Penicillosis marneffei: Serological and exoantigen studies*

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Abstract

An immunodiffusion (ID) test has been developed to diagnose infections caused by *Penicillium marneffei.* A 20 X concentrated culture-filtrate of six-week-old shake cultures (25 C) of *P. marneffei* was employed as an antigen. This preparation was found to be better in quality than that from still cultures of the same age (30 C). Anti-*P. marneffei* rabbit sera were produced by injecting rabbits with increasing dosages of the inoculum for at least six weeks. These sera demonstrated two to three precipitin lines following their reaction with their antigens for 24–48 h at 25 C. The *P. marneffei* antigenic preparations did not react with rabbit antisera to five species of *Aspergillus,* which commonly cause aspergillosis, or to antisera for four dimorphic systemic fungi. Similarly, the antigens of these other fungi did not react against the anti-*P. marneffei* rabbit serum. However, the anti-*P. marneffei* rabbit sera demonstrated antibody titres (1:32 to 1:64) to histoplasmin, blastomycin and coccidioidin in the complement fixation test. Cross-reactions were not observed with any of the human sera in the suspected or proven cases of opportunistic or systemic mycotic infections. Therefore, the ID test for penicillosis marneffei is considered to be highly specific. Exoantigen studies demonstrated that, of a total of 34 isolates of ten species of *Penicillium* tested, only the extracts of *P. marneffei* (6 isolates) and one isolate (PLM 771) of a *P. species* reacted positively with the anti-*P. marneffei* rabbit serum, giving at least two lines of identity with reference reagents. Based on this analysis, the *P. species* (PLM 771) was identified as *P. marneffei.* The exoantigen test is considered to be a specific and rapid method for the identification and confirmation of *P. marneffei* isolates.

Zusammenfassung

Eine Immun-Verbreitung (ID) Test war ausgearbeitet fur die Diagnose der Infektionen die *Penicillium marneffei* verursachen. Die 20 X conzentrierte Kultur-Fieltrier von der sechs Wochen alten schuttelten Kulturen (25 C) von *P. marneffei* war gebraucht fur Antigen. Diese Preparation war besser in Qualitat denn die stillen Kulturen von dem selben Alter (30 C). Die Anti-*P. marneffei* Kaninchen Sera wurde produziert durch die Injektion der Kaninchen mit immer vergrösserden Dosen von dem Imfstoff-mindestebs wahrend sechs Wochen. Diese Sera zeigte zwei – drei Ubere sturzung Linien durch die Reaktion von ihrer Antigenen wahrend 24–48 Stunden im 25 C. Die Antigen-Preparationen von *P. marneffei* gaben keine Reaktionen mit

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Kaninchen Anti-sera fur funf Species von *Aspergillus,* die stellen an gewohnlich Aspergillosis, oder zu der Antisera fur fier dimorpische systemische Pilze. Gleichweis, die Antigenen von diese Plize gaben keine Reaktionen gegen die Kaninchen Antisera von *P. marneffei.* Jedoch die Kaninchen Antiserum von *P. marneffei* zeigte Anti-Korper Titer (1:32 bis 1:64) zu Histoplasmin, Blastomycin und Coccidioidin in der Complement (Erganzung) Fixing Test. Kreuz-Reaktionen wurden nicht observiert mit keine menschlichen Sera in verdachtigen oder erwiesenen Krankengeschichten von passenden oder systematischen mycotischen Infectionen. Deswegen die ID Test fur Penicillosis marneffei ist sehr spezifisch. Die Untersuchungen haben demonstriert, dass 34 isolationen von zehn Species von *Penicillium* – die untersucht waren – nur der Auszug von *P. marneffei* (6 Isolationen) und ein Isolation (PLM 771) von *P. species* eine positive Reaktion mit der Kaninchen Anti-Serum von *P. marneffei,* und es gab mindestens zwei identische Linen mit die Referenz Reagenten. An diese Analyse grunden wir dass, die *P. species* (PLM 771) war identifiziert wie *P. marneffei.* Die Exo-Antigen Test ist anbetrachtet wie eine spezifische und schnelle Methode fur die Identifikation und Bestatigung der Isolierten von *P. marneffei.*

