科技部補助專題研究計畫成果報告
期末報告

黴菌毒素之單株及單鏈抗體生產及快速檢測多重毒素之免疫奈米試紙開發(第3年)

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執 行 期 間 ： 105年08月01日至106年10月31日
執 行 單 位 ： 中山醫學大學生物醫學科學學系（所）

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中 華 民 國 107 年 01 月 31 日

中 文 摘 要 ： 橘黴素(citrinin, CTN)與脫氧雪腐鐮刀菌烯醇(deoxynivalenol, DON)主要分別由黴菌
Penicillium與Fusarium 屬等常產生的黴菌毒素，此類毒素泛存於食品與穀物中，食用遭受污
染的食品或穀物導致人類或動物許多疾病及癌症的生成。我們將橘黴素接合血藍蛋白質
(CTN-KLH)抗原分別打入老鼠體內或兔子體內來生產橘黴素的抗體，目前以CTN-KLH免疫的
兔子抽取血清所純化抗體，我們已經建立了敏感性相當不錯的直接競爭型酵素免疫分析法，
在直接競爭型酵素免疫分析法中，其抑制50%的橘黴素-酵素接合物與抗體結合所需橘黴素的
濃度(IC50)為5.0 ng/mL。此外我們將橘黴素之抗體與奈米金粒子與相接合形成探針來
開發出橘黴素免疫層析試紙(immunochromatographic strip)。以紅色奈米金粒子與抗體結合形成抗
體奈米金探針，藉由免疫層析試紙的裝置使得檢測耗時1~2小時之ELISA免疫分析縮短至10~20分
鐘內完成，目前開發出來之免疫層析試紙其cut-off值約為20 ng/mL，並且用來快速檢測分析19種
紅麴樣品中橘黴素之含量，其中紅麴米樣品1-6號遭受1.6~9.5 ppm橘黴素之汙染。此外在老鼠血清
中其抑制50%的橘黴素-酵素接合物與抗體結合所需 橘黴素的濃度(IC50)為10~20 ng/mL，目前我
們仍持續進行融合瘤的篩選來生產橘黴素之單株抗體。有關脫氧雪腐鐮刀菌烯醇抗體的生產，我
們將脫氧雪腐鐮刀菌烯醇接合牛血清蛋白質(DON-BSA, DON-KLH)抗原分別打入老鼠體內或兔
子體內來生產脫氧雪腐鐮刀菌烯醇的抗體，雖然動物體內抗體效價有上升中，但是兔子體內所產
生的抗體對脫氧雪腐鐮刀菌烯醇的專一性均不足，老鼠體內之專一性抗體已逐漸產生。目前也正
改進各種的接合方法來得到脫氧雪腐鐮刀菌烯醇與蛋白質的接合物來提升老鼠體內抗體之效價。

中 文 關 鍵 詞 ： 橘黴素

英 文 摘 要 ： Citrinin (CTN) and deoxynivalenol (DON) are mycotoxins that are produced by Penicillium and
Fusarium and. They are commonly found in wheat, cereals, and food products, which cause toxic
effects and cancer in human and animal. Antibodies specific to CTN were generated from rabbit and
mouse immunized with CTN-KLH respectively. By using these antibodies from the rabbit with CTN-KLH, a sensitive competitive direct and indirect enzyme-linked immunosorbent assay
(cdELISA) for detecting CTN were established for food

samples. In the cdELISA, CTN at a concentration of 5.0 ng/ml causes 50% inhibition (IC50) of binding CTN-horseradish peroxidase to the antibodies. In addition, we conjugate the antibody to gold nanoparticles to form the probe the immunochromatographic strip was developed. This immunochromatographic test strips could reduce the detection time from 1-2 hours of ELISA to 10-20 minutes. The cut-off value for citrinin of the immunochromatographic test strip is about 20 ng/mL. These strips were applied to the rapid detection of 19 red yeast samples. CTN content in the sample of red yeast rice CTN content in the sample of red yeast rice No. 1-6 were contaminated 1.6 ~ 9.5 ppm of citrinin. Moreover, the antibody in the mouse serum in which the concentration of citrinin required to inhibit the binding of 50% (IC50) of the citrinin-HRP to the antibody is 10 to 20 ng / mL,
About the screening the hybridoma cell for secreting monoclonal antibody for CTN is proceedin For production of antibody for DON, the rabbit and mouse were injected the DON-BSA or DON-r-globulin, respectively. Both antibody titers in the animals arose gradually, only the antiserum from mouse did produce specific antibody for DON. Currently, we will use different conjugation methods to increase the antibody titer specific for DON.

英文關鍵詞：citrinin

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

徽菌毒素之單株及單鏈抗體生產及快速檢測多重毒素之免疫層析試紙開發

計畫類別：■個別型計畫　□整合型計畫
計畫編號：MOST　103－2313－B－040－002－MY3
執行期間：　2014 年 8 月 1　日至 2017 年 10 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：吳仕偉, 鐘唯恆

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□移地研究心得報告
■出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

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中　華　民　國 107　年 01 月 30 日

行政院國家科學委員會專題研究計畫期中報告

黴菌毒素之單株及單鏈抗體生產及快速檢測多重毒素之免疫層析試紙開發

計畫編號：MOST 103－2313－B－040－002－MY3

執行期限：103 年 8 月 1 日至 106 年 10 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:

橘黴素(citrinin, CTN)與脫氧雪腐鐮刀菌烯醇(deoxynivalenol, DON)主要分別由黴菌 *Penicillium*與*Fusarium* 屬等常產生的黴菌毒素，此類毒素泛存於食品與穀物中，食用遭受污染的食品或穀物導致人類或動物許多疾病及癌症的生成。我們將橘黴素接合血藍蛋白質(CTN-KLH)抗原分別打入老鼠體內或兔子體內來生產橘黴素的抗體，目前以CTN-KLH免疫的兔子抽取血清所純化抗體，我們已經建立了敏感性相當不錯的直接競爭型酵素免疫分析法，在直接競爭型酵素免疫分析法中，其抑制50%的橘黴素-酵素接合物與抗體結合所需橘黴素的濃度(IC50)為5.0 ng/mL。此外我們將橘黴素之抗體與奈米金粒子與相接合形成探針來開發出橘黴素免疫層析試紙(immunochromatographic strip)。以紅色奈米金粒子與抗體結合形成抗體奈米金探針，藉由免疫層析試紙的裝置使得檢測耗時1~2小時之ELISA免疫分析縮短至10~20分鐘內完成，目前開發出來之免疫層析試紙其cut-off值約為20 ng/mL，並且用來快速檢測分析19種紅麴樣品中橘黴素之含量，其中紅麴米樣品1-6號遭受1.6~9.5 ppm橘黴素之汙染。此外在老鼠血清中其抑制50%的橘黴素-酵素接合物與抗體結合所需 橘黴素的濃度(IC50)為10~20 ng/mL，目前我們仍持續進行融合瘤的篩選來生產橘黴素之單株抗體。有關脫氧雪腐鐮刀菌烯醇抗體的生產，我們將脫氧雪腐鐮刀菌烯醇接合牛血清蛋白質(DON-BSA, DON-KLH)抗原分別打入老鼠體內或兔子體內來生產脫氧雪腐鐮刀菌烯醇的抗體，雖然動物體內抗體效價有上升中，但是兔子體內所產生的抗體對脫氧雪腐鐮刀菌烯醇的專一性均不足，老鼠體內之專一性抗體已逐漸產生。目前也正改進各種的接合方法來得到脫氧雪腐鐮刀菌烯醇與蛋白質的接合物來提升老鼠體內抗體之效價。

