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ASPERGILLUS ANTIGEN AND ANTIBODY PREPARATION AND THEIR VALUE FOR DETECTION OF ASPERGILLOSIS

การเตรียมแอนติเจนและแอนติบอดีจากเชื้อแอสเพอจิลัสพร้อมทั้งประโยชน์ในการใช้วินิจฉัยโรคแอสเพอจิโลซิส

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ABSTRACT

The serodiagnosis of aspergillosis was studied by producing antigens and antisera for detection of aspergillosis by immunodiffusion test. The antigens were prepared from static culture filtrate of 5-week-old Aspergillus *using acetone precipitation method and stored by freeze drying. The antisera were obtained 4 weeks after immunizing rabbits with self-prepared antigens of* A. fumigatus, A. flavus *and* A. niger. *Self-prepared antigens and antisera were reacted with reference antisera and antigens obtained from the Center for Disease Control (CDC), Atlanta, Georgia, until they met the standard potency and specificity as required by the CDC. Moreover, in this study 16 patients with aspergillosis and 133 with non-aspergillosis such as tuberculosis, melioidosis, pneumonia, vaginal candidosis, pulmonary mycoplasmosis, miscellaneous diseases, and 134 cases of apparently normal individual were tested by immunodiffusion (ID). Positive results were obtained in only 10 cases (62.5%) of*

aspergillosis group. This finding indicated that immunodiffusion serodiagnosis technique is helpful for diagnosing antibodies in aspergillosis cases. Especially the reference antigens and antisera can be easily prepared in Thailand.

บทคัดย่อ

ได้ศึกษาการผลิตและการพัฒนาแอนติเจนและแอนติบอดีของเชื้อแอสเพอจิลัส เพื่อที่จะนำไปใช้ในการช่วยวินิจฉัยโรคแอสเพอจิโลซิสด้วยวิธีทางเซรุ่ม โดยการเตรียมแอนติเจนจากวิธีตกตะกอนน้ำเลี้ยงเชื้อ (culture filtrate) ของเชื้อแอสเพอจิลัสที่มีอายุ 5 สัปดาห์ ด้วยอะซีโตนแล้วเก็บในสภาพแช่แข็งแห้ง (freeze dried) ส่วนแอนติบอดีเตรียมได้จากกระต่ายที่ผ่านการกระตุ้นด้วยแอนติเจนเป็นเวลา 4 สัปดาห์ด้วยเชื้อ 3 ชนิด คือ *A. fumigatus A. flavus* และ *A. niger* ผลการศึกษาพบว่าแอนติเจนและแอนติบอดีที่เตรียมได้เมื่อนำมาตรวจสอบฤทธิ์กับแอนติเจนและแอนติบอดีอ้างอิง (reference antigens and antisera) ของ Center for Disease Control (CDC) เมืองแอตแลนตา รัฐจอร์เจีย ประเทศสหรัฐอเมริกา พบว่าจะมีฤทธิ์ (potency) และความจำเพาะ (specificity) ตรงตามมาตรฐานของ CDC กำหนดไว้ นอกจากนี้ยังได้ใช้แอนติเจนที่เตรียมได้นี้มาทำการตรวจหาแอนติบอดีในผู้ป่วยด้วยโรคแอสเพอจิโลซิส 16 ราย และโรคอื่น ๆ เช่น วัณโรค ปอดอักเสบ เมลิออยโดซิส ช่องคลอดอักเสบจากเชื้อราแคนดิดา ปอดอักเสบจากเชื้อไมโคร-พลาสมา ฯลฯ รวม 133 ราย และคนปกติอีก 134 ราย โดยใช้วิธีการทดสอบปฏิกิริยาการเกิดเส้นตะกอนในเนื้อวุ้น (immunodiffusion test, ID) ผลการตรวจได้ผลบวกในผู้ป่วยด้วยโรคแอสเพอจิโลซิส 10 ราย (62.5%) แสดงว่าแอนติเจนและแอนติบอดีที่เตรียมได้เองนี้ สามารถใช้ในการช่วยวินิจฉัยโรคแอสเพอจิโลซิสได้เป็นอย่างดี

