±0.06	1.43±0.06	NG*	-
7.	DP1/2/ macropod	2.13±0.06	3.45±0.06	1.03±0.06	+
8.	B6/woodlice	2.97±0.12	3.93±0.06	1.30±0.20	+
9.	B4/toad	2.40±0.06	2.86±0.13	0.63±0.12	+
10.	B1/bearded dragon	3.36±0.06	3.67±0.06	1.76±0.06	+
11.	B30/gecko	3.23±0.06	4.43±0.06	1.70±0.00	+
12.	B15/woodlice	2.03±0.06	1.70±0.12	NG*	-
13.	B2A/macropod	2.03±0.21	2.10±0.00	1.0±0.00	-
14.	B29/macropod	1.67±0.21	2.20±0.17	0.95±0.05	+

Mean±SE, n=3.

* N G = No growth

Table 2 : Characteristics of heat- tolerant isolates inoculated intracerebrally into suckling mice.

No.	Isolate	Zygospore wall*	Clinical signs: Hydro-cephalus	Ner-vous	Loss of-condition	No dead/no tested (Mortality %) week 1	6	Reisola-tion
1.	Eq7	S	-	+	+	11/17 (65)	11/17 (65)	+
2.	B51	S/U	+	-	-	7/16 (44)	9/16 (56)	+
3.	B46	U	+	-	-	1/12 (8.5)	5/12 (34)	-
4.	B7	S	+	+	+	2/6 (33)	3/6 (50)	+
5.	DP1/2	U	+	+	+	12/14 (86)	12/14 (86)	+
6.	B6	U	-	+	+	12/22 (54)	12/22 (54)	+
7.	B4	S	+	+	+	13/21 (62)	15/21 (71)	+
8.	B1	S	+	+	-	10/12 (82.5)	10/12 (82.5)	+
9.	B30	S	-	-	+	9/12 (74.5)	10/12 (82.5)	+
10.	B29	S	-	-	-	1/16 (6)	1/16 (6)	-
11.	Control		-	-	-	0/12 (0)	0/12 (0)	-

* S = Smooth, U = Undulate

Tabel 3 : Characteristics of non-heat - tolerant isolates inoculated intracerebrally into suckling mice.

No.	Isolate	Zygospore Wall*	Clinical signs: Hydrocephalus	Nervous	Loss condition	No dead/no tested (Mortality %) week 1	week 6	Reisolation
1.	B2A	S/U	-	-	-	1/14 (7)	2/14 (14)	-
2.	B16	S	-	-	+	4/9 (44)	4/9 (44)	-
3.	B25	S/U	-	-	+	6/13 (46)	8/13 (60)	-
4.	B15	S	-	-	+	1/13 (8)	4/13 (31)	-
5.	Control	-	-	-	-	0/12 (0)	0/12 (0)	-

* S = Smooth, U = Undulate