英文摘要:

Citrinin (CTN) and deoxynivalenol (DON) are mycotoxins that are produced by *Penicillium* and *Fusarium* and. They are commonly found in wheat, cereals, and food products, which cause toxic effects and cancer in human and animal. Antibodies specific to CTN were generated from rabbit and mouse immunized with CTN-KLH respectively. By using these antibodies from the rabbit immunized with CTN-KLH, a sensitive competitive direct and indirect enzyme-linked immunosorbent assay (cdELISA) for detecting CTN were established for food samples. In the cdELISA, CTN at a concentration of 5.0 ng/ml causes 50% inhibition (IC_{50}) of binding CTN-horseradish peroxidase to the

antibodies. In addition, we conjugate the antibody to gold nanoparticles to form the probes and the immunochromatographic strip was developed. This immunochromatographic test strips could reduce the detection time from 1-2 hours of ELISA to 10-20 minutes. The cut-off value for citrinin of the immunochromatographic test strip is about 20 ng/mL. These strips were applied to the rapid detection of 19 red yeast samples. CTN content in the sample of red yeast rice No. 1-6 were contaminated 1.6 ~ 9.5 ppm of citrinin. Moreover, the antibody in the mouse serum in which the concentration of citrinin required to inhibit the binding of 50% (IC50) of the citrinin-HRP to the antibody is 10 to 20 ng / mL, About the screening the hybridoma cell for secreting monoclonal antibody for CTN is proceeding right now. For production of antibody for DON, the rabbit and mouse were injected the DON-BSA or DON-r-globulin, respectively. Both antibody titers in the animals arose gradually, only the antiserum from mouse did produce specific antibody for DON. Currently, we will use different conjugation methods to increase the antibody titer specific for DON.

【I】前言與目的:

橘黴素(Citrinin，縮寫 CTN，分子量 250)是由 *Aspergillus*，*Penicillium* 和 *Monascus* 麴菌株所產生的二級代謝物，常在玉米、小麥、稻米、裸麥和堅果類等穀物及糧食中被發現 (Trantham and Wilson, 1984)。由於紅麴食品含有抗血壓及降膽固醇等有效成分而成為近年來熱門的健康食品，但是 Citrinin 卻是紅麴製品製程中難以完全避免的的二級代謝物。Citrinin 已知會造成多種動物在肝臟與腎臟方面的傷害，可能具有基因毒性，影響細胞正常分裂以及潛在的致癌能力，甚至發現與其他黴菌毒素共同作用而導致人類的 endemica Balkan nephropathy (Vrabcheva *et al.*, 2000)，因此臺灣食品藥物管理署(TFDA) 針對橘黴素 CTN 分別設有紅麴色素 0.2 ppm; 紅麴米 5 ppm; 紅麴原料製成之食品 2 ppm 的含量限制。TFDA(廖等, 2010)調查 2009 年台灣市售產品中，45%的紅麴膠囊和 100%的紅麴米樣品檢出橘黴素，而在港口抽樣進口紅麴米，則發現 30.8 %的檢體超過台灣管制限量。最近 TFDA(Liao et al., 2014)分析在 2009-2012 年所採集的 84 個市售紅麴米樣品中有 58 個樣品遭受到 0.4 – 93.5 ppm 橘黴素的污染，77 個健康食品補充物則有約 35%檢出橘黴素，其污染情形可謂相當嚴重; 本實驗室亦曾利用 HPLC 分析發現 6 個市售紅麴樣品都有遭受到 0.28~6.29 ppm 不等的橘黴素污染(Liu et al., 2005)。此外，2004 年寶路飼料引起數千隻寵物犬腎衰竭事件裡，生產寶路飼料的艾芬公司亦公開承認飼料遭受到橘黴素的污染，由此可知橘黴素的污染情形在食品安全中是個重要議題，需要有效而且持續的監控檢測。

脫氧雪腐鐮刀菌烯醇 (Deoxynivalenol, DON) ，其化學式為 ($C_{15}H_{20}O_6$)，它是一個極性的有機結構，其分子量為 296.3 也是個小分子毒素，屬於鐮刀單端孢黴菌毒素(Trichothecene mycotoxin) B 族，為全球穀物污染較嚴重而且相當廣泛的毒素，主要是由 *F. graminearum* 和 *F. culmorum* 等鐮刀菌屬的絲狀真菌所產生的代謝產物 (Cahill et al., 1999)。除了產生次級代謝物導致穀物被黴菌毒素污染之外，也會造成穀物疾病，最常見的穀物疾病為赤黴病 (Fusarium Head Blight, FHB)，常在美國中西部地區上造成重大經濟損失。DON 雖然並非致癌物，但具有神經毒性並且會抑制人體的免疫系統，當誤食 DON 汙染的食品會引起腹痛、腹瀉、腸胃炎、出血性腹瀉等急性毒性，以及厭食、體重減輕、消化能力下降等慢性中毒症狀 (Li et al., 2007)。此外 DON 具有強烈的皮膚毒性和細胞毒性，也會干擾蛋白質和 DNA 合成 (Ueno and Fukushima, 1968)。因此許多國家對於對於嘔吐毒素(deoxynivalenol, DON)設有 300-2000 ppb 左

右之含量限制，目前臺灣對於 DON 則尚未有含量限制。臺灣食品藥物管理署最近針對穀物中 DON 檢驗，檢出 DON 之檢出率約為 5%，其含量介於 148-2133 ppb (陳等, 2012)。因上述兩種毒素對人體健康都有非常嚴重的危害，因此需要生產專一性高之抗體及開發快速檢測分析法來分析此類毒素。

二、 材料與方法

2.1 Materials.

Materials Citrinin (CTN), and Deoxynivalenol (DON)**,** Bovine serum albumin (BSA), γ-globulin, gelatin, ovalbumin (OVA), ammonium biocarbonate, Tween 20, dimethyl sulfoxide (DMSO), 1,1′-carbonyldiimidazole (CDI), 1-ethyl-3- [3-dimethylaminopropyl]- carbodimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse-peroxidase conjugate and keyhole limpet hemocyanin (KLH) were obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase (HRP) was obtained from Roche (Mannheim, Germany). HRP substrate solution 3, 3', 5, 5'-tetramethylbenzidine (TMB) was obtained from Neogen Corp (Lexington, KY). Ammonium sulfate, absolute ethanol and HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Microtiter plates and strips (low and high protein binding) were obtained from Nunc (Roskilde, Demark). ELx 50 ELISA washer was purchased from Bio-Tek instruments (Winooski, VT). Vmax automatic ELISA reader was purchased from Molecular Devices Co. (Menlo Park, CA). Virus-free, 9–10-week-old, female BALB/c mice were obtained from National Animal Research Center (Taipei, Taiwan). All other chemicals and organic solvents used were of reagent grade or better.