INTRODUCTION

Aspergillosis is caused by several species of aspergilli, most commonly by *Aspergillus fumigatus*. The disease is characterized by granulomatous or necrotizing lesions which may involve any tissue of the body. Lung is a common site of significant infection. Aspergillosis varies in severity from an incidental, saprophytic relationship to a fulminating to fatal infection. The disease is found in all races throughout the world.[4]

The accurate diagnosis of aspergillosis, in most instances, is based on a combination of clinical, cultural and histologic findings. However, to establish the diagnosis on the basis of cultural findings may be difficult due to frequent false-positive and false-negative

results. In many particular cases histologic materials may not be available for diagnosis. Moreover, the organism may not always be definitively identified, since *Aspergillus*, *Penicillium, Scopulariopsis*, and *Petriellidium* are indistinguishable in stained tissue sections. In such instances, other methods, particularly serologic tests may be of value. Many serologic tests have been developed for detecting antibodies: for example, agar gel immunodiffusion (ID), immunoelectrophoresis (IE), complement fixation (CF), latex agglutination (LA), indirect immunofluorescence (IF), and indirect hemagglutination (IHA). Of these procedures, immunodiffusion is the simplest[7] and most widely performed test for the detection of Aspergillus antibodies. The specificity of the test is in the range between 90-100% and its sensitivity is between 50-93% in the patients with allergic bronchopulmonary aspergillosis (ABPA), invasive aspergillosis and aspergilloma.[2,8,10]

Even though pulmonary aspergillosis is apparently common in Thailand, as indicated by the high incidence of aspergillosis seen at Siriraj Hospital during A.D. 1959-1979. The Department of Pathology, Faculty of Medicine, Siriraj Hospital found that 1% (165 cases) of 16, 219 autopsy cases showed deep fungal infection and about 30.63% of these 165 cases had fungal pulmonary infection, 37.29% from *Aspergillus* and 35.03% from *Candida.*[11] The facilities for serodiagnosis are generally lacking. The required antigens are not available locally and have to be imported.

This research was a study of how to prepare standard antigens and antisera for use in immunodiffusion test (ID) for detecting aspergilli specific antibodies in the sera of suspected cases of pulmonary aspergillosis and to evaluate the use of immunodiffusion method in antemortem diagnosis of aspergillosis.

MATERIALS AND METHODS

Materials

Patients with aspergillosis

Patients with aspergillosis: 16 patients (10 males, 6 females) identified by physicians from the evidence based on a respiratory and constitutional symptoms plus radiographic findings compatible with sputum culture or biopsy yielding *Aspergillus* spp. In one case mycelia was seen in tissue section.

Patients with non-aspergillosis

Patients with tuberculosis: 21 patients (17 males, 4 females) diagnosed by physicians based on a respiratory and constitutional symptoms plus radiographic findings compatible with Acid Fast Bacilli (AFB) positive.

Patients with melioidosis: 21 patients (11 males, 10 females) diagnosed by the indirect hemagglutination test.

Patients with vaginal candidosis: 24 single serum specimens from 24 female patients diagnosed as vaginal candidosis based on symptoms and presence of budding yeast and hyphae from fresh of vaginal discharge.

Patients with pulmonary mycoplasmosis: 15 patients (7 males, 8 females) diagnosed by complement fixation test.

Patients with pneumonia: 5 patients (2 males, 3 females) caused by the Respiratory Syncytium Virus were diagnosed by complement fixation test.

Patients with miscellaneous diseases: 42 patients with lung cavity (18), systemic candidosis (6), allergic bronchiolitis (4), cryptococcal meningitis (4), nocardiosis (2), aplastic anemia (2), systemic lupus erythrematosus (2), endocarditis (1), acute promegaloblastic leukemia (1), diabetes mellitus (1) and liver abscess (1).

Normal control

Normal control consisted of 134 medical students (94 males, 40 females) of the Faculty of Medicine, Siriraj Hospital with an average age of 20 years.

Methods

Antigen preparation

Standard strains: Standard strains of *Aspergillus* spp from Center for Disease Control (CDC), were chosen as follows: *A. fumigatus* (B-1172), *A. flavus* (B-15), *A. flavus* (85-031124), *A. niger* (107). One strain from Ruhr University at Bochrum (RUB), West Germany was *A. niger* (AP-1/4 V).

