行政院國家科學委員會專題研究計畫 成果報告

黴菌毒素單株和單鏈抗體之生產及奈米免疫檢測試劑之開發及應用(第 3 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 96-2628-B-040-001-MY3
執 行 期 間 ：98 年 08 月 01 日至 99 年 07 月 31 日
執 行 單 位 ：中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人：余豐益

計畫參與人員：碩士級-專任助理人員：王敬之

報 告 附 件 ：出席國際會議研究心得報告及發表論文

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中 華 民 國 99 年 10 月 31 日

行政院國家科學委員會補助專題研究計畫成果報告

黴菌毒素單株與單鏈抗體之生產及奈米免疫檢測試劑之開發及應用

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC　96－2628 －B －040－001－MY3
執行期間：　96 年 8 月 1　日至 99 年 7 月 31　日

執行機構及系所：中山醫學大學生物醫學科學系

計畫主持人：余豐益
共同主持人：
計畫參與人員：王敬之, 曹子杰

成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

本計畫除繳交成果報告外，另須繳交以下出國心得報告：
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■出席國際學術會議心得報告
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中　華　民　國　99　年 10　月 31 日

行政院國家科學委員會專題研究計畫期中報告

黴菌毒素單株與單鏈抗體之生產及奈米免疫檢測試劑之開發及應用

Production of monoclonal and ScFv antibodies and development of nanogold rapid immunostrip assay for ochratoxin A and patulin

計畫編號：NSC 96-2628-B-040-001-MY3

執行期限：96 年8 月1 日至99 年7 月31 日

主持人：余豐益 中山醫學大學生物醫學科學系

中文摘要:

赭麴毒素A 為一個廣泛污染飼料和食物的黴菌毒素，為了發展一快速且敏感的方法來檢測赭麴毒素A，本實驗室用免疫OTA-KLH 的BALB/c 品種老鼠的脾臟細胞與NS-1 骨髓瘤細胞融合，篩選得到一個穩定的融合瘤細胞株H9，用來生產抗赭麴毒素A 的單株抗體。我們用赭麴毒素專一性的單株抗體建立了敏感性高的直接競爭型酵素免疫分析法和以奈米金粒子為標記物的快速免疫層析試紙分析法。直接競爭型酵素免疫分析法中，抑制50%的OTA-HRP 與抗體結合所需OTA 的濃度(IC50)為0.32 ng/mL。利用這個單株抗體與奈米金粒子相結合，形成抗體奈米金探針開發出赭麴毒素A 的快速免疫層析試紙，利用此一試紙來檢測咖啡中赭麴毒素A 的含量，此試紙最低限制為5 ng/mL，可在10 分鐘完成檢測結果，不需任何儀器可進行當場的赭麴毒素A 檢測。此外本計劃下我們也開發了黴菌毒素伏馬鐮孢毒素之奈米金快速免疫檢測試紙，利用此一試紙來檢測食品與飼料中伏馬鐮孢毒素的含量，此試紙最低限制為1 ng/mL，目前此一文稿也已經發表於Journal of Science of Food and Agriculture 2010 年。

英文摘要:

A monoclonal antibody (mAb) specific to ochratoxin A (OTA) was produced from a stable hybridoma cell line, 9C9H9, generated by the fusion of P3/NS1/1-AG4-1 myeloma cells with spleen cells isolated from a Balb/c mouse immunized with OTA-keyhole limpet hemocyanin (KLH). The 9C9H9 mAb belongs to the immunoglobulin G1 (kappa chain) isotype. A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA (ciELISA) were established for antibody characterization. The concentration causing 50% inhibition (IC50) of binding of OTA-horseradish peroxidase to the antibody by OTA, OTB, and OTC was found to be 0.32, 0.17, and 0.28 ng/mL, respectively. A sensitive and rapid mAb–based gold nanoparticle immunochromatographic strip was also developed using this mAb. This strip has a detection limit of 5 ng/mL for OTA and can be completed in 10 min. Analysis of OTA in coffee samples revealed that data obtained from immunochromatographic strip were in a good

agreement with those obtained from cdELISA. The mAb–based cdELISA and immunochromatographic strip assay established in this study were sensitive and accurate for rapid screening of OTA in coffee samples. Under this project, we have also developed immunochromatographic strip assay for fumonisin B1. This strip has a detection limit of 1 ng/mL for fumonisin B1 and can be completed in 10 min.

