出國報告（出國類別：研究）

參加「國際公定分析化學家協會(AOAC) 2011 年年會」出國報告

服務機關：行政院衛生署食品藥物管理局
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報告日期：100年12月13日

目　次

摘　要

國際公定分析化學家協會 2011 年年會(125th AOAC Annual Meeting)，於 2011 年 9 月 8 日至 21 日於美國路易斯安那州紐奧良市之的喜來登飯店(Sheraton New Orleans Hotel)舉行。本次會議筆者代表TFDA研檢組二科出席，並於大會的壁報論文發表會展出本局研究成果壁報兩篇，其一是針對動物用藥—抗生素多重殘留檢驗方法開發，其二則是針對食品中毒案例之水樣檢體中病毒之檢驗。本屆AOAC年會之主要活動內容包括：座談會、科學性專題研討會、壁報論文發表會、儀器及試藥試劑廠商展示、及贊助廠商大型專題研討會等，會議中可直接與全球各分析檢驗領域之專家學者進行交流，有任何問題亦可當面請益，即時獲得各種科學性新知，也可建立與國外專家學者的聯繫管道。大會期間參加儀器廠商的大型發表會，掌握未來先進快速分析設備的發展現況，並藉由聆聽廠商所邀請的大陸食品管理領域之專家學者的報告，瞭解陸方食品檢驗技術相關的機構資訊，做為本局與陸方進行食品檢驗交流的參考資訊。筆者亦協助辦理 20 日晚間所舉辦的AOAC台灣分會會議事宜，會議由孫璐西理事長主持，並以台灣食品中塑化劑污染事件之始末進行專題報告，眾多參與台灣分會會議的華裔專家學者針對此事件熱烈討論，亦對本局合宜的處置措施及檢驗能力表示敬佩。

目　的

國際公定分析化學家協會(Association of Official Analytical Chemists Communities International，AOAC) 創始於 1884 年，最早是由美國農業部 (USDA) 所創辦,是全球最早成立的有關食品及農業檢驗檢測技術國際標準化的組織，該組織致力於分析方法的驗證及認可，並制訂各種具國際公信力的檢驗檢測標準。經過一百多年的發展 AOAC 所頒佈各種國際標準已成為全球通行的技術標準，並為世界各國廣泛採用。AOAC 每年皆於美國境內舉辦大型的年會活動，會議中所安排的各種科學性專題研討會及壁報論文發表等活動，早已成為全球從事科學性檢驗研究工作人員重要的學術發表、教育訓練、交流學習、建立聯繫管道等的重要平台。筆者奉派參加本年度於美國路易斯安那州紐奧良市所舉辦的 AOAC 第 125 屆年會，並於會中發表兩篇壁報論文，其一為有關多重動物用藥抗生素方法開發，另一則為水中食因性病毒的檢測技術，展示本局檢測技術的研發成果。同時藉由聆聽各領域的科學性專題研討會、觀摩來自全球實驗室最新的檢驗研究成果壁報論文發表、與其他專家學者進行面對面討論交流等活動，廣泛收集國際間新式檢驗檢測技術發展趨勢，並可建立與不同國家學者的聯繫管道，期望參與此次 AOAC 年會中所獲得的科學性資訊，可運用於筆者所服務單位的業務發展，為國內檢驗研究工作提供助益。

過　程

本年度國際公定分析化學家協會年會(125th AOAC Annual Meeting)，於2011年9月19日在美國路易斯安那州紐奧良市的喜來登飯店(Sheraton New Orleans Hotel)舉行，正式會議時間為9月18日至21日共計四天，而主要的會議活動則集中在19日至21日三天。筆者於台灣時間9月16日出發，途經美國洛杉磯機場轉機後於9月17日抵達紐奧良。9月18日大會開放辦理報名人員的註冊手續，大會於當日安排數場次的 Committee meeting ，此類會議是由AOAC所聘請的各領域專家學者組成委員會並進行定期性會議，非一般人士可以參加。筆者於當天午後完成報到註冊程序，並領取大會手冊資料帶等。本屆AOAC年會之主要活動內容包括：座談會、科學性專題研討會、壁報論文發表、儀器及試藥試劑廠商展示及贊助廠商年度大型專題研討會等。各種會議活動與議程，如附件一所示。

9月19開始的會議議程，安排有一系列的科學性專題研討會(Scientific Session)及壁報論文發表(Poster Presentations)。大會所排定的科學性專題研討會涵蓋眾多領域，場次多達26場，與會者可預先篩選想要聆聽的場次並到場聆聽。26場研討會的內容，含蓋微生物之檢測、全球肉品業微生物方法標準、抗氧化物質之測定、海洋生物毒素之檢測方法、動物用藥分析研究之發展趨勢、食品中微小分子污染物質之分析、天然毒素檢測的發展趨勢、食品中及膳食補給品中為量金屬的先進分析技術、貝毒的化學穩定

性與代謝途徑、食品過敏原之篩檢與分析、食品過敏原標準品的製備與研發、食品與飼料中植物毒素Pyrrolizidine alkaloids之研究、分析方法的國際調和、化學分析方法確校及方法效能的標準、分析方法不確定度的用途等，其演講內容的性質從最基本的檢驗技術開發，檢驗方法的評估，到管理法規層面、檢驗方法的調和等，包羅萬象。每個時段筆者皆挑選業務相關的研討會出席，聆聽各國專家學者的研究成果與發現，獲取最新科學資訊，對於檢驗技術的研究發展方向，提供許多的新的觀念，並開拓檢驗研究的視野，獲益良多。

壁報論文展示分 3 個群組，分別於 9 月 19、20、21 日三天分三個時段進行張貼發表，9 月 19 日第一群組共有 93 篇壁報論文發表，本日展示的壁報主題包含五項：

A. Analysis of Foodborne Contaminants and Residues（食因性污染物及殘留物之分析）： 共24篇。

B. Analysis of Non-Foodborne Contaminants and Residues（非食因性污染物及殘留物之分析）： 共31篇。

C. Microbiological Methods（微生物檢驗方法）： 共20篇。

D. Pharmaceutical Analysis, Authenticity and Safety（藥物分析之可信度與安全性）： 共計11 篇。

E. Plant Food, Pet Food and Animal Feed Nutritives, Additives and Contaminants（植物性食品，寵物食品，動物飼料之營養、添加劑及

污染物）： 共計7篇。

9月20日第二群組共有73篇壁報論文發表，本日展示的壁報主題包含三項：

F. Detection and Measurement of Natural Toxins（天然毒性物質之檢測）：共29篇。

G. Emerging Issues in Food Safety and Security（食品安全性之突發問題）： 共11篇。

H. Food Nutrition and Food Allergens(食物營養及食物過敏原)： 共33篇。

9月21日第三群組共有84篇壁報論文發表，本日展示的壁報主題包含三項：

I. General Methods, Quality Assurance and Accreditation（一般共同分析法、品質保證與信賴度）： 共34篇。

J. Botanicals and Dietary Supplements（植物性營養補充劑）： 共40篇。

K. Performance Tested Methods（經效能測試方法）： 共10篇。

與會者可於大會規定的時段（中午 11:10~13:00）於壁報展示區與壁報作者進行面對面的溝通交流，藉由專家間的問答與資訊交換，提升精進各研究領域的技術。筆者代表 TFDA 研究檢驗組第二科，發表兩篇壁報論文，第一篇是：Simultaneous Determination of Multiclass Veterinary Drug Residues in Porcine Liver by Liquid Chromatography-Electrospray Tandem Mass Spectrometry（以利用液相層析串聯質譜儀檢測豬肝中動物用藥殘留之多重分析法）；第二篇則是：Viruses in Water Samples from Foodborne Outbreaks in Taiwan (2010)（2010

年台灣食因性中毒案例中水樣檢體之病毒檢測)。筆者所發表的壁報論文摘要及壁報論文如附件二。

出席學術性國際研討會，參觀來自全球專家學者的最新研究成果展示是學習新知並瞭解科學技術發展趨勢的最佳管道，筆者把握機會閱覽業務相關領域的壁報論文，期望能收集到對筆者單位業務相關且具有發展檢驗研究效益的科學新知。如土耳其的學者所發表的論文：Identification of animal species origin of gelatine used in processed foods in Turkey & Germany（鑑別土耳其及德國市售加工食品中所使用之明膠成分的動物來源）。明膠成分具有凝膠特性，廣泛使用於糕餅甜點或糖果的製造上，明膠的來源可分為幾種，有豬皮來源、牛皮來源、魚皮來源、海草來源等，然土耳其等回教國家或猶太教徒因宗教教義的關係，不允許食用含有豬來源的食物成分，但甜點中的明膠成分因為經過酸鹼水解等的萃取程序，要鑑別其源自於何種物種，難度極高。作者選用SureFood 這家公司所生產的DNA萃取套組，搭配該公司所生產的Real-Time PCR檢測套組，可在合理的確校範圍內精確的檢測甜點糖果中的明膠成分是否含有豬成分〈如附件三〉。

日本厚生勞動省國立醫藥品食品衛生研究所 (National Institute of Health Science/Ministry of Health, Labour and Welfare) 的中村公亮研究官，發表 Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus TK Strain in Processed Food（加工食品中基因改造木瓜 PRSV-YK 品系之檢測方法）。筆者曾數次與日本從事基因改造食品檢驗的

政府機構進行交流，知悉該篇作者所服務的機構致力於基因改造食品檢驗技術的開發，筆者之所以對本篇論文特別感到興趣的原因是因為其文中所提到的基因改造木瓜 PRSV-YK 品系正是源自於台灣，亦即日本市售木瓜產品中驗出含有台灣學術界所研發的基因改造木瓜，由於該項作物在日本是屬於未經核准的基因改造作物，故曾在日本政府及社會引起極大的關注。作者於木瓜輪點病毒外鞘蛋白的保守區域基因序列(highly conserved sequence)及 35S 啟動子序列間設計引子及探針，開發完成可檢測未經核准基改木瓜的檢測技術。筆者於會場與作者進行討論，作者解釋該檢驗技術並非只針對台灣所發展的 PRSV-YK 基改木瓜進行檢測，而是可篩選出所有具 PRSV 抗性的基改木瓜，其所驗得的加工食品有木瓜葉茶包，木瓜醃漬物及木瓜果醬，主要集中在沖繩地區，且這些產品的原料都不是使用進口的木瓜，而是使用日本當地所生產的木瓜，因此可推測目前日本境內已有未經日本政府核可的基改木瓜種植。

來自日本農林水產省食品總和研究所的真野潤一博士，發表 Development of Group Testing Method to Evaluate Weight per Weight GMO Content in Maize Grains(玉米穀物中所含基因改造成分重量百分比的群集測試方法)。由於分子生物技術的進步，現今全球的作物市場已經充斥各種的基改作物，其中又以玉米及黃豆為最大宗。台灣因為地小人稠，因此每年都需進口大量的玉米供給國內龐大的食品及飼料市場需求，因此亦引進不少的基改玉米。隨著越來越多的基改玉米商業化種植，如何有效採樣及檢

驗體積龐大的進口原料玉米成為各國檢驗機構的重要課題。本篇作者即運用群集檢測(group testing)的方式，開發一套較為快速又相對比較可行的基改玉米定量檢驗技術。其方法是使用一種極容易於市面上購得的小型粉碎機，於玉米樣品中隨機選取 20 顆玉米粒作為一個群集檢測單位，以小型粉碎機一起粉碎、抽取 DNA 並以 Real time PCR 定性檢測是否含有基改玉米成分，於第一輪迴篩選測試時，共需執行 10 個群集檢測單位的測試，如果在第一輪迴篩選測試中測得 7 個以上的群集檢測單位含有基改玉米成分，則需再另外執行第二輪迴篩選測試，亦需進行 10 個群集檢測單位的基改玉米定性測試。測試結果如果在這總共 20 個群集檢測單位，驗得超過 12 個群集檢測單位含有基改玉米成分，則經由統計程式分析即可計算該批玉米樣品中所含基改玉米成分，是否超過日本基改食品標示制度法規所規定的5%上限。筆者所發展的技術已經通過日本國內實驗室共同試驗確效程序，其準確度與目前現行官方方法不相上下，但操作實驗上則更為簡便有效率，作者表示未來希望將此方法推展成為日本官方公告標準方法，以取代現行較為繁瑣的檢驗方法〈如附件四〉。

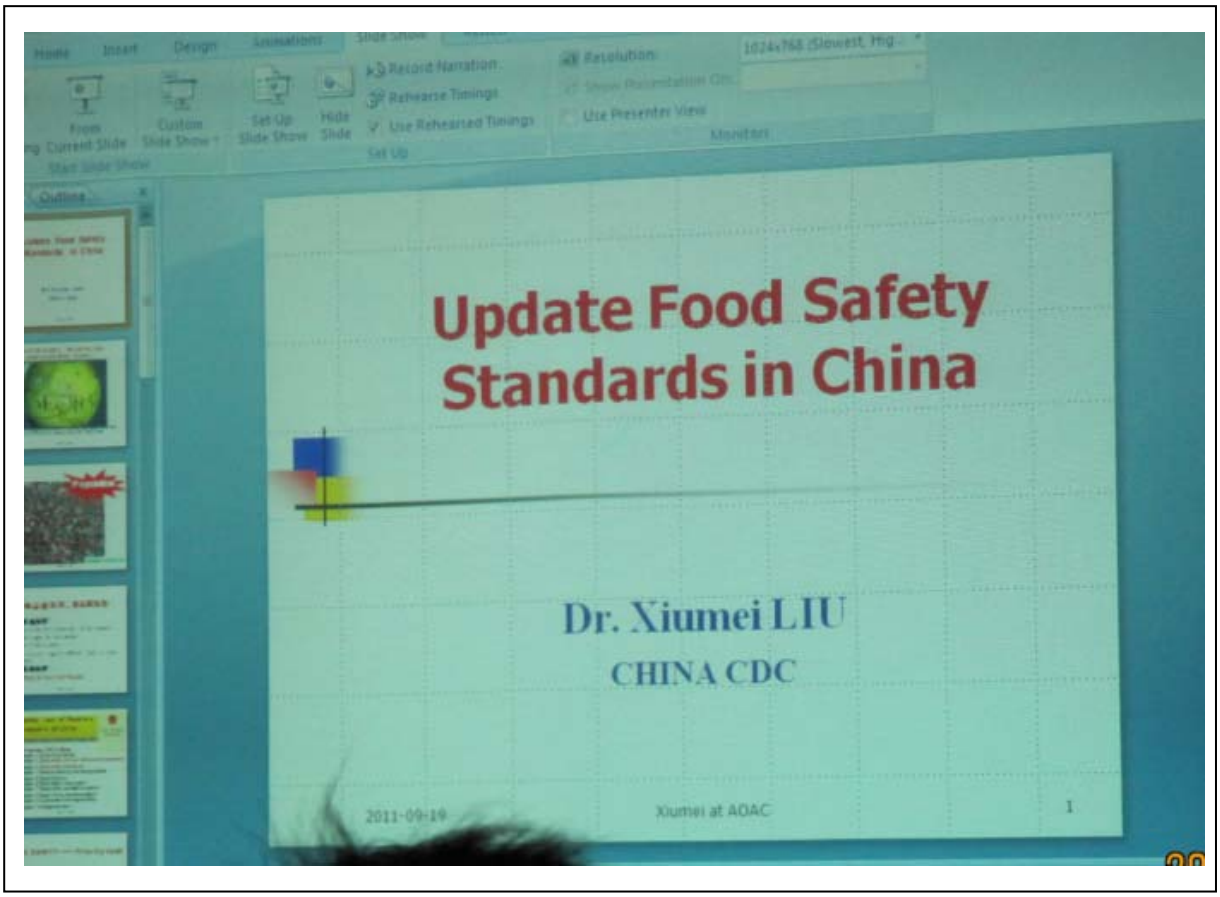
AOAC 台灣分會會議（Taiwan Section Business Meeting）於9月19日晚間6 點舉行，由AOAC台灣分會理事長台大教授孫璐西博士主持，筆者亦協助會議的準備工作，幫忙布置會場、準備、發送會議資料及致贈與會來賓紀念品等。會議約有20多位來賓出席，幾位服務於美國FDA及其他州政