Introduction

Species of the genus *Penicillium* are basically saprophytes or plant parasites and their isolation from clinical specimens usually has no medical significance. In recent years, the use of cytotoxic drugs, antibacterial antibiotics, steroid compounds, antimetabolites, immunosuppressive compounds, and radiation therapy has contributed to a rapid increase in infections caused by the so-called 'opportunistic fungi'. Included in this group is an infection by *P. citrinum* of the urinary tract (5). Huang and Harris (6) reported a combined pulmonary and meningeal infection caused by *P. commune,* in a patient suffering from acute leukemia. They cited 10 cases of bronchopulmonary penicillosis, caused by *P. bertai, P. bicolor, P. crustaceum, P. glaucum, P. spinulosum* and *P. species,* in their survey of the literature since 1918. Among these 10 cases, four patient had some other primary disease and the remainder had no known predisposing factor or underlying disease. It is well known that various species of *Penicillium* can cause mycokeratitis, external ear infections, allergic type reactions, granuloma of the lung and endocarditis of aortic valve prosthesis (13, 14, 16, 23).

The first report of the isolation of *P. marneffei* from the liver of a bamboo rat *(Rhizomyces sinensis)* was presented by Capponi *et al.* (2) Three years later, Segretain (17) published the description of this new species and also recorded an accidental human infection due to *P. marneffei.* In 1973, DiSalvo, Fickling and Ajello (3) reported the first known natural infection in a human to be caused by *P. marneffei.* Their patient's primary illness was Hodgkin's disease for which he had received radiation treatment and some surgical intervention. Another human case caused by *P. marneffei* has been recently diagnosed in Indonesia (Dr. A. A. Padhye, Centers for Disease Control, Atlanta, Georgia 30333, U.S.A., personal communication).

It is now clear that *P. marneffei* and some other species of *Penicillium,* viz. *P. chrysogenum, P. citrinum, P. commune, P. expansum* and *P. spinulosum,* are capable of producing disease of various types in humans who may or may not have primary illnesses or predisposing factors. The present investigation was undertaken to develop a serological procedure for the (i) diagnosis of infection caused by *P. marneffei* and (ii) to develop an exoantigen test for the rapid and specific identification or confirmation of *P. marneffei* isolates. These studies appeared desirable because (i) *P. marneffei* could well be mistaken for the tissue form of *Histoplasma capsulatum in vivo* (3) and (ii) the diagnosis of mycotic infections by conventional means is always time-consuming and an immunological test, if available, would provide rapid and presumptive evidence of infection (8).

Materials and methods

Organisms

Details relating to the sources of *P. marneffei* and the other species of *Penicillium* used in the study are given in Table 1.

Inoculum production

Five ml of sterile distilled water were pipetted

Table 1. Sources of the isolates of *P. marneffei, P. citrinum, P. commune* and two species of *Penicillium* studied.