Preparation of Various CTN Conjugates.

Conjugation of CTN to KLH/BSA. CTN was conjugated to KLH or BSA by the mannrich method under the following conditions. The formaldehyde solution (37%, 0.32 mL) were freshly prepared and then added to an CTN solution (1.0 mg of OA in 0.2 mL of methanol). The mixture was added slowly to 5.0 mg of KLH, which was dissolved in 0.8 mL of 0.1 M sodium acetate buffer and kept at room temperature for 72 h. After reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15M NaCl (PBS, pH 7.5) for 72 h with two exchanges of PBS and then lyophilized for storage at –20 ^{o}C.

Conjugation of CTN to OVA for Indirect competitive ELISA. CTN was conjugated to OVA by the mannich method and used as a solid-phase antigen for the indirect competitive ELISA[31]. In a typical reaction, 0.5 mg of CTN in 0.1 mL of methanol was mixed with 4 mg of OVA first, and then the formaldehyde solution (37%, 0.32 mL) were added to the mixture with constant stirring. After the coupling reaction was carried out at 25 ^{o}C for 72h, the mixture was dialyzed against PBS for 72 h and then lyophilized for storage.

Preparation of OA-Peroxidase. CTN (0.2 mg) in 0.1 mL of methanol was mixed with 0.04 ml formaldehyde solution (37%), and then a HRP solution (0.8 mg of HRP in 0.3 mL of 0.1 M sodium acetate) was added. After being stirred at room temperature for 72 h, the mixture was dialyzed against PBS for 72 h and then lyophilized.

Production of polyclonal antibody. The rabbit was injected intradermally at multiple sites on the shaved back (about 30 sites) with 250 μg of immunogen (CTN-KLH or CTN-BSA conjugates) in 1 ml of 0.01M PBS mixed with 1 ml of Freund Complete adjuvant. For booster injections, the same amount

of immunogen was mixed with an equal volume of incomplete Freund adjuvant and injected subcutaneously at 4 sites on the thigh of the rabbit. The antisera were precipitated with $(NH_4)_2SO_4$ to a final concentration of 50% and 35% in sequence by using a 100% saturated $(NH_4)_2SO_4$ solution. The precipitate was redissolved in distilled water equal to half of the original volume and then dialyzed against 2 L of PBS for 72 hr at 4 ^oC with two changes of buffer. Final added 0.01 M PBS to the original volume.

Characterization of Polyclonal Antibodies

Competitive Indirect ELISA (ciELISA)

Each well of a microtiter plate was coated with 0.1 mL of the CTN-OVA and kept at 4 ^oC overnight. After the plate had been washed four times with Tween-PBS (0.35 mL per well; 0.05% Tween 20 in PBS) using an automated ELISA washer, 0.17 mL of gelatin-PBS (0.17 mL per well; 0.1% gelatin in PBS) was added and allowed to incubate at 37 ^oC for 30 min. The plate was washed again and 0.05 mL of CTN standard with concentrations from 0.01 to 100 ng/mL in PBS or extracted samples were added to each well, and then the anti-CTN pAb was added to all wells and incubated at 37 ℃ for 50 min. After incubation, the plate was washed four times with Tween-PBS, and 0.1 mL of goat anti-rabbit IgG-HRP conjugate (1:20000 dilution) was added and incubated at 37℃ for 45 min. The plate was washed four times with Tween-PBS, and 0.1 mL of TMB substrate solution was added. After 10 min of incubation at room temperature, 0.1 mL of 1 N hydrochloric acid was added to stop the reaction. Absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

cdELISA. The anti-CTN pAb was diluted in PBS (1:1000 dilution; 1 μg/mL) and 0.1 mL of the diluted solution was used to coat each well. After the plate had been incubated at 4 ^oC overnight, it was washed with Tween-PBS followed by blocking with BSA-PBS at 37 ^oC for 30 min. The plate was washed again with Tween-PBS four times, and then CTN standard (0.05 mL per well in PBS) concentrations from 0.01 to 100 ng/mL or samples together with the CTN-HRP conjugate (10 ng/mL, in PBS, 0.05 mL per

0.02 well) was added and incubated at 37^{o}C for 50 min. The plate was washed four times with 350 μL PBS-Tween , and 0.1 mL of TMB substrate solution was added. After incubation at room temperature in the dark for 10 min, the reaction was terminated by adding 0.1 mL of 1 N HCl. The absorbance at 450 nm was determined in the Vmax automatic ELISA reader.

Production of Monoclonal Antibody (mAb).

Immunization

To generate an immune response against CTN, four female BALB/c mice (9–10 weeks old of age) were intraperitoneally (ip) injected with 50 μg of CTN-KLH or CTN-BSA which had been emulsified with 0.1 mL of

Freund's complete adjuvant. Two weeks following the first injection, sequential boosts were performed weekly with 50 μg of the same immunogen in PBS without adjuvant. After each booster, blood samples were collected from the tail vein of each mouse and serum was collected by centrifugation.

A competitive direct ELISA (cdELISA) described below was used to determine the specificity of the antibody in the serum.

Fusion and cloning.

The mouse that exhibited the highest antibody specificity, 15 weeks after the initial immunization, was chosen in the subsequent fusion reaction. The selected mouse was primed with a total of 50 μg CTN-KLH four days before fusion, and was euthanized on the day of fusion. The entire spleen was removed aseptically, mashed with a glass pestle, and then passed through a sieve-tissue grinder kit with a mesh 80 (CD-1, Sigma). A spleen cell suspension in the single-cell stage was mixed with 1 x 10^{7} of myeloma cells. The cells were fed every five days with freshly prepared HAT medium. When the cell colonies reached at least half-confluence in the well, cdELISA described below was applied to screen the hybridomas that secreted specific antibodies against CTN.

DON

Preparation of Various DON Conjugates.

Conjugation of DON to BSA/r-globulin. DON was conjugated to BSA or r-globulin by the 1,1 carbonyldiimidazole (CDI) method under the following conditions. 1.0 mg of DON in 0.3 mL of acetone was mixed with 2 mg of CDI, and then 4 mg of BSA or r-globulin in 0.5 ml of carbonate buffer was added, respectively. The mixture was stired at room temperature for 2 h. The mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15M NaCl (PBS, pH 7.5) for 72 h with two exchanges of PBS and then lyophilized for storage at –20 ^{o}C.