府部門的台灣專家如周家璜博士等也到場參與開會討論，另有許多則是來自大陸的各界人士以及同樣服務於美國FDA的大陸籍專家學者，其中，劉秀梅女士則是中國現任國際食品添加劑法典委員會秘書處（Secretariat in General, Codex Committee of Food Additives）的負責人。會議開場由現任AOAC台灣分會理事長孫璐西教授以台灣今年所發生的食品中違法添加塑化劑事件為題進行專題報告，本局研檢組曾素香科長則接續進行補充說明，將塑化劑事件發生的始末及TFDA如何經由檢驗發現飲料中含塑化劑、擴大調查規模、後續處置、警檢調介入調查、可疑食品查扣等程序做了系統性的說明，出席者對於此食品危安事件都具有極大的興趣，會議中踴躍的針對此議題發問及討論，而孫教授及曾素香科長的說明，也讓與會者清楚瞭解到TFDA在塑化劑事件中所扮演的角色，更加肯定衛生署的處理原則及TFDA的在此事件中所做的努力。當討論到TFDA整併之後，檢驗部門面臨到預算與人力嚴重吃緊的問題時，與會專家也深表認同並表達憂心的看法。周家璜博士以美國FDA的例子說明食品檢驗業務在美國受到重視的實況，周博士講述近年來美國政府的財政狀況也不佳，政府部門裁減預算時有所聞，幾年前部分FDA實驗室也因經費的不足問題面臨關閉實驗室的壓力，就在檢討實驗室存廢之際，2008年大陸發生不肖商人於奶粉中添加三聚氰胺化工原料事件，影響所及，許多使用此種有毒奶粉成分進行加工製作的食品被銷往世界各國，引起全球性食品安全的恐慌，包括美國在內，

此時美國政府也正視到食品檢驗實驗室是無法以實際產出效益作為績效評估的依據，實驗室的存在價值是當發生事件的當時適時發揮其檢驗檢測的功能，找出致病因子進而進行預防，避免事件擴大或防止其再發生影響更多人民健康，而在事件發生之前，實驗室則必須不斷進行檢驗研究實力的培養，所謂練兵千日用在一時的概念。因此在食品三聚氰胺事件之後，美國國會即做成一項決議，無論國家財政如何拮据，FDA及FBI兩個單位的實驗室絕對不可以關閉，因為民生議題永遠是影響國家安全的首要問題，美國政府的考量正好可以提供台灣規劃成立食品藥物管理署時的參考，會議召開的實況，如附件五所示。

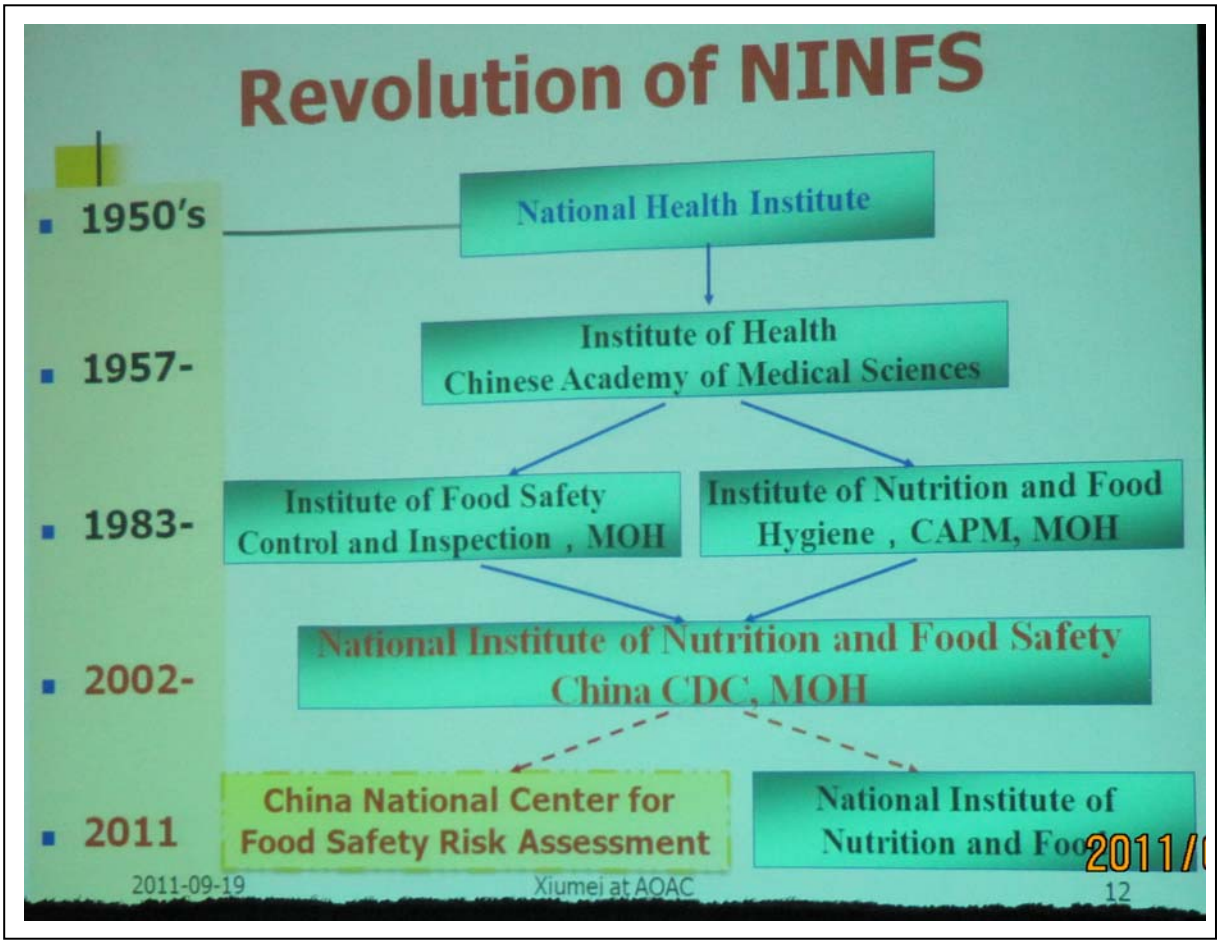
9 月 19 日，筆者出席由Waters公司專為華人所舉辦的晚宴說明會，時間正好安排在AOAC 台灣分會會議結束後的時間，Waters公司針對其公司之沿革及若干新式儀器先行做了一番介紹，由於此宴會是由Waters公司所舉辦，故商業宣傳與廣告自是在所難免，在一段公司宣傳活動後，接下來則是邀請中國疾病預防控制中心營養與食品安全所（National Institute of Nutrition and Food Safety, Chinese. Center for Disease Control and Prevention）的劉秀梅女士進行演說，劉女士是中國現任國際食品添加劑法典委員會秘書處(Secretariat in General, Codex Committee of Food Additives)的負責人，其在中國食品界甚至全球性有關食品添加劑的標準及規範的制訂，具重要之影響力，因此筆者出席AOAC大會期間，台大孫璐西教授就特別說明這位劉

秀梅女士是所有在食品界華人都應該要認識的重要人物。劉女士以「中國食品安全標準的現況」發表短暫演說，讓出席晚宴的華人對目前中國食品安全標準規範有一些概略性瞭解（如下圖）。



依據筆者所知，中國目前與食品安全標準與檢驗技術標準最為相關的中央部會，就屬劉女士所任職的中國疾病預防控制中心營養與食品安全所，劉女士簡報中也介紹了中國疾病預防控制中心營養與食品安全所的組織沿革，該單位的前身最早可溯到 1950 年代的中央衛生實驗院（National Health Institute），1957～1983 年改制為中國醫學科學院衛生研究所（Chinese Academy of Medical Sciences），於 1983 年中國成立了衛生部食品衛生監督

檢驗所（Institute of Food Safety Control and Inspection, MOH）及中國預防醫學科學院營養與食品衛生研究所（Institute of Nutrition and Food Hygiene, CAPM, MOH），承接食品衛生和營養領域相關任務業務。2002 年中國成立中國疾病預防控制中心（CDC），上述兩個單位再次進行業務整併，成為CDC下轄的營養與食品安全所（NINFS），專司中國食品安全相關工作。劉女士並指出，2011 年營養與食品安全所又將會分割成為兩個單位，分別是中國食品安全風險評估中心（China National Center for Food Safety Risk Assessment）及營養與食品所（National Institute of Nutrition and Food），這些資訊成為筆者瞭解中國食品衛生管理機構的重要資訊（如下圖）。



本年度 AOAC 年會會議的所有議程於 9/21 日下午全部結束，大會當天晚上七點於喜來登飯店舉辦盛大的閉幕晚宴，犒賞來自全球各地的專家學者四天來的辛勞，同時也歡慶這次大會的順利成功及收穫滿滿，並預告明年的國際公定分析化學家協會 2012 年年會將於美國西岸的拉斯維加斯市舉行。筆者 9/22 日即由紐澳良國際機場出發，經由洛杉磯機場轉機後於 9/23 日順利返抵台灣，結束此趟行程。

心得與建議

一、加強建立與國際專家學者持續性聯繫，獲取食品領域重要資訊

筆者於會議期間瀏覽各國專家學者所發表的壁報論文，壁報會場中閱讀到日本厚生勞動省國立醫藥品食品衛生研究所－中村公亮研究官所發表發表 Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus TK Strain in Processed Food（加工食品中基因改造木瓜 PRSV-YK 品系之檢測方法），由於本論文檢測的 PRSV-YK 基改木瓜是源自於台灣，筆者正好可以藉此機會當面請教作者關於該事件的始末，作者瞭解筆者所服務的單位為台灣負責基改食品檢驗方法開發的單位，也很熱切與筆者交換名片及討論（如附件六）。筆者表示論文中所發現的含基改木瓜的市售產品包含木瓜葉茶包，木瓜醃漬物及木瓜果醬，產品都集中在沖繩地區，且所運用的原料皆為日本當地栽種的木瓜，顯示日本境內有未經核准的基改木瓜種植，作者所研發的篩檢技術亦可提供本局檢驗研究參考。透過作者的介紹，也清楚瞭解目前日本厚生勞動省國立醫藥品食品衛生研究所負責基改食品檢驗的單位及其主管，雙方同意日後如有基改食品方面的問題，可藉由 E-mail 進行進一步的聯繫。筆者於會議結束後回到台灣，亦收到中村公亮透過 E-mail 所提供的有關基改木瓜 PRSV-YK 檢測檢驗的發表文獻及日本厚生勞動省食品安全局所公告的有關基改木瓜 PRSV-YK 的檢驗方法，提供筆者參考（如附件七）。現今由於貿易全球化，許多食品

安全的議題都是跨國性的問題，隨時掌握全球所發生的食安議題，並收集來自各國專家的資訊與意見，才能掌握先機，以利第一時間擬定因應方案以維護國家民眾安全，AOAC 大會集中來自全球產官學界的專家學者，是交換資訊及建立聯繫管道的最佳平台，本局身負國內食品藥物化妝品等的安全事務，更應持續參與此類國際性學術會議，廣泛結識各國各領域專家，充實本局國際資訊的來源管道。

二、食品過敏原為國際關注的食品安全重點項目之一，應持續投注相關檢驗研究

食品中的成分引發人體過敏反應一直時醫學界及食品界重視極為重視的食品安全議題之一，食物過敏是導因於攝入口中的食物含有會誘導人體免疫系統反應的的成分，亦即生理上對於攝入的食物產生了免疫反應。這種免疫反應輕則於皮膚上出現紅腫，重則造成呼吸困難進而死亡，因此不可不慎。由於地域及人種的差異，不同國家或地區的民眾對於可能引發食品過敏反應的食物種類也不盡相同，因此為維護民眾飲食安全，目前許多重視食品安全的先進國家，已先後實施食品中過敏原成分的標示制度，如美國、加拿大、英國、歐盟、日本、韓國等國家，因此，建立食品過敏原檢測技術亦成為食品檢驗領域重要的課題。本次大會安排的壁報論文發表活動中，9月20日所排定的三大主題其中之一即為Food Nutrition and Food Allergens（食物營養及食物過敏原），本主題中共有33篇壁報，其中16篇是

有關各種食品過敏原成分的相關研究論文。由於引發過敏反應的食品成分中，蛋白質是主要的致病因子之一，因此多篇論文是研究以ELISA原理開發快速檢測方法，另有幾篇是針對麵粉中麩質(gluten)致過敏成分的研究，此外尚有針對芥菜(mustard)致過敏蛋白質的研究、米中的過敏原物質及蛋白質的研究、甲殼類動物成分之研究、黑巧克力及甜餅乾中多重致過敏成分之檢測研究等等，更有學者發表以LC/MS/MS儀器開發檢測麵包及義大利麵中致過敏成分之檢驗技術。本署近年來已積極研究規劃適合我國國情的食品過敏原標示制度，在各種先期的調查與研究中研檢組也積極從事於食品過敏原分子生物快速檢測方法的開發，本次會議中所收集到有關各種食品過敏原的檢驗技術研究，以及以LC/MS/MS原理檢測食品過敏原成份等的科學新知，正可提供作為本局研檢組構思規劃未來從事食品過敏原檢驗研究的方向。同時，從國際性學術會議對於食品過敏原研究主題的重視程度，亦清楚點出本署應更積極構思與研擬適合台灣國情的食品過敏原標示制度，而對於台灣食品業者而言，更是必須認清實施過敏原標示制度是台灣食品產業未來必須一定要走的路。

三、應持續擴增檢驗分析精密儀器，建構符合世界水準的檢驗技能，以維護民眾安全並促進食品國際流通

隨者科技進步，越來越多的食品成分的檢驗技術都必須運用高科技精密儀器設備，並開發更為精良的分析檢驗技術。本次會議多篇針對食品中

動物用藥殘留之檢驗技術研究報告，都著重於以高效液相層析串聯質譜儀 LC/MASS/MASS 或 Q-TOF MASS 開發多重殘留的檢測技術；食品中真菌毒素殘留的檢測研究，著重於開發 LC/MASS/MASS 技術；食品中真海洋毒素之研究，著重於開發 LC、LC/MASS、Tandem MASS 的技術；甚至食品過敏原成分之檢驗研究，亦有使用 LC/MASS/MASS 儀器設備，顯見在越來越講求快速、精確及低檢出值的發展趨勢下，使用更為先進的儀器設備以制訂檢驗方法已經是必然的潮流，本局研檢組為我國的國家實驗室，具技術領導之檢驗地位，更必須洞燭先機，規劃購置各領域先進精密儀器，開發建立相關的檢驗技術，以利與國際接軌。而本局近年來已先後購置一些精密分析儀器，如高效液相層析串聯質譜儀(LC/MASS/MASS)、液相層析四極柱式/飛行式串聯質譜儀(LC/Q TOF MS)、即時聚合酶連鎖反應器(Real-Time PCR)等，並也都投入於相關檢驗方法的開發，未來仍需請支持本局持續購置各種精密儀器設備，建立相關檢驗技術與能力，加強為全體國民的健康安全把關，亦能促進台灣食品產業的國際貿易流通，以檢驗技術協助解決跨國性貿易障礙。

四、檢驗技術人才培育不易，應持續培植及維持實驗室檢驗人才與人力

AOAC 台灣分會會議（Taiwan Section Business Meeting）於 9 月 19 日晚間舉行，多位服務於美國 FDA 及其他州政府食品衛生相關部門的台灣或華裔專家學者皆到場參加，多位出席的專家學者對於台灣 FDA 目前的檢驗

人力編制及規劃中之食品藥物管理署的檢驗人力編制都相當關心，對於本局檢驗研究部門面臨預算與人力吃緊的問題，大家都深表關心與憂心，以美國 FDA 為例，目前從事檢驗技術研究工作的實驗室編制內人員，就達到千人的規模。而食品檢驗技術與人才建立不易，其能力是必須靠經年累月實際從事實驗工作的經驗累積而成，如此才能在任何重大食品危安事件發生時，立即發揮其功能，這些檢驗技術人員一旦離職或轉職，當面臨需求再來重建實驗能力之際，所需耗費的經費與時間將會是數倍計，因此多位專家學者皆表達本局應持續投注心力與經費維持檢驗技術人才及實驗室規模，以利面臨越來越嚴苛與多樣化的國際化食品安全與衛生議題。