Isolate	Source		
PLM 759	Nasal swab (6 145–78)	IMI 235 429	as *P. cyclopium*
PLM 760	Catheter site (5 168–78)	IMI 235 430	as *P. verrucosum,* var. *cyclopium*
PLM 761	Drainage (6 466–78)	IMI 235 434	as *P. cyclopium*
PLM 762	Knee fluid (6 340–78)	IMI 235 431	as *P. chrysogenum*
PLM 763	Bronchial aspirate (6 063–78)	IMI 235 432	as *P. chrysogenum*
PLM 764	Eye swab (0 536–79)	IMI 235 433	as *P. chrysogenum*
PLM 765	Bronchial washing (0 345–79)	IMI 235 435	as *P. chrysogenum*
PLM 150	Not known	IMI 235 436	as *P. chrysogenum*
PLM 398	Not known	UAMH 2 219	as *P. claviforme*
PLM 382	Not known	UAMH 485	as *P. notatum*
PLM 554	The Pasteur Institute, Paris, France. Received as ≠ 560	UAMH 2 993	as *P. marneffei* (1)[b]
PLM 689	Human; from Dr. A. F. DiSalvo	ATCC 24 100	as *P. marneffei* (2)[b]
PLM 767	From Dr J. E. Ellis	NRRL 805	as *P. citrinum*
PLM 768	(Id.)	NRRL 6 184	as *P. marneffei* (3)[c]
PLM 769	(Id.)	NRRL 1 843	as *P. citrinum*
PLM 771	From Dr R. A. Samson	CBS 549.77	as *P. species*[a] (4)[b]
PLM 772	(Id.)	CBS 311.48	as *P. commune*
PLM 773	From Dr R. A. Samson; rat (= CMI 68 794; = ATCC 18 224)	CBS 334.59	as *P. marneffei* (5)[c] Segretain
PLM 774	(Id.)	CBS 668.77	as *P. citrinum*
PLM 780	From Commonwealth Mycological Institute, Kew, U.K.	IMI 6 199	as *P. citrinum*
PLM 781	(Id.)	IMI 49 122	as *P. citrinum*
PLM 782	(Id.)	IMI 87 247	as *P. commune*
PLM 783	(Id.)	IMI 92 215	as *P. commune*
PLM 784	(Id.)	IMI 91 918	as *P. commune*
PLM 785	(Id.)	IMI 39 812	as *P. commune*
PLM 786	(Id.)	IMI 24 307	as *P. citrinum*
PLM 787	(Id.)	IMI 68 214	as *P. citrinum*
PLM 833	From Dr A. A. Padhye, Centers for Disease Control, Atlanta, Georgia, U.S.A.	B 3 189 (= NRRL 890)	as *P. commune*
PLM 834	(Id.)	B 3 190 (= NRRL 806)	as *P. citrinum*
PLM 835	(Id.)	B 3 191 (= NRRL 843)	as *P. citrinum*
PLM 836	(Id.)	B 3 192 (= NRRL 5 927)	as *P. citrinum*
PLM 837	(Id.)	B 3 193 (= NRRL 6 184)	as *P. marneffei* (6)[c]
PLM 895	(Id.) Human; Indonesia	B 3 420	as *P. marneffei* (7)[b]
PLM 898	From Dr M. A. Gordon, State of New York Department of Health, Albany, N.Y.	M 961	as *P. species*

ATCC = American Type Culture Collection, Rockville, Md., U.S.A.
CBS = Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands
IMI = Imperial Mycological Institute, Kew, United Kingdom
NRRL = Northern Regional Research Laboratory, Peoria, Illinois, U.S.A.
PLM = Provincial Laboratory of Public Health (Mycology Number), The University of Alberta, Edmonton, Alberta
UAMH = University of Alberta Mold Herbarium and Culture Collection, The University of Alberta, Edmonton, Alberta, Canada
[a]Based on exoantigenic analysis, this isolate (PLM 771; received from Dr R. A. Samson) was identified as *P. marneffei.* In a recent personal communication, Dr. R. A. Samson informed Dr Libero Ajello that he had received PLM 771, P.sp., from Dr A.F. DiSalvo. Thus PLM 771 is a subculture of PLM 689.
[b]Human isolates of *P. marneffei.*
[c]Indochina rat isolates.

into each culture (7-day-old), grown on slants of cereal agar (18). The tubes were shaken vigorously for about 5 min. Microscopic examination of the resulting suspension revealed a mixture of conidia and mycelial fragments.

Antigen production

For antigen production, liquid stationary and shake cultures were used. Each Erlenmeyer flask, containg Sabouraud's dextrose broth (300 ml) (16), was inoculated with the fungal suspension (5 ml/flask). One set of inoculated flasks was incubated at 30 C and the second set was placed on a gyrotary shaker (150 r.p.m.) kept at room temperature (25 C) for six weeks. At the end of the incubation period, both the still and shake cultures, were killed by adding a 1% aqueous solution of merthiolate (Thimerosal, Sigma Chemical Company, St. Louis, Mo.; approximately 30 mg/flask). The stationary cultures were returned to the incubator and held at 30 C for 72 h. During their growth period of six weeks, the still cultures did not quite form a mycelial mat; instead, various sized clumps of mycelium developed. No diffusible pigment was released into the culture medium by the two isolates of *P. marneffei* (PLM 554 & 689) grown in still cultures.