Conjugation of DON to OVA for Indirect competitive ELISA.

DON was conjugated to OVA by the CDI method and used as a solid-phase antigen for the indirect competitive ELISA. In a typical reaction, 0.5 mg of DON in 0.2 mL of acetone was mixed with 1 mg of CDI, and then 4 mg of OVA in 0.5 ml of carbonate buffer was added, The mixture was stired at room temperature for 2 h. The mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15M NaCl (PBS, pH 7.5) for 72 h with two exchanges of PBS and then lyophilized for storage at –20 ^{o}C.

Derivatization of carbonyl group on DON to CMO (Carboxymethoxylamine hemihydrochloride, CMO) to prepare DON-CMO

One mg of DON was dissolved in 2 ml reflux solution (methanol : pyridine : d_2H_2O, 4:1:1, v/v/v) and then added 1.5 mg CMO in ml reflux solution. The mixture was refluxed and heated to 60℃ in dark for 36h. After reaction, the reflux solution was rotary evaporated to dry and stored in -20℃ for use.

Conjugation of DON-CMO to horse radish peroxidase (HRP) *for direct competitive ELISA*

DON-CMO was conjugated to HRP by carbodiimide method under the following conditions. DON-CMO (0.2 mg) was dissolved in 50 μl DMF, then added 1.2 mg EDC in 12 μl DMF solution and 1 mg NHS in 10 μl DMF. The mixture was reactedt at room temperature for 2 hours in the dark. After the completion of the reaction, the solution was removed, slowly dropped into 100 μl of carbonate buffer (0.1 M, pH 9.6) containing 0.8 mg of HRP, incubated at room temperature for 2 hours in the dark, placed in the dark at 4 ° C for 18 hours , And finally dialyzed four times in PBS (0.01 M, pH 7.4) and the finished product was placed at -20 ° C for use.

Production of polyclonal antibody. The rabbit was injected intradermally at multiple sites on the shaved back (about 30 sites) with 250 μg of immunogen (DON-BSA or r-globulin conjugates) in 1 ml of 0.01M PBS mixed with 1 ml of Freund Complete adjuvant. For booster injections, the same amount of immunogen was mixed with an equal volume of incomplete Freund adjuvant and injected subcutaneously at 4 sites on the thigh of the rabbit. The antisera were precipitated with $(NH_4)_2SO_4$ to a final concentration of 50% and 35% in sequence by using a 100% saturated $(NH_4)_2SO_4$ solution. The precipitate was redissolved in distilled water equal to half of the original volume and then dialyzed against 2 L of PBS for 72 hr at 4 ^{o}C with two changes of buffer. Final added 0.01 M PBS to the original volume.

三. 結果(Results)

1. 橘黴素(CTN)多株抗體的生產

將兔子分別免疫CTN-KLH 或CTN-BSA 後，經四週後分別以相同抗原加強注射後，從第五週開

始採血進行抗體的效價測試，由非直接型酵素免疫分析法可以得知抗體效價從第六週開始出現，然後逐漸上升至第十二週達到一個高峰，其中又以免疫CTN-KLH 的兔子抗體效價較佳。因此為了使紐西蘭大白兔抗體效價可以往上升，目前以純化的兔子血清已經來建立一套橘黴素的直接競爭型酵素免疫分析法(dcELISA)，此一直接競爭型酵素免疫分析法其最低偵測限制約為0.2 ng/mL，50%毒素抑制濃度(IC50) 大約為5.0 ng/ml (圖一)，以已非直接競爭型酵素免疫分析法其50%毒素抑制濃度(IC50) 大約為4.6 ng/ml (圖二)。目前抗體仍持續生產當中，目前已可開發一套可以檢測橘黴素之直接酵素免疫分析法。

2. 橘黴素(CTN)單株抗體的生產

Balb/c 老鼠分別打入CTN-KLH 或CTN-BSA 的抗原，第三週經過加強免疫注射後, 並且每隔一周進行加強注射。每週以尾巴採集老鼠血清以非直接競爭型酵素免疫分析法檢測老鼠血清是否對橘黴素有專一性抗體生成，目前兩組老鼠皆可產生對橘黴素的專一性抗體。其中以注射CTN-KLH 抗體專一性較好，其非直接競爭性酵素免疫分析法得到的老鼠血清針對橘黴素的50%毒素抑制濃度(IC50) 為10~20 ng/ml 見圖三。計畫執行三年內，總共執行四次細胞融合實驗，取出小鼠脾臟細胞與老鼠骨隨瘤細胞(NS-1/SP2)進行細胞融合試驗，雖然每次都耗費許多人力及物力，但是仍無法篩選到具有專一性之融合瘤細胞株來生產橘黴素單株抗體。目前仍持續免疫小鼠，老鼠融合瘤的篩選以生產單株抗體仍持續進行中。

3. 利用多株抗體開發橘黴素免疫奈米層析試紙

我們將CTN-OVA 蛋白質接合物附到呈色區硝酸纖維膜上的下端，作為一個競爭物來與加樣區(sample pad)樣品中的毒素彼此競爭CTN-pAb gold nanoparticle 抗體金奈米探針(conjugation pad)。在呈色區內，同一硝酸纖維膜上(nitrocellulose)進行CTN 之奈米快速檢測技術開發，因此在呈色區第一區吸附CTN-蛋白質接合物。在呈色區內，任何未與樣品中分析物反應之抗體奈米金探針會分別與鍵結位於呈色區之CTN-蛋白質，而停留於此區呈色，抗體奈米金呈色強弱跟樣品中分析物的含量呈反比(即樣品中的所含毒素愈多，所呈顏色愈少或沒有顏色)，剩餘之抗體奈米金探針則會繼續前進至第四區，第四區由吸附物(absorbent pad)所組成，具有良好的吸水能力用以促進毛細管作用，使整體反應完全(圖四)。

4. 以橘黴素免疫奈米層析試紙建立橘黴素之快速分析法

目前以多株抗體已經建立橘黴素免疫層析試紙，當毒素濃度小於20 ng/ml 時，其test line 呈現紅色一條線，當毒素量大於20 ng/ml 時其test line 紅色一條線消失，因此免疫層析試紙最低偵測限制約為20 ng/ml (圖五),此試紙也用來進行市售各項紅麴樣品之橘黴素之檢測，市售樣品19個先經酵素免疫分析法先進行檢測，其結果如表一，紅麴米樣品1-6號遭受1.6~9.5 ppm橘黴素之汙染，其中有兩個樣品超過5 ppm限制含量，其餘樣品污染仍在限制含量以下尚在。此外我們也利用此一免疫層析進行市售紅麴樣品之檢測。結果顯示紅麴米樣品1-6號大多受到橘黴素之汙染。其中以紅麴米所含橘黴素之含量較高（圖六），相關酵素免疫分析法分析結果及免疫層析試紙結果將以HPLC螢光檢測法進一步確認所得之結果。