附　件

附件一

2011 Annual Meeting Schedule At A Glance

Saturday, September 17, 2011

7:30 am - 12:00 pm	Rhythms 1	Editorial Board Meeting
9:00 am - 5:00 pm	Napoleon Foyer	Registration Open
5:00 pm - 6:00 pm	Evergreen	Journal Section Editors Meeting

Sunday, September 18, 2011

7:30 am - 7:00 pm	Napoleon Foyer	Registration Open
8:00 am - 9:00 am	Rhythms 1	Finance Committee Meeting
9:00 am - 11:00 am	Rhythms 1	AOAC INTERNATIONAL Board of Directors Meeting
12:00 pm - 4:00 pm	Nottoway	Methods Committee on Antimicrobial Efficacy Testing Meeting, Part 1
12:00 pm - 4:00 pm	Rhythms 3	Dietary Supplements Task Force and Community Meeting
1:00 pm - 4:00 pm	Oak Alley	Community Leadership Training
2:00 pm - 4:00 pm	Napoleon A	Standard Methods Performance Requirements Education
4:00 pm - 6:00 pm	Waterbury	Methods Committee on Microbiology Meeting
6:00 pm - 8:00 pm	Napoleon	Exhibit Hall Grand Opening Reception
8:00 pm - 10:00 pm	Rhythms	President's Welcome Reception

Monday, September 19, 2011

7:00 am - 8:00 am	Bayside B	TDRM Executive Committee Meeting
7:30 am - 8:00 am	Armstrong Foyer	Continental Breakfast
7:30 am - 5:00 pm	Napoleon Foyer	Registration Open
8:00 am - 10:30 am	Armstrong	Keynote Address and Awards Ceremony
10:00 am - 5:00 pm	Napoleon	Exhibit Hall Open
10:00 am - 5:00 pm	Napoleon	POSTER PRESENTATIONS: Analysis of Foodborne Contaminants and Residues, Analysis of Non-Foodborne Contaminants and Residues, Microbiological Methods, Pharmaceutical Analysis, Authenticity and Safety, and Plant Food, Pet Food and Animal Feed Nutritives, Additives, and Contaminants
10:30 am - 1:00 pm	Borgne	Latin America Section Business Meeting
10:45 am - 11:15 am	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Roka Bioscience
11:30 am - 1:00 pm	Napoleon	Poster Author Presentations
11:45 am - 12:15 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Biotage AB
1:00 pm - 1:30 pm	Rhythms 1/2	H.W. Wiley Award Address
1:00 pm - 5:15 pm	Bayside C	AOAC Expert Review Panel
1:30 pm - 3:00 pm	Rhythms 1/2	Wiley Award SYMPOSIUM: Partners in Chasing and Tracing Chemicals in Food
1:30 pm - 3:00 pm	Napoleon A	ROUNDTABLE: Comprehensive Screening for Food Allergens in One Shot - Just a Nice Dream or a Feasible Project?
1:30 pm - 5:00 pm	Waterbury	ROUNDTABLE: Hot Areas of Interest in Botanicals for Dietary Supplement Industry
3:00 pm - 3:30 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Dionex - Part of Thermo Fisher Scientific
3:00 pm - 3:30 pm	Napoleon	Refreshment Break
3:30 pm - 5:00 pm	Rhythms 1/2	SYMPOSIUM: New Blood 2011 - Developing Methods for the Detection of Chemical Contaminants
3:30 pm - 5:00 pm	Napoleon A	ROUNDTABLE: What Do You Mean You Can't Clean It? Validation of Allergen Sanitation SOP in Food Establishments
4:30 pm - 5:30 pm	Bayside B	Laboratory Proficiency Testing Program Advisory Committee Meeting
4:30 pm - 6:30 pm	Southdown	Methods Committee on Antimicrobial Efficacy Testing Meeting, Part 2
5:00 pm - 6:00 pm	Rhythms 3	Media Reporting on Science: Implications for the Analytical Community, Supported by The Coca-Cola Company
5:00 pm - 6:30 pm	Gallery	New Member Welcoming Reception, Sponsored by MATHESON
5:00 pm - 7:00 pm	Oak Alley	Chemical Contaminants and Residues in Food Community Meeting
5:00 pm - 7:30 pm	Grand Chenier	Marine and Freshwater Toxins Community Meeting
5:15 pm - 8:15 pm	Maurepas	Food Allergen Community Meeting
6:00 pm - 7:00 pm	Bayside C	Taiwan Section Business Meeting
6:00 pm - 7:00 pm	Bayside A	Japan Section Business Meeting
6:00 pm - 8:00 pm	Gallier	Agricultural Materials Community Meeting
6:30 pm - 7:30 pm	Lagniappe	Reception for TDLM Members, Co-Sponsored by Microbiologics®, Inc.
7:00 pm - 8:00 pm	Rhythms 3	Joint Asian Sections Meeting

Tuesday, September 20, 2011

7:15 am - 8:15 am	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Waters Corporation
7:15 am - 8:15 am	Edgewood	Nominating Committee Meeting
7:30 am - 5:00 pm	Napoleon Foyer	Registration Open
7:45 am - 8:15 am	Napoleon Foyer	Refreshment Break
8:00 am - 12:00 pm	Bayside A	AAFCO Meeting
8:00 am - 12:00 pm	Maurepas	AOAC Expert Review Panel
8:15 am - 9:45 am	Napoleon A	SYMPOSIUM: Inorganic Speciation Topics
8:15 am - 9:45 am	Waterbury	SYMPOSIUM: Reference Materials for Food Allergens... Heaven Must Wait?
8:15 am - 9:45 am	Rhythms 1/2	SYMPOSIUM: Alternative Methodology and Method Validation - Building an Internationally Harmonized Approach
9:00 am - 11:00 am	Southdown	Water/Waste Water Community Meeting
9:45 am - 10:15 am	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Thermo Scientific
10:00 am - 10:30 am	Napoleon	Refreshment Break
10:00 am - 12:00 pm	Bayside C	Committee on Statistics Meeting
10:00 am - 5:00 pm	Napoleon	Exhibit Hall Open
10:00 am - 5:00 pm	Napoleon	POSTER PRESENTATIONS: Detection and Measurement of Natural Toxins, Emerging Issues in Food Safety and Security, and Food Nutrition and Food Allergens

10:15 am - 11:45 am	Napoleon A	SYMPOSIUM: Cosmetics at AOAC
10:15 am - 11:45 am	Rhythms 1/2	SYMPOSIUM: Chemoinformatic Aided Compound Identification in Mass Spectrometry - Applications, Challenges, and the Future Development
10:15 am - 11:45 am	Waterbury	TDRM/TDLM SYMPOSIUM: The Many Uses of Measurement Uncertainty
11:30 am - 1:00 pm	Napoleon	Poster Author Presentations
11:45 am - 1:15 pm	Bayside B	Contaminants Subgroup Meeting - Pesticides
12:00 pm - 1:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Agilent Technologies
12:30 pm - 2:30 pm	Southdown	Committee on Sections and Membership Meeting
1:00 pm - 3:00 pm	Edgewood	Methods Committee on Pesticides and Disinfectant Formulations Meeting
1:00 pm - 5:00 pm	Maurepas	AOAC Expert Review Panel
1:30 pm - 2:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Microbiologics®, Inc.
1:30 pm - 3:00 pm	Bayside B	Contaminants Subgroup Meeting - Unknowns
2:30 pm - 3:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Pickering Laboratories
2:30 pm - 3:00 pm	Napoleon	Refreshment Break
2:30 pm - 4:30 pm	Cornet	Veterinary Drug Residues Expert Review Panel
3:00 pm-4:30 pm	Napoleon A	SYMPOSIUM: Modern Analytical Techniques Used in Evaluating the Concentration of Trace Metals in Foods and Dietary Supplements
3:00 pm - 4:30 pm	Waterbury	TDRM/TDLM WORKSHOP: Reference Materials and Laboratory Accreditation
3:00 pm-4:30 pm	Rhythms 1/2	SYMPOSIUM: Experts Take on Detection of Non-O157:H7 STEC
4:30 pm - 5:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: LECO Corporation
4:30 pm - 6:00 pm	Bayside B	Contaminants Subgroup Meeting - Veterinary Drugs
4:30 pm - 7:30 pm	Oak Alley	Mycotoxin Community Meeting
5:00 pm - 6:00 pm	Bayside C	TDRM Members Meeting
5:00 pm - 7:00 pm	Bayside A	Committee on Safety Meeting
5:30 pm - 6:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Advanced Chemistry Development, Inc. (ACD/Labs)
6:00 pm - 7:00 pm	Lagniappe	TDRM Members Reception, Co-Sponsored by Silliker
6:00 pm - 7:30 pm	Ellendale	Europe Section Executive Committee Meeting
6:15 pm - 7:45 pm	Bayside B	Contaminants Subgroup Meeting - Metals
7:00 pm - 8:00 pm	Southdown	China Section Business Meeting

Wednesday, September 21, 2011

7:15 am - 8:15 am	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Phenomenex
7:30 am - 5:00 pm	Napoleon Foyer	Registration Open
7:45 am - 8:15 am	Napoleon Foyer	Refreshment Break
8:15 am - 9:45 am	Rhythms 1/2	Hot Topic SYMPOSIUM: Antioxidant Testing - "Fit for Purpose" Goes Beyond Statistics
8:15 am - 9:45 am	Waterbury	SYMPOSIUM: Method Validation and Method Performance Criteria of Chemical Analysis
8:15 am - 9:45 am	Napoleon A	SYMPOSIUM: Methods Harmonization - CURRENT Global Validation Schemes
9:45 am - 10:15 am	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: Covance Laboratories
9:45 am - 10:15 am	Napoleon	Refreshment Break
10:00 am - 12:00 pm	Nottoway	AOAC Research Institute Board of Directors Meeting
10:00 am - 12:00 pm	Gallery	Collaborative Study for Pesticide Multiresidues in Tea - Progress Report
10:00 am - 5:00 pm	Napoleon	POSTER PRESENTATIONS: General Methods, Quality Assurance and Accreditation, Botanicals and Dietary Supplements, and *Performance Tested Methods*℠
10:15 am - 11:45 am	Rhythms 1/2	SYMPOSIUM: Unique Challenges of Dietary Fiber Assays
10:15 am - 11:45 am	Waterbury	SYMPOSIUM: Pyrrolizidine Alkaloids, Undesirable Plant Toxins in Food and Feed
10:15 am - 11:45 am	Napoleon A	SYMPOSIUM: Microbial Method Criteria Used in the Global Meat Industry
11:30 am - 1:00 pm	Napoleon	Poster Author Presentations
11:45 am - 1:00 pm	Borgne	Technical Programming Council Meeting
12:00 pm - 1:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: AB SCIEX
1:00 pm - 2:00 pm	Bayside B	TDLM Executive Committee Meeting
1:00 pm - 2:30 pm	Rhythms 1/2	SYMPOSIUM: Refining, Validating and Implementing LC and LC-MS Methods for Marine Toxins
1:00 pm - 2:30 pm	Napoleon A	SYMPOSIUM: New Methods - New Surrogates for Efficacy Testing of Antimicrobials
1:00 pm - 2:30 pm	Waterbury	SYMPOSIUM: Recent Advances in Veterinary Drug Research and Analysis
2:30 pm - 3:00 pm	Napoleon	Refreshment Break
2:30 pm - 3:00 pm	Rhythms 3	EXHIBITOR/PARTNER PRESENTATION: bioMérieux
2:30 pm - 3:30 pm	Jefferson Suite #4904	Meet Your Board of Directors
2:30 pm - 4:30 pm	Nottoway	AOAC Research Institute Advisory Council Meeting
3:00 pm - 4:30 pm	Waterbury	SYMPOSIUM: Best Practices for the Determination of Small Molecule Contaminants in Foods
3:00 pm - 4:30 pm	Napoleon A	SYMPOSIUM: New Trends in Natural Toxins for Targeted/Non-Targeted Analysis by Using Mass Spectrometers
3:00 pm - 4:30 pm	Rhythms 1/2	SYMPOSIUM: Chemical Stability and Metabolism of Shellfish Toxins
4:30 pm - 6:00 pm	Maurepas	AOAC INTERNATIONAL Business Meeting
7:00 pm - 10:00 pm	Armstrong	Annual Meeting Closing Reception

Thursday, September 22, 2011

8:30 am - 1:00 pm	Bayside A	Grocery Manufacturers Association Meeting
9:00 am - 4:00 pm	Bayside C	Official Methods Board Meeting
1:00 pm - 5:00 pm	Southdown	Juice and Juice Products Community Meeting

附件二

Simultaneous Determination of Multiclass Veterinary Drug Residues in Porcine Liver by Liquid Chromatography-Electrospray Tandem Mass Spectrometry

Hsin-Fang Lu[a], Yu-Yun Tang[ab], Che-Yang Lin[a], Hsu-Yang Lin [a,*], Deng-Fwu Hwang[b], Lih-Ching Chiueh[a], Daniel Yang-Chih Shih[a]

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[b] Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan

Abstract

The aim of this study was to develop a rapid method by using liquid chromatography tandem mass spectrometry (LC/MS/MS) combined with positive electrospray ionization to identify different classes of 15 antibiotics in porcine liver (macrolides, β-lactam antibiotics, lincosamides and miscellaneous antibiotics). Sample preparation was included liquid/liquid extraction followed by methanol, Na_2EDTA and acetonitrile. The extracts were cleaned up by hyflo supercel, and were evaporated to dryness under a stream of nitrogen. The chromatography was carried out on a Waters Acquity UPLC HSS T3 column, mobile phase component A was water with 0.005% formic acid, while component B was acetonitrile. The average recoveries were 50.2% to 115.8 %, and coefficients of variation were from 2.8% to 15.1 %. Estimated limits of quantification were 0.25- 10 *ppb*.

Key words: Liquid Chromatography Tandem Mass Spectrometry; LC/MS/MS, macrolides, β-lactam, lincosamides

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Introduction

Veterinary drugs are widely used for the treatment and prevention of disease in livestock . Main veterinary drugs used today include β-lactams, sulfonamides, tetracyclines aminoglycosides, chloramphenicol, macrolides and quinolones. Despite the positive effects of these drugs, inadequate use of antibiotics poses a potential health risk to consumers. Multiclass, multi residue methods are gaining importance for residue control in food products. Microbiological, immunological assays and liquid chromatography (LC) with ultraviolet (UV) or fluorimetric detecter are the traditional screening techniques, but they are often lengthy and not sufficiently specific for analytical purposes. Liquid chromatographic–tandem mass spectrometric (LC–MS/MS) affords a highly specific and rapid method for simultaneous determination of a number of residual veterinary drugs in foods.

In this report, a rapid and simple method for simultaneous determinaion of 15 antibiotics in porcine liver, from different classes of antibiotics namely, macrolides, β-lactam antibiotics, lincosamides and miscellaneous antibiotics, is described.

Materials and Methods

Materials

Porcine liver was purchased from supermarkets.

Sample preparation

Weight 1g of homogenized sample and mix with with 10 mL methanol and 0.5 mL 0.1M Na_2EDTA. The mixture was centrifuged at 3,200 *x g* for 10 min, and then the supernatant was decanted to the new centrifuge tube. The pellet was homogenize with 15 mL acetonitrile, and the homogenate was centrifuge for 10 min at 3,200 *x g*. The supernatant was combined with the first extraction portion, and the solid remnant was discarded. 2 g Hyflo Super-Cel was added to the extract and shake vigorously for 5 min The mixture was centrifuged at 3,200 *x g* for 10 min, and then the supernatant was decanted to the new centrifuge tube. The pellet was washed with 10 mL acetonitrile, centrifuge for 10 min at 3,200 *x g*. The extract was combined and dryness with a stream of N_2 at 35℃ water bath. The residue was dissolved with 1 mL 50% acetonitrile and was filtered through a 0.2 μm PVDF syringe filter for LC-MS/MS analysis.