一、 前言與目的

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum*. It is mainly found as a contaminant of cereals, cereal products and coffee beans. Toxicological studies indicate that OTA is generally absorbed from the gastrointestinal tract in animals and shows strong toxic effects their livers and kidneys. Several studies have revealed that OTA was the major causative factor in mycotoxic porcine nephropathy and Balkan Endemic Nephropathy in many European countries. In addition, the toxin is also considered to be teratogenic, mutagenic and immunosuppressive in certain animal models. Since consumption of food contaminated with OTA is associated with an increased incidence of upper urinary tract tumors in human; the International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). To protect human and animal exposure to OTA, the European Union enacted a regulatory limit for the levels of OTA in cereals (5 μg/kg), roasted coffee (5 μg/kg) and instant coffee (10 μg/kg). In order to determination the OTA levels in foods and feeds, several research efforts have been conducted to develop sensitive and specific methods for OTA detection. Currently high-performance liquid chromatography (HPLC)- fluorescence detection with good accuracy and reproducibility is the most widely employed method for monitoring OTA. However, HPLC requires the involvement of highly qualified personnel and extensive sample cleanup as well as expensive equipment. Development of immunochemical analysis has led to many rapid and sensitive methods for monitoring and quantifying OTA in contaminated food. Several groups have established immunoassays for OTA, but most of the assays are not suitable for on-site detection because of the long incubation time, tedious washing steps and the application of instrumentation. Recently, the immunostrip test that based on the use of colored gold nanoparticle-antibody conjugates as detector reagent is an emergent immunoassy. The principle of immunostrip relies on the migration of test samples and antibody-gold nanoparticle conjugates along membrane strips on which the binding interactions take place. The results of immunostrip could be examined visually, thus providing fast and simple on site detection in less than 10 min without the need of skilled personnel and any instruments. Since coffee samples collected from different regions have been reported to be contaminated with various amounts of OTA, an effective on-site detection of OTA in coffee samples is needed. In the present study, a mAb against OTA was generated, and then a sensitive cdELISA and a mAb-based gold nanoparticle immunostrip were established for such purposes.

三、Result

Production of Monoclonal Antibodies Mice were injected and boostered with antigen OTA-KLH, and then the mouse with serum of highest affinity to OTA was tested by ciELISA and selected for hybridoma screening. After spleen/NS-1 cell fusion and cloning, the ciELISA with OTA-OVA as a coated reagent was used for screening the hybridoma cells which was able to produce monoclonal antibodies specific to OTA. Of the 630 wells examined, only two clones gave strong positive signals in the ciELISA; among them, the clone, 9C9, showed the highest affinity for OTA. Therefore, the supernatant of 9C9 culture was aspirated from the fusion well and subjected to limiting dilution for monoclonal selection. After limiting dilution and ELISA screening, clone 9C9H9 showing the highest affinity against OTA was selected for production of culture supernatant and ascites fluid.

Characterization of Antibodies.

Determination of isotypes. The isotype of mAb produced by cell line 9C9H9 was found to be immunoglobulin G1, -light chain.

Competitive direct and indirect ELISA. Both the cdELISA and ciELISA were used to determine the specificity of 9C9H9 mAb. As shown in in Figure 2A, the concentrations causing 50% inhibition (IC_{50}) of binding of OTA-HRP with the mAb by OTA, OTB, and OTC were found to be 0.32, 0.17, and 0.28 ng/mL, respectively in cdELISA (Figure 1). A similar result was also found in the ciELISA, in which OTA-OVA were coated onto the wells of ELISA plates to serve as solid-phase antigen. The IC_{50} of binding of mAb to OTA-OVA by free OTA, OTB, and OTC were calculated to be 0.28, 0.35, and 0.25 ng/mL, respectively (Figure 2). However, phenylalanine and citrinin,(*14*) two molecules with chemical structures similar to a part of OTA molecule, did not inhibit the binding of mAb to the marker antigen (OTA-HRP or OTA-OVA) in either ELISA system even at a concentration as high as 100 μg/mL.

