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(出國類別：研究實習)

食品中毒原因微生物之分子生物檢驗技術

服務機關：衛生署藥物食品檢驗局

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主辦機關:

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關鍵詞: 志賀氏桿菌

內容摘要: 爲能提升本局食品中毒原因菌之分子生物檢驗技術及檢驗品質、加強對食品中毒事件的防治，以維護國民飲食安全與健康，藥物食品檢驗局特別派員赴日本獨立行政法人食品綜合研究所(National Food Research Institute, NFRI)研習相關檢驗技術。本次研習之主要重點爲爲(1)研習食品中毒原因微生物之分子生物檢驗技術相關檢驗技術，作爲研訂方法之參考依據(2)蒐集日本食品中毒微生物、食品中生物毒素之最新檢驗方法及相關資訊與未來檢驗技術發展之趨勢，並建立藥檢局與日本學界之交流。滯日考察期間與負責專家一色賢司(Kenji Isshiki)教授深入研討及溝通，建立溝通及聯絡之管道，對於未來食品中毒微生物之監測、防治及法規研訂方面皆有實質助益。本次考察收獲良多，除帶回日本最新之志賀氏桿菌檢驗方法外、並能研習到最先進之食品中毒原因菌及總生菌數之快速檢測法，包括(1)聚合酶鏈鎖反應(polymerase chain reaction, PCR) (2)Micro Foss 法(3)測溶氧量（DOX method）(4)Menadione-Catalyzed Luminol Chemiluminescent assay。除此，有幸在Dr. Latiful Bari的指導下接觸日本目前用來抑制食品中病原性微生物生長的最新科技產物－－酸性電解水(Electrolyzed acidic water)及Calcinated calcium；並且在一色賢司(Kenji Isshiki)教授等鼎力幫助下，出席日本社團法人舉辦之「日本食品衛生學會第八十五回學術演講會」；參觀先進儀器及設備；參觀食品綜合研究所P2級實驗室。並能了解日本飲食文化，及其易於引起食品中毒事件之食品。 日本開發之酸性電解水(Electrolyzed acidic water)可結合上述微生物快速檢驗方法，搭配HACCP (Hazard analysis critical control point system)系統，應用在餐飲業，爲食品衛生把關。可把檢驗時間縮短到8-24小時以內。其應用於食品衛生方面的潛力不容忽視。在日本食品綜合研究所有關食品中毒原因菌的實驗，基於防護安全上的理由，均在P-2級實驗室內進行。並且，門禁控管嚴格。此

外，對於不同防護層級（如P-1 level或P-2 Level）之實驗室亦有黏貼標籤加以標示，依層級高低對其垃圾或廢棄物作妥善之管理，在微生物實驗室的一些品管措施亦值得我們學習改進。

本文電子檔已上傳至出國報告資訊網

食品中毒原因微生物之分子生物檢驗之檢驗技術

摘　　要

為能提升本局食品中毒原因菌之分子生物檢驗技術及檢驗品質、加強對食品中毒事件的防治，以維護國民飲食安全與健康，藥物食品檢驗局特別派員赴日本獨立行政法人食品綜合研究所(National Food Research Institute, NFRI)研習相關檢驗技術。本次研習之主要重點為為(1)研習食品中毒原因微生物之分子生物檢驗技術相關檢驗技術，作為研訂方法之參考依據(2) 蒐集日本食品中毒微生物、食品中生物毒素之最新檢驗方法及相關資訊與未來檢驗技術發展之趨勢，並建立藥檢局與日本學界之交流。滯日考察期間與負責專家一色賢司(Kenji Isshiki)教授深入研討及溝通，建立溝通及聯絡之管道，對於未來食品中毒微生物之監測、防治及法規研訂方面皆有實質助益。

本次考察收獲良多，除帶回日本最新之志賀氏桿菌檢驗方法外、並能研習到最先進之食品中毒原因菌及總生菌數之快速檢測法，包括(1)聚合酶鏈鎖反應(polymerase chain reaction, PCR) (2)Micro Foss 法(3)測溶氧量（DOX method）(4)Menadione-Catalyzed Luminol Chemiluminescent assay。除此，有幸在 Dr. Latiful Bari 的指導下接觸日本目前用來抑制食品中病原性微生物生長的最新科技產物－－酸性電解水(Electrolyzed acidic water)及 Calcinated calcium；並且在一色賢司(Kenji Isshiki)教授等鼎力幫助下，出席日本社團法人舉辦之「日本食品衛生學會第八十五回學術演講會」；參觀先進儀器及設備；參觀食品綜合研究所 P2 級實驗室。並能了解日本飲食文化，及其易於引起食品中毒事件之食品。

日本開發之酸性電解水(Electrolyzed acidic water)可結合上述微生物快速檢驗方法，搭配 HACCP(Hazard analysis critical control point system)系統，應用在餐飲業，為食品衛生把關。可把檢驗時間縮短到 8-24 小時以內。其應用於食品衛生方面的潛力不容忽視。

在日本食品綜合研究所有關食品中毒原因菌的實驗，基於防護安全上的理由，均在 P-2 級實驗室內進行。並且，門禁控管嚴格。此外，對於不同防護層級（如 P-1 level 或 P-2 Level）之實驗室亦有黏貼標籤加以標示，依層級高低對其垃圾或廢棄物作妥善之管理，在微生物實驗室的一些品管措施亦值得我們學習改進。