The shake cultures, following merthiolation, were further shaken for a period of 72 h at 25 C. These cultures of *P. marneffei* (PLM 554 & 689) produced a brick red pigment within 3 days of their initial incubation period. It became dull red as the cultures aged.

Antigen extraction

Culture filtrates of the still and shake cultures were freed of mycelium and other fungal elements by passing them through Whatman filter paper (grade 202) under suction. The clear filtrates were then membrane sterilized (0.45 u). These sterile filtrates were treated with chilled acetone (1:3, v/v), shaken vigorously, and left at 0–4 C for approximately 18 h. The precipitate of each flask was dissolved in phosphate buffered saline (pH 7.2), equal to approximately 1/20th the volume of the original sterilized culture filtrates. This 1/20th or 20 X-concentrated preparation following its merthiolation served as an antigen.

Exoantigenic preparations

One-week-old cultures of *P. marneffei* (7 isolates) and the other isolates of *Penicillium species* (see Table 1), grown on cereal agar at 25 C, were flooded with 1% aqueous merthiolate (8 ml) and incubated for 24 h. These extracts were centrifuged at 18 000 × g for 10 min at 0 C, and concentrated 25 ×, using Minicon B-15 concentrators (Amicon Corp., Denver, Mass.). These concentrated preparations, after merthiolation, were stored (0–4 C) until used.

Antiserum production

Rabbits were injected subcutaneously at weekly intervals with 2.5 ml of the inoculum suspensions mixed with equal volumes of Freund's incomplete adjuvant. The immunization schedules were continued for at least six weeks with increasing numbers of fungal elements (8 500–51 000), or until a satisfactory immune response was acheived. One week after the final injection, blood was collected by cardiac puncture. The blood was allowed to stand at room temperature for 3–4 h. The antiserum was separated from the blood components, centrifuged at 18 000 × g for 10 min (0 C), separated from the sediment and then merthiolated (1:10 000) to prevent microbial growth. The antisera were then stored at –30 C in 5 ml portions.

Performance of the serological tests

Reactions between the antigens and antisera were observed by performing macro- and micro-immunodiffusion (micro-ID) tests in 1% noble agar prepared in 0.1 M veronal buffer, pH 8.6 (15).

Determination of Cross-reactions

Anti-*P. marneffei* sera were tested, using the micro-ID technique, against *Aspergillus fumigatus, A. flavus, A. nidulans, A. niger, A. terreus, Blastomyces dermatitidis, Coccidioides immitis, Sporothrix schenckii,* and *Micropolysporafaeni, Thermopolyspora candidus* and *T. vulgaris* antigens, prepared by the methods described elsewhere (1, 9, 12). Anti-*P. marneffei* sera were also investigated against histoplasmin, blastomycin and coccidioidin with the complement fixation technique. Similarly, the antisera to all the above organisms were tested against the *P. marneffei* antigen.

Results

Serological studies

When the *P. marneffei* antigen from liquid stationary cultures was reacted against the antiserum to this fungus, one strong precipitin line was observed following incubation of the agar gel plates for 48 h at 25 C (Fig. 1). However, when the *P. marneffei* antigen, produced by the shake culture method, was similarly used, three or four precipitin lines were observed in both the micro-ID (Fig. 2) and macro-ID procedures.

