Home-made *Aspergillus* antigens and antisera for serodiagnosis of aspergillosis

นิตยา ไตรภิญโญภาพ, วทม. (จุลชีววิทยา) *
อังคณา ฉายประเสริฐ, ปร. ด**

บทคัดย่อ Aspergillosis เป็นโรคที่เกิดจากราสายในสกุล *Aspergillus* โดยพบว่าก่อโรคในระบบทางเดินหายใจบ่อยที่สุด และ aspergillosis ยังพบบ่อยที่สุดโรคหนึ่งของโรคติดเชื้อราในระบบอวัยวะภายในจากการชันสูตรศพ การวินิจฉัย จำเป็นจะต้องอาศัยข้อมูลจากอาการผู้ป่วย ภายถ่ายรังสีทรวงอก รวมทั้งผลการเพาะเชื้อจากสิ่งส่งตรวจและผลจากการตรวจทางน้ำเหลืองวิทยา ในการศึกษาครั้งนี้จึงได้ผลิตชุดน้ำยาสำหรับตรวจวินิจฉัยทางน้ำเหลืองวิทยา ด้วยวิธี double immunodiffusion ตามมาตรฐานของ CDC สหรัฐอเมริกากำหนด อันประกอบด้วย culture filtrate (CF) antigens และ homologous antisera ของเชื้อ *Aspergillus fumigatus* B - 1172, *A. flavus* B - 15, *A. niger* 107, A. *nidulans* B - 1390 และ *A. terreus* B - 985 เพื่อใช้สำหรับตรวจช่วยการวินิจฉัยโรค aspergillosis ในบ้านเรา

Abstract **Home-made *Aspergillus* antigens and antisera for serodiagnosis of aspergillosis**

Nitaya Tripinyopap, MS. (Microbiology)*
Angkana Chaiprasert, Ph.D.**

Aspergillosis is a disease caused by ubiquitous filamentous fungus belonging to the genus *Aspergillus*. It frequently affects respiratory tract and is of the most common systemic mycoses reported from autopsy cases. The definitive diagnosis needs information from clinical picture, chest roentgenogram together with culture and serological results. In this study, a home-made battery of reagents, composed of culture filtrate (CF) antigens and their homologous rabbit antisera of *Aspergillus fumigatus* B-1172, *A. flavus* B-15, *A. niger* 107, *A. nidulans* B-1390 and *A. terreus* B-985 was prepared according to the CDC standards for being used in conventional double immunodiffusion method for aid the diagnosis of aspergillosis.

(MJS 1996 ; 3 : 67 - 74)

* ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ประสานมิตร
** ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล
* Department of Microbiology, Faculty of Medicine, Srinakharinwirot University.
** Department of Microbiology, Faculty of Medicine, Mahidol University, Siriraj Hospital.

Introduction

Aspergillosis is a disease caused by ubiquitous filamentous fungus belonging to the genus *Aspergillus*. It is one of the most common systemic mycoses reported from autopsy cases. Aspergillosis frequently affects respiratory tract[1-4] and can be defined according to its different forms as extrinsic asthma, extrinsic alveolitis, allergic bronchopulmonary aspergillosis (ABPA), invasive aspergillosis, aspergilloma and chronic necrotizing pulmonary aspergillosis (CNPA).

The definitive diagnosis by demonstration of fungus either by culture or staining methods performed on the specimens taken from the affected lesions is difficult and not practical.[3-5] Besides this, the results from cultures of sputum are usually not reliable. Therefore, the diagnosis needs information from clinical pictures, chest roentgenogram together with culture and serological results.[4,6-7,11]

The nonspecific clinical and radiological pulmonary manifestations of aspergillosis create diagnosis difficulty. In this study, a double immunodiffusion method was developed by preparing a battery of home-made reagents according to the CDC standards for being used in conventional double immunodiffusion method.[10]

Materials

1. Microorganisms

1.1 *Aspergillus fumigatus* B - 1172

1.2 *Aspergillus flavus* B - 15

1.3 *Aspergillus niger* 107

1.4 *Aspergillus nidulans* B - 1390

1.5 *Aspergillus terreus* B - 985

All *Aspergillus* species were provided by the Center for Disease Control (CDC), Atlanta, Georgia, USA.