Analysis of OTA in Coffee Samples with mAb-based cdELISA.

Fifteen coffee samples were collected from local food stores and subjected to cdELISA for determining the contaminated levels of OTA; the results are presented in Table 1. Eleven of the fifteen examined samples were found to be OTA positive in our detection system. Among them, the extract of sample 3 showed the highest OTA level at 10.97 ng/mL (54.85 ng/g) and samples 14 and 15 also had the OTA levels higher than 1.0 ng/mL. The remaining eight positive samples were lower than 1.0 ng/mL.

Construction of Immunochromatographic Strip

MAb was applied to construct an effective immunochromatographic strip, in which the OTA-OVA conjugate competes with OTA in the sample solution for the antibody-gold nanoparticle label. A schematic description of the immunochromatographic strip test format is

shown in Figure 3. In the absence of OTA in the sample solution, the antibody-gold nanoparticle conjugate was bound and trapped by the OTA-OVA conjugate to form a visible spot on the test zone. In contrast, if sufficient concentration of OTA is present in the sample solution, the toxin would occupy the antigen binding sites on the antibody-gold nanoparticle conjugates; consequently, the limited antibody-gold nanoparticle conjugates failed to bind with the OTA-OVA conjugate on the test zone. The absence of color spot on the test zone indicated a positive result (Figure 3). Control zone coated with rabbit-anti-mouse secondary antibody was constructed to verify whether the assay has been performed properly; this control zone should always show a red color spot under an accurate operation regardless the presence of OTA or not. An OTA-free sample shows two red spots, whereas a positive sample with OTA presents only one red spot on the membrane.

Characterizaion of OTA Immunochromatographic Strip.

Various concentrations of OTA standard solution (0-100 ng/mL) were subjected to immunochromatographic strip test. The whole assay could be completed in less 10 min, and the detection limit of immunochromatographic strip test for OTA was about 5 ng/mL (Figure 4). In order to characterize and define the cutoff level for each selected concentration more than n=10 measurements were tested in our immunochromatographic strip detection system.

OTA at a concentration above 5 ng/mL occupied all the antibody-gold nanoparticle conjugates and prevented the antibody-gold nanoparticle conjugates from binding with the OTA-OVA on the test zone, but the antibody-gold nanoparticle conjugates were captured by the rabbit anti-mouse antibody on the membrane resulted in only one spot on the control zone.

Analysis of OTA in Coffee Samples with Immunochromatographic Strip Assay

We furthermore applied immunochromatographic strip to analyze OTA contamination in coffee samples. Results were shown in Table 1. Sample 3 containing 10.97 ng/mL of ochratoxins in ELISA gave a positive result with only one spot on the strip membrane (Figure 5). All the remaining coffee samples with toxin levels lower than 5.0 ng/mL demonstrated two red spots on the membrane, indicating that they were negative in our immunochromatographic strip assay.

四、計畫期末成果自評

本研究的主要目的以生產黴菌毒素之單株抗體並且開發酵素免疫分析法及快速免疫檢測試紙來檢測分析食品或咖啡中赭麴毒素的含量，目前有關赭麴毒素的融合瘤細胞已經獲得並且開始生產單株抗體並建立了敏感性高的直接競爭型酵素免疫分析法。有關棒麴毒素融合瘤細胞之篩選進行的並不十分順利，主要是免疫老鼠不容易有棒麴黴素的抗體產生出來，因此阻礙了融合瘤篩選計畫。目前赭麴毒素A 的單株抗體正進行美國專利的申請，此一赭麴毒素A的酵素免疫分析法與奈米金粒子快速免疫檢測試紙已經開發完成。目前此一部份文稿已經發表在美國化學學會分析化學期刊 (Anal. Chem. 2008,80,7029-7035)。此外本計