LC-MS-MS analysis

The LC separation was performed on Waters ACQUITY UPLC System with a HSS T3 column (1.8 μm, 2.1*100 mm). The mobile phase consisted of a gradient of 0.005% formic acid solution (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 0.3 mL/min showed in Table 1. The mass spectrometry measurement was performed on a triple quadrupole mass spectrometer XevoTM TQ from WATERS. The instrument was working with an electrospray ion source (ESI) in positive mode under multiple reaction monitoring (MRM) conditions which are shown in Table 2. The following mass spectrometer parameters were used for all substances: capillary voltage, ion source temperature, desolvation temperature, desolvation flow, con gas flow were 2.5 kV, 150℃, 600 ℃, 1200 L/hr and 26 L/hr respectively.

Method validation

Recovery was performed in triplicate by analysing blank samples, which was fortified at four concentration levels (25, 50, 100 and 200 ng/g) by using matrix-matched calibration spiking blank extracts at six different concentration levels (from 10 to 300 ng/g). Intra-day precision was studied at four concentration levels (25, 50, 100 and 200 ng/g), using triplicate per concentration level. Inter-day precision was studied spiking blank samples at the same concentration levels, and they were analysed at three different days. Limits of detection (LOD) and limit of quantification (LOQ) were determined as the minimum concentration of analyte providing a signal to noise (S/N) ratio with 3 and 10 as the minium.

Table 1. Parameters of liquid chromatography conditions

Mobile phase	A: Water, containing 0.005% formic acid. B: Acetonitrile		
Gradient program	Time (min)	A (%)	B (%)
	0.0	100	0
	0.0→1.5	100→100	0→0
	1.5→4.0	100→50	0→50
	4.0→7.0	50→20	50→80
	7.0→9.0	20→20	80→80
	9.0→13.0	20→5	80→95
	13.0→14.0	5→5	95→95
	14.0→14.1	5→100	95→0
	14.1→20.0	100→100	0→0
Flow rate	0.3 mL/min		
Injection volume	10 μL		
Analysis time	20 min		

Table 2. The MRM transitions and parameters of 15 veterinary drugs and internal standard

Compound	Abbre-viation	Retention time (min)	Parent ion (m/z)	Transition 1 (CE)	Transition 2 (CE)	Transition 3 (CE)	Cone voltage	Ion ratio (%)**
clarithromycin	CLA	4.7	748.7	115.9(44)	**158.0*(32)**	590.5(20)	28	26.2 (0.7)
erythromycin	ERY	4.4	734.6	116.0(46)	**158.1*(32)**	576.5(18)	26	38.4 (1.3)
natamycin	NAT	4.4	666.5	463.3(32)	485.3(14)	**503.3*(12)**	54,18,18	55.9 (4.1)
oleandomycin	OLE	4.3	688.6	116.0(42)	**158.0*(28)**	544.5(16)	24	46.4 (1.8)
tilmicosin	TIL	4.1	869.8	115.9(64)	132.0(50)	**174.1*(46)**	70	28.5 (3.2)
troleandomycin	TRO	5.0	814.7	**200.1*(26)**	158.0(46)	116.0(46)	34	15.3 (0.5)
virginiamycin M1	VIR	5.4	526.4	231.0(36)	337.1(22)	**355.2*(18)**	24	82.7 (2.0)
cefoperazone	CEO	4.3	646.4	**143.1*(40)**	526.2(10)	530.2(18)	18,18,28	63.9 (4.4)
cloxacillin	CLO	5.5	436.2	114.0(34)	160.0(12)	**277.1*(14)**	16	100.7 (4.7)
mecillinam	MEC	3.8	326.3	122.1(36)	139.1(30)	**167.1*(22)**	32	13.0 (0.3)
oxacillin	OXA	5.3	402.3	114.1(32)	160.0(12)	**243.1*(12)**	16	41.9 (4.0)
clindamycin	CLI	4.1	425.3	**126.1*(28)**	377.2(20)	389.3 (18)	30	5.9 (0.3)
lincomycin	LIN	3.5	407.3	**126.1*(30)**	172.1(22)	359.3(18)	32	7.9 (0.3)
morantel	MOR	4.0	221.1	**122.9*(34)**	111.0(26)	164.0(28)	42	97.7 (3.0)
orbifloxacin	ORB	3.9	396.3	226.1(42)	267.1(36)	**295.1*(24)**	32	16.7 (0.4)
roxithromycin (I.S.)	ROX	4.7	837.8	**158.1*(36)**	679.5(22)	522.4(26)	32	63.7 (3.6)

* Transitions with bold numbers were used for quantification.
** Relative standard deviation (RSD) is given in parentheses (n=18).

Result and Discussion

Extraction solvent comparison

Initial experiments were aimed at finding the best solvent in term of recovery of the analytes. Methanol , acetonitrile, and 0.1M Na_2EDTA, methanol, acetonitrile were selected for this study. A porcine liver samples were spiked with solution of standard mixture then extracted with different extraction solvent, and results are shown in Fig. 1. The results showed that, compared to methanol, acetonitrile afforded much higher analyte recovery to most of macrolides but the result of β-lactam antibiotics are opposite. Therefore, the suitable solvent for the subsequent experiments were performed by using methanol, 0.1M Na_2EDTA and acetonitrile.

Validation of method

Validation parameter including recoveries, intra-day and inter-day coefficient of variation, LODs and LOQs. Calibration was performed by use of matrix-matched calibration standards. The average recoveries of macrolides were 50.2-104.8%, β-lactam antibiotics were 77.4-104.1%, lincosamides were 100.9-115.8%, orbifloxacin was 105.9% and morantel was 114.5%, the results are summarized in Table 3. LODs and LOQs were tested by analysing blank samples, which was fortified seven concentrations (0.25, 0.5, 1, 2.5, 5, 10 and 20 ng/g). The LOD values of macrolides were between 1-10 ng/g, β-lactam antibiotics were between 2.5-10 ng/g, licosamides were between 0.5-2.5 ng/g, morantel was 5 ng/g and orbifloxacin was 2.5 ng/g, the results are summarized in Table 4. Four concentrations of mixed standard solutions of the fifteen antibiotics were used for analyzing the intra-day and inter-day repeatability. Each concentration was analyzed three times for intra-day repeatability. For inter-day repeatability, each concentration was analyzed three times for three days. The coefficients of variation of intra-day and inter-day assays were lower than 15.8% and 18.6%, respectively (Table 5).

Conclusions

A multiresidue method was developed for rapid and simultaneous determination of 15 antibiotics in porcine liver by LC/MS/MS. The rapid extraction and the appropriate clean-up procedure provide good validation parameters, make it suitable for the routine residue monitoring.

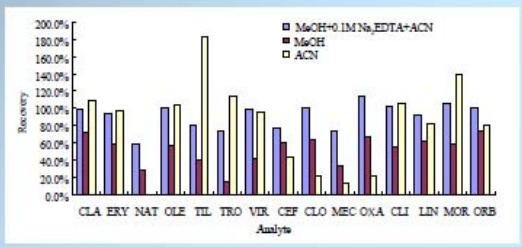

Fig 1. The recoveries of 15 veterinary drugs in porcine liver with different extraction buffers.

Table 3 The recoveries of 15 veterinary drugs in porcine liver sample

Drug / Spiked standard (ppb)	Recovery (%) n = 3				
	25	50	100	200	average
Macrolides					
clarithromycin	100.8	102.8	102.2	103.7	102.4
erythromycin	109.1	107.0	101.1	101.9	104.8
natamycin	53.7	51.0	45.3	50.6	50.2
oleandomycin	99.5	103.2	96.3	100.2	99.8
troleandomycin	62.7	65.4	60.4	59.6	62.0
tilmicosin	75.7	91.5	88.0	84.2	84.9
virginiamycin M1	74.3	77.2	72.0	73.2	74.2
β-lactam					
cefoperazone	75.6	88.2	74.2	71.7	77.4
cloxacillin	84.9	109.6	109.1	112.6	104.1
mecillinam	76.7	81.3	76.5	76.1	77.7
oxacillin	82.6	109.6	110.1	106.4	102.2
Licosamides					
clindamycin	116.6	121.9	113.2	111.5	115.8
lincomycin	106.4	101.6	97.4	98.3	100.9
Miscellaneous					
Orbifloxacin	111.5	104.8	100.4	106.9	105.9
morantel	117.9	118.8	110.4	110.7	114.5

Table 4 Limits of detection (LODs) and limits of quantitation (LOQs) of 15 veterinary drugs in porcine liver

Drug	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)
Macrolides		
clarithromycin	0.5	1
erythromycin	1	2.5
natamycin	5	10
oleandomycin	0.5	1
troleandomycin	1	2.5
tilmicosin	2.5	5
virginiamycin M1	5	10
β-lactam		
cefoperazone	5	10
cloxacillin	2.5	5
mecillinam	1	2.5
oxacillin	5	10
Licosamides		
clindamycin	1	2.5
lincomycin	0.25	0.5
Miscellaneous		
morantel	2.5	5
orbifloxacin	1	2.5

Table 5 Intra-day and inter-day coefficient of variation of 15 veterinary drugs in porcine liver at various spiked levels

Drug / Spiked standard (ppb)	Intra-day / Inter-day, C.V. (%) n = 3				
	25	50	100	200	average
Macrolides					
clarithromycin	5.8/17.0	9.3/11.2	8.8/11.6	5.8/10.9	7.4/12.7
erythromycin	3.7/10.8	7.9/9.3	6.8/7.2	7.7/8.4	6.6/8.9
natamycin	10.2/13.0	14.4/12.7	9.5/8.0	9.6/6.3	10.9/10.0
oleandomycin	15.8/11.2	11.9/10.6	12.0/10.4	13.1/7.5	13.2/9.9
troleandomycin	7.9/18.6	6.4/13.0	13.5/15.1	9.5/13.7	9.3/15.1
tilmicosin	7.6/12.1	2.3/4.8	0.5/4.0	0.9/3.6	2.8/6.1
virginiamycin M1	10.6/4.0	14.7/10.0	1.0/5.8	3.7/7.3	7.5/6.8
β-lactam					
cefoperazone	14.8/13.3	11.5/12.0	13.3/10.1	4.6/11.9	11.0/11.8
cloxacillin	7.4/9.4	10.6/5.8	7.6/11.8	8.0/12.8	8.4/10.0
mecillinam	11.7/9.2	8.9/9.4	9.6/7.8	9.9/5.4	10.0/8.0
oxacillin	6.2/14.3	12.2/5.2	12.5/8.7	9.8/13.4	10.2/10.4
Licosamides					
clindamycin	6.1/6.8	6.5/9.6	6.8/8.5	7.3/7.4	6.7/8.1
lincomycin	8.8/9.1	5.8/5.0	8.7/7.8	8.2/8.3	7.9/7.6
Miscellaneous					
orbifloxacin	5.5/10.3	7.3/9.4	9.9/7.8	8.4/5.4	7.8/8.2
morantel	6.8/7.4	6.4/6.9	6.8/5.1	7.9/4.2	7.0/5.9

Viruses in Water Samples from Foodborne Outbreaks in Taiwan (2010)

Kao-Shien Hsu, Che-Yang Lin, Shu-Wan Wang, Tsui-Ping Huang, Hsu-Yang Lin, Lih-Ching Chiueh, Daniel Yang-Chih Shih
Section of Food Biology Analysis, Division of Research and Analysis, Food and Drug Administration, Department of Health, Republic of China.

Abstract

Enteric viruses cannot multiply in the environment, but they may survive longer in water than most intestinal bacteria and are more infectious than most other microorganisms. In many respects, PCR is more effective than conventional cell culture and has proven to be a rapid, sensitive, specific and inexpensive method for detecting viruses. We developed methods to concentrate and detect viruses in water. Water sample 100 mL was concentrated by Amicon ultra-15 centrifugal filter units. For 1000 mL water, it was filtered through negatively charged membrane first, then the eluate was further concentration by using Amicon ultra-15 centrifugal filter units. Viral RNA was detected by reverse transcription PCR (RT-PCR) after RNA extraction. While HAV (strain HM175) were inoculated into 15 and 1000 mL distilled water, the detection limits were 50 and 100 genome equivalents, respectively. Thirty-two water samples, from foodborne outbreaks in Taiwan throughout 2010, were examined. The results showed, among norovirus GI, norovirus GII, HAV and astroviruses, the detected ratios were 3.1%, 9.4%, 12.5%, and 25%, and sapovirus, rotavirus (A~C), and HEV were non-detected at all.

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ABSTRACT

Enteric viruses cannot multiply in the environment, but they may survive longer in water than most intestinal bacteria and are more infectious than most other microorganisms. In many respects, PCR is more effective than conventional cell culture and has proven to be a rapid, sensitive, specific and inexpensive method for detecting viruses. We developed methods to concentrate and detect viruses in water. Water sample 100 mL was concentrated by Amicon ultra-15 centrifugal filter units. For 1000 mL water, it was filtered through negatively charged membrane first, then the eluate was further concentration by using Amicon ultra-15 centrifugal filter units. Viral RNA was detected by reverse transcription PCR (RT-PCR) after RNA extraction. While HAV (strain HM175) were inoculated into 15 and 1000 mL distilled water, the detection limits were 50 and 100 genome equivalents, respectively. Thirty-two water samples, from foodborne outbreaks in Taiwan throughout 2010, were examined. The results showed, among norovirus GI, norovirus GII, HAV and astrovirus, the detected ratios were 3.1%, 9.4%, 12.5%, and 25%, and sapovirus, rotavirus (A~C), and HEV were non-detected at all.

INTRODUCTION

Enteric viruses and enterically transmitted hepatitis viruses have been associated with many outbreaks of nonbacterial gastroenteritis or hepatitis in different countries every year. These viruses are transmitted by the human fecal-oral route either via contaminated food and water or person to person spread through body contact. Enteric viruses are high stable in the environment, maintaining their infectivity even after exposure to treatment processes. Viral contamination of wastewater, recreational water, drinking water, irrigation water, ground or subsurface water has been frequently reported. Considering the low infectious dose of these viruses, only a small amount, present in the contaminated water, is usually sufficient to infect a human host. Thus, it is important to develop sensitive and efficient methods to detect viruses in water. Several concentration methods for viruses, in water samples, have been described. Among them, the filtration technique seems promising, since it enables the filtration of a large amount of water, while eliminating simultaneously potential inhibitors present in the sample. Food-borne viruses are the second most important cause of food-borne outbreaks in the European Union (EU) after Salmonella. In 2009, they were responsible for 19% of all outbreaks in the EU causing over 1000 outbreaks and affecting more than 8700 citizens. The total number of outbreaks caused by viruses has been increasing since 2007. In the United States, approximately 21 million illnesses attributable to norovirus are estimated to occur annually. According to data from the Real-time Outbreak and Disease Surveillance System operated by the Taiwan Centers for Disease Control (Taiwan CDC), there were around 50% reported diarrhea clusters tested positive for gastroenteritis virus infection, and caused by a variety of viruses, among them rotavirus and norovirus were the two most common agents. We had established a rapid method and applied to detect viruses in water samples from foodborne outbreaks in Taiwan.

MATERIALS AND METHODS

Viral strain. HAV RNA working reagent containing approximately 2000 genome equivalents of HAV strain HM175 (purchased from NIBSC) was used to spike into 15 and 1000 mL distilled water.

Concentration methods. For 100 mL samples, inoculated water was concentrated using Amicon ultra-15 centrifugal filter units. For 1000 mL samples, inoculated water was filtered through negatively charged membrane and the eluate was further concentrated using Amicon ultra-15 centrifugal filter units. The procedures were shown in Figure 1.

RT-PCR assay. One-step RT-PCR was performed in a reaction mixture (25 μL) contained 5 μL of RNA sample, 1.5 μL of each primer (10 μM), 5 μL of QIAGEN OneStep RT-PCR Buffer, 1 μL of dNTP Mix, 1 μL of Enzyme Mix and 10 μL of RNase-free water. The RT-PCR program was as following: reverse-transcription at 50℃ for 30 min, followed by 40 cycles of 95℃ for 30 s, 50℃ for 30 s, 72℃ for 1 min and final extension at 72℃ for 10 min. The primers used in this study was shown in Table 1.