2. Reference reagents

CDC - reference reagents composed of culture filtrate (CF) antigens and rabbit antisera of *A. fumigatus* B - 1172, *A. flavus* B - 15 and *A. niger* 107. All of them were provided by the Center for Disease Control (CDC), Atlanta, Georgia, USA.

Methods

1. Preparation of Home-made *Aspergillus* culture filtrate (CF) antigens comprised of

A. fumigatus B - 1172 CF antigen

A. flavus B - 15 CF antigen

A. niger 107 CF antigen

A. nidulans B - 1390 CF antigen

A. terreus B - 985 CF antigen

The cultures of *A. fumigatus* B - 1172, *A. flavus* B - 15, *A. niger* 107, *A. nidulans* B - 1390 and *A. terreus* B - 985 were separately grown in 400 ml Sabouraud Dextrose Broth (SDB) in a liter Erlenmeyer screwcapped flask for 5 weeks (static) at room temperature. The cultures obtained were killed by adding formalin to concentration of 0.5 %, manually shaked to wet mycelial mat and let stand at room temperature for overnight.

The killed cultures were filtered through Whatman filter paper No. 40. The supernate were then collected and further precipitated at 4°C by adding 2 volumes of cold acetone dropwise while simultaneously stirring. After all acetone had been added, the mixtures were continually stirred for another 15 minutes.

The mixture were then centrifuged at 10,000 rpm for 20 minutes at 4°C then decanted, the retained precipitate was left dry overnight in an evacuated dessicator at room temperature. The dry

precipitate was resuspended in steriled distilled water to 1:10 volume of the original culture filtrate. The suspensions were then filtered through Whatman filter paper No. 1 to remove undissolved precipitate.

The determination of carbohydrate content of the CF antigens was performed according to the Phenol Sulfuric acid test, and it should be adjusted to 1,000 - 1,500 μg carbohydrate/ml for routine testing. The CF antigen were aliquot and stored at-20°C after lyophilization.

2. Preparation of *Aspergillus* rabbit antisera

comprised of

Rabbit antiserum of *A. fumigatus* B-1172 CF antigen

Rabbit antiserum of *A. flavus* B-15 CF antigen

Rabbit antiserum of *A. niger* 107 CF antigen

Rabbit antiserum of *A. nidulans* B-1390 CF antigen

Rabbit antiserum of *A. terreus* B-985 CF antigen

The carbohydrate content of each *Aspergillus* CF antigen was adjusted to 4,000 μg/ml. Each CF antigen was emulsified with equal volume of Freund's incomplete adjuvant by forcing through a 21 G hypodermic neddle with a syringe until the emulsion attained the consistency of thick cream.

Each CF antigen was immunized into 2-3 rabbits weighing 2-2.5 kg by injecting 1.0 ml. antigen intramuscularly into the thigh of each rabbit on Tuesday, Wednesday and Thursday during the first week.

Booster dose were given on Wednesday during the second and third week with 0.5 ml. of CF antigen alone intravenously at the marginal ear vein.

The rabbits were test bled on the fourth week, and the sera obtained were to perform double immunodiffusion test with CDC-reference antigens. If any antiserum did not reveal the reactivity given in the evaluation, the immunization had to be continued with intravenous injection of 0.5 ml. of CF antigen alone. The rabbit were bled again 7-10 days after the last injection.

3. Double Immunodiffusion procedure

The double immunodiffusion method was performed according to Coleman and Kaufman in 1972.[10]

4. Interpretation of double immunodiffusion test results

The presence of one or more precipitin lines was indicative of either a fungal ball, allergic bronchopulmonary aspergillosis or invasive aspergillosis.