劃下我們也開發了黴菌毒素伏馬鐮孢毒素之奈米金快速免疫檢測試紙，目前此一文稿也已經發表於Journal of Science of Food and Agriculture 2010 年。

五、重要參考文獻

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Table 1.ELISA and Immunochroatographic Strip Analysis of OTA in Coffee Samples

Samples	ELISA [a]		Immunochromatographic strip[a]
	(ng/mL ± SD)	(ng/g± SD) [b]	
Green coffee beans			
1.	ND	ND	—
2.	ND	ND	—
3.	10.97 ± 0.27	54.85 ± 1.35	+
4	ND	ND	—
5	ND	ND	—
Roasted coffee beans			
6	0.46 ± 0.03	2.30 ± 0.15	—
7.	0.41 ± 0.04	2.05 ± 0.20	—
8.	0.87 ± 0.09	4.35 ± 0.45	—
9.	0.6 ± 0.04	3.0 ± 0.2	—
10.	0.72 ± 0.03	3.6 ± 0.15	—
Instant coffee			
11.	0.6 ± 0.04	3.0 ± 0.20	—
12 .	0.77 ± 0.15	3.83 ± 0.75	—
13.	0.86 ± 0.12	4.3 ± 0.6	—
14.	1.19 ± 0.08	5.93 ± 0.40	—
15.	1.36 ± 0.2	6.8 ± 1.0	–

[a] Each sample was extracted twice and each extract was analyzed in duplicate.

[b] One mL extract solution contains 0.2 g of coffee samples.