Patient strains: There were 5 strains: *A. fumigatus* (Metinee), *A. fumigatus* (n18), *A. fumigatus* (Pranom), *A. fumigatus* (Patient) and *A. fumigatus* (Juan).

Food strains: One strain of *Aspergillus* sp isolated from food was found to be *A. niger* (r2).

The acetone precipitation of culture filtrate method[2] was chosen for preparation of aspergillus antigens from standard strains, patient strains and food strains. Determine the carbohydrate content of antigen by the Phenol Sulfuric acid test,[3] and adjust to 1,000-1,500 μg carbohydrate/ml for routine testing.

Immunodiffusion test

Immunodiffusion test for Aspergillus was two dimensional double diffusion-in - gel qualitative technique of the type as described in detail by Ouchterlony.[10]

Preparation of Aspergillus rabbit antisera

Preparation of antigen: Procedure was the same as the preparation of immunodiffusion antigen by using acetone precipitation[2] method but it had 2 steps difference that, the precipitate was suspended to 1/25 of the original culture filtrate volume and the carbohydrate content was adjusted to 4,000 μg/ml.

Immunization schedule: Antigens from *A. fumigatus* (Pranom), *A. fumigatus* (B-1172), *A. flavus* (B-15), *A. niger* (107) and *A. niger* (r2) were used to immunize 1 rabbit, 2 rabbits, 2 rabbits and 1 rabbit, respectively using the method as follows:

1st week - On Tuesday, Wednesday and Thursday a 1.0 ml of antigen suspended in Freund's incomplete adjuvant was intramuscular injected.
2nd and 3rd weeks - On Thursday, a 0.5 ml of the antigen was intravenous injected.
4th week - Rabbits' blood and sera were obtained to ID Test using homologous antigen. If the antigen did not give reactivity, continued immunization with weekly injection of 0.5 ml of the antigen intravenously.

The rabbits were exsanguinate 7 to 10 days after the last inoculation.

RESULTS

A comparison between acetone precipitation method and dialysis method in preparation of antigens

Culture filtrate of *A. fumigatus* (Metinee), *A. fumigatus* (B-1172), *A. flavus* (B-15) and *A. niger* (107) were each divided into 2 portions to be used for preparing antigens and

were compared in 2 different methods in the pattern of precipitating lines after reacted with reference antisera (Ab) and carbohydrate content at the concentration 1/10 of the original culture filtrate volume. The comparison is shown in Table 1.

Standard curve for carbohydrate determination

Phenol-Sulfuric Acid Test was used for determination of carbohydrate content in each antigen preparation and glucose in concentration of 10-75 μg/ml was used as standard reagent (Figure 1).

Comparison of self-preparation of antigens

The antigens of 11 *Aspergillus* strains (3 obtained from CDC, 8 from patients and food) were prepared by acetone precipitation method and reacted with reference homologous antisera from CDC. The numbers of precipitation lines obtained in each species were compared in Table 2.

Detection of the serum specific antibodies against Aspergillus antigen by ID in patients and normal control

Patients with aspergillosis: Sixteen sera of suspected aspergillosis patients were reacted with reference antigens and self-prepared antigens. Ten sera of those gave positive result which were about 62.5%. The results are shown in Table 3. And the 10 patients' sera that gave positive result were diluted by two-fold dilution and reacted with reference antigen and self-prepared antigen, the results are shown in Table 4.

Sera from non-aspergillosis patients and normal control: Serum specimens collected from non-aspergillosis patients and healthy control were reacted with pooled reference antigens and pooled self-prepared antigens. The results are shown in Table 5. The pooled reference antigens were prepared by mixing equal volumes of reference *A. fumigatus* Ag, *A. flavus* Ag, and *A. niger* Ag. The pooled self-prepared antigens comprised equal volumes of *A. fumigatus* (Pranom) Ag and *A. fumigatus* (B-1172) Ag mixed together with two volume of *A. flavus* (B-15) Ag and *A. niger* (107) Ag. The pooled reference antisera were prepared by mixing equal volumes of reference *A. fumigatus* Ab, *A. flavus* Ab and *A. niger* Ab.