Results

1. Determination of the potency of the home-made *Aspergillus* Culture filtrate (CF) antigens and homologous rabbit antisera against CDC *Aspergillus* antigens and antisera

1.1 *Aspergillus fumigatus* B - 1172

According to the CDC requirement for determining the home-made *A. fumigatus* B-1172 CF antigen and its homologous rabbit antiserum to be used as reference rengents in double immunodiffusion method, the home-made *A. fumigatus* B-1172 CF antigen and rabbit antiserum must react with the CDC *A. fumigatus* B-1172 rabbit antiserum and CF antigen respectively and provide three or more identity precipitin lines. The results are shown in Fig. 1 and Fig. 2, which can be seen that the home-made reagents of *A. fumigatus* B-1172 meet the CDC requirement.

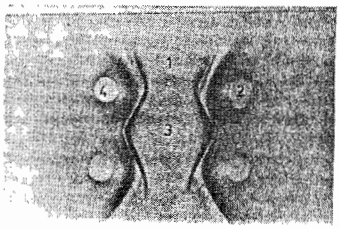

Fig.1 Number of precipitin lines occurred between the wells of home-made *A. fumigatus* B-1172 CF antigen and CDC-reference *A. fumigatus* antigen and antisera.

well No. 3 CDC-reference *A. fumigatus* Ag
well No. 2,4 CDC-reference *A. fumigatus* Ab
well No. 1 home-made *A. fumigatus* Ag

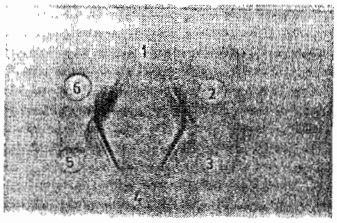

Fig.2 Number of precipitin lines occurred between the wells of home-made *A. fumigatus* rabbit antiserum and CDC-reference *A. fumigatus* antigen and antiserum.

central well CDC-reference *A. fumigatus* Ag
well No. 3,5 CDC-reference *A. fumigatus* Ab
well No. 2,6 home-made *A. fumigatus* Ab

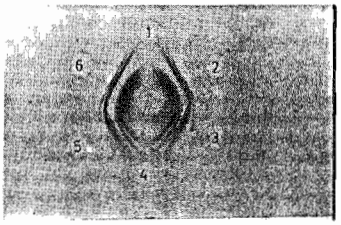

Fig.3 Number of precipitin lines occurred between the wells of home-made *A. flavus* B-15 CF antigen and CDC-reference *A. flavus* antigen and antiserum.

central well CDC-reference *A. flavus* Ab
well No. 3,5 CDC-reference *A. flavus* Ag
well No. 2,6 home-made *A. flavus* Ag

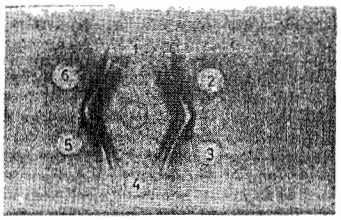

Fig.4 Number of precipitin lines occurred between the wells of home-made *A. flavus* rabbit antiserum and CDC-reference *A. flavus* antigen and antisera.

central well CDC-reference *A. flavus* Ag
well No. 3,5 CDC-reference *A. flavus* Ab
well No. 2,6 home-made *A. flavus* Ab

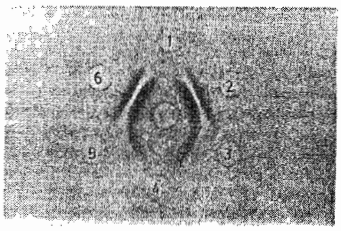

Fig.5 Number of precipitin lines occurred between the wells of home-made *A. niger* 107 CF antigen and CDC-reference *A. niger* antigen and antiserum.

central well CDC-reference *A. niger* Ab
well No. 3,5 CDC-reference *A. niger* Ag
well No. 2,6 home made *A. niger* Ag

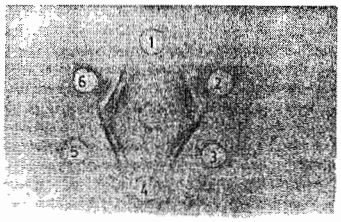

Fig.6 Number of precipitin lines occurred between the wells of home-made *A. niger* rabbit antiserum and CDC-reference *A. niger* antigen and antiserum.

central well CDC-reference *A. niger* Ag
well No. 3,5 CDC-reference *A. niger* Ab
well No. 2,6 home-made *A. niger* Ab