5. 脫氧雪腐鐮刀菌烯醇(DON)多株抗體的生產

將兔子分別免疫 DON-BSA 或DON-r-globulin 後，經四週後分別以上次相同的抗原加強注射後，

從第五週開始採血進行抗體的效價測試，由非直接型酵素免疫分析法以DON-OVA coating plate 進行抗體效價測試，雖然抗體效價隨著週次而上升。但是以非直接型競爭型酵素免疫分析法進行抗體專一性的測試，不論DON 濃度加到500 ng/mL 仍無法取代抗體鍵結到固相之DON-OVA。由此可以得知此類DON-KLH 或DON-r-globulin 抗原並無法誘發兔子體內免疫系統產生對DON 具有專一性之抗體。因此目前正利用不同之接合方法來接合DON 與載體蛋白質再進行動物的免疫。

6. 脫氧雪腐鐮刀菌烯醇(DON)單株抗體的生產

Balb/c 老鼠分別打入DON-BSA 的抗原，第三週經過加強免疫注射後，並且每隔一週進行加強注射。每週以尾巴採集老鼠血清以非直接競爭型酵素免疫分析法檢測老鼠血清是否對脫氧雪腐鐮刀菌烯醇有專一性抗體生成，目前兩組老鼠皆已對脫氧雪腐鐮刀菌烯醇產生專一性抗體。老鼠血清抗體效價隨著 週次而上升(圖七)，以非直接型競爭型酵素免疫分析法進行抗體專一性的測試，DON 濃度加到10 ng/mL 已經可以取代抗體鍵結到固相之DON-OVA(圖八)。由此可以得知此DON-BSA 可以誘發Balb/c 老鼠體內免疫系統產生對DON 具有專一性之抗體。 但是其抗體效價並不夠好，其中可能原因為脫氧雪腐鐮刀菌烯醇與蛋白質接合之方法並不理想，或是老鼠飼料可能遭受DON 毒素之汙染，使得老鼠早已長期食用遭受DON 汙染之飼料中，已對DON 具免疫力，因此對於外來之DON 抗原無法誘發免疫系統產生抗體之能力。此外我們也嘗試了各種不同衍生接合不同載體蛋白質如DON-SH-BTG ; DON-SH-EDA-BTG 作為抗原免疫小鼠，並於第三週後持續每週利用競爭型酵素連結免疫法 (ciELISA, cdELISA) 對小鼠抗體進行檢測，持續檢測至第三十四週，發現無專一性忼體產生。故於第三十五週改變抗原接合比例 (1:8 改為 1:2, w/w)，並混合費氏不完全佐劑注射入小鼠，且持續利用競爭型酵素連結免疫法對小鼠抗體進行檢測，然而，至第三十九週仍無專一性忼體產生。因此決定更換接合蛋白為 EDA-BTG，並持續利用競爭型酵素連結免疫法對小鼠抗體進行檢測，但至第五十週仍無專一性抗體產生。

DON-CDI-OVA 作為抗原免疫小鼠

本實驗選用 DON-CDI-OVA (DON : OVA, 1:8, w/w) 作為小鼠之免疫抗原，並於第三週後持續每週利用競爭型酵素連結免疫法 (ciELISA, cdELISA) 對小鼠抗體進行檢測，持續檢測至第二十週，發現無專一性忼體產生。故於第二十一週改變抗原接合比例 (1:8 改為 1:2, w/w)，並混合費氏不完全佐劑注射入小鼠，且持續利用競爭型酵素連結免疫法對小鼠抗體進行檢測，然而，至第四十週仍無專一性忼體產生。

DON-CMO-BTG ; DON-CMO-EDA-BTG 作為抗原免疫小鼠

本實驗選用 DON-CMO-BTG (DON : BTG, 1:2, w/w) 作為小鼠之免疫抗原，並於第三週後持續每週利用競爭型酵素連結免疫法 (ciELISA, cdELISA) 對小鼠抗體進行檢測，持續檢測至第七週，發現無專一性忼體產生。故於第八週混合費氏不完全佐劑注射入小鼠以加強免疫，且持續利用競爭型酵素連結免疫法對小鼠抗體進行檢測，然而，至第二十七週仍無專一性忼體產生。因此決定更換抗原為 DON-CMO-EDA-BTG (DON : EDA-BTG, 1:2, w/w)，並持續利用競爭型酵素連結免疫法對小鼠抗體進行檢測，但截至第四十四週，其仍無專一性忼體產生。

7 利用不同接合 DON 之辣根過氧化氫酵素檢測抗體專一性

本實驗於檢測前，利用先前生產之 DON 之多株抗體，進行三種接合 DON 之辣根過氧化

氫酵素 (AcDON-CMO-HRP, DON-CMO-HRP, DON-CDI-OVA-HRP) 之測試，由圖九得知 DON-CMO-HRP 及 DON-CDI-OVA-HRP 有競爭效果出現，因此判斷兩者皆接合成功，其中又以 DON-CMO-HRP 競爭效果較為出色，其 IC50 約在 5-10 ng/ml。我們利用上述 DON-CMO-HRP，檢測現階段實驗小鼠血清抗體 (抗體稀釋倍率 ：1:500 – 1:2,000，接合 DON 之辣根過氧化氫酵素稀釋倍率 ：1:500 – 1:2,500) 以檢測抗體專一性。然而，經過多次檢測，小鼠之抗體皆僅有微弱抗體生產，其競爭效果尚無法進行細胞融合實驗。目前 DON 免疫小鼠實驗仍持續進行中。

四、計畫成果自評

本研究主要目的是分別針對橘黴素(CTN)與脫氧雪腐鐮刀菌烯 (DON)建立融合瘤細胞株以生產單株抗體以及開發單鏈抗體及免疫層析法，此一計畫是三年期計畫，目前有關橘黴素的多株抗體已經產生成功，並且利用此一抗體建立了敏感性高的直接競爭型酵素免疫分析法，並且已經建立橘黴素快速免疫奈米層析試紙法來分析橘黴素，此一橘黴素之快速免疫層析試紙，目前市面上尚無此類產品，相當具有產業應用價值。至於橘黴素單株抗體，計畫執行三年內，總共執行四次細胞融合實驗，取出小鼠脾臟細胞與老鼠骨隨瘤細胞(NS-1/SP2)進行細胞融合試驗，雖然每次都耗費許多人力及物力，但是仍無法篩選到具有專一性之融合瘤細胞株來生產橘黴素之單株抗體。此外對於脫氧雪腐鐮刀菌烯醇抗體的生產，老鼠血清雖然已慢慢獲得專一性抗體之抗體，也一直持續加強老鼠免疫，但老鼠抗體效價仍無法達到標準效價來進行融合瘤篩選，此外我們也利用不同接合方法來進行脫氧雪腐鐮刀菌烯醇與蛋白質的接合物以重新進行免疫，但是效果仍然不太理想。綜合言之，此計畫所進行之單株抗體開發並不太順利，使得許多接續工作無法達成，但是我們仍會持續努力來完成。