目次

前言與目的

食品中毒檢驗為本局重要業務之一，去年(九十一年度)之食品中毒案件數更是創下歷年新高。綜觀國內食品中毒的發生原因殆以微生物為主，而目前傳統之檢驗技術仍有效能不佳，耗費時日與人力之缺點。因此，應用生物技術以解決傳統檢驗方法的缺點，係當前食品病原菌檢驗發展之趨勢。日本臨近台灣，二國飲食習慣互相影響，致食品中毒原因微生物與國內檢出情況相似，其發展之技術亦為本局檢驗方法之重要參考依據。為防治食品中毒原因微生物之散播，冀藉此次赴日本相關研究所研習機會，汲取最新生物技術與經驗，提升檢驗效能。

本次出國考察之主要目的為(1)研習食品中毒原因微生物之分子生物檢驗技術相關檢驗技術，作為研訂方法之參考依據(2) 蒐集日本食品中毒微生物、食品中生物毒素之最新檢驗方法及相關資訊與未來檢驗技術發展之趨勢，並建立藥檢局與日本學界之交流。

內　容

此次赴日研習行程共計 13 日，扣除去返日與假日，實際研習天數為 9 天。研習的機關為農林水產省獨立行政法人食品總合研究所企畫調整部、東京都立衛生研究所、日本厚生省國立醫藥品食品衛生研究所。研習內容包括食品中毒病原菌之快速檢驗方法、食品中志賀氏桿菌之檢驗流程以及參加日本食品衛生檢驗相關研討會，了解其他與食品中毒病原菌相關之訊息等。行程內容整理後摘要如下所述：

五月五日（一）：啟程

上午十點搭乘日本亞細亞航空班機啟程赴日，中午二點抵達日本成田機場。下午五點半左右抵達獨立行政法人食品綜合研究所(National Food Research Institute, NFRI)，由食品企畫部一色賢司(Kenji Isshiki)博士接待。於下午六點半下榻築波國際旅館(Tsukuba International House, TIH)。

七月六日（二）：

1.考察首日，拜會食品綜合研究所企畫調整部長春見隆文(Takafumi Kasumi)、田中健治(Kenji Tanaka)博士、永田忠博(Tadahiro Nagata)博士、日野明寬(Akihiro Hino)博士、食品工學部計測工學研究室長大谷敏郎(Toshio Ohtani)、食品工學部製造工學研究室長五十部誠一郎(Seiichiro Isobe)博士、生物機能開發部細胞機能研究室長矢部希田子(Kimiko Yabe)博士、山本和貴(Kazutaka Yamamoto)博士及一色賢司博士之研究室成員包括橘田和美(Kazumi Kitta)、Dr. Latiful Bari、川崎晉(Sasumu Kawasaki)博士、稻津康弘(Yasuhiro Inatsu)。

2.研讀「食品衛生學」（一色賢司）等相關期刊報告。

五月七日（三）：

1. 研習抑制食品中病原性微生物生長的新科技——Electrolyzed acidic water 的製造原理與其在食品工業上之應用。
2. 專題討論「從微生物觀點日本新興食品 Kimchi(韓國泡菜)的潛在危機」。

五月八日（四）：

1.研習抑制食品中病原性微生物生長的新科技－－Calcinated calcium 在食品工業上之應用。

2.研習應用分子生物技術分型及食品中毒原因菌之快速檢測方法－－Micro Foss 法、測溶氧量（DOX method）、Menadione-Catalyzed Luminol Chemiluminescent assay。

五月九日（五）：

1.參觀食品綜合研究所 P-2 級實驗室。

2.參觀 Electrolyzed acidic water 製造機與設置場所。

五月十~五月十一日（六、日）：假日

1.整理出國考察資料。

2.研讀「食品衛生學」（一色賢司）等相關期刊報告。

五月十二日（一）：

1.考察東京都立衛生研究所，拜會甲斐明美(Akemi Kai)博士並與其研討日本目前志賀氏桿菌之檢驗方法。

2.國立醫藥品食品衛生研究所，拜會山本茂貴博士(Shigeki Yamamoto)、食品部長米谷民雄博士、食品添加物部第三室長河村葉子(Yoko Kawamura)博士、安全情報部第二室室長春日文子(Fumiko Kasuga)博士、Kumiko Sasaki 博士、Tamio Maitani 博士。

3.參觀 Ochanomizu 女子大學，拜會村田容常(Masatsune Murata)博士及森光

康次郎(Yasujiro morimitsu)博士。

五月十三日（二）：

專題演講及討論「日本發酵食品中黴菌毒素的檢出情形」。

五月十四日（三）~五月十五日（四）：

參加日本食品衛生檢驗相關研討會**(Meeting of Food Hygienic Society of Japan)**。

五月十六日（五）：

1.整理出國考察資料。

2.研讀「食品衛生學」（一色賢司）等相關期刊報告。

五月十七日（六）： 歸國

整理行李。搭乘 10 點 25 分日本亞細亞航空班機歸國，於午後一點抵達中正機場。

心 得

一、抑制食品中病原性微生物生長的新科技

(一)酸性電解水(Electrolyzed acidic water)

近年來，在日本由於部份營養學家強調飲食中蔬果對防治心血管疾病的重要性及生機飲食的保建防癌效果，有愈來愈多的人嗜食生菜。然而這也使得食用生鮮蔬果而引起食品中毒的案例愈來愈多。有鑑於此，日本開發一種能製造酸性電解水的機器。該機器的設計是包括(1)12%鹽水(加鹽自來水)入口管路(2)電解槽部份，中間有隔膜區隔正負極(3) 出口管路部份，