[c] ND, not detected

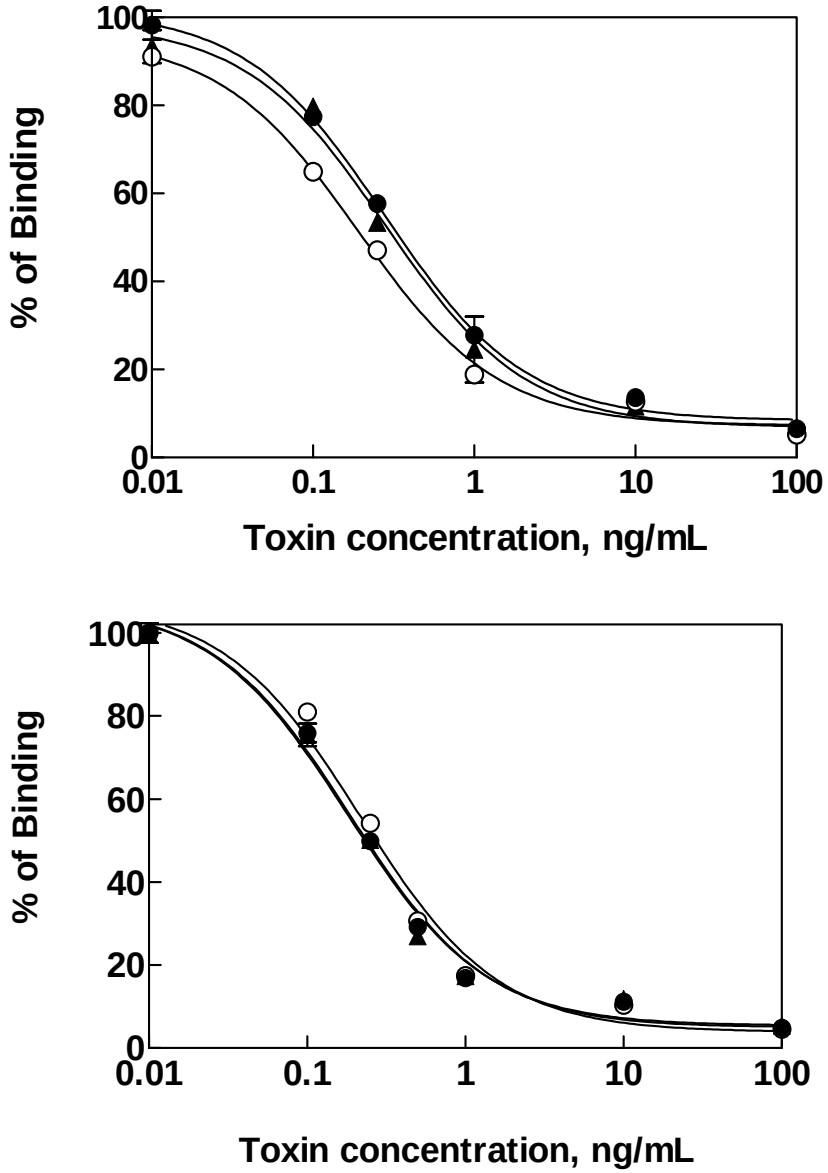

Figure 2. A. Cross-reactivity of anti-OTA antibodies with OTA (●), OTB (○) and OTC(▲) in a cdELISA. **B.** Cross-reactivity of anti-OTA antibodies with OTA (●), OTB (○) and OTC (▲) as determined by a ciELISA. All data were obtained from the average of three sets of experiments.