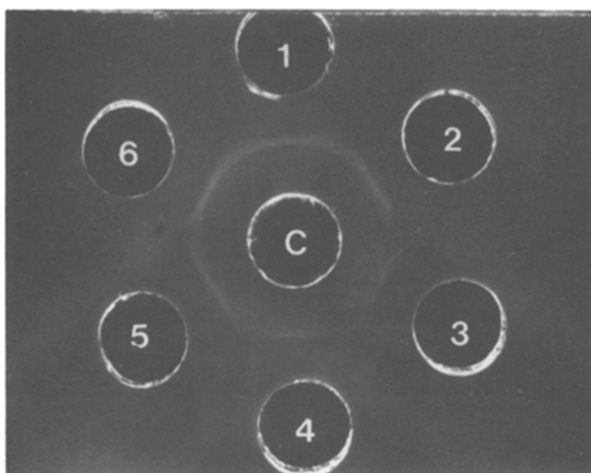

Fig. 1. P. marneffei antigen (wells 1–6), of liquid still cultures, following its reaction with anti-*P. marneffei* rabbit serum (well C). This antigen produced only one precipitin in the macro-ID test.

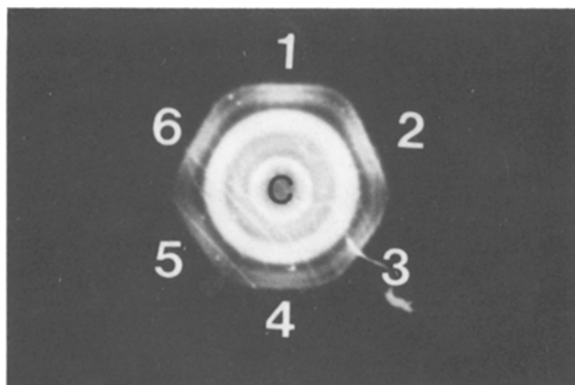

Fig. 2. P. marneffei antigen (wells 1–6), of liquid shake cultures, following its reaction with anti-*P. marneffei* rabbit serum (well c). This antigen produced at least three to four precipitins in the micro-ID test.

Exoantigenic analyses

Exoantigenic preparations, obtained from the 6 *P. marneffei* isolates, 10 *P. citrinum* isolates, 6 *P. commune* isolates, 5 *P. chrysogenum* isolates, 2 *P. cyclopium* isolates, 2 *P. species* isolates, and one isolate of each of *P. verrucosum, P. claviforme* and *P. notatum* (see Table 1), were tested against the anti-*P. marneffei* rabbit serum in the presence of the *P. marneffei* antigen. Of these 34 isolates belonging to ten species of *Penicillium*, only the *P. marneffei* and *P. species* (PLM 771) extracts reacted positively, giving at least two lines of identity with the *P. marneffei* reference reagents. None of the extracts of the other eight species of *Penicillium* had precipitins to anti-*P. marneffei* serum (Fig. 3).