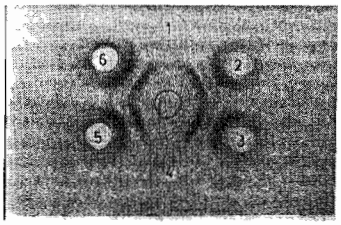

Fig.7 Number of precipitin lines occurred between the wells of home-made *A. nidulans* B-1390 CF antigen and its homologous rabbit antiserum determination by double immunodiffusion method.

central well home-made *A. nidulans* Ag
well No. 2,3,5,6 home-made *A. nidulans* Ab

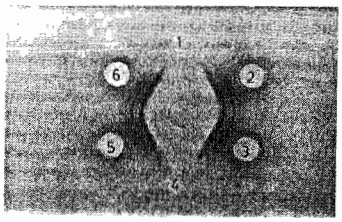

Fig.8 Number of precipitin lines occurred between the wells of home-made *A. terreus* B-985 CF antigen and its homologous rabbit antiserum determination by double immunodiffusion method.

central well home-made *A. terreus* Ag
well No. 2,3,5,6 home-made *A. terreus* Ab

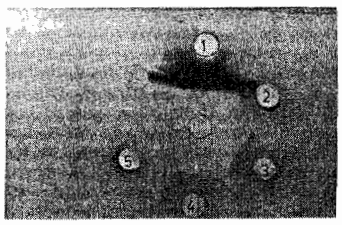

Fig.9 Determination of cross-reactivity of the home-made CF antigen of *A. fumigatus* B-1172 with the home-made rabbit antisera.

central well	home-made *A. fumigatus* Ag
well No. 1	home-made *A. fumigatus* Ab
well No. 2	home-made *A. flavus* Ab
well No. 3	home-made *A. niger* Ab
well No. 4	home-made *A. nidulans* Ab
well No. 5	home-made *A. terreus* Ab

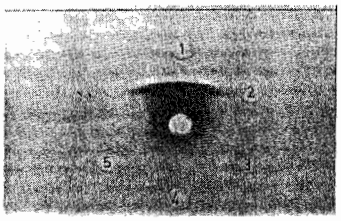

Fig.10 Determination of cross-reactivity of the home-made rabbit antiserum of *A. fumigatus* with the home-made CF antigens.

central well	home-made *A. fumigatus* Ab
well No. 1	home-made *A. fumigatus* Ag
well No. 2	home-made *A. flavus* Ag
well No. 3	home-made *A. niger* Ag
well No. 4	home-made *A. nidulans* Ag
well No. 5	home-made *A. terreus* Ag

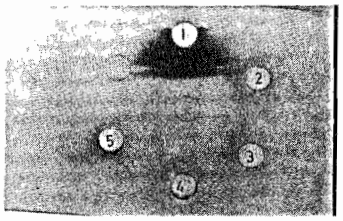

Fig.11 Determination of cross-reactivity of the home-made CF antigen of *A. flavus* B-15 with the home-made rabbit antisera.

central well	home-made *A. flavus* Ag
well No. 1	home-made *A. flavus* Ab
well No. 2	home-made *A. fumigatus* Ab
well No. 3	home-made *A. niger* Ab
well No. 4	home-made *A. nidulans* Ab
well No. 5	home-made *A. terreus* Ab

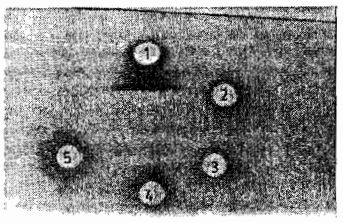

Fig.12 Determination of cross-reactivity of the home-made rabbit antiserum of *A. flavus* with the home-made CF antigens.

central well	home-made *A. flavus* Ab
well No. 1	home-made *A. flavus* Ag
well No. 2	home-made *A. fumigatus* Ag
well No. 3	home-made *A. niger* Ag
well No. 4	home-made *A .nidulans* Ag
well No. 5	home-made *A. terreus* Ag