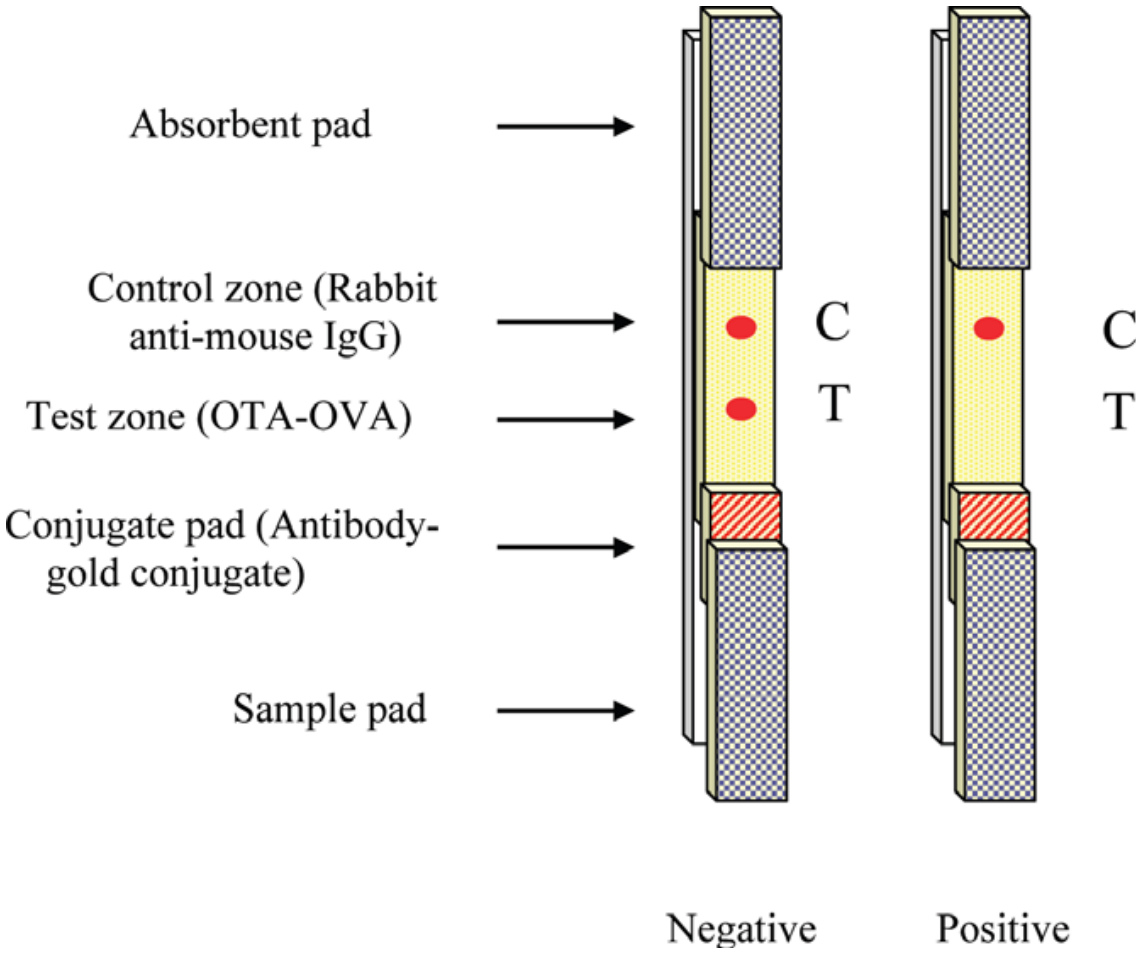

Figure 3. The schematic illustration of immunochromatographic strip. C, control zone (rabbit anti-mouse IgG); T, test zone (OTA-OVA).

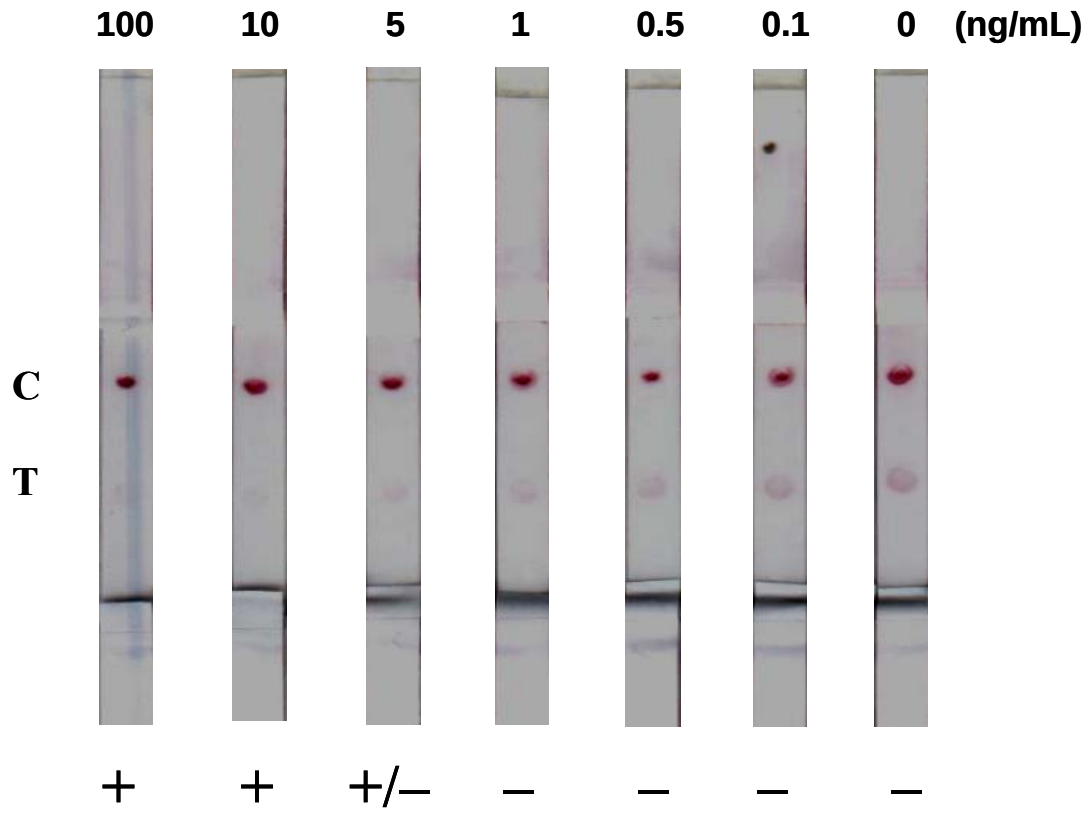

Figure 4. Detection limit of OTA toxin with immunochromatographic strip. A series of dilution (0-100 ng/mL) of standard OTA was dissolved in PBS. OTA toxin concentration higher than 5 ng/mL was found to cause a disappearance of red spot on the test zone.