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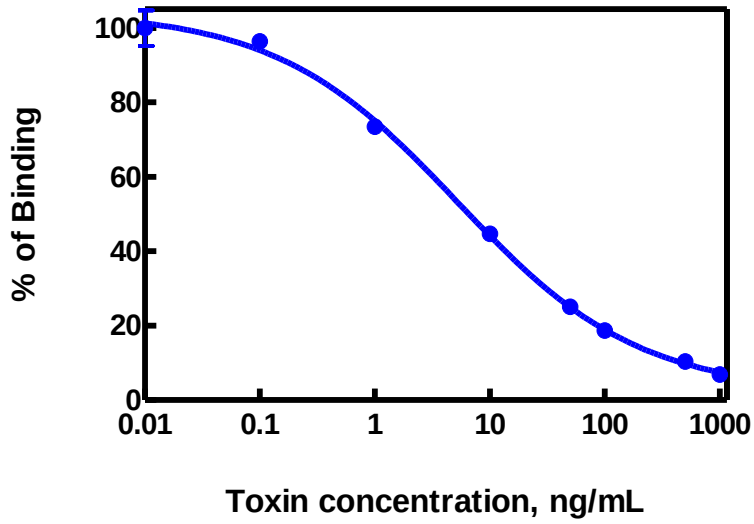
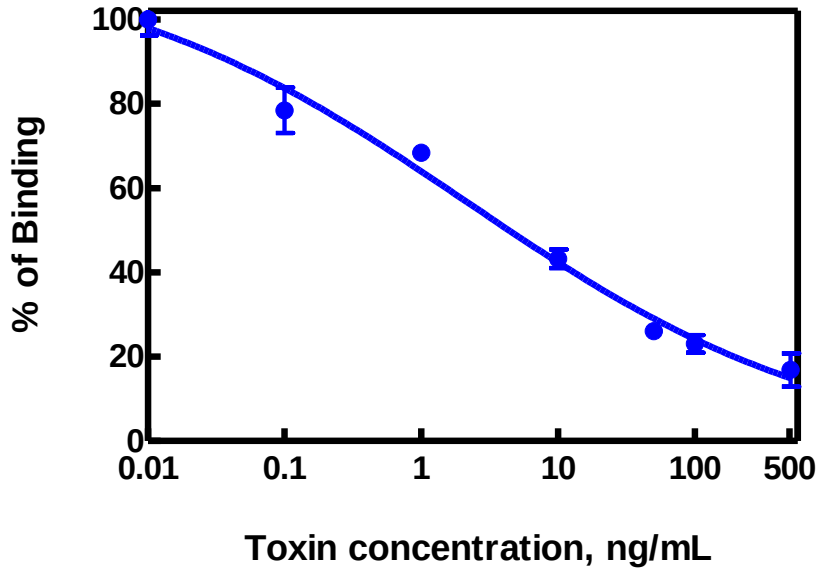
表一 紅麴產品樣品以 ELISA 及免疫層析試紙檢測結果

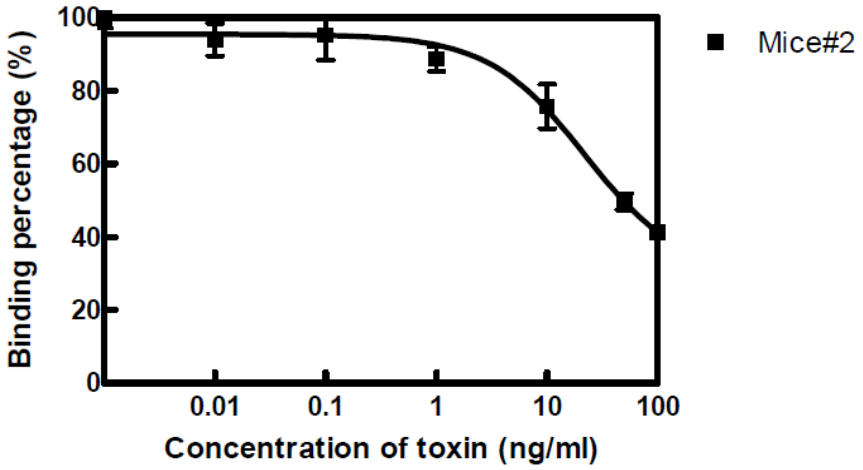
Samples	Food	ELISA (ng/g)	Immunochromatography strip assay
1	Rice	1677 ± 41	+
2	Rice	4683 ± 335	+
3	Rice	3065 ± 247	+
4	Rice	1628 ± 75	+
5	Rice	9454 ± 806	+
6	Rice	6987 ± 23	+
7	Cookie	12.3± 1.5	-
8	Cookie	61.7 ± 8.4	-
9	Cookie	16.1 ± 3.8	-
10	Wine	42.1 ± 2.9	-
11	Sauce	20.1 ± 2.2	-
12	Bean	34.2 ± 1.3	-
13	Sauce	28.6 ± 1.4	-
14	Sauce	43.6 ± 1.1	-
15	Sauce	18.4 ± 2.7	-
16	Sauce	33.5 ± 1.0	-
17	Sauce	49.3 ± 2.0	-
18	Capsule	20.7 ± 2.1	-
19	Sauce	64.4 ± 5.3	-
20	Honey	ND	
21	Beef	ND	