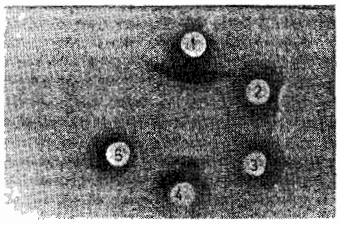

Fig.13 Determination of cross-reactivity of the home-made CF antigen of *A. niger* 107 with the home-made rabbit antisera.

central well	home-made *A. niger* Ag
well No. 1	home-made *A. niger* Ab
well No. 2	home-made *A. fumigatus* Ab
well No. 3	home-made *A. flavus* Ab
well No. 4	home-made *A. nidulans* Ab
well No. 5	home-made *A. terreus* Ab

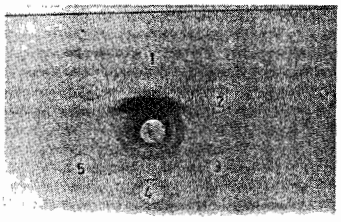

Fig.14 Determination of cross-reactivity of the home-made rabbit antiserum of *A. niger* with the home-made CF antigens.

central well	home-made *A. niger* Ab
well No. 1	home-made *A. niger* Ag
well No. 2	home-made *A. fumigatus* Ag
well No. 3	home-made *A. flavus* Ag
well No. 4	home-made *A. nidulans* Ag
well No. 5	home-made *A. terreus* Ag

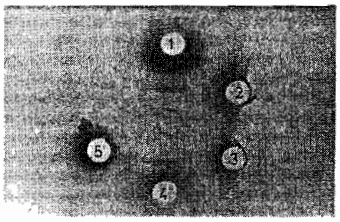

Fig.15 Determination of cross-reactivity of the home-made CF antigen of *A.nidulans* B-1390 with the home-made rabbit antisera.

central well	home-made *A. nidulans* Ag
well No. 1	home-made *A. nidulans* Ab
well No. 2	home-made *A. fumigatus* Ab
well No. 3	home-made *A. flavus* Ab
well No. 4	home-made *A. niger* Ab
well No. 5	home-made *A. terreus* Ab

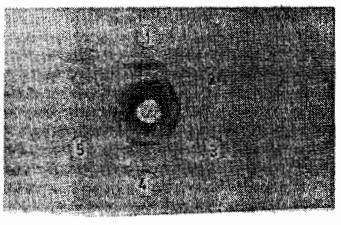

Fig.16 Determination of cross-reactivity of the home-made rabbit antiserum of *A. nidulans* with the home-made CF antigens.

central well	home-made *A. nidulans* Ab
well No. 1	home-made *A. nidulans* Ag
well No. 2	home-made *A. fumigatus* Ag
well No. 3	home-made *A. flavus* Ag
well No. 4	home-made *A. niger* Ag
well No. 5	home-made *A. terreus* Ag

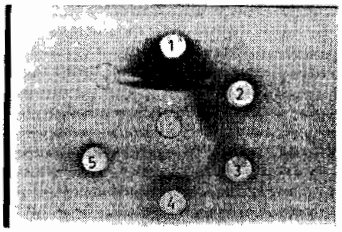

Fig.17 Determination of cross-reactivity of the home-made CF antigen of *A. terreus* B-985 with the home-made rabbit antisera.

central well	home-made *A. terreus* Ag
well No. 1	home-made *A. terreus* Ab
well No. 2	home-made *A. fumigatus* Ab
well No. 3	home-made *A. flavus* Ab
well No. 4	home-made *A. niger* Ab
well No. 5	home-made *A. nidulans* Ab

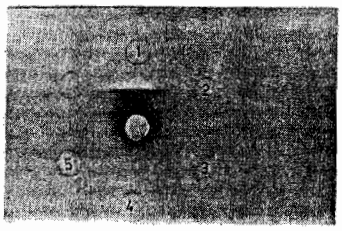

Fig.18 Determination of cross-reactivity of the home-made rabbit antiserum of *A. terreus* with the home-made CF antigens.

central well	home-made *A. terreus* Ab
well No. 1	home-made *A. terreus* Ag
well No. 2	home-made *A. fumigatus* Ag
well No. 3	home-made *A. flavus* Ag
well No. 4	home-made *A. niger* Ag
well No. 5	home-made *A. nidulans* Ag

1.2 *Aspergillus flavus* B - 15

To determine the home - made *A. flavus* B - 15 reagents to be used as reference reagent, the home - made *A. flavus* B - 15 CF antigen and rabbit antiserum must react with the CDC *A. flavus* B - 15 rabbit antiserum and CF antigen respectively and provide one or more identity precipitin lines. The results are shown in Fig. 3 and Fig. 4, which can be seen that the home - made *A. flavus* B-15 reagents meet the CDC requirement.