Comparison of the number of precipitating lines occurring during the reaction between self-prepared antisera and reference homologous antigens.

Immunized antisera from rabbits were checked with reference homologous antigen from CDC. The numbers of precipitation lines were compared in Table 6.

Pooled antisera previously immunized with the same species but different strain of *Aspergillus* were reacted with the reference homologous antigen from CDC. The numbers of precipitating lines were compared in Table 7.

Evaluation of standard and self-prepared antigens for the detection of antibodies in heterologous antisera

Antisera from immunized rabbits and standard antisera against *A. fumigatus*, *A. flavus*, *A. niger* from CDC were reacted with different self-prepared antigens and standard antigens from CDC to check cross reaction interspecies (Table 8).

Evaluation of self-prepared antigens and antisera

Pooled self-prepared antigens were reacted with pooled self-prepared homologous antisera. The results are shown in Table 9.

Proposed ID test-set for using in the diagnosis of aspergillosis

For developing ID test-set in laboratory diagnosis of aspergillosis first antigens and antisera to Aspergillus species should be prepared and tested until they met the CDC-potency. Later, all antigens, antisera together with 1% nobel agar, 5% sodium citrate coomassie blue, filter paper punch set and pattern were packed. The direction for using this ID test-set was included in the set.

DISCUSSION

Evaluation of preparation method of antigen

Antigens which were used in immunological test for diagnosis of aspergillosis were soluble substance obtained from culture filtrates or cell extracts. In *Aspergillus* the use of culture filtrates was recommended.[2,7,9,10,13] The filtrates were usually required to be concentrated before the optimal dilution for each immunological test was determined. Varieties of concentration methods had been used, for example, dialysis followed by freeze drying[7,9,13] and acetone precipitation.[2,10] In this study the acetone precipitation method was used in the filtrate concentration, as shown in Table 1. The numbers of precipitation lines and carbohydrate content using acetone precipitation method in 3 out of total 4 strains of *Aspergillus* were greater than when dialysis method was used. Another reason for choosing precipitation method

was due to its low cost. To concentrate each 400 ml of culture filtrates, about 800 ml of acetone was used which costed 115.2 baht while in dialysis followed by freeze drying method a dialysis bag (about 377 cm long containing 400 ml of filtrate) costed 652.21 baht. So it was 5.66 times cheaper in acetone precipitation method than dialysis method. Reference antigens antisera used in this study were also prepared by acetone precipitation method.

Evaluation of self-prepared antigens

The potency of each self-prepared antigen was evaluated according to the number of precipitin lines occurred in ID test between each antigen and homologous reference antisera. The results revealed that different numbers of precipitin lines occurred in Table 2 indicating the variation in each strain of *Aspergillus* in producing antigens. This might be due to the concentration of antigens in each strain that was not optimum. The CDC used the strains of *A. fumigatus* (B-1172), *A. flavus* (B-15) and *A. niger* (107) for preparing the antigens and recommended that the carbohydrate content in each batch should be within the range of 1,000-1,500 μg/ml which was suitable for ID test. In this experiment the carbohydrate content of each antigen was adjusted to 1,000-1,500 μg/ml and then used to react with the reference homologous antisera. Some of self-prepared antigens might not require the carbohydrate content of 1,000 - 1,500 μg/ml. Another reason why self-prepared antigens gave different numbers of precipitin lines might be due to the ability of each individual strain in producing unequal components of antigens.