Electrophoresis and sequencing. PCR products were analyzed on 2% agarose gel and electrophoresis was carried out. The PCR amplicons were directly sequenced by ABI 3730 XL DNA Analyzer. The resulting sequences were compared with other nucleotide sequences in GenBank.

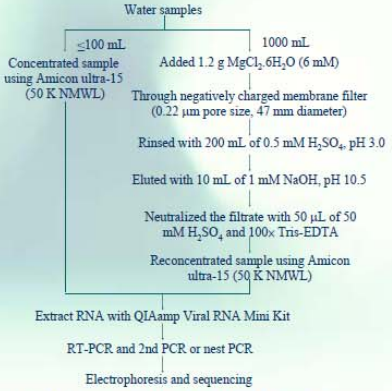

FIGURE 1. Steps to concentrate and detect viruses from water samples.

TABLE 1. Primer pairs used in this study

Target virus	Primer set	Sequence 5' to 3'	Product size (bp)
Norovirus GI	COG1F/ G1SKR	5'-CGYTGGATGCGNTTYCATGA-3' 5'-CCAACCCARCCATRTACA-3'	383
Norovirus GII	QNIF2D/ G2SKR	5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3' 5'-CCRCCNGCATRHCCRTTRTACAT-3'	391
Astrovirus	MON340/348	5'-CGTCATTATTTGTTGTCATACT-3' 5'-ACATGTGCTCGCTGTTACTATG-3'	289
Enterovirus	EV05/EV06	5'-CACGGACACCCAAAGTA-3' 5'-CAAGCACTTCYGTTCCCCGG-3'	400
HAV	VP1-4/5	5'-CGTTGCTTCCCATGTCAGAG-3' 5'-GACCTTCCCATAAACTTGTAG -3'	369
HEV	HEV-F/R	5'-CCTTGGGCCTAGAGTGTGCT-3' 5'-ACCGGCGAAGCGCACGACA-3'	406
Sapovirus	SLV5317/ SLV5749	5'-CTCGCCACCTACRAWGCBTGGTT-3' 5'-CGGRCYTCA AAVSTACCBCCCCA-3'	434
Rotavirus A	Beg9/VP7-1	5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3' 5'-ACTGATCCTGTTGGCCATCCTTT-3'	395
Rotavirus B	ADG9-1F/1R	5'-GGCAATAAAATGGCTTCATTGC-3' 5'-GGGTTTTTACAGCTTCGGCT-3'	814
Rotavirus C	G8NS1/G8NA2	5'-ATTATGCTCAGACTATCGCCAC-3' 5'-GTTTCTGTACTAGCTGGTGAA-3'	351

Detection limit. 50 and 100 genome equivalents of HAV were inoculated into 15 mL and 1000 mL distilled water and obtained final concentrations of 3.3 genome equivalents/mL for 15 mL samples and 10^{-1} genome equivalents/mL for 1000 mL. The preparation was then handled by methods described above.

Determination of occurrence of viruses in water samples from foodborne outbreaks. The occurrence of norovirus GI, norovirus GII, astrovirus, enterovirus, HAV and HEV in 32 suspicious water samples of foodborne outbreaks was determined by using the methods we developed.

RESULTS AND DISCUSSION

Concentration methods. When using negatively charged membrane to concentrate virus, addition of cation to a freshwater sample was necessary in the virus adsorption to a membrane. The concentration of the cation also played an important role. The optimized concentration of cation in our study was about 6 mM. Higher and lower concentration both decreased absorption.

RT-PCR assay. The lowest detection limit of the RT-PCR assay was approximately 4 genome equivalents/reaction, as shown in Figure 2. The size of PCR amplicons was 396 bp.

FIGURE 2. Detection limit of RT-PCR. Lane M: 100 bp marker; Lane 1-4: 4,8,12,16 genome equivalents of HAV/reaction; Lane N: no template control, NTC.

Detection limit. Since the levels of human enteric virus in water are normally low, detection methods with a high sensitivity are needed. The detection limits of the developed methods were 50 genome for 15 mL samples and 100 genome for 1000 mL samples, (Figure 3 and 4).

FIGURE 3. Detection limit for 15 mL samples. Lane M: 100 bp marker; lane 1-2: 50, 100 genome equivalents of HAV/15mL; lane N: NTC.

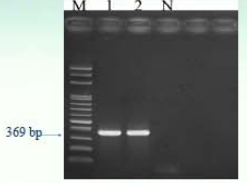

FIGURE 4. Detection limit for 1000 mL samples. Lane M: 100 bp marker; lane 1-2: 100 genome equivalents of HAV/L; lane N : NTC.

TABLE 2. Occurrence of virus in water samples

	Percentage of positive sample	
Year	2009	2010
Norovirus GI	6.8%	3.1%
Norovirus GII	25%	9.4%
Astrovirus	0%	25%
Enterovirus	34%	0%
HAV	2.3%	12.5%
HEV	0%	0%
Sapovirus	0%	0%
Rotavirus(A,B,C)	0%	0%
Sample number	44	32

Occurrence of viruses in water samples. By means of the developed methods, GI norovirus, GII norovirus, astrovirus, enterovirus and HAV were detected in real water samples. The results were showed in Table 2.

CONCLUSION

In summary, we developed sensitive RT-PCR methods for detecting virus in water samples. The developed methods were applied to the detection of viruses in 32 suspicious water samples from 2010 foodborne outbreaks in Taiwan and effectively detected GI norovirus, GII Norovirus, astrovirus and HAV in these samples. Hopefully, it could help to increase the detecting rate of etiology of foodborne outbreaks.

teamwork

Food authenticity ®

LATEST NEWS; According to Berita Harian newspaper published on 2011.03.09 in Malaysia, at the request of Deputy Minister of Science, Technology and Innovation Minister, a survey of, three of 15 gelatin capsules pharmaceutical were positive in terms of pig DNA. (http://www.bharian.com.my/bharian/articles/SaintisMuslimbertanggungjawabbangunkansumberhalalkapsulgelatin/Article/)

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Identification of animal species origin of gelatine used in processed foods in Turkey & Germany

Turkish delight and marshmallow demonstrated that the method could also detect an addition of 1% by weight of porcine gelatine. In a survey of 29 retail gelatine products from Turkey 1 positive sample was found which was non-compliant, and in 14 samples from Germany 2 were positive but....

By P. Ulca, H.Balta, İ.Çağın & H.Z. Şenyuva

There are very detailed and specific provisions concerning authentication of foods as Halal and/or Kosher, such as the prohibition of products derived from certain animal species such as pork. The inadvertent inclusion of material which is not Halal and/or Kosher might occur with gelatine products (including confectionary and bakery products), where it can be difficult to verify the authenticity of the gelatine. For the detection of pork DNA in samples of gelatine a PCR method has been validated involving DNA extraction (using SureFood® Prep Animal X) and amplification using the SureFood® Animal ID Pork Sens Plus kit. The viability of the method was demonstrated by spiking porcine gelatine into authenticated samples of bovine, fish and seaweed gelatine. No false positives were found, but the false negative rate was around 2%. Adulteration of Halal and/or Kosher gelatine could be reliably detected by rt-PCR at a level of 1% w/w. Spiking into blank gelatine-based products such gumdrops, Turkish delight and marshmallow demonstrated that the method could also detect an addition of 1% by weight of porcine gelatine. In a survey of 29 retail gelatine products from Turkey 1 positive sample was found which was non-compliant, and in 14 samples from Germany 2 were positive but these were in conformity with labeling.

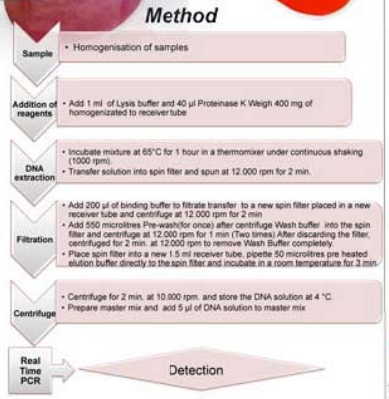
Gelatine is 83% protein produced by partial hydrolysis of collagen extracted from bones, connective tissues and skin of animals or fish. An EU Regulation (EC) No 853/2004 permits the use of bones, hides and skins of farmed ruminant animals, pig skins, poultry skins, tendons and sinews, wild game hides and skins and fish skin and bones for the manufacture of gelatine. The majority of edible gelatine in Europe (about 80%) is derived from pig skin. However, a significant portion (about 15%) of edible gelatine is of bovine origin, coming from the hide splits. The remaining 5% is of bone origin, extracted from porcine, bovine, poultry, or fish species.

Once gelatine has been manufactured and purified, it is virtually impossible to tell its origin without sophisticated biochemical analysis. Gelatine is widely used as a gelling agent (E441) in a diverse range of food products such in desserts, aspic, marshmallows, and confectioneries such as 'gumdrops'. For these types of food to qualify as Halal or Kosher, the gelatine must obviously come from non-porcine sources, and must come from animals slaughtered ritually. With a global trade in raw materials and food ingredients such as gelatine, it is very difficult to ensure authenticity and thus it is of some interest to be able to carry out rigorous tests of processed foods in Turkey.

Samples

Twelve gelatine samples of known species origin (bovine, porcine or seaweed) were obtained in powder or sheet form. Fifty five of the soft and fruity chew confectionery (gumdrop). Turkish delight and marshmallow containing gelatine were provided from markets in Turkey and Germany. For gelatine, samples of known species origin (i.e., cattle, pig, or dried seaweed) supplied in granules, powders or bar were mixed to form binary gelatine admixtures containing 0.1, 0.3, 1, 5, 10 or 100% (wt/wt) pork species gelatine within porcine or other gelatine backgrounds, and then genomic DNA template extraction was performed. The samples were stored at -20°C before the extraction of DNA to facilitate homogenization with High Throughput Homogeniser.

Method



Results and Discussion

DNA template isolation. Gelatine is highly difficult processes that involves controlled acidic or basic hydrolysis of connective tissue raw material, high temperature extraction with water, sterilization, and drying. In the final gelatine product (which are the sample matrices for laboratory), both proteins and nucleic acids are highly degraded. We began by attempting to obtain enough template DNA from gelatine for analysis.

Selectivity and sensitivity of the real-time- PCR assays for gelatine material
The percentage of pork amount in the samples are variables to verify the sensitivity of this method. Analyzing of gumdrop containing 0% pork gelatine, 3% pork gelatine, 5% pork gelatine as five parallels for each samples. And marshmallow containing 0% pork gelatine, 0.1% pork gelatine, 0.5% pork gelatine and 3% pork gelatine were analyzed as five parallels. Control samples were detected as a negative. Gumdrop including 0.5% pork gelatine was not detected each parallels. Additionally, Expected results were obtained from gumdrop containing 3 and 5% pork gelatine.
Results of marshmallow including 0.1% pork gelatine were not detected but 0.5, 3 and 5% pork gelatine were detected in terms of pork DNA.
To confirm the effectiveness and the specificity of these results, 55 products obtained from Turkey and Germany, of 14 retail products purchased in Germany, 2 samples were found to contain porcine gelatine, with the remaining 12 samples being negative. The 2 positive samples had Ct values of 30.04 and 43. Of a total of 29 samples from Turkey 28 were found to be negative. However, one product (cake covered with gelatine) from the Turkish retail market was found to have a Ct value of 36.3 clearing indicating that this product contained porcine DNA. This product was therefore not Halal and the labeling failed to indicate the use of porcine gelatine. Spiking of all samples with 5% porcine gelatine provided confirmation that the test was functioning adequately, although one false negative was detected for a sample remained negative even after spiking.

Quality Assurance
Control parameters were performed. Negative control and extraction control were detected as a negative. Positive control was also positive.
According to **TS EN 24276**, false negative rate must be ≤ 5%. False negative rate was calculated as 1.96% for containing 5% pork gelatin.

100% pork gelatin	10 % pork gelatin	1% pork gelatin
28.07	32.09	33.55
28.57	32.29	41.89
28.44	32.48	37.04
28.34	32.21	36.14
28.87	33.92	41.59
28.43	31.63	37.32

Table 1. Ct values of 100%, 10% and 1% pork gelatine

Products	Total no samples	Porcine negative	Porcine positive
Powder gelatine	8	2	6
Sheet gelatine	4	-	4
Marshmallow/cake	17	16	1
Gum-drops	22	20	2
Jelly	3	3	-
Turkish delight	1	1	-
Total	55	42	13

Table 2 Results of survey of samples obtained from Turkey and Germany

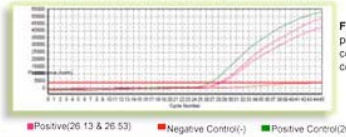


Figure 1. PCR amplification plots for samples of gelatine containing positive sample and controls .

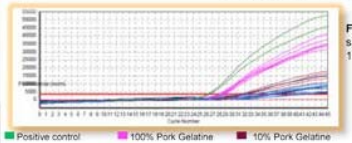


Figure 2. PCR amplification plots for samples of gelatine containing 100%, 10% and 1% pork gelatine .

Acknowledgement
The authors gratefully acknowledge the support of CONGEN Biotechnologie GmbH (Berlin, Germany) and Sincer Dis Ticaret (Izmir, Turkey) for the supply of SureFood® kits used for the development and validation work.

Designed and prepared by hamide.senyuva@foodlife.com

附件四

Development of group testing method to evaluate weight per weight GMO content in maize grains

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Introduction

Food labeling regulations in some countries refer to the genetically modified (GM) material on a weight/weight (w/w) percentage, while the most commonly used technique for GM organism (GMO) quantification in grain is the quantitative real-time PCR analysis of bulk sample homogenates, and the analysis typically measures GMO contents based on the ratio of GM DNA to plant species DNA. Due to the increasing use of GM stacked events which have GM DNA corresponding to two or more single events, the commonly used bulk sample methods for the PCR quantification of GM maize in non-GM maize are prone to overestimate the GMO content, compared to the actual w/w percentage of GM maize in the grain sample. Recently a single kernel-based analytical system was developed and implemented in Japan as an official method, as one possible solution for the potential overestimation due to stacked events[1]. In this method, individual maize kernels are analyzed to determine their GM or non-GM status, and the w/w GMO content is evaluated based on a postulation that the ratio of GM kernels relative to the total number of kernels equals the w/w ratio. As an alternative method, we examined a group testing strategy in which the GMO content is statistically evaluated based on qualitative analyses of multiple small pools of maize kernels. This approach enables the efficient GMO content evaluation on a w/w basis, irrespective of the presence of stacked event kernels. In fact, the theoretical applications of the group testing strategy to GMO analysis have been investigated[2-4]. To improve the user-friendliness of the method for routine application, we attempted to devise an easy-to-use PCR-based qualitative analytical method comprised of a sample preparation step in which 20 maize kernels are ground in a lysis buffer, and a subsequent PCR assay in which the lysate is directly used as a DNA template[5].

Testing protocol based on Ampdirect technology

Step 1
Preparation of groups
(5 min)

Groups comprised of 20 maize kernels in glass vessels with the capacity of 75 mL for use with a Milser 600-DG household food processor (Iwatani, Tokyo, Japan).