即正極流出液(Electrolyzed acidic water)及負極流出液(Electrolyzed alkaline water)出口管路(廢水)。正極流出液即是所謂的 Electrolyzed acidic water，pH 值≦2.7，氧化還原電位(oxidation reduction potential)＞1100 mV，含有游離態氯(free-chlorine)約 10~80ppm 、0.1% NaCl 及 HCl、HOCl 等抑菌成份。其正負極電化學反應原理以化學方程式表示如下：

正極：$2OH^- \rightarrow H_2O + 1/2O_2 + 2e^-$

$2Cl^- \rightarrow Cl_2 + 2e^-$

$Cl_2 + H_2O \rightarrow HOCl + HCl$

負極：$2H^- + 2e^- \rightarrow H_2$

正負極間電位差 10V，電流設定為 19.8 A(安培)。

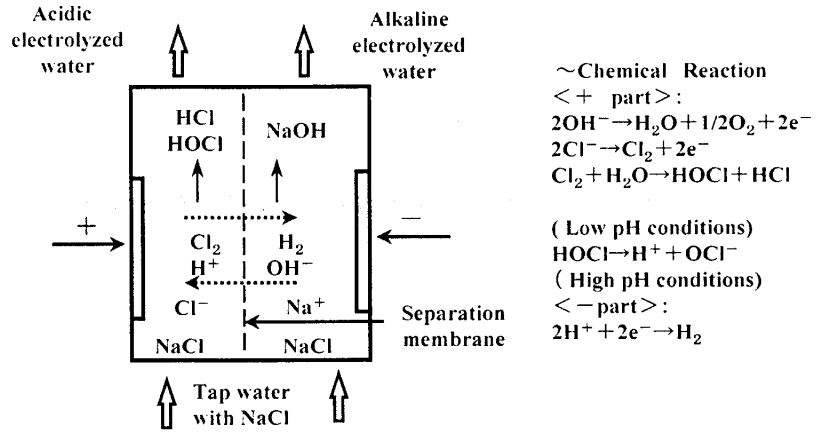
經 Dr. Bari 及一色賢司教授等學者實驗證實，Electrolyzed acidic water 可在模擬洗滌生鮮蔬果的試驗中，有效降低或不活化番茄表面的出血性大腸桿菌血清型 O157:H7 (*Escherichia coli* O157:H7)、沙門氏菌(*Salmonella* Enteritidis)、單核增多性李斯特菌(*Listeria monocytogenes*)；其洗滌及降低上述病原菌的能力優於含 200 ppm 之氯水(chlorine water)。

由於 Electrolyzed acidic water 能有效地抑制食品中病原性微生物在生鮮蔬果上生長。在日本牙醫學界已經有人將它應用在處理病人傷口、消毒醫療器材上。更有學者建議將它應用在餐飲業結合 HACCP(Hazard analysis critical control point system)概念，以去除或不活化食品病原菌。

(二)Calcinated calcium

Calcinated calcium 是一種商品化產品，在日本它已被應用在生鮮食品的清洗以降低菌數。Bari 博士及一色賢司教授實驗亦證實，0.5% (wt/vol) Calcinated calcium 在模擬洗滌生鮮番茄的試驗中，有效降低或不活化番茄表面的出血性大腸桿菌血清型 O157:H7 (*Escherichia coli* O157:H7)、沙門氏菌

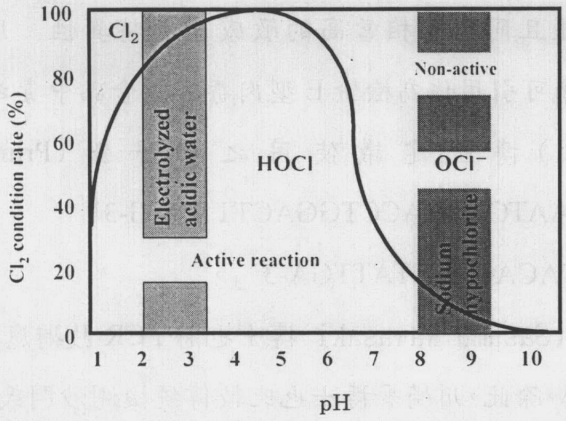
(*Salmonella*)、單核增多性李斯特菌(*Listeria monocytogenes*)；其洗滌及降低上述病原菌的能力優於含 200 ppm 之氯水(chlorine water)。



Principle of electrolyzed oxidizing water

圖一、酸性電解水(Electrolyzed acidic water)之製造原理

(Bari, 2003)



Cl_2 profile change with pH

圖二、酸性電解水(Electrolyzed acidic water)中氯濃度與 pH 值之關係

(Bari, 2003)

二、食品中毒原因菌的快速檢測法

(一)聚合酶鏈鎖反應(polymerase chain reaction, PCR)

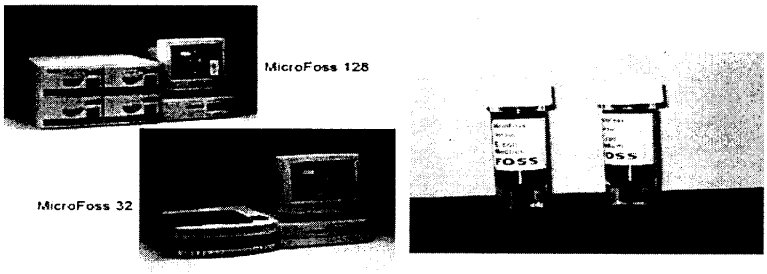
川崎晉(Sasumu Kawasaki)博士研究以定量 PCR 方法，檢測調氣包裝魚製品(modified-atmosphere-packaged fish)中 E 型肉毒桿菌(*Clostridium botulinum* Type E)之生長情形。經實驗評估結果發現定量 PCR 方法可應用於魚製品中 E 型肉毒桿菌(*Clostridium botulinum* Type E)之檢測，其敏感度及特異性均非常高。例如在低接種量(85 CFU/g)的添加試驗中，有 35.3~120.6 %的回收率(recovery)；在高接種量($8.5x10^4$ CFU/g)時，仍有 19.7~173.5 %的回收率。在定性試驗方面，6 株 E 型肉毒桿菌均可以 PCR 方法測得，且與 18 株非 E 型肉毒桿菌及 24 株非肉毒桿菌屬之其它食品中毒原因菌亦無非特