According to the CDC reference reagents, satisfactory antigens of *A. fumigatus* must give at least 3 distinct precipitin lines, while that of *A. flavus*, and *A. niger* must give one or more distinct precipitin lines with homologous reference sera. As shown in Table 2 the antigens of *A. flavus* (B-15) gave more antigenic composition than *A. flavus* (85-031124) and showed more than one precipitin line which indicated its suitability to be used later. In the same way the antigens prepared from *A. niger* (107) was better than *A. niger* (r2) and *A. niger* (AP114V). Owing to the result, it revealed that *A. flavus* (B-15) and *A. niger* (107) should be selected as reference strains in further antigen preparations using in diagnosis of aspergillosis. According to CDC requirement *A. fumigatus* (B-1172) should give 3 precipitin lines but it gave only 2. The CDC prepared culture filtrate of *A. fumigatus* (B-1172) by incubating it at 31°C for 5 weeks in screwcapped Erlenmeyer flask while a preparation culture filtrate was incubated at room temperature for 5 weeks in Erlenmeyer flask with cotton plug. The temperature and aerosol may influence the metabolic antigen produced by *A. fumigatus* (B-1172). However, result showed that *A. fumigatus* (Pranom) gave 3 precipitin lines according to CDC requirement.

Self-prepared antigens from *A. fumigatus* isolated from the patients in Thailand and reference antigens obtained from the CDC had different antigenic compositions (Table 3) and the numbers of the patients whose sera formed precipitin lines with each of these antigens

were not equal. One positive result was obtained when the antigens of *A. fumigatus* (Juan), *A. fumigatus* (n18) was used while 7 and 10 positive results were obtained when *A. fumigatus* (Pranom) and *A. fumigatus* (B-1172) were used, respectively. Kurup and Fink[6] showed in their investigations that the reactivity of individual antigens varied from 42 to 87% which indicated that mixed antigen of more than one strain of *A. fumigatus* might be efficiently used. In the same way mixed antigen of *A. fumigatus* (Pranom) and *A. fumigatus* (B-1172) was recommended to be used in ID test in further diagnosis of aspergillosis in the test set.

Evaluation of self-prepared antisera

Antigens from *A. fumigatus* (Pranom), *A. fumigatus* (B-1172), *A. flavus* (B-15), *A. niger* (107), and *A. niger* (r2) were used to immunized rabbits. Pooled antisera from the rabbits immunized with the same strain of antigen were reacted with reference homologous antigen. The results were as shown in Table 6. The antisera obtained from the rabbits immunized with *A. fumigatus* (B-1172) and *A. fumigatus* (Pranom) did not give the CDC potency. This might be due to individual variation in the response of the rabbits or might be due to the properties of antigens. Self-prepared antigen of *A. fumigatus* (Pranom) gave 3 lines when reacted with reference homologous antiserum (Table 2) and was used to immunized a rabbit. The antiserum obtained from the rabbit should also give 3 lines when reacted with reference homologous antigen but according to the finding it gave only 2 lines (Table 6). This might be due to the effect of individual variation in the response of the rabbits, or some labile components might be lost during the process of lyophilization. Self-prepared antigen of *A. fumigatus* (B-1172) gave 2 lines when reacted with reference homologous antiserum (Table 2) and the antisera from an immunized rabbit with this antigen also gave 2 lines when reacted with reference homologous antigen. Such antiserum did not reach CDC potency which might be due to the effect of antigen properties that lack some components to make the rabbit produce perfect antiserum with complete components as required by the CDC indicating by the presence of 3 lines.

When the antisera from the rabbits immunized with the antigen of the same species of Aspergillus were pooled and reacted with reference homologous antigen (Table 7), all of them met CDC potency. No cross reaction among interspecies of Aspergillus were noted (Table 8). Self-prepared antisera did not react with heterologous antigens but reference antisera of *A. niger* and *A. fumigatus* could react with reference antigen of *A. flavus*.

Pooled self-prepared antigens reacted with the antisera and met the CDC potency (Table 9). In *A. flavus* and *A. niger* the ID test revealed 2 and 3 precipitation lines respectively, whereas in *A. fumigatus* 3 precipitation lines could be observed.