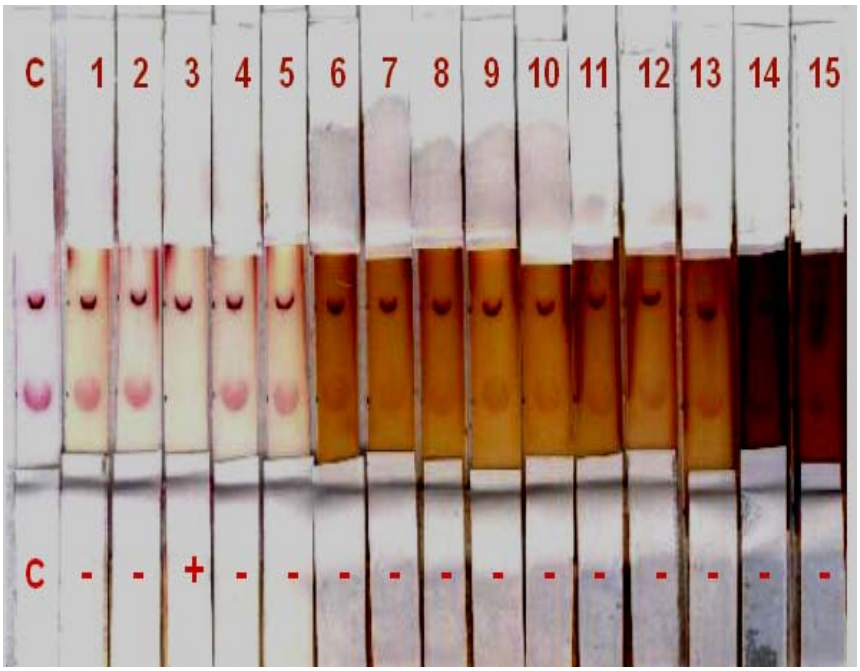

Figure 5. Detection of OTA with immunochromatographic strip in control and 15 coffee samples. Control strip containing no OTA shows two red spots on the membrane. Sample 3 containing ochratoxins more than 10 ng/mL was found to cause a disappearance of red spot at the test zone.

國科會補助專題研究計畫項下出席國際學術會議心得報告

日期：99 年 10 月 31 日

計畫編號	NSC　　96－2628－B－040－001－MY3		
計畫名稱	黴菌毒素單株與單鏈抗體之生產及奈米免疫檢測試劑之開發及應用		
出國人員 姓名	余豐益	服務機構 及職稱	中山醫學大學生物醫學系
會議時間	99 年 3 月 22 日 至 99 年 3 月 24 日	會議地點	中國北京
會議名稱	(中文)第二屆世界抗體大會 (英文)2nd International Congress of Antibody		
發表論文 題目	(中文)抗體與奈米科技應用於環境毒素之偵測 (英文)Antibody and nanotechnology used in environmental toxin detection		

一、參加會議經過

此次第二屆世界抗體大會應主辦單位 BIT Life Science 邀請前往中國北京參加會議並進行口頭演講, 演講題目為 Antibody and nanotechnology used in environmental toxin detection,此次會議於北京會議中心舉行，時間為 3 月 22 日至 3 月 24 日.此會議中心正位於北京奧運競賽場左前方, 我於 3 月 21 日下午到達此中心並完成報到手續,展開三天的會議行程.

二、與會心得

此次會議共有 800 多位來自世界各地的抗體與疫苗地專家學者與會，能夠與來自世界之抗體專家齊聚一堂進行討論,實在是一個難得之機會,感謝國科會經費補助, 使得筆者能參與此一盛會. 此次會議由於與會者相當多人,口頭演講採同一時段有 10 個場次同時進行,因此每一場次參與之人數並不多,而且往往錯過許多精采之演講. 此乃此會議一美中不足之處.