1.3 *Aspergillus niger* 107

In the same as above, the home-made *A. niger* 107 reagents must react with the CDC reagents and provide one or more identity precipitin lines. The results are shown in Fig. 5 and Fig. 6, which can be seen that the home-made *A. niger* 107 CF antigen and its homologous rabbit antiserum meet the CDC requirement.

1.4 *Aspergillus nidulans* B-1390 and *A. terreus* B-985

Since *A. nidulans* and *A. terreus* rarely encountered in aspergillosis but they had been reported as causative agents in Thailand[11] and according to the CDC recommendation, detection of specific antibody in patients with aspergillosis by double immunodiffusion method, might include both of them in the battery reagents. In the present study, *A. nidulans* B-1390 and *A. terreus* B-985 CF antigens and their homologous rabbit antisera were prepared according to the procedure previously described.

The result of the reaction between the home -made *A. nidulans* B-1390 CF antigen and its homologous rabbit antiserum is shown in Fig.7, which can be seen that it provides three precipitin lines. Whereas the reaction between the home-made *A. terreus* B-985 CF antigen and its homologous antiserum provide four precipitin lines and the result is demonstrated in Fig.8.

2. Determination of inter - species cross - reactivity of the home-made *Aspergillus* CF antigens and rabbit antisera

To determine the specificity of the home-made reagents by performing double immunodiffusion between each home-made CF antigen and the five home-made rabbit antisera, and between each home-made rabbit antiserum and the five home-made CF antigen. The results are as followed :

2.1 The home-made CF antigen of *A. fumigatus* B-1172

provided slight cross-reactions by showing one faint precipitin line with the home-

made rabbit antisera of *A. niger* 107 and *A.nidulans* B-1390. The results are shown in Fig. 9. And the results of the home-made rabbit antiserum of *A. fumigatus* B-1172 are shown in Fig.10, which can be seen that the home-made rabbit antiserum of *A. fumigatus* B-1172 provided slight cross-reactions by showing one faint precipitin line with the home-made CF antigens of *A. flavus* B - 15 and *A. niger* 107.

2.2 The home-made CF antigen of *A. flavus* B-15 provided slight cross-reactions by showing one faint precipitin line with the home-made rabbit antisera of *A. fumigatus* B-1172, *A.niger* 107 as shown in Fig.11. The home-made rabbit antiserum of *A. flavus* B-15 provided slight cross-reactions by showing one precipitin line with the home-made CF antigen of *A. nidulans* B-1390 as shown in Fig. 12.

2.3 The home-made CF antigen of *A. niger* 107 provided slight cross-reactions by showing one precipitin line with the home-made rabbit antisera of *A. fumigatus* B-1172, *A. flavus* B-15 and *A. nidulans* B-1390 as shown in Fig.13. The home-made rabbit antiserum of *A. niger* 107 provided slight cross-reactions by showing one faint precipitin line with all the home-made CF antigens of *A. fumigatus* B-1172, *A. flavus* B-15, *A. nidulans* B-1390 and *A. terreus* B-985 as shown in Fig.14.

2.4 The home-made CF antigen of *A. nidulans* B-1390 provided slight cross-reactions by showing one faint precipitin line with the home-made rabbit antiserum of *A. niger* 107 as shown in Fig.15. The home-made rabbit antiserum of *A. nidulans* B-1390 provided slight cross-reactions by showing one faint precipitin line with all the other four home-made CF antigens as shown in Fig.16.