Evaluations of the use of immunodiffusion method as an aid in antemortem diagnosis of aspergillosis

The results of the ID test as shown in Table 5 indicated that the test was specific. Aspergillus precipitins were not detected in any of the 34 serum specimens from patients with other mycotic infections (30 with candidosis, 4 with cryptococcosis) or 59 sera from cases with bacterial diseases (21 with melioidosis, 21 with tuberculosis, 15 with pulmonary mycoplasmosis, and 2 with nocardiosis) or 10 sera from viral diseases (RSV pneumonia) or 22 sera from pulmonary diseases with unknown etiology (18 with cavity lung, 4 with allergic bronchitis) or 8 sera from miscellaneous diseases (2 with aplastic anemia, 2 with SLE, 1 with endocarditis, 1 with APL, 1 with DM, 1 with liver abscess) or 134 sera from apparently normal persons. Aspergillus precipitin could be detected by this method in 10 patients out of 16 with clinically suspected aspergillosis. This investigation indicated that the ID test for detection of Aspergillus specific antibodies could be used as an aid in the antemortem diagnosis of aspergillosis. Gerber and Jones suggested that antibodies to *A. fumigatus* were detectable by ID test and they also reflexed recent or active infection.[5] Only 62.5% of the patients with clinically suspected aspergillosis gave positive result for specific antibodies to Aspergillus by the ID test which might be due to the following reasons:

1. The patients were diagnosed as aspergillosis according to the clinical signs and symptoms without any evidence on histologic findings, therefore, false or missed diagnosis is also possible.

2. From the studies done by other groups,[14] it was shown that the patients with proven invasive aspergillosis could give negative result due to the suppression of immune response.

In this study the conclusion of the sensitivity of the ID test could not be made because in the 10 positive cases, the test was performed with prospectively not retrospectively, except in one proven case of aspergillosis. A patient who showed the opacity inside the lung cavity in the chest X-ray film had severe haemoptysis. The physician suspected aspergillosis and, therefore, sent the patient's serum for the ID test which gave positive result. The physician then performed a surgical resection and the removed mass was sent to a pathologist for diagnosis, and the result came out with aspergillosis. *A. fumigatus* was isolated from two of three sputum cultures of this patient. That was only one case proven by the pathologist that the patient with aspergillosis gave positive ID test.

In Table 4, when the patients' sera were diluted to 1:4 and 1:8, positive result dropped to 50% and 10% suggesting that the sensitivity of the ID test was low. There must be a large quantity of antibodies in the serum in order to be detectable.

According to this study the ID test had high specificity, as no cross-reactions with

other 267 proven cases of non-aspergillosis and with apparently normal subjects was observed. This finding was supported by Coleman and Kaufman[2] who also reported negative ID test in 55 patients with other mycotic or bacterial infections or neoplastic diseases, or healthy subjects. This is in contrast with the results obtained by the more sensitive following methods : indirect immunofluorescence (IF),[12] indirect radioimmunodiffusion (RID),[1] which could detect such antibodies in all cases of the control group. This suggests that although sensitivity of ID test in this investigation was not high but its specificity was reliable and should be recommended to use as an aid in antemortem diagnosis of aspergillosis.

CONCLUSIONS

The results of this research study can be concluded as follows:

1. Acetone precipitation method is suitable for concentration of the culture filtrates used in the self-preparation of antigens.

2. There are variations in composition and concentration of self-prepared antigens among aspergilli strain. The antigens of *A. fumigatus* (Pranom), *A. fumigatus* (B-1172), *A. flavus* (B-15) and *A. niger* (107) showed many precipitin lines (more than two) were chosen for preparing self-reference antigens.

3. Then out of total 16 serum specimens (62.5%) obtained from aspergillosis patients yielded positive immunodiffusion test. Negative result was obtained in all 133 non-aspergillosis cases and in 134 apparently normal persons. The specificity of immunodiffusion test is reliable.

4. Self-prepared antisera has no cross reaction with heterologous antigens whereas reference antisera of *A. fumigatus* and *A. niger* could react with reference *A. flavus* antigen.

5. Although self-prepared antisera reached the CDC potency, it lacked some components when compared with reference antisera.

The antigens and antisera which were important in serodiagnosis technique were not locally available. They had to be imported with high cost. Immunodiffusion test can be easily performed but some needed reagents were expensive and not available in the rural laboratories. Immunodiffusion kit is, therefore, proposed as it contains all necessary reagents and simple equipments for immunodiffusion performance.

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Table 1. Showing the comparison of the two methods used for preparing the antigen of each strain.