三、考察參觀活動(無是項活動者略)

四、建議

由參加此次會議之經驗,筆者認為中國可以舉辦此種大型會議,為何台灣似乎還沒有能力舉辦如此大型會議並且邀請到世界各地如此多的專家學者與會,此是一個需要深思的問題,如何集思廣益來籌辦大型會議跟世界知名學者交流以增加台灣學者之曝光率.

五、攜回資料名稱及內容

抗體大會議程,各個演講者 CV 介紹.

六、其他

國科會補助計畫衍生研發成果推廣資料表

日期 2010年11月01日

國科會補助計畫	計畫名稱: 黴菌毒素單株和單鏈抗體之生產及奈米免疫檢測試劑之開發及應用 計畫主持人: 余豐益 計畫編號: 96 -2628-B -040 -001 - 學門領域: 食品及農化		
研發成果名稱	(中文) 赭麴毒素A 單株抗體與快速檢測技術開發		
	(英文) Production of monoclonal antibody and development of ELISA and immunochromatographic strip for ochratoxin A		
成果歸屬機構	中山醫學大學	發明人 (創作人)	余豐益
技術說明	(中文)赭麴毒素A 為一個廣泛污染飼料和食物的黴菌毒素，為了發展一快速且敏感的方法 來檢測赭麴毒素A，本實驗室用免疫OTA-KLH 的BALB/c 品種老鼠的脾臟細胞與NS-1 骨 髓瘤細胞融合，篩選得到一個穩定的融合瘤細胞株H9，用來生產抗赭麴毒素A 的單株抗 體。我們用赭麴毒素專一性的單株抗體建立了敏感性高的直接競爭型酵素免疫分析法和以 奈米金粒子為標記物的快速免疫層析試紙分析法。直接競爭型酵素免疫分析法中，抑制50% 的OTA-HRP 與抗體結合所需OTA 的濃度(IC50)為0.32 ng/mL。利用這個單株抗體與奈米 金粒子相結合，形成抗體奈米金探針開發出赭麴毒素A 的快速免疫層析試紙，利用此一試 紙來檢測咖啡中赭麴毒素A 的含量，此試紙最低限制為5 ng/mL，可在10 分鐘完成檢測結 (英文)A monoclonal antibody (mAb) specific to ochratoxin A (OTA) was produced from a stable hybridoma cell line, 9C9H9, generated by the fusion of P3/NS1/1-AG4-1 myeloma cells with spleen cells isolated from a Balb/c mouse immunized with OTA-keyhole limpet hemocyanin (KLH). A sensitive ELISA and rapid mAb-based gold nanoparticle immunochromatographic strip was also developed using this mAb. This strip has a detection limit of 5 ng/mL for OTA and can be completed in 10 min.		
產業別	農業		
技術/產品應用範圍	赭麴毒素A 的酵素免疫分析法與快速免疫層析試紙/應用於食品飼料檢測		
技術移轉可行性及預期效益	酵素免疫分析法快速、免疫層析試紙簡便不需要任何儀器可做赭麴毒素A 現場檢測		

註：本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。

96 年度專題研究計畫研究成果彙整表

計畫主持人：余豐益	計畫編號：96-2628-B-040-001-MY3
計畫名稱：黴菌毒素單株和單鏈抗體之生產及奈米免疫檢測試劑之開發及應用	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	0	100%		
國外	論文著作	期刊論文	2	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	1	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 ■申請中 □無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

本計畫生產出針對赭麴毒素 A 具有高度專一性之單株抗體,利用此一抗體我們開發出赭麴毒素 A 的酵素免疫分析法，並且利用單株抗體與奈米金粒子接合開發出赭麴毒素快速免疫層析試紙來快速檢測分析食品或穀物中赭麴毒素的含量。學術成果發表論文於 Analytical Chemistry 期刊。技術創新開發赭麴毒素 A 奈米檢測試紙。目前此一針對赭麴毒素 A 的單株抗體正進行美國專利的申請。