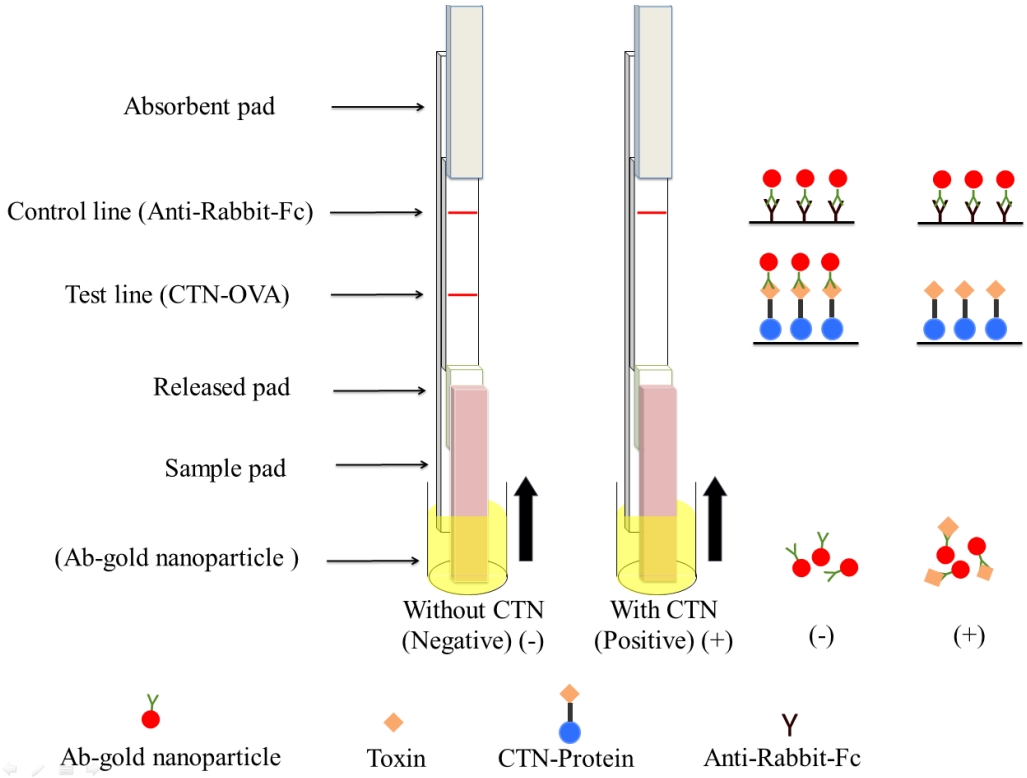
圖一、以直接競爭性酵素免疫分析法得到的兔子血清(抗原CTN-KLH)針對橘黴素的50%抑制濃度為5 ng/mL。



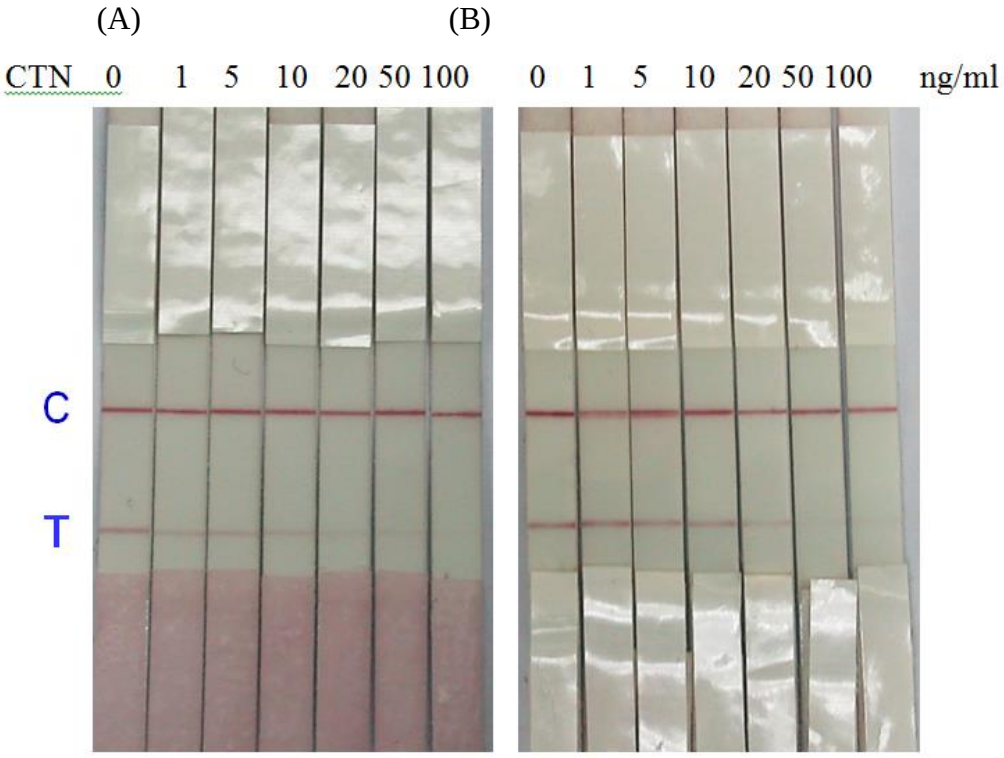
圖二、非直接競爭性酵素免疫分析法得到的老鼠血清(抗原CTN-KLH)針對橘黴素的50%抑制濃度4.6 ng/mL。



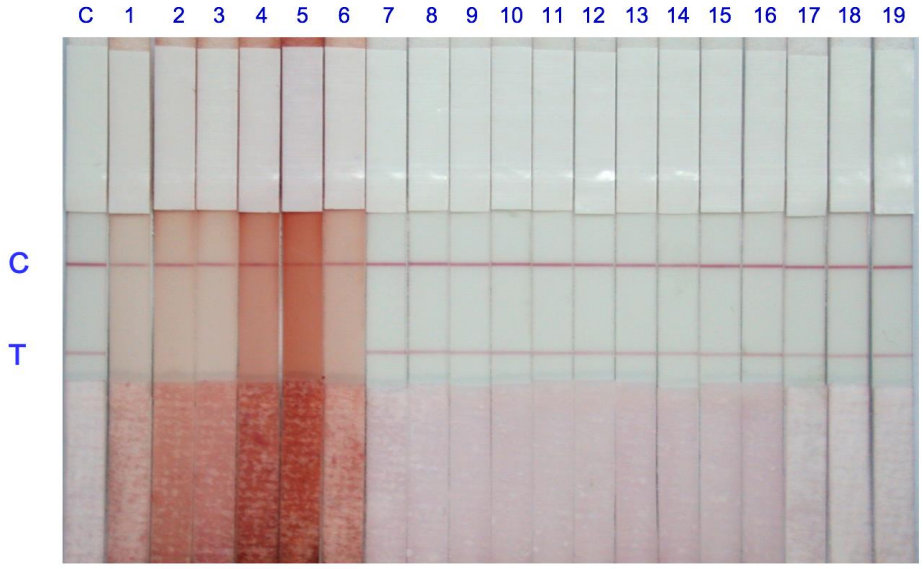
圖三、 以直接競爭性酵素免疫分析法得到的老鼠血清(抗原CTN-BSA)針對橘黴素的50%抑制濃度為10-20 ng/mL。