Strain	Method	CHO content at 10x	No. of bands
A. fumigatus (Metinee)	acetone dialysis	3,350 μg/ml 940 μg/ml	1 none
A. fumigatus (B – 1172)	acetone dialysis	4,250 μg/ml 1,860 μg/ml	2 1
A. flavus (B – 15)	acetone dialysis	1,300 μg/ml 1,400 μg/ml	3 1
A. niger (107)	acetone dialysis	3,300 μg/ml 1,150 μg/ml	2 1

Table 2. Showing in comparison the numbers of the precipitating lines obtained from different species and strains.

Species	Strain	No. of bands
A. fumigatus	Metinee	1
	n 18	1
	Pranom	3
	Patient	2
	Juan	0
	B – 1172	2
A. flavus	85 – 031124	2
	B – 15	3
A. niger	Ap 114V	1
	r2	2
	107	3

Table 3. Showing the numbers of the precipitating lines occured when patients' sera reacted with reference and self-prepared antigens.

Serum	*A. fumigatus* antigens							Reference antigens	
	Reference	Metinee	n18	Pranom	Patient	Juan	B-1172	*A.flavus*	*A.niger*
Ms. Maliga	1	0	0	0	0	0	1	0	0
Mr. Manop	1	0	0	0	0	0	1	0	0
Mr. Saelymp	1	0	0	1	0	0	1	0	0
Ms. Peknai	1	0	0	1	0	0	1	0	0
Mr. Boonchai	2	0	0	1	0	0	1	0	0
Mr. Sumrit	2	0	0	1	0	0	1	0	0
Mr. Luxana	1	0	0	1	0	0	1	0	0
Mr. Taweepon	1	0	1	2	1	1	1	0	0
Ms. Pranom	1	0	0	0	0	0	1	0	0
Ms. Jomsri	1	0	0	1	1	0	1	0	0
Reference Ab									
A. fumigatus	2	1	1	3	2	0	2	2	0
A. flavus	0	0	1	0	1	0	0	3	0
A. niger	0	0	0	0	0	0	0	2	2

Table 4. Showing the numbers of patients' sera which still showed positive precipitating lines with *A. fumigatus* Ag when they were diluted.

Antigen	No. of patients sera which showed positive results in different titers					
	Undilute	1:2	1:4	1:8	1:16	1:32
Reference *A. fumigatus*	10	9	5	1	1	0
A. fumigatus (Metinee)	0	0	0	0	0	0
A. fumigatus (n18)	1	0	0	0	0	0
A. fumigatus (Pranom)	7	7	3	1	0	0
A. fumigatus (Patient)	2	1	0	0	0	0
A. fumigatus (Juan)	1	0	0	0	0	0
A. fumigatus (B – 1172)	10	8	5	0	0	0

Table 5. Showing the results of immunodiffusion test between different sera and pooled Aspergilli antigens.

Diagnosis	No. of total sera	No. of positive sera react with antigens	
		Pooled reference antigen	Pooled self-prepared antigen
Candida vaginitis	24	0	0
Tuberculosis	21	0	0
Melioidosis	21	0	0
Cavity lung	18	0	0
Pulmonary mycoplasmosis	15	0	0
Respiratory syncytium virus (RSV) pneumonia	10	0	0
Systemic candidosis	6	0	0
Allergic bronchiolitis	4	0	0
Cryptococcal meningitis	4	0	0
Nocardiosis	2	0	0
Aplastic anemia	2	0	0
Systemic lupus erythrematosus (SLE)	2	0	0
Endocarditis	1	0	0
Acute promegaloblastic leukemia (APL)	1	0	0
Diabetes mellitus	1	0	0
Liver abscess	1	0	0
Normal controls	134	0	0

Table 6. Showing immunized antisera reacted with reference homologous antigens.

Antisera from rabbit immunized with	No. of bands reacted with reference homologous antigens
A. fumigatus Pranom	2
B – 1172	2
A. flavus B – 15	2
A. niger r2	1
107	1

Table 7. Showing pooled immunized antisera reacted with reference homologous antigens.