- To add 20 ml of a lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 400 mM NaCl, 0.3% SDS)
- To grind samples by the Milser 600-DG for 20 s
- To incubate samples for 10 min at room temperature
- To shake samples vigorously by hand
- To stand samples statically for 10 min at room temperature for solid-liquid separation

Step 2
Sample grinding and cell lysis
(30 min)

50 µL of supernatant in plastic tube

- To add 50 µL of sterile water for dilution
- To centrifugate with more than 1,000 × g by a personal benchtop centrifuge for 1 min

Template DNA solution for PCR

Step 3
Real-time PCR assays
(140 min)

PCR mixtures

	For 7900HT	For 7500
Ampdirect plus buffer (Shimadzu corporation)	12.5 µl	12.5µl
Primer probe solution	5µl	5µl
50 × ROX dye	0.5µl	0.05µl
dDW	4.375µl	4.825µl
RicTaq HS polymerase	0.125µl	0.125µl
Template DNA sol.	2.5µl	2.5µl
	25µl	25µl

For GM maize screening assay,
primer probe solution was comprised of
2.5 µM P35S 1-5', 2.5 µM P35S 1-3' 0.5 µM P35S-taq (FAM-BHQ1)
2.5 µM TNOS 2-5', 2.5 µM TNOS 2-3', and 0.5 µM TNOS-taq (see ref. 6)

For experimental control assay,
primer probe solution was comprised of
2.5 µM IPC 1-5', 2.5 µM IPC 1-3' 0.5 µM IPC 1-taq (FAM-BHQ1)
2.5 µM SSIIb 1-5', 2.5 µM SSIIb 1-3', 0.5 µM SSIIb-taq (HEX-BHQ1),
and theoretical 8 copy of pART plasmid shown on the right side. (see ref. 6)

Thermal cycling by real-time PCR ABI7900HT or 7500
95°C 10min → (95°C 15 sec → 60°C 1 min) × 45 cycles

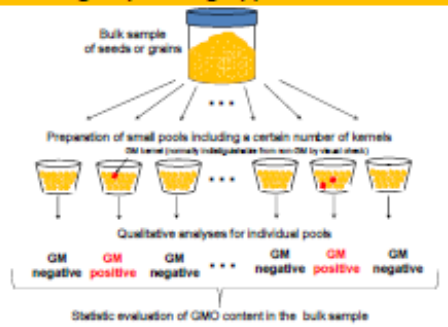
GM maize screening assay (Duplex real-time PCR): P35S (FAM-BHQ1) / TNOS (FAM-BHQ1)
Experimental control assay (Duplex real-time PCR): SSIIb (HEX-BHQ1) / IPC* (FAM-BHQ1)
Detection of all GM maize events commercially distributed
Confirmation of PCR inhibition and sufficient DNA extraction
*Internal Positive Control

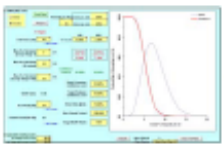
Data analysis

Step 4
GM positive/negative determination
(5 min)

Software: Sequence Detection Software Version 2.3 for 7900HT system
Sequence Detection Software Version 1.4 for 7500 system
Settings: The manual Ct mode (threshold; 0.258 for FAM and 0.084 for HEX)
The manual baseline mode (start of baseline; 3; end of baseline; 15)
at "Delta Rn vs. Cycle" view of the "Amplification Plot" feature
Judgement: DNA amplifications with Ct values below 40 were determined to be positive. If the SSIIb or IPC detection is determined to be negative by the experimental control assay, the group in question is rejected. If both SSIIb and IPC are positive, the group is determined to be either GM positive or GM negative based on the result of the GM maize screening assay.

Overview of group testing applied to GMO testing

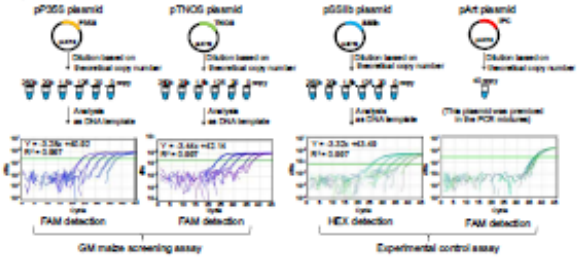




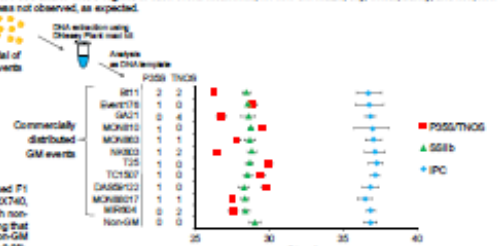
The judgement of acceptance or rejection are performed based on the preliminarily determined criterion. The established Excel spread applications, such as Seedcalc from International Seed Testing Association, facilitate the statistic calculation and the set of criterion. Based on the Seedcalc program, we can design the group testing which has the approximately same accuracy of judgments in the single kernel based method as a Japanese official testing method. The designed sampling plan is as follows: A group is comprised of 20 maize kernels and 10 groups are applied for the first screening. If there are 7 or more GM positive groups in the first screening, another set of 10 groups will be tested. If the total numbers of GM positive groups in the two tests (20 groups) combined are 12 or less, the GMO content of the bulk sample is determined to be below 4%.

Performance evaluation of the real-time PCR assays

The pUC19 plasmids harboring each of the target sequences, P35S, TNOS, SSIIb, and IPC, were prepared and named pP35S, pTNOS, pSSIIb, and pART, respectively. The plasmids were purified by cesium chloride/ethidium bromide equilibrium centrifugation and then diluted to the given concentration based on the theoretical copy numbers. We confirmed the amplification linearity by using dilution series of plasmids except for pART. Regression lines were calculated by the mean values of triplicate analyses, and their parameters were shown in the individual amplification plot. Detection results for P35S, TNOS, and SSIIb showed high coefficient values (> 0.990).

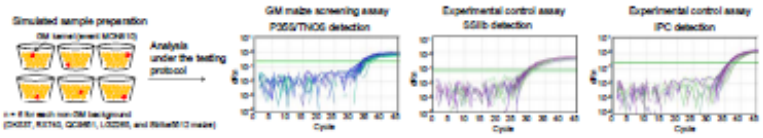


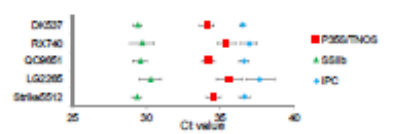
Subsequently, we confirmed the detection specificity with genomic DNAs from commercially distributed GM maize events and non-GM crops. P35S and/or TNOS regions were detected for all GM maize events, and these Ct values roughly corresponded to the copy numbers of P35S and/or TNOS regions in each event. Meanwhile, for non-GM maize, soy, wheat, barley, and rice, non-specific detection was not observed, as expected.



Demonstration using simulated samples

We performed analyses with the simulated groups of a maize sample that included one GM kernel among 20 kernels. As the GM maize kernel, we used F1 generation seeds of the MON810 event, which has the lowest copy number of the target of the GM maize screening assay. As non-GM materials, DK537, RX740, QC9651, LG2295, and Strike5512 maize were individually used. The results of real-time PCR assays for all samples and summary of Ct value data for each non-GM background (means ± standard deviations, n = 8) are shown below. No false negative result was observed in the GM maize screening assay, suggesting that the testing protocol had the capacity to detect at least one GM kernel in a group. Homogeneity of Ct value-variances of SSIIb detection between different non-GM materials was confirmed by Bartlett's test ($\alpha = 0.05$), and one-way analysis of variance (ANOVA) ($\alpha = 0.05$) of the data showed no significant differences ($p = 0.66$). This indicated that stable DNA extraction was achieved by the testing protocol, irrespective of maize materials.





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5) Yamamura, K. and Hino, A. Commun. Stat. Simulat. 2007, 36, 949-957.
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Acknowledgements
We thank Monsanto, Pioneer Hi-Bred International, and Syngenta Seeds for providing plant materials. This research was funded by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan ("Assurance of Safe Use of Genetically Modified Organisms" and "Research Project for Genomics for Agricultural Innovation GAM-211") and by a grant from the Ministry of Health, Labour and Welfare of Japan.

附件五


BAYSIDE C
AOAC
INTERNATIONAL
Taiwan Section
Business Meeting
PEPSICO
2011/09/20


2011/09/20


2011/09/20


2011/09/20

附件六


220
Development of group testing method
to evaluate weight per weight GMO content in maize grains
AOAC
AOAC


AOAC
Annual Meeting
AOAC
Annual Meeting

附件七

8月23日 初校

Identification and Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus YK Strain

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Unauthorized genetically modified (GM) papaya (*Carica papaya* LINNAEUS) was detected in a commercially processed product, which included papaya as a major ingredient, in Japan. We identified the transgenic vector construct generated based on resistance to infection with the papaya ringspot virus (PRSV) YK strain. A specific detection method to qualitatively monitor papaya products for contamination with the GM papaya was developed using the real-time polymerase chain reaction.

Key words genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* LINNAEUS) is an important fruit crop in tropical and subtropical areas.[1)] Infection with the papaya ringspot virus (PRSV) causes disastrous damage to papaya harvests.[2)] In response to this problem, genetically modified (GM) papayas have been developed in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, Philippines and Vietnam.[2)]

Japan has announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients, and the importation of any unauthorized GM foods to Japan has been prohibited since April 1, 2001. Therefore, the establishment of qualitative detection methods for unauthorized GM foods was required for monitoring purposes. We previously developed and reported qualitative detection methods for various GM crops, such as potato,[3,4)] maize,[5—8)] rice,[9)] and flax,[10)] using polymerase chain reaction (PCR) methods. In the case of papaya, we established a qualitative detection method for GM papaya (Line 55-1), which was the first commercialized PRSV-resistant GM papaya developed in Hawaii, using a PCR test and a histochemical assay.[11—13)] Line 55-1 was authorized in Japan after a safety assessment by the Ministry of Health, Labor and Welfare of Japan in 2009.[14)] Since Japan imports many papayas from Southeast Asia, we are required, in Japan, to monitor commercially processed products that include papaya as a major ingredient for contamination with other unauthorized GM papayas generated in the region.

GM papayas carry the transgenic vector construct generated based on resistance to PRSV infection by expressing the PRSV's coat protein (CP) gene. Since the other unauthorized GM papayas developed may differ in the transgenic vector construct of the authorized GM papaya (Line 55-1), we developed a method for detecting contamination with unauthorized GM papaya. In the present study, we found the unauthorized GM papaya, PRSV-YK, in processed products containing papaya as a major ingredient, papaya-leaf-tea, pickles and jam, and developed a method for the detection of PRSV-YK using the real-time PCR.

MATERIALS AND METHODS

Papaya Samples Papaya products were purchased through the internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

Purification of DNA Dried papaya leaves in papaya-leaf-tea, papayas in pickles and Sunset sarcocarp were ground using a mixing mill. Papaya jam was used for purification of DNA without grinding. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; QIAGEN, Hilden, Germany) as follows: 30 ml Buffer G2 (QIAGEN), 20 μl 100 mg/ml RNase (QIAGEN) and 500 μl cellulase (Sigma-Aldrich, St. Louis, MO, U.S.A.) were added to the sample and vortexed thoroughly, then incubated at 50 °C for 1 h. The mixture was incubated at 50 °C for another 1 h after the addition of 200 μl Proteinase K (QIAGEN). During the incubation, the samples were mixed several times by inverting the tubes. The samples were then centrifuged at 3000×***g*** at 4 °C for 20 min. The supernatant was applied to a Genomic-tip 100/G column (QIAGEN), which was pre-equilibrated with 4 ml Buffer QBT (QIAGEN). The tip was washed three times with 7.5 ml Buffer QC (QIAGEN) and transferred to a fresh centrifuge tube, and 3 ml pre-warmed Buffer QF (QIAGEN) (50 °C) was added to elute the DNA. The DNA sample was transferred to a centrifuge tube, an equal volume of isopropyl alcohol was added, and the sample was mixed thoroughly. DNA was collected by centrifugation at 12000×***g*** for 15 min. The pellet was rinsed with 1 ml 70% (v/v) ethanol and centrifuged at 12000×***g*** for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 20 μl water for use in analyses. The DNA was quantified by measuring UV absorption at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

PCR Each PCR reaction mixture (25 μl) contained 2.5 μl 10×PCR buffer II (Life Technologies, Carlsbad, CA, U.S.A.), 0.16 mM deoxyribonucleotide triphosphate (dNTP) (Life Technologies), 1.5 mM $MgCl_2$, 1.2 μM forward and reverse primers, 0.8 U AmpliTaq Gold (Life Technologies) and 25 ng template DNA. The PCR conditions were as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s and terminal elongation at 72 °C for 7 min. PCRs were carried out using the GeneAmp PCR System 9700 (Life Technologies). To determine the nucleotide sequence of the transgenic vector construct harbored in GM papaya, DNA

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fragments were amplified by PCR using the following primer set. Forward primer: 5′-GACATCTCCACTGACGTAAGGG-3′ (p324). Reverse primer: 5′-CTATCRCTCTCTCCAGTTT-TTG-3′ (p323).

DNA Sequencing The PCR-amplified DNA fragments were extracted from the agarose-gel and purified using a QIAquick PCR purification kit (QIAGEN). The DNA fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide sequences were analyzed using Lasergene version 7.2 software (DNASTAR Inc., Madison, WI, U.S.A.).

Real-Time PCR Real-time PCR assays were performed using the ABI PRISM™ 7900 Sequence Detection System (Life Technologies). The 25 μl reaction mixture consisted of 2.5 μl sample DNA solution (25 ng), 12.5 μl Universal Master Mix® (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. The PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. GM papaya was detected using the following primers and probe. Forward primer: 5′-GATCCCCGGGTGGTCAGT-3′ (YK-1F). Reverse primer: 5′-CCGGTATCCACAGCTTCATTTT-3′ (YK-1R). Probe: 5′-FAM-AGACGCCATGGAAGG-MGB-3′ (YK-P).

For detecting the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession No.: AY803756), we designed the following primers and probe referring to published report.[15] Forward primer: 5′-CCATGCGATCCTC-CCA-3′ (Q-Chy-1F2). Reverse primer: 5′-CATCGTAGCCA-TTGTAACACTAGCTAA-3′ (Q-Chy-2R). Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCATTCCCACTCTTGAGA-3′ (Q-Chy-P). BHQ1 (black-hole quencher 1) was labeled for Q-Chy-P at the underlined thymidine in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for ABI PRISM™ 7900 Sequence Detection System.

Real-Time PCR Data Analysis Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting the cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reactions with Ct values of less than 48 and exponential amplification plots were scored as positive. If the Ct value could not be obtained, the reaction was

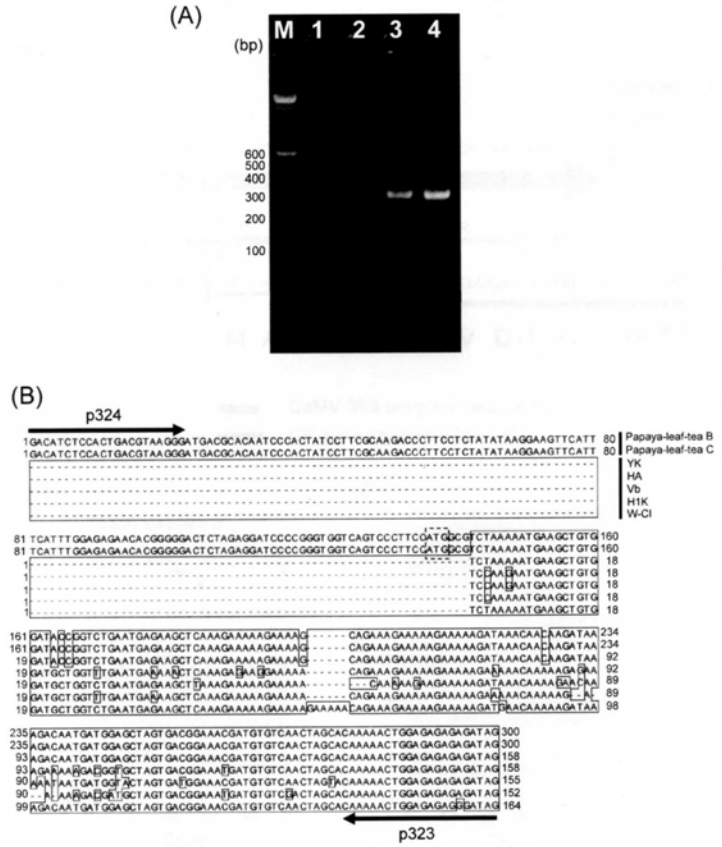

Fig. 1. PCR Targeting Construct Specific Sequence of PRSV-Resistant GM Papaya

(A) DNA templates (lane 1, non-GM papaya (Sunset); lane 2, papaya-leaf-tea A; lane 3, papaya-leaf-tea B; lane 4, papaya-leaf-tea C) were used for the PCR test using the p324 and p323 primer set. The PCR-amplified products were run on a 3% (w/v) agarose-gel. Lane M, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from YK, HA, Vb, H1K, W-CI strains and the PCR products obtained using papaya-leaf-tea B and C. Homologous sequences were boxed. The initiation codon for the GM papaya was boxed with a dashed line. Numerals beside the sequence indicate the numbers of nucleotides from the 5′ terminus.

scored as negative. Reactions with Ct values of less than 48, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multi-component plots were scored as negative.