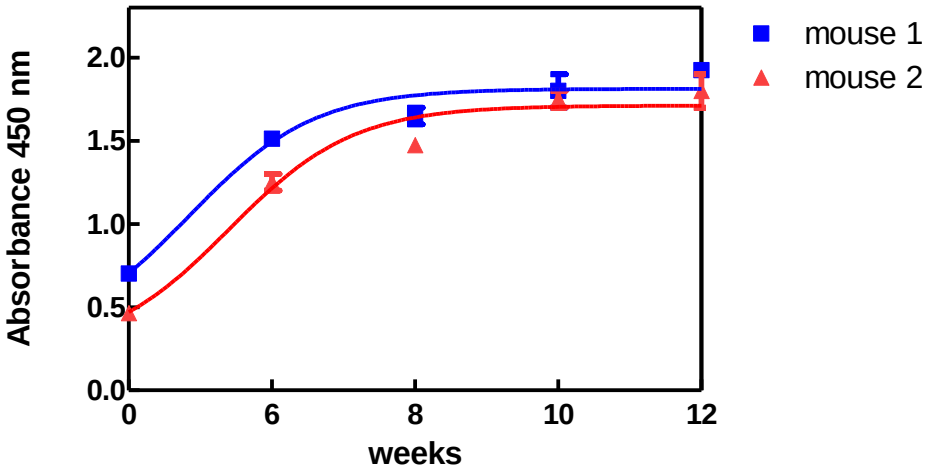
圖四、免疫層析試紙各成份示意圖



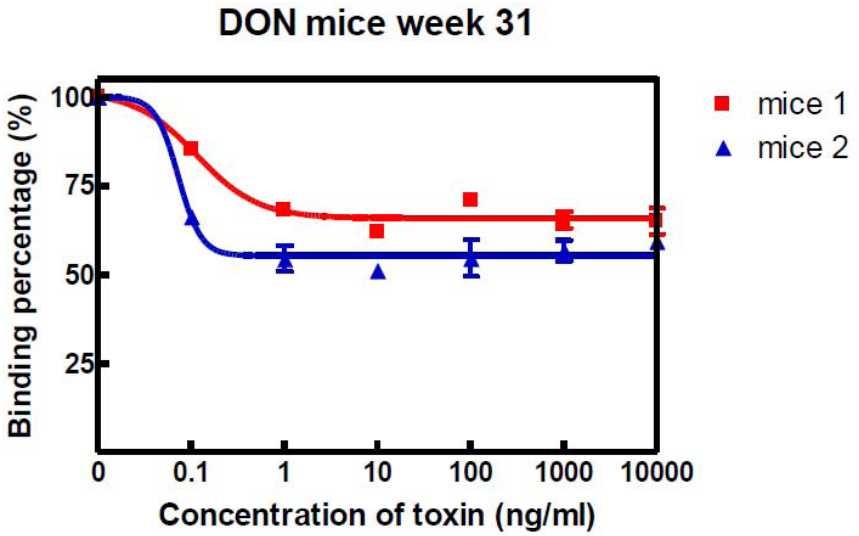
圖五、比較不同組裝方式之呈色及競爭效果(A) 抗體奈米金粒子接合物與樣品先在樣品處反應 (B) 抗體奈米金粒子接合物在 release pad 位置



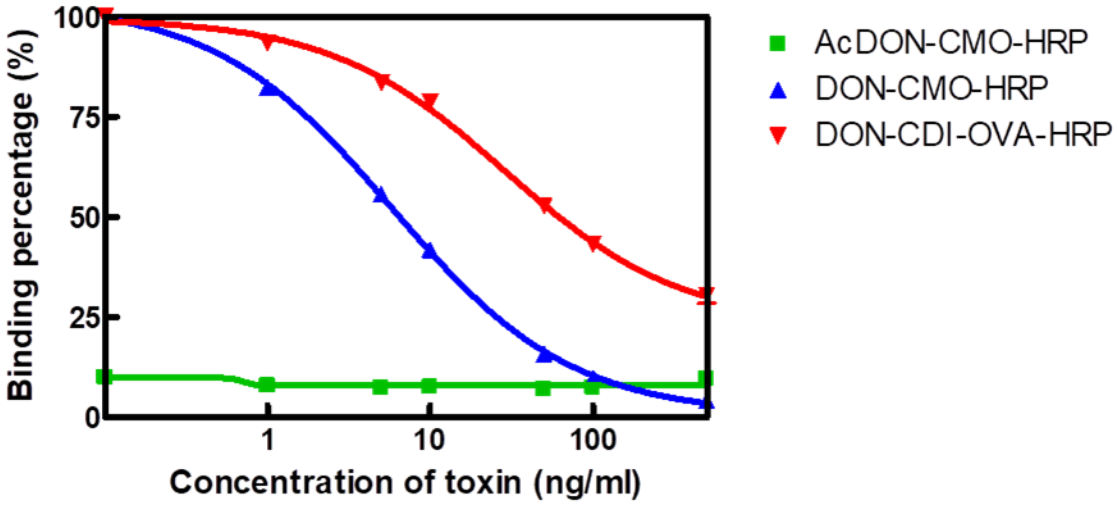
圖六、以免疫層析試紙進行紅麴樣品測試, No 1-6 為紅麴米樣品, 7-19 為其他紅麴產品



圖七、以直接競爭性酵素免疫分析法檢測的老鼠血清(抗原DON-BSA)對脫氧雪腐鐮刀菌烯醇之抗體



圖八、以直接競爭性酵素免疫分析法檢測量的老鼠血清(抗原DON-BSA)對脫氧雪腐鐮刀菌烯醇抗體之專一性，當DON毒素濃度加到10 ng/mL 已可以取代抗體鍵結到固相之DON-OVA。



圖九 利用先前生產之 DON之多株抗體，進行三種接合 DON 之辣根過氧化氫酵素 (AcDON-CMO-HRP, DON-CMO-HRP, DON-CDI-OVA-HRP)之測試，其中以DON-CMO-HRP 效果最佳，其IC50約5-10 ng/ml

103年度專題研究計畫成果彙整表

計畫主持人：余豐益	**計畫編號：**103-2313-B-040-002-MY3
計畫名稱：黴菌毒素之單株及單鏈抗體生產及快速檢測多重毒素之免疫奈米試紙開發	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			1		1.參加於7月25-27於日本大阪舉行Drug discovery and technology 會議發表海報論文一篇。題目為Rapid ELISA and Gold Nanoparticle immunostrip for detection of mycotoxin citrinin.
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		

		著作權	0		
		品種權	0		
		其他	0		
	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	2	人次	大學部學生2人參與2年
		碩士生	2		碩士班學生參與2人三年
		博士生	1		博士班學生參與1人三年
		博士後研究員	0		
		專任助理	2		專任助理2人三年
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		

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。）	目前有關橘黴素的多株抗體已經產生成功，並且利用此一抗體建立了敏感性高的直接競爭型酵素免疫分析法，並且已經建立橘黴素快速免疫奈米層析試紙法來分析橘黴素，此一橘黴素之快速免疫層析試紙，目前市面上尚無此類產品，相當具有產業應用價值。

科技部補助專題研究計畫成果自評表

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（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）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（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   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200字為限）
   目前研究結果正投稿中

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）
   此一計畫是三年期計畫，目前有關橘黴素的多株抗體已經產生成功，並且利用此一抗體建立了敏感性高的直接競爭型酵素免疫分析法，並且已經建立橘黴素快速免疫奈米層析試紙法來分析橘黴素，此一橘黴素之快速免疫層析試紙，目前市面上尚無此類產品，相當具有產業應用價值。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否　□是
   說明：（以150字為限）