Pooled antisera from rabbits immunized with the antigens of	No. of bands reacted with reference homologous antigens
A. fumigatus	3
A. flavus	2
A. niger	2

Table 8. Showing cross reaction between Ag & Ab among interspecies of Aspergillus

Ab from	Ag / Strain	*A. fumigatus*			*A. flavus*		*A. niger*		
		Reference	B-1172	Pranom	Reference	B-15	Reference	r2	107
A. fumigatus	Reference	+	+	+	+	–	–	–	–
	B – 1172	+	+	+	–	–	–	–	–
	Pranom	+	+	+	–	–	–	–	–
A. flavus	Reference	–	–	–	+	+	–	–	–
	B – 15	–	–	–	+	+	–	–	–
A. niger	Reference	–	–	–	+	+	+	+	+
	r2	–	–	–	–	–	+	+	+
	107	–	–	–	–	–	+	+	+

\+ mean $\geqslant$ 1 bands are seen

– mean band absent

Table 9. Showing the numbers of precipitating lines when self-prepared Ag and Ab were reacted in the ID.

Pooled self-prepared Ag of		No. of bands reveal when reacted with pooled self-prepared rabbits antisera
Species	Strain	
A. fumigatus	B – 1172 Pranom	3
A. flavus	B – 15	2
A. niger	107 r2	3

Standard glucose (μg/ml)	10	25	50	75
OD at 490 nm	0.10	0.27	0.56	0.77

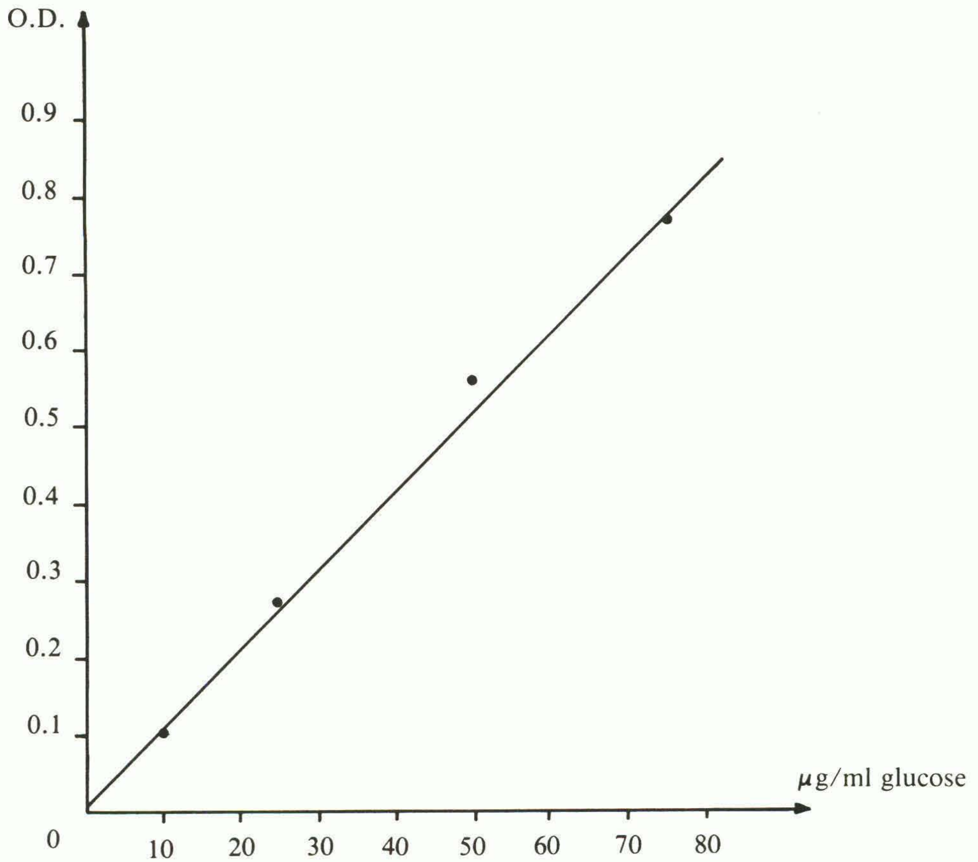

Fig. 1 Showing a standard glucose curve as determined by phenol-sulfuric acid test