2.5 The home-made CF antigen of *A. terreus* B-985 provided slight cross-reactions by showing two precipitin lines with the home-made rabbit antisera of *A. fumigatus* B-1172 and one faint precipitin line with the home-made rabbit antisera of *A. flavus* B-15 and *A. niger* 107 as shown in Fig.17. However, the home-made rabbit antiserum of *A. terreus* B-985 did not provided cross-reaction with any home-made CF antigen as shown in Fig.18.

Discussion

In this study, double immunodiffusion method for detection of precipitating antibodies in patients with aspergillosis has been developed by preparation of home-made reagents according to the CDC requirement. The home - made antigens used in this system were prepared from culture filtrates of the fungi (4-6 weeks old). They were prepared during the autolytic phase of growth and believed to contain not only extracellular (metabolic) components but also intracellular (cytoplasmic) components as a results of lysis and therefore, consist of a full spectrum of antigens likely to be encountered in vivo.[8-9,14-16]

The results from determination of inter-species cross-reactivity of the home-made reagents showed that the home-made CF antigens of *A. fumigatus* B-1172, *A. flavus* B-15, *A. niger* 107, *A. nidulans* B-1390 and *A. terreus* B-985 had some common antigenic determinants which can be illustrated one line of identity by double immunodiffusion method.

References

1. Brouwer J. Detection of antibodies against *Aspergillus fumigatus* : comparison between double immunodiffusion, ELISA and immunoblot analysis. Int Arch Allergy appl Immun 1988 ; 85:244-49.
2. Beaumont F. Clinical manifestations of pulmonary *Aspergillus* infections. Mycoses 1988;31:15-20.
3. Rippon JW. Medical mycology. 3rd ed. Philadelphia : W.B. Saunders company, 1988;618-50.
4. Campbell MJ, Clayton YM. Bronchopulmonary aspergillosis. A correlation of the clinical and laboratory findings in 272 patients investigated for bronchopulmonary aspergillosis. Am Rev Respir Dis 1964;89:186-96.
5. Schonheyder H. Pathogenic and serological aspects of pulmonary aspergillosis. Scand J Inf Dis 1987;1-62.
6. Khan ZU, Richardson MD, Warnock DW. Evaluation of a rapid enzyme-linked immunosorbent assay for IgG antibodies to *Aspergillus fumigatus* in the serological diagnosis of allergic aspergillosis. Arch Allergy app Immun 1984;73:205-11.
7. Hearn VM, Wilson EV, Proctor AG, et al. Preparation of *Aspergillus fumigatus* antigens and their analysis by two-dimensional immunoelectrophoresis. J Med Microbiol 1980;13:451-58.
8. Taylor ML, Longbottom JL. Partial characterization of a rapidly released antigenic/ allergenic component (Ag5) of *Aspergillus fumigatus*. J Allergy Clin Immun 1988;81:548-57.
9. Harvey C, Longbottom JL. Characterization of a major antigenic component of *Aspergillus fumigatus*. Clin exp Immunol 1986;65:206-14.
10. Coleman RM, Kaufman L. Use of the immunodiffusion test in the serodiagnosis of aspergillosis. Appl Microbiol 1972;23:301-8.
11. Sukrungreung S. Pathogenic fungi and fungal diseases. 1 st ed. Sarnmualchon, 1986:1-283.
12. Smith JMB, Derks A. Serological tests in the diagnosis of pulmonary aspergillosis. Pathology 1984;16:184-88.
13. Hearn VM, Mackenzie DWR. The preparation and chemical composition of fractions from Aspergillus fumigatus wall and protoplasts possessing antigenic activity. J Gener Microbiol 1979;112:35-44.
14. Harvey C, Longbottom JL. Release of antigens and allergins during shake-culture of Aspergillus fumigatus. Allergy 1987;42: 359-65.
15. Kaufman HF, Heide S, Laan S, et al. Standardization of allergenic extracts of *Aspergillus fumigatus*. Liberation of IgE-binding components during cultivation. Int Archs Aller appl Immun 1985;76:168-73.
16. Kaufman HF, Vries K. Antibodies against *Aspergillus fumigatus*. I. Standardization of the antigenic composition. Int Aller appl Immun 1980;62:252-64.