RESULTS AND DISCUSSION

Detection of Unauthorized GM Papaya To investigate the contamination with unauthorized GM papaya in commercially processed products, containing papaya as a major ingredient, in Japan, we used genomic DNA purified from the papaya-leaf-tea products as a template for the PCR test. The forward primer (p324) was designed to hybridize in the cauliflower mosaic virus (CaMV) 35S promoter sequence, which is the most common promoter used in the transformation of papaya for various GM papaya traits,[2] and the reverse primer (p323) was designed in the highly conserved sequence of the CP gene, which is cloned from various strains of PRSV (GenBank accession no.: YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). Electrophoresis of the PCR products using p324 and p323 primers showed a single band of about 300 bp in length using DNA purified from two of the three papaya-leaf-tea products (papaya-leaf-tea B and C) (Fig. 1A). The DNA purified from non-GM papaya (Sunset) as a control and papaya-leaf-tea A generated no PCR products with the identical length. Direct sequence analysis of the PCR product and BLASTn analysis indicated that the 3′ end sequence was identical to the CP gene in a Taiwan isolate of PRSV (PRSV YK strain)[16] (Fig. 1B). Furthermore, the multiple cloning site (containing restriction sites for *Bam*HI and *Nco*I) and two amino acid mutations (methionine and alanine) between the CaMV 35S promoter and the N-terminus of CP gene were detected (Fig. 2A). According to the literature,[17] the design of this transgenic vector construct was identical to that of the GM papaya, which was generated to resist infection of the PRSV YK strain. These results suggest that the papaya-leaf-tea products were contaminated with the unauthorized GM papaya (PRSV-YK).

Development of a Construct-Specific Detection Method for PRSV-YK In order to qualitatively detect PRSV-YK in processed products, containing papaya as a major ingredient, with high specificity and sensitivity, we designed specific primers and a probe for a real-time PCR assay producing a short amplicon (57 bp), based on the detected transgenic construct sequence. The forward (YK-1F) and the reverse (YK-1R) primers were designed in the region between the transgenic vector backbone and the CP gene sequence. The probe

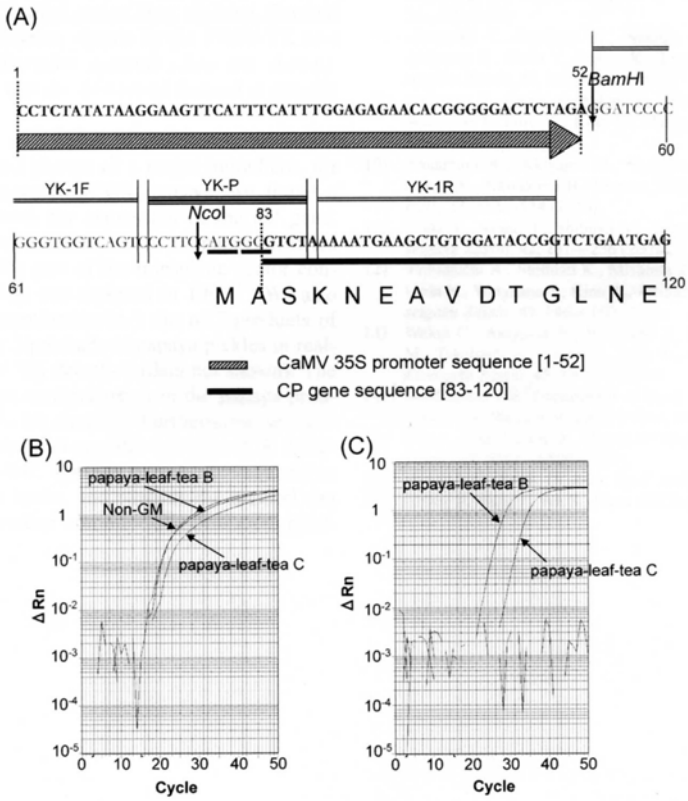

Fig. 2. Detection of PRSV-YK Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence was obtained and restriction sites were marked by vertical arrows. Design of the primers (YK-1F and YK-1R) and the probe (YK-P) for detecting construct-specific sequence of PRSV-YK is indicated by lines above the sequence. Numerals indicate the numbers of nucleotides from the 5′ terminus. (B) Endogenous *Chy* detection using a primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) (C) PRSV-YK detection using a primer set (YK-1F and YK-1R) and probe (YK-P). The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

(YK-P) was designed on the site of the initiation codon of the CP gene (Fig. 2A).

Since the forward primer sequence for detecting the papaya endogenous internal control gene, *Chy*, had an unintentional error of a single nucleotide sequence in the previous report[15] (according to personal communication), we used the right sequence for the forward primer (Q-Chy-1F2), the reverse primer (Q-Chy-2R) and the probe (Q-Chy-P). The real-time PCR assay for PRSV-YK detection confirmed that the papaya-leaf-tea products B and C were positive for PRSV-YK, producing Ct values of 25.93 and 31.88 with a threshold value of 0.2, respectively. Endogenous *Chy* detection was positive for all samples, with the papaya leaf-tea product B, C and the non-GM papaya (Sunset) producing Ct values of 21.55, 23.82 and 21.45, respectively, with a threshold value of 0.2 (Figs. 2B, C). The copy numbers of PRSV-YK construct and *Chy* sequence were calculated from Ct values using standard curves which were generated using the positive control plasmid. Papaya-leaf-tea products B and C contained 1 copy of PRSV-YK construct sequence in 27 copies and 167 copies of *Chy* sequence, respectively (data not shown). Because the genetic background of PRSV-YK used in the processed papaya products was unknown, estimation of the content of PRSV-YK in a papaya product was not possible. The non-template control and the genomic DNA derived from other crops, such as maize, rice, soybean, flax and canola, gave no amplification signals in the PRSV-YK and the endogenous *Chy* detection systems (data not shown). These results indicated that the developed method is specific for detecting PRSV-YK.

In the present study, as a result of monitoring processed products, which included papaya as a major ingredient, for contamination with unauthorized GM papaya, we found a transgenic vector construct for expression of the CP gene, which was cloned from the YK strain, in papaya-leaf-tea products. The design of a part of the transgenic vector construct was identical to the one reported in 1996.[17] We also detected PRSV-YK contamination in 1 out of 7 products of papaya jam and 2 out of 3 products of papaya pickles in real-time PCR test for PRSV-YK detection (data not shown). The origin of the GM papaya contamination in the papaya products in Japan remains to be clarified. Furthermore, we successfully developed a construct-specific real-time PCR detection method for PRSV-YK. Further studies are required to determine the detection limits, and whether the method can be used for detection in other commercially processed products containing papaya as a major ingredient.

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附件八

食安監発０２２２第３号
平成２３年２月２２日

各（都道府県／保健所設置市／特別区）衛生主管部（局）長　殿

厚生労働省医薬食品局食品安全部監視安全課長

安全性未審査の遺伝子組換えパパイヤ（PRSV-YK）の
暫定検査法について

このたび、国立医薬品食品衛生研究所において、台湾で開発され、国内においては食品衛生法上、安全性未審査である遺伝子組換えパパイヤ(PRSV-YK)の検査法を検討し、その暫定検査法を別添のとおり策定しましたので、検査にあたっては本検査法により実施してください。

安全性未審査の遺伝子組換えパパイヤ(PRSV-YK)の検査

1. 検体採取方法

1.1 生鮮パパイヤの検体採取

生鮮パパイヤの検体採取については、対象となるロットの大きさに応じて以下の表に従い検体採取を行うこと。

ロットの大きさ	検体採取のための開梱数	検体採取量(個)
≦ 50	2	2
51 ～ 500	3	3
501 ～ 35,000	5	5
≧ 35,001	8	8

1.2 パパイヤ加工食品の検体採取

パパイヤ加工食品の検体採取については、対象となるロットの大きさに応じて以下の表に従い検体採取を行うこと。

以下の表に従って検体採取を行う。

ロットの大きさ	検体採取のための開梱数	検体採取量(g)※1	検体数
≦ 15	2	120	1
16 ～ 50	3	120	1
51 ～ 150	5	120	1
151 ～ 500	8	120	1
501 ～ 3,200	13	120	1
3,201 ～ 35,000	20	120	1
35,001 ～ 500,000	32	120	1
≧ 500,001	50	120	1

※1果汁・飲料製品、氷菓等製品については、検体採取量を480gとする。

また、パパイヤの含有量が少ない加工品について実施する場合は、製品分類ごとに複数回の前処理試行が可能となるよう適宜検体採取量を増やして採取する。

2. 検査原則

当検査は、生鮮パパイヤ及び種々の加工食品が検査対象検体として想定されるため、その性状により測定結果は変動する。これらを縮小するための原則について記す。

- 検査対象検体は、一検体数を一単位とする。
- 検査対象検体の食さない部分を廃棄した可食部を試料とする。（生鮮パパイヤについては種子・果皮を除いた果肉部分）

・ 試料中の成分は、不均一に分布すると考えられるため、検査に供する前に試料全量を粉砕器等*1で十分に破砕し、均質混和して調製試料とする。
・ 検査に供する調製試料は固体や液体の性状に関わらず、重量測定にて一定量を採取する。
・ 試料調製を含む検査全般は、空気の動きがなく温度・湿度の変動が少ない区切られた空間で行い、コンタミネーションを防ぐよう実施する。
・ 微量測定のため、粉砕用器具*1容器、秤量用器具、凍結乾燥瓶は中性洗剤等で洗浄後、アルカリ洗剤に一晩浸け置きする。あるいは超音波洗浄機を用い、30 分間の超音波処理を行う。

*1 レッチェ GM200（レッチェ社製）、Millser(Iwatani 社製)、磁製乳鉢・乳棒及び同等の結果が得られるものを用いる。

3. リアルタイム PCR 法を用いた定性 PCR 法

本法では生鮮パパイヤおよびパパイヤ加工食品を検査対象とし、DNA抽出精製は、以下の陰イオン交換樹脂タイプキット法（QIAGEN社製Genomic-tip 100/G）を用いる。1検体からDNAを抽出し、DNA試料液を得る。そのDNA試料液を用いてリアルタイムPCRを用いた定性PCR法を実施する。生鮮パパイヤおよびパパイヤ加工食品は以下の７種類の製品に細分類し、「3.1 試料前処理」に示したそれぞれの試料前処理プロトコルに従ってDNA抽出精製前の試料調製を行う。

① 生鮮および調味漬け製品 （生鮮パパイヤ、缶詰、漬物など乾固されていないある程度パパイヤの原型を保持している試料）
② 乾物製品 （乾燥パパイヤ）
③ 砂糖漬け乾燥製品 （ドライフルーツ）
④ 乾燥製品 （健康食品、お茶など）
⑤ 果肉含有ゲル状製品 （ジャム、ピューレなど）
⑥ 果汁・飲料製品 （フルーツミックスジュース、ドリンク剤など）
⑦ 氷菓等製品 （アイス、シャーベットなど）

3.1 試料前処理

① 生鮮および調味漬け製品

製品から目視でパパイヤと判断されるもののみを全て取り出し(生鮮パパイヤについては種子・果皮を除いた果肉部分)、その重量の 2 倍以上の滅菌蒸留水で 3 回洗浄した後、よく水分をきり、Millser 等で粉砕する(生鮮パパイヤに関しては果肉を洗浄せず粉砕する)。粉砕した試料 10g をポリプロピレン製遠沈管（50mL 容）に量りとり、G2 緩

衝液[*1]30 mL を加え、よく転倒混和して均質とする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い、以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

なお、生鮮パパイヤについては、平成 13 年 3 月 27 日付食発第 110 号「組換え DNA 技術応用食品の検査方法について」の 2.2.2.に示す方法により DNA 抽出精製を行うことも可能である。

② 乾物製品

製品から目視でパパイヤと判断されるもののみを全て取り出し、Millser 等で粉砕する。粉砕した試料 2g をポリプロピレン製遠沈管（50mL 容）に量りとり、G2 緩衝液[*1]30mL を加え、よく転倒混和して均質にする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

③ 砂糖漬け乾燥製品

製品から目視でパパイヤと判断されるもののみを全て取り出し、その重量の 2 倍以上の滅菌蒸留水で 3 回洗浄した後、等重量分の滅菌蒸留水を加え、Millser 等で粉砕する。粉砕した試料 10g をポリプロピレン製遠沈管（50mL 容）に量りとり、G2 緩衝液 30mL[*1]を加え、よく転倒混和して均質にする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い、以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

④ 乾燥製品

Millser 等で粉砕し均質にした試料 2g をポリプロピレン製遠沈管（50mL 容）に量りとり、G2 緩衝液 30mL[*1]を加え、よく転倒混和して均質にする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

⑤ 果肉含有ゲル状製品

Millser 等で粉砕し均質にした試料 10g をポリプロピレン製遠沈管（50mL 容）に量りとり、G2 緩衝液 30mL[*1]を加え、よく転倒混和して均質にする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

⑥ 果汁・飲料製品

開封前によく転倒混和して均質にした製品 100mL をメスシリンダーで量りとり、凍結乾燥用容器（500mL 容）に移し、傾けた状態で-80℃冷凍庫中で 2 時間凍結させる。その後、凍結乾燥機にセットし、24 時間乾燥後、試料 30g を乳鉢に量りとり G2 緩衝液*1 20mL に乳棒を用いて溶解させる。次いで全量をポリプロピレン製遠沈管（50mL 容）に移し、乳鉢と乳棒の残存試料を新たに G2 緩衝液*1 10mL を追加し洗いいれ、よく転倒混和して均質にする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い、以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

⑧ 氷菓等製品

試料 100g を凍結乾燥用容器に量りとり、24 時間凍結乾燥する。その後、試料 10g を先に G2 緩衝液*1 30mL を入れたポリプロピレン製遠沈管（50mL 容）に少しずつ加えながら溶解させ、よく転倒混和して均質にする。イオン交換樹脂タイプの DNA 抽出精製キット（QIAGEN Genomic-tip）を用い、以下の「3.2 パパイヤ試料からの DNA の抽出精製」に従い DNA を抽出精製する。

*1 G2 緩衝液は QIAGEN 社（Cat. No. 19060）に付属しているが、足りない場合には単品で購入するかキットの説明書に従って調製可能である。

3.2 パパイヤ試料からの DNA の抽出精製

3.2.1 DNA の抽出精製

「3.1 試料前処理」を行った試料に、100mg/mL RNase*1 20μL、cellulase*2 500μL を加えて（なお⑤果肉含量ゲル状製品のジャム製品に限り、α-Amylase*3 20μL も同時に加える）、転倒混合し均質化した後、50℃で 1 時間放置する。その間 2～3 回遠沈管を反転させて試料を転倒混和する。次いで Proteinase K*4 200μL を加え 50℃で 1 時間放置する。その間も 2～3 回遠沈管を反転させて試料を転倒混和する。次いで、その遠沈管を 3,000×g、低温下(4℃)、20 分間遠心し、得られた上清（約 25mL～35mL）を採取し、あらかじめ QBT 緩衝液*5 4mL を用い平衡化した QIAGEN Genomic-tip 100/G に負荷する。次いで、100/G を QC 緩衝液*5 で 7.5mL ずつ 3 回洗浄した後、あらかじめ 50℃に温めておいた QF 緩衝液*5 1mL を負荷し、はじめの溶出液は捨てる。新しい遠沈管に移し、再度 50℃に温めておいた QF 緩衝液*5 2mL を負荷し、DNA を溶出する。溶出液と等量のイソプロピルアルコールを加えよく混合し、遠沈管（1.5mL 容もしくは 2.0mL 容）に移し、10,000×g 以上で、低温下（4℃）15 分間遠心後、上清を捨てる。この際、上清を極力除去する*6。70%エタノール 1 mL を加え、さらに 10,000×g 以上で、低温下（4℃）5 分間遠心する。さらに上清を捨て*6、残った沈殿を、乾燥させた後、予め 50℃に温めた滅菌蒸留水 50μL に溶解し、DNA 試料原液とする。