異性之交叉反應。相較於傳統方法(mouse assay)有節省時間、人力、無須使用動物等優點，並且同樣有相當高的敏感度及特異性。川崎晉(Sasumu Kawasaki)博士建議可引用作為檢驗E型肉毒桿菌食品中毒之參考。川崎晉(Sasumu Kawasaki)博士建議使用之引子對(Primers)序列為「5'-GTGAATCAGCACCTGGACTTTCAG-3'」及「5'-GCTGCTTGCACAGGTTTATTGA-3'」。

另外，川崎晉(Sasumu Kawasaki)博士也將PCR技術應用在沙門氏菌(*Salmonella*)的檢測。除此，川崎晉博士也比較傳統檢測沙門氏菌(*Salmonella*)與其自行研究開發之PCR方法，其間檢出率之差異。實驗結果發現，PCR方法的檢出結果與併用4種傳統沙門氏菌檢驗方法之檢出結果吻合，並且無交叉反應現象。由是觀之，PCR方法的敏感性與特異性均佳，以PCR方法來篩檢可能受污染之可疑食品檢體，是相當實用與值得評估的。

(二)Micro Foss 法

本法限定使用Micro Foss公司開發之test vial及Micro Foss reader，依檢測的目的微生物大腸桿菌(*Escherichia coli*)或大腸桿菌羣(coliform)，其test vial中所含之選擇性培養液亦不同，例如檢測大腸桿菌用之test vial是使用「E. coli medium」(商品名)。該產品的設計目的是為能在短時間內測出食品中的衛生指標菌大腸桿菌(*E. coli*)或大腸桿菌羣(coliform)。其原理是將待測食品接種於含有酸鹼指示劑選擇性培養液的test vial中，於特定溫度培養，並於固定時間間隔偵測培養液之顏色變化情形。若食品中污染之大腸桿菌或大腸桿菌羣菌數愈多，其生長代謝產物造成培養基變色的速率愈快。計算其造成培養基變色的時間，即可估算食品中的大腸桿菌或大腸桿菌羣菌數。川崎晉(Sasumu Kawasaki)博士表示，依其個人經驗Micro Foss法與傳統方法比較其相關性亦高。

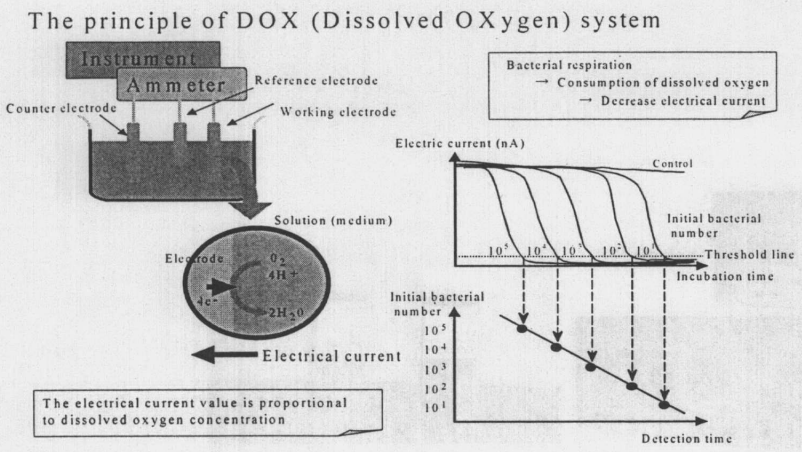


圖三、Micro Foss 套組之所需搭配之相關儀器及培養基

(Kawasaki, 2003)

(三)測溶氧量（DOX method）

DOX 法測菌數的原理是利用微生物在培養基中生長時，會呼吸改變培養基中的溶氧量，並產生代謝產物，改變培養基的導電度。由於培養基中的起始菌數愈多，相對其培養基導電度的變化愈快。故利用此特性記錄培養基導電度發生明顯變化的時間(detection time, Dt)即可反推培養基中可能含有的起始菌數(the viable bacterial cell number)。川崎晉(Sasumu Kawasaki)博士表示，依其研究結果，培養基導電度發生明顯變化的時間與培養基中可能含有的起始菌數呈現很好的相關性。大 DOX 法與傳統方法比較其相關性尚佳，應用在大腸桿菌群時其相關係數為 0.85。DOX system 包括三組各別獨立的培養箱及 180(60 vials x 3)管可測溶氧量的氧電極棒。

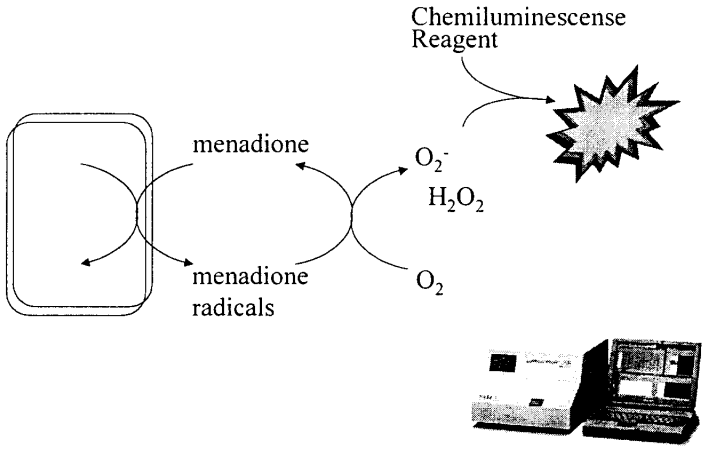


圖四、 DOX (Dissolved OXygen) system 之反應原理

(Kawasaki, 2003)

(四)Menadione-Catalyzed Luminol Chemiluminescent assay

Menadione-Catalyzed Luminol Chemiluminescent assay 法測菌數的原理主要是利用大腸桿菌*(E. coli)* 在含有 Menadione 的培養基中生長，當 Menadione 與細胞中的 H^+及 NAD(P)H 作用時會代謝轉變為 $NAD(P)^+$、semiquinone radicals 及 menadiol；當 semiquinone radicals 及 menadiol 轉換回 Menadione 時會將氧(O_2)轉換成活化狀態(O_2^-)並產生過氧化氫(H_2O_2)。大腸桿菌在生長對數期(exponential phase)時之菌落數(colony-forming unit, CFU)與 O_2^-所能催化產生螢光反應之強度成正比。利用 Menadione-Catalyzed Luminol Chemiluminescent assay 法可迅速有效地檢測活菌數。目前已有學者專家將它應用在抗生素最小抑菌濃度(minimal inhibitory concentration, MIC)的檢測上。



圖五、Menadione-Catalyzed Luminonl Chemiluminescent assay 套組之反應原理

(Kawasaki, 2003)

三、日本特殊食品中毒案例

日本一向嗜食生鮮食品，近年來，由於世界貿易潮流的衝擊與飲食文化的改變，在日本有愈來愈多的人喜歡「Kimchi」。所謂「Kimchi」其實就是韓式泡菜。目前「Kimchi」在日本的消費量仍在成長當中，導致日本傳統的發酵食品消費量年年減少。由於近年來「Kimchi」類製品就曾因汙染病原性大腸桿菌 *E. coli* O157：H7，而在日本當地引起重大食品中毒事件。因此，食品綜合研究所稻津康弘(Yasuhiro Inatsu)先生從微生物觀點，研究日本新興食品「Kimchi」的潛在危機。結果發現，沙門氏桿菌、病原性大腸桿菌及金黃色葡萄球菌在韓國泡菜販售及儲藏期間中仍然可以存活。反觀國內亦有該類食品，其食品衛生方面之潛在危機亦不容忽視。

此外，東京都立衛生研究所甲斐明美(Akemi Kai)博士指出，許多日本

人喜歡生食雞肝或牛肝片。生鮮肝臟片也曾在日本引起重大的病原性大腸桿菌 *E. coli* O157：H7 食品中毒事件。

四、出席日本社團法人舉辦之「日本食品衛生學會第八十五回學術演講會」參觀先進儀器及設備

「日本食品衛生學會第八十五回學術演講會」訂於 2003 年 5 月 14 日及 5 月 15 日在日本東京中央區銀座「中央會館」盛大舉行，當日除日本食品衛生界的學者專家到場演說發表論文外，另有邀請產官學界蒞臨指導，並有邀請儀器商聯合展出先進儀器及設備。演講內容十分豐富，主題大都均與日本當前的食品衛生有關。其性質與本局主辦之「食品檢驗科技研討會」相似。例如「腸炎弧菌(*Vibrio parahaemolyticus*)於調理過程中菌數的變化」、「國內魚介類水產品中 TDH 產生性腸炎弧菌(*Vibrio parahaemolyticus*)之分型」、「以定量 PCR 方法檢測及定量 A 型及 B 型肉毒桿菌(*Clostridium botulinum*)之研究」、「以新規遺傳子增幅法(Loop-Mediated Isothermal Amplification, LAMP)檢測沙門氏菌(*Salmonella*)之研究」、「食品中志賀氏桿菌之檢驗方法靈敏度評估」、「以微生物方法檢測食品中之組織氨(Histamine)及其方法評估」、「產氣莢膜桿菌(*Clostridium perfringens*)之食品中毒案例」、「市售雞肉中曲狀桿菌(*Campylobacter*)之定量檢測及其分離菌株之抗藥性研究」等，頗具參考價值。當日的行程是由一色賢司(Kenji Isshiki)教授特別安排帶領，並由川崎晉(Sasumu Kawasaki)博士陪同參觀。

是日在會場另有安排日本部分食品業相關廠商參展。與食品衛生檢驗較有關的有以下幾個攤位：

一是由日本 SAKAMI 公司展出的嫌氣菌培養用「P.T. Pouch」、「P.T. Pouch 電子封口機」、「P.T. Pouch holder stand 培養架」(依規格分 15mL x

10、150mL x 10、250mL x 10、)及高效過濾「N-95 口罩」(過濾率>96%)。P.T. Pouch 是一特殊材質，具有防水、防漏、隔菌、阻氣、熱融熱封等特性，目前在日本廣泛應用在肉毒桿菌(*Clostridium botulinum*)及產氣莢膜桿菌(*Clostridium perfringens*)的分離與培養。與目前國內嫌氣菌培養方法比較其優點為(1)不需要特殊氣體或產氣包 GasPak (2)不需要特別的培養基(3)不須要嫌氣菌培養罐或特殊的嫌氣培養箱或容器(4)為可拋棄式(5)可直接以接種針或環在 P.T. Pouch 表面挑選可疑菌落(6)攜帶方便(7)價格便宜。

此外，Applied Biosystem, AB 公司展出「定量 PCR (Real-time PCR)」及「MicroSeq® System」亦是令人印象深刻。定量 PCR (Real-time PCR) 本局第五組業已採購，目前已應用於 GMO 食品其 DNA 含量之定性及定量。而「MicroSeq® System」則是該公司開發出來的一套微生物鑑定系統。該系統需搭配 Applied Biosystem, AB 公司所開發的鑑定試劑套組(名為「16S Bacterial rDNA & D2 Fungal rDNA Seguencing kits」)及軟體資料庫。

其他重要的參展廠商及產品尚有以下幾家：如關東化學株式會社(Kanto Co.)代理的曲狀桿菌(*Campylobacter*)分離培養基(CCDA agar、Karmali agar、Preston medium、Bolton medium)、快速鑑定套組及殘留農藥試驗用「農藥混合標準液」；CHROMagar™ Microbiology 針對食品中毒原因菌所開發的新一代選擇性分離培養基，例如 CHROMagar Salmonella、CHROMagar Vibrio、CHROMagar Listeria 等。

五、參觀 P2 級實驗室

目前食品綜合研究所進行中的所有關於食品中毒原因菌的實驗均在 P2 級實驗室內進行。該實驗室之硬體規格實際上已符合 P3 級，但目前仍編

列在 P2 級。目前，該實驗室之負責人為一色賢司博士。為防止汙染性微生物的污染，P2 級實驗室係採獨立棟建築，並與行政大樓分開。

參觀當日，是由 Dr. Bari 帶領介紹該實驗室的所有軟應體設備。Dr. Bari 表示該實驗室當時係委由日本三洋公司(SANYO Co.)設計承建，實驗室內用的實驗操作台、空調、空氣濾網等設備均採用三洋公司的產品。而在維修方面則是與三洋公司簽立合約，定期維修，按時檢修更換濾網等耗材設備。欲進出 P2 實驗室必須先向食品綜合研究所權責單位申辦感應磁卡；進出所有的門禁、出入口均需要刷卡及簽名確認，並且要依規定換上拋棄式實驗衣。P2 實驗室內的所有實驗用具(包括拋棄式實驗衣、拋棄式接種針及接種環)及垃圾，均須經實驗室內殺菌釜滅菌後方能移出。其進入 P2 實驗室的流程如後圖所示：

食品綜合研究所的 P2 級微生物實驗室人員進出之控管相當嚴格，無磁卡的非工作人員無法進入；即使是工作人員，倘若未依規定使用卡片，或借用他人卡片違規進入，都可由其書面簽名及卡片的刷卡電腦紀錄偵測出。違規進入者也會受到處罰。因此，在食品綜合研究所不按規定進出的個案很少。

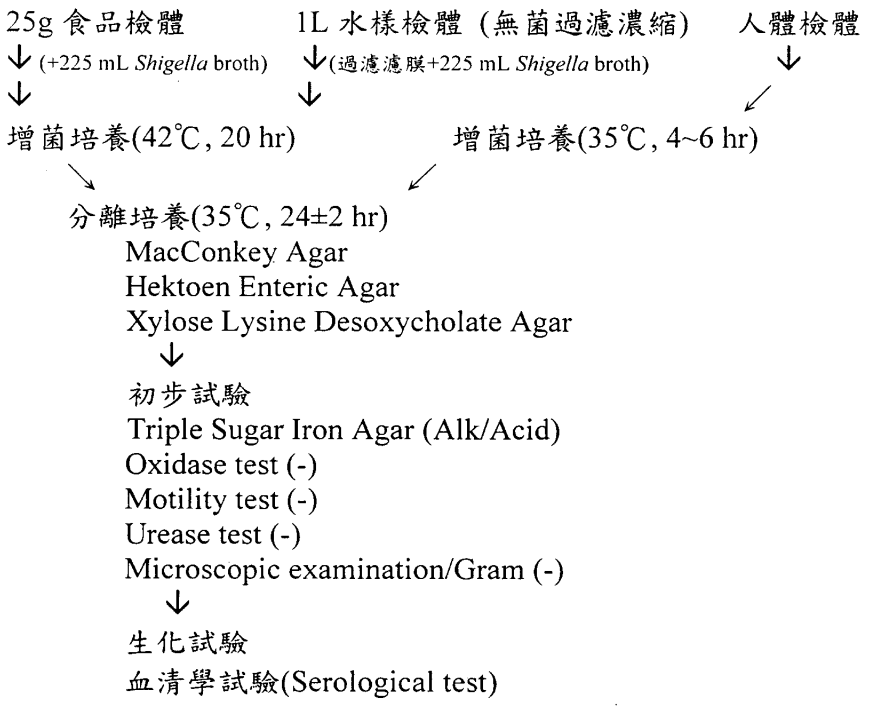
申請門禁控管磁卡
↓
入口(第一道門)，刷卡及簽名
↓
刷卡資料傳輸到「電算機中心」確認身份及密碼
↓→若資料不符或已過核准進出期限；無法進入
若資料無誤；開門
↓
更換拖鞋
↓
出入口(第二道門)，進入 P1 級區域
↓更換拖鞋，通過 Air shower
出入口(第三道門)，進入 P2 級區域
(溫度、濕度及負壓監控之前置室)
↓更換拖鞋、拋棄式實驗衣
出入口(第四道門)，進入 P2 級區域
↓
實驗操作台(實驗進行區域)
(P2 級區域：溫度、濕度及負壓監控)
↓
實驗完成
↓
出入口(第四道門)，
↓
出入口(第三道門)
↓出 P2 級區域
進入洗滌滅菌區
P2 實驗室內的所有實驗用具(包括拋棄式實驗衣、拋棄式接種針及接種環)及垃圾，均須經實驗室內殺菌釜滅菌後，方能移出。
↓
出入口(第二道門)
↓
入口(第一道門)
↓
刷卡、簽名及紀錄離開時間

圖六、食品綜合研究所 P2 級微生物實驗室人員進出之控管流程圖

六、日本志賀氏桿菌的最新檢驗方法

九十二年五月十二日誠蒙一色賢司(Kenji Isshiki)教授居中協助與安排，由川崎晉博士陪同參訪東京都立衛生研究所甲斐明美(Akemi Kai)博士、國立醫藥品食品衛生研究所山本茂貴博士(Shigeki Yamamoto)及Ochanomizu University 村田容常(Masatsune Murata)博士，考察日本現行食品中毒原因微生物之檢驗及研擬、實驗室規模與檢驗研究執行狀況。

是日並與甲斐明美(Akemi Kai)博士針對我國、日本與美國FDA志賀氏桿菌檢驗方法，三者間之利敝得失等議題(詳如以下圖二至四)，共同討論，並交換檢驗心得與經驗。甲斐明美博士提供二種日本現行宋志賀氏桿菌(*Shigella sonnei*)檢驗方法(圖三及圖四)及相關研究報告作為本局往後修訂志賀氏桿菌檢驗方法之參考。甲斐明美博士建議增加使用其它選擇性培養基(CHROMagar O157 TAM、DHL agar、*Salmonella* agar acc. to ONOZ 、*Salmonella-Shigella* agar)及併用PCR方法，以提高檢出率。



圖七、目前國內志賀氏桿菌檢驗之流程圖

25g 食品檢體

↓ (+225 mL *Shigella* broth)

↓混合均勻，於室溫放置 10 min

嫌氣增菌培養(44℃, 20 hr)

↓————————————————→PCR 試驗

分離培養(35~37℃, 18~24 hr)

MacConkey Agar No.3 (OXOID)

CHROMagar O157 TAM

DHL agar

Xylose Lysine Desoxycholate Agar

Salmonella agar acc. to ONOZ (Merck)

Hektoen Enteric Agar

Salmonella-Shigella agar

↓自以上各種選擇性分離培養基各至少挑 5 個菌落

一般非選擇性培養基(TSA agar)

↓ 35~37℃培養 18~24 hr

確認試驗(生化試驗)

↓

PCR 試驗

↓

血清型別試驗(Serological test)

圖八、日本宋志賀氏桿菌(*Shigella sonnei*)檢驗方法(一)

25g 食品檢體

↓ (+225 mL Buffered Peptone Water)
↓ 混合均勻，於室溫放置 10 min
培養(35~37℃, 18~24 hr) ——————→PCR 試驗
↓ (取 1 mL 培養液+10 mL *Shigella* broth)
嫌氣增菌培養(44℃, 20 hr)
↓——————————————————→PCR 試驗
分離培養(35℃, 24±2 hr)
MacConkey Agar No.3 (OXOID)
CHROMagar O157 TAM
DHL agar
Xylose Lysine Desoxycholate Agar
Salmonella agar acc. to ONOZ (Merck)
Hektoen Enteric Agar
Salmonella-Shigella agar
↓自以上各種選擇性分離培養基各至少挑 5 個菌落
一般非選擇性培養基(TSA agar)
↓ 35~37℃培養 18~24 hr
確認試驗(生化試驗)
↓
PCR 試驗
↓
血清型別試驗(Serological test)

圖九、日本冷凍食品中宋志賀氏桿菌(*Shigella sonnei*)檢驗方法(二)

建　議

(1)酸性電解水(Electrolyzed acidic water)及微生物快速檢驗方法可結合HACCP (Hazard analysis critical control point system)系統，應用在餐飲業，為食品衛生把關。就食品衛生的觀點，目前 HACCP 系統應用在餐飲業及食品工業的最大問題是無法以傳統微生物的檢驗方法，在短時間內發現某個管制點的衛生指標菌、總生菌數或大腸桿菌群菌數已超過容許範圍。例如以傳統方法測總生菌數須耗費 24-48 小時；測大腸桿菌或大腸桿菌群菌數則更是需要三天以上的培養時間。是故等待檢驗結果出來後，往往原料已加工做成待售之成品了。反觀部份微生物快速檢驗法，例如 PCR 法、Menadione-Catalyzed Luminol Chemiluminescent assay 、及 Micro Foss 法、可把檢驗時間縮短在 8-24 小時以內。其應用於食品衛生方面的潛力不容忽視。

(2)在日本食品綜合研究所有關食品中毒原因菌的實驗，基於防護安全上的理由，均在 P-2 級實驗室內進行。並且，實驗室與辦公室分開，防止微生物外流。建議本局應比照食品綜合研究所著手建立 P-2 級以上之實驗室。此外，對於不同防護層級（如 P-1 level 或 P-2 Level）之實驗室在日本亦有黏貼標籤加以標示，並且依層級高低對其垃圾或廢棄物作妥善之管理；而使用過之試管架亦整架滅菌。在這方面很值得我們學習改進。

(3)目前本組的研究計畫主題較少涉略有關食品中毒防治方面的計畫，建議宜適度的增列。

(4)日本都立衛生試驗所在食品中毒病原菌方面的檢驗頗具規模。建議本局每年定期邀請在這方面學有專精的專家（例如一色賢司、甲斐明美博

士)，蒞局演講有關食品中毒病原菌的最新檢驗技術與方法，並可作為本局檢驗方法修訂之參考。

(5)本局為處理食品中毒案件之權責單位，擁有各種汙染來源之食品及環境分離菌株。以往，本組對於分離菌株雖然都有加以冷凍保存。但由於歷經颱風斷電或冰櫃故障等因素，而發生部份庫存菌株無法活化情形，無形中造成研究資源損失，此殆因本組只使用冷凍法保存菌株使然。綜觀一色賢司博士之研究團隊，今日能有如此的豐碩的研究成績，不外乎是團隊的合作無間。因此，建議本局宜增購菌株保存設備，鼓勵同仁妥善保存各種食品或環境來源之分離菌株，並且集中妥善保存，以供學術研究之用。如此，除可提升本局之研究層次外，亦能建立庫存菌株，提供予臨床或學界。此對本土菌株之分型、流病分析及疾病控管方面有莫大幫助。

(6)實驗室間合作與交流，有助於迅速獲得最新檢驗資訊提升檢驗品質。建議往後能加強與其他先進實驗室間之交流及合作。

(7)日本食品綜合研究所之研究成果斐然，除歸功於研究人員的努力外，儀器設備完善也是重要因素之一。冀望局內於將來儀器採購與經費編列等事項能予支持，以助檢驗工作順利進行。

附　件

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Effectiveness of Electrolyzed Acidic Water in Killing *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* on the Surfaces of Tomatoes

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ABSTRACT

A study was conducted to evaluate the efficacy of electrolyzed acidic water, 200-ppm chlorine water, and sterile distilled water in killing *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on the surfaces of spot-inoculated tomatoes. Inoculated tomatoes were sprayed with electrolyzed acidic water, 200-ppm chlorine water, and sterile distilled water (control) and rubbed by hand for 40 s. Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in the rinse water and in the peptone wash solution were determined. Treatment with 200-ppm chlorine water and electrolyzed acidic water resulted in 4.87- and 7.85-$\log_{10}$ reductions, respectively, in *Escherichia coli* O157:H7 counts and 4.69- and 7.46-$\log_{10}$ reductions, respectively, in *Salmonella* counts. Treatment with 200-ppm chlorine water and electrolyzed acidic water reduced the number of *L. monocytogenes* by 4.76 and 7.54 $\log_{10}$ CFU per tomato, respectively. This study's findings suggest that electrolyzed acidic water could be useful in controlling pathogenic microorganisms on fresh produce.

Fresh fruits and vegetables are an essential part of the diets of people around the world. Nutritionists emphasize the importance of fruits and vegetables in a healthy diet, and researchers have recommended the consumption of at least five servings per day *(16)*. Possibly as a result of these efforts, over the past decade there has been an increase in the consumption of fresh fruits and vegetables, concurrent with increased global distribution, which has made more varieties of produce available year-round *(5)*. An increased number of microbial infections associated with the consumption of fresh fruits and vegetables also have been documented in recent years. Enterohemorrhagic *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* are foodborne pathogens of major public health concern worldwide. A variety of foods, including poultry, eggs, meat, milk, fruits, and vegetables, have been implicated as vehicles for one or more of these pathogens in outbreaks of foodborne illness *(2, 6, 8)*. Effective methods of reducing or eliminating pathogens in food are important for the successful implementation of hazard analysis critical control point programs by the food industry and for the establishment of critical control points in restaurants, homes, and other food service units. Raw agricultural produce is washed with water in the industry; however, washing alone does not render a product completely free of pathogens. Although many chemicals that are generally recognized as safe, including organic acids, have antimicrobial activity against foodborne pathogens, none, when used individually at concentrations acceptable in foods, can eliminate large populations of pathogens. The treatment of fruits and vegetables with water containing sanitizers, including chlorine, may reduce but not eliminate pathogens on the surfaces of produce *(2, 29)*. Hence, there is a need for, and interest in, the development of practical and effective antimicrobial treatments for the inactivation of pathogenic microorganisms on foods.

Electrolyzed acidic water is the product of a new concept developed in Japan. Research carried out in Japan revealed that electrolysis of deionized water containing a low concentration of sodium chloride (0.1%) in an electrolysis chamber where anode and cathode electrodes were separated by a diaphragm imparted strong bactericidal and virucidal properties to the water collected from the anode. Water from the anode normally has a pH of ≤2.7, an oxidation reduction potential (ORP) of >1,100 mV, and a free-chlorine concentration of 10 to 80 ppm *(23)*. Electrolyzed acidic water has been used experimentally in Japan by medical and dental professionals for treating wounds or disinfecting medical equipment. The objective of this study was to evaluate the efficacy of electrolyzed acidic water in killing *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* on fresh produce with a view to its potential application to foods and food contact surfaces as an antimicrobial treatment.

MATERIALS AND METHODS

Test strains. The strains studied and their sources were as follows. Enterohemorrhagic *E. coli* O157:H7 strains CR-3, MN-28, MY-29, and DT-66 were isolated from bovine feces. *Salmonella* Enteritidis strains SE-1, SE-3, and SE-4 (from chicken feces)

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