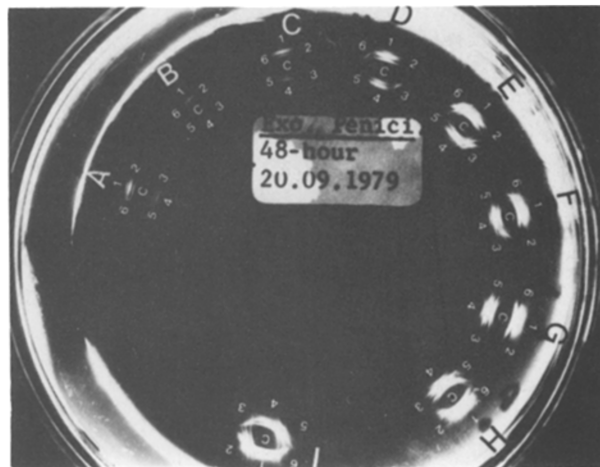

Fig. 3. Micro-ID patterns (A – I) of the isolates of *P. marneffei, P. Commune, P. citrinum* and the *P. species* (PLM 771) produced during analysis of their exoantigenic extracts. Wells C, and 1 and 4 in the A – I patterns contained anti-*P. marneffei* rabbit serum and *P. marneffei* antigen respectively. The lateral wells of pattern A contained extracts of *P. commune* (PLM 783 and 784). Pattern B: wells 2 and 5, *P. commune* (PLM 782); wells 3 and 6, *P. citrinum* (PLM 781). Pattern C: wells 2 and 5, *P. citrinum* (PLM 774); wells 3 and 6, *P. citrinum* (PLM 780). Pattern D: wells 2 and 5, *P. commune* (PLM 772); wells 3 and 6, *P. marneffei* (PLM 773). Pattern E: wells 2 and 5, *P. citrinum* (PLM 769); wells 3 and 6, *P. species* (PLM 771; note the lines of identity with the reference system). Pattern F: wells 2 and 5, *P. citrinum* (PLM 767); wells 3 and 6, *P. marneffei* (PLM 768). Pattern G: wells 2, 3, 5 and 6, P. citrinum (PLM 786, 787). Pattern H: wells 2 and 5, *P. marneffei* (PLM 689); wells 3 and 6, *P. commune* (PLM 785). Pattern I: wells 2 and 5, *P. species* (PLM 771; the lines of identity are seen); wells 3 and 6, *P. marneffei* (PLM 773). Note only the extracts of the *P. marneffei* isolates and the *P. species* (PLM 771) contained specific antigen.

Cross-reaction studies

The *P. marneffei* antigen did not react with the anti-rabbit sera to *A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus,* or to the antisera of the four systemic fungi or the antisera to the three thermophilic actinomycetes. Similarly, the anti-*P. marneffei* rabbit serum was negative for precipitins when reacted against the antigens of five species of *Aspergillus,* the four systemic fungi and the three thermophilic actinomycetes.

A total of 411 human sera, received for routine serology, were examined for their reactivity against the *P. marneffei* antigen and antiserum. Among these sera, 84 contained one to three precipitins to one or more species of *Aspergillus sp.* tested. Two hundred eighteen of the sera came from suspected cases of aspergillosis and contained no precipitins to the *Aspergillus* species. One hundred seven sera were from suspected cases of histoplasmosis. They had no antibody to histoplasmin. One serum was from a proven case of coccidioidomycosis and it produced precipitin against coccidioidin. Another serum was from a suspected case of blastomycosis but it contained no precipitins to the *B. dermatitidis* antigen. The results of this study indicated that none of the 411 heterologous sera produced reactions against the *P. marneffei* antigen.

When tested by the complement fixation procedure, anti-*P. marneffei* rabbit sera demonstrated titres of 1:32, 1:64 and 1:64 to blastomycin, coccidioidin and histoplasmin, respectively.

Discussion

An ID test, in a macro and micro form, specific for penicillosis marneffei has been developed. Antigen prepared from stationary cultures was inferior to that prepared from shake cultures of the same age (Fig. 2). The ID test proved to be highly specific; neither the antigen nor antiserum to *P. marneffei* reacted against the antigens or antisera of five species of *Aspergillus,* thermophilic actinomyces, and the systemic pathogenic fungi. Furthermore, the ID test was found not to cross-react with sera from cases of aspergillosis and other mycoses. However, anti-*P. marneffei* rabbit sera were found to cross-react with histoplasmin, blastomycin and coccidioidin, in appreciably high titres (1:32 to 1:64), in the complement fixation procedure. Cross-reactions of the sera from individuals having systemic fungal infections are well known when tested by the complement fixation technique (18). This finding suggests that the sera demonstrating complement fixation titres to histoplasmin, blastomycin and coccidioidin, but negative in the ID test with these antigens, should be further tested against *P. marneffei* antigen in the ID test.

The exoantigen studies showed that the *P. marneffei* isolates produced specific antigens. The other species of *Penicillium* tested, with the exception of one isolate (PLM 771) of *P. species,* lacked the antigens extracted from *P. marneffei.* The exceptional isolate (PLM 771), which had been received with the label *P. sp.* from Dr R. A. Samson, produced antigens that were identical to those produced by the isolates of *P. marneffei.* These results indicate that exoantigenic analysis can be used as an additional aid in the rapid identification of isolates of *P. marneffei.*

Based on exoantigenic analysis, the *Penicillium species* isolate (PLM 771) was identified as *P. marneffei.* Application of exoantigens of *C. immitis, H. capsulatum, B. dermatitidis* and *Paracoccidioides brasiliensis* has been found to be a highly successful and rapid procedure in the specific identification of these fungi (10, 11, 7, 3, 19, 20, 21, 22).

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