*1 QIAGEN 社（Cat. no. 1018048）のもの又は同等の効力を持つものを用いる。

*2 Sigma-Aldrich 社（Cat. no. C2730-50ML）のもの又は同等の効力を持つものを用いる。

*3 ニッポン・ジーン社（Cat. no. 312-06671）のもの又は同等の効力を持つものを用いる。

*4 Promega 社（Cat. no. V3021）100mg を滅菌水 5mL に溶解したもの又は同等の効力を持つものを用いる。

*5 QBT 緩衝液、QC 緩衝液及び QF 緩衝液は QIAGEN 社（Cat. No. 19060）に付属しているが、足りない場合には単品で購入するかキットの説明書に従って調製可能である。

*6 沈殿物が見えない場合でも、遠沈管内の底部付近にはできるだけ触れないように、上清を除去する。

3.2.2 DNA 試料原液中の DNA の純度の確認並びに DNA 試料液の調製と保存

DNA試料原液の適当量を取り、滅菌蒸留水を用いて適宜希釈*1し、200～320nmの範囲で紫外部吸収スペクトルを測定し、260nm及び280nmの吸光度*2（O.D.260及びO.D.280）を記録する。次いでO.D.260の値1.0を50ng/μL DNAと換算し、DNA濃度を算出する。またO.D.260/O.D.280を計算する。この比が1.7～2.0になれば、DNAが十分に精製されている

ことを示す*3。得られたDNA濃度から、DNA試料原液を10ng/μLに滅菌蒸留水で希釈して調製し、DNA試料液とする。DNA試料液は40μLごとにマイクロ試料管に分注後、－20℃以下で冷凍保存する。分注したDNA試料液は、融解後直ちに使用し、残った溶液は再度保存せず廃棄する。なお、DNA試料原液の濃度が10ng/μLに達しないときは、そのままDNA試料液として用いる。

*1 希釈する場合には、滅菌蒸留水を用いる。また、希釈倍率は、吸光度測定装置により適切な測定に要する液量及び濃度域が異なるため、適宜とする。
*2 O.D.260 がDNA 由来の吸光度、O.D.280 がタンパク質等不純物由来の吸光度と考える。
*3 O.D.260/O.D.280の比が1.7～2.0の範囲外であっても精製等の更なる操作は要さない。

3.3 リアルタイムPCR 法

遺伝子組換えパパイヤ(PRSV-YK)検知試験用として、カリフラワーモザイクウイルス35Sプロモーター配列とPapaya Ringspot Virus coat protein (PRSV-cp)遺伝子の境界領域を検知するプライマー、プローブを用いる。カリフラワーモザイクウイルス35Sプロモーター配列 (CaM) 検知試験用として、CaMを検知するプライマー対、及び、プローブを用いる。また、パパイヤ陽性対照試験用として、Chymopapain (Chy) 遺伝子配列を検知するプライマー、プローブを用いる。各プライマー、プローブは滅菌蒸留水に溶解する。プライマー、プローブの塩基配列は以下のとおりである。

遺伝子組換えパパイヤ(PRSV-YK)検知試験用プライマー対、及び、プローブ
YK-2F: 5’-ACA CGG GGG ACT CTA GAG -3’
YK-2R: 5’-ACC GGT ATC CAC AGC TTC -3’
YK-2P: 5’-FAM- TCC CTT CCA TGG CGT C-TAMRA-3’

CaM配列検知試験用プライマー対、及び、プローブ
35S-F：5’-GCC TCT GCC GAC AGT GGT -3’
35S-R：5’-AAG ACG TGG TTG GAA CGT CTT C-3’
35S-P：5’-FAM- CAA AGA TGG ACC CCC ACC CAC G-TAMRA-3’

パパイヤ陽性対照試験用プライマー対、及び、プローブ*1
Q-Chy-1F2: 5’-CCA TGC GAT CCT CCC A-3’
Q-Chy-2R: 5’-CAT CGT AGC CAT TGT AAC ACT AGC TAA-3’
Q-Chy-P: 5’-FAM-TTC CCT TCA T(BHQ1)CC ATT CCC ACT CTT GAG A-3’

*1 Q-Chy-Pプローブのクエンチャー（消光物質）は、T-baseのblack-hole quencher 1

(BHQ1)を使用する。

3.3.1 リアルタイム PCR 法(Applied Biosystems 7900HT, Applied Biosystems 7500)

3.3.1.1 PCR 用反応液の調製

PCR用反応液は25μL/wellとして調製する。組成は以下のとおりである。TaqMan Gene Expression Master Mix*1 12.5μL、対象プライマー対溶液（各プライマー、50μmol/L）各0.4μL、対象プローブ溶液（10μmol/L）0.25μLを混合し、DNA試料液5μLを添加し滅菌蒸留水で全量25μLに調製する。PCRのブランク反応液として、必ずDNA試料液を加えないものについても同時に調製する*2。分注操作終了後、真上からシール*3し、完全にウェルを密閉する。このとき、しわが寄らないよう注意し、専用のシーリング用アプリケーターを用いて行う。最後にウェルの底を観察し、底に気泡がある場合は、プレートの縁を軽く叩いて気泡を抜いておく。プレートの確認後、ABI PRISM Optical Cover Compression Pad*4を茶色の面が上になるよう、プレートの上面にセットする。DNA試料液あたりパパイヤ陽性対照試験、遺伝子組換えパパイヤ(PRSV-YK)検知試験、およびCaM配列検知試験をそれぞれ2ウェル並行して行うものとする。

*1 TaqMan Gene Expression Master Mix
本試薬は粘性が高いため、混合操作を行う際には、混合が確実に行われるように注意する。不十分な場合には、PCR がうまくいかない場合がある。使う直前には必ず軽く攪拌後、遠心し、溶液を試料管の底に集めておいてから使用する。また、ウェルに分注する際は、以後攪拌、遠心が困難なことを考慮し、ウェルの底に確実に入れる。

*2 Non-Template Control (NTC)
DNA 試料液の添加の際、NTC には DNA 試料液の代わりに滅菌蒸留水をウェルに 5μL 添加する。

*3 96 ウェルプレート、シール、及び、シーリングアプリケーター
MicroAmp Optical 96-Well Reaction Plate （Life Technologies 社)、及び、ABI PRISM Optical Adhesive Cover （Life Technologies 社）を使用する。シーリングの詳細については製品付属のマニュアルを参考のこと。

*4 ABI PRISM Optical Cover Compression Pad
ABI PRISM Optical Cover Compression Pad （Life Technologies 社）を使用する。Applied Biosystems 7500 では使用しない。

3.3.1.2 プレート情報の設定

反応に際しては、プレート情報の設定を行わなければならない。設定を行う項目は、検体の配置と種類、及び、プローブ特性である。具体的には新規シート上で、調製したプレートの配置に対応するように気を付けながら、検体の種類（「NTC」：Non-Template

Control、「UNKN」: DNA 試料液）の設定を行う。またプローブ特性に関しては、YK-2P、35S-P、Q-Chy-P ともに Reporter が「FAM」、Quencher が YK-2P は「TAMRA」、35S-P は「TAMRA」、Q-Chy-P は「Non Fluorescent」となるように設定する。また、Passive Reference は「ROX」に設定する。なお、ランモードの設定は 9600 emulation モードを選択する。Sample Volume は 25μL に設定する。

3.3.1.3 PCR 増幅

装置にプレートをセットし、反応とデータの取り込みを開始する。反応条件は以下のとおりである。50℃、2 分間の条件で保持した後、95℃で 10 分間加温し、ホットスタート法で反応を開始する。その後、95℃ 15 秒間、60℃ 1 分間を 1 サイクルとして、50 サイクルの増幅反応を行う。Remaining time が 0 分となっていることを確認し、反応を終了させた後、測定結果の解析を行う。

3.4 結果の解析と判定

遺伝子組換えパパイヤ(PRSV-YK)検知試験、CaM配列検知試験およびパパイヤ陽性対照試験のいずれについても、結果の判定はAmplification plot上で指数関数的な増幅曲線とCt値の確認、及び、multicomponent上での対象色素由来の蛍光強度(FAM)の指数関数的な明確な増加の確認をもって行う。

遺伝子組換えパパイヤ(PRSV-YK)検知試験およびCaM配列検知試験の両試験とも目視でAmplification plot上に指数関数的な増幅曲線が確認された場合には、遺伝子組換えパパイヤ(PRSV-YK)陽性を疑う。次いで、ベースライン(3サイクルから15サイクル)のΔRnのノイズ幅の最大値の上側で、安定した指数関数的な増幅曲線上で交わるThreshold line (Th. line)を選択する*1。そのTh. lineからCt値が得られるか否かを解析する。DNA試料液においてパパイヤ陽性対照試験の2ウェル並行の少なくとも一方で48未満のCt値が得られ、かつ遺伝子組換えパパイヤ(PRSV-YK)検知試験およびCaM配列検知試験の両試験とも各2ウェル並行全てで48未満のCt値が得られた場合(①)に、当該試料は陽性と判定する。パパイヤ陽性対照試験の2ウェル並行の少なくとも一方で48未満のCt値が得られ、遺伝子組換えパパイヤ(PRSV-YK)検知試験及びCaM配列検知試験の両試験の2ウェル並行全てにおいて48未満のCt値が得られない場合(②)には、遺伝子組換えパパイヤ(PRSV-YK)陰性と判定する(図1参照)。

パパイヤ陽性対照試験の2ウェル並行の少なくとも一方で48未満のCt値が得られ、遺伝子組換えパパイヤ(PRSV-YK)検知試験あるいはCaM配列検知試験の結果の組み合わせが①又は②のいずれにも該当しない場合は、粉砕・均質後の当該試料から改めて2回目*2のDNA抽出精製を行い、さらに「3.3 リアルタイムPCR法」以降の操作を実施して、判定を行う。2回目のDNA試料液を用いた場合でも陽性又は陰性の判定が得られない場合には、改めて3回目*2のDNA抽出精製を行い、さらに「3.3 リアルタイムPCR法」以降の操作を実施して、判定を行う。3回目のDNA試料液を用いた場合でも陽性の判定が得られない場合には、遺伝子組換えパパイヤ(PRSV-YK)陰性と判定する。なお上記により陽性と判定された結果についてmulticomponentを解析し、目視でFAMの蛍光強度の指数関数的な増加が観察でき、ROXの蛍光強度の明確な下降やFAMの蛍光強度の緩やかな上昇がないことを確認する(図1参照)。

また、パパイヤ陽性対照試験の2ウェル並行の両方で48未満のCt値が得られないDNA試料液については、再度、粉砕・均質後の当該試料から改めて2回目のDNA抽出精製を行い、さらに「3.3 リアルタイムPCR法」以降の操作を行い、それでもパパイヤ陽性対照試験の2ウェル並行の両方で48未満のCt値が得られない場合には、粉砕・均質後の当該試料から改めて3回目のDNA抽出精製を行い、さらに「3.3 リアルタイムPCR法」以降の操作を実施して、判定を行う。3回目のDNA試料液を用いた場合でもパパイヤ陽性対照試験の2ウェル並行の両方で48未満のCt値が得られない場合には、本試料からの検知は不能とする(図1参照)。

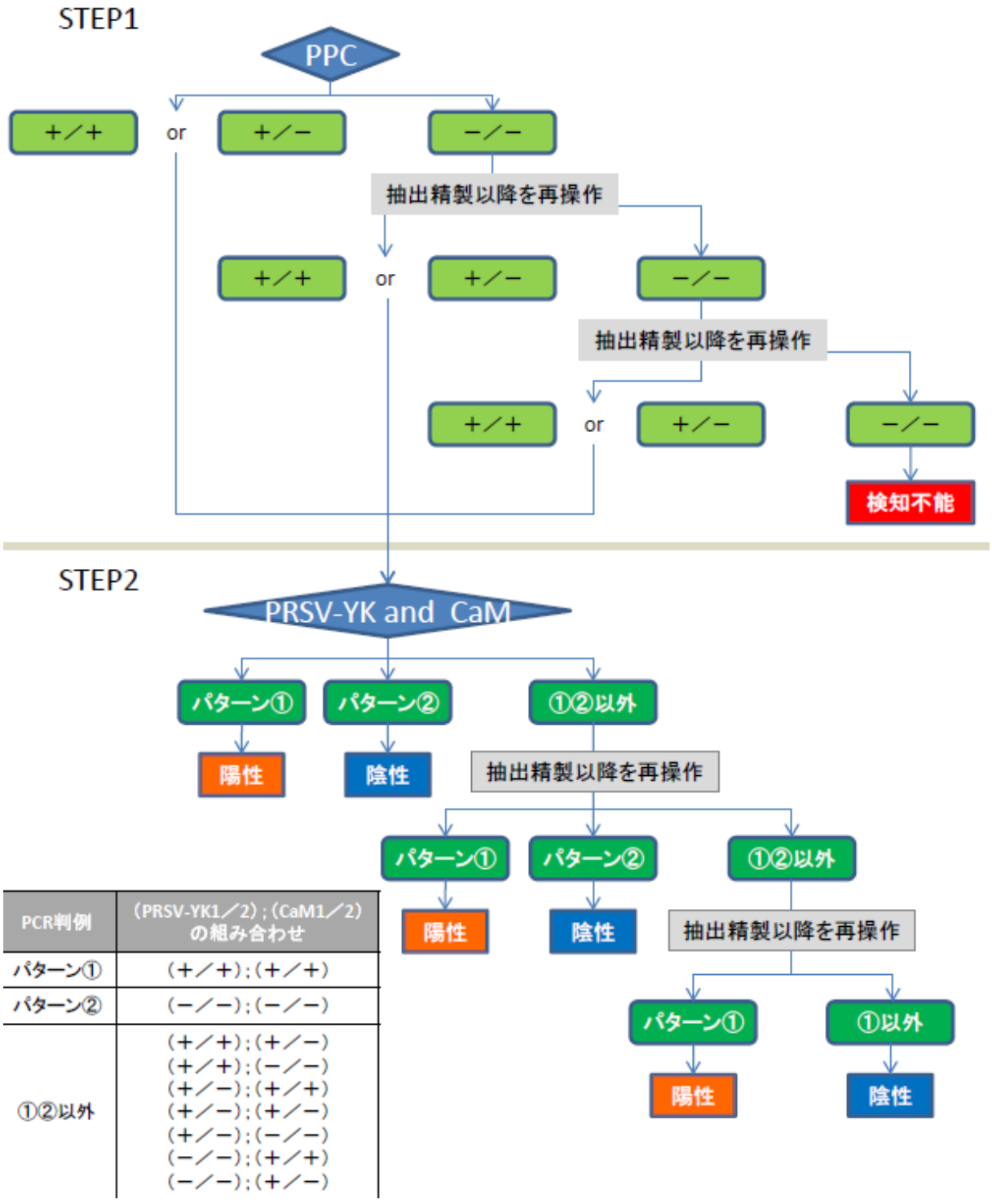
*1 個々の機種の状態によってAmplification plot上のΔRnが変動することから、普遍的なTh.

lineの設定の数値を示すことが困難である。従ってAmplification plot上でベースライン(3サイクルから15サイクル)のΔRnのノイズ幅の最大値の上側で、安定した指数関数的な増幅曲線上で交わるTh. lineを選択する。参考としてApplied Biosystems 7900HT, 及びApplied Biosystems 7500ともに0.2-0.5の範囲であると考えられる。

*2DNA 抽出精製を行うために必要な試料量が不足している場合には、「3.1 試料前処理」から実施する。

図1　結果の判定スキーム

PPC；パパイヤ陽性対照試験
PRSV-YK；遺伝子組換えパパイヤ(PRSV-YK)検知試験
CaM；CaM配列検知試験



PCR判例	(PRSV-YK1／2)；(CaM1／2)の組み合わせ
パターン①	(＋／＋)；(＋／＋)
パターン②	(－／－)；(－／－)
①②以外	(＋／＋)；(＋／－) (＋／＋)；(－／－) (＋／－)；(＋／＋) (＋／－)；(＋／－) (＋／－)；(－／－) (－／－)；(＋／＋) (－／－)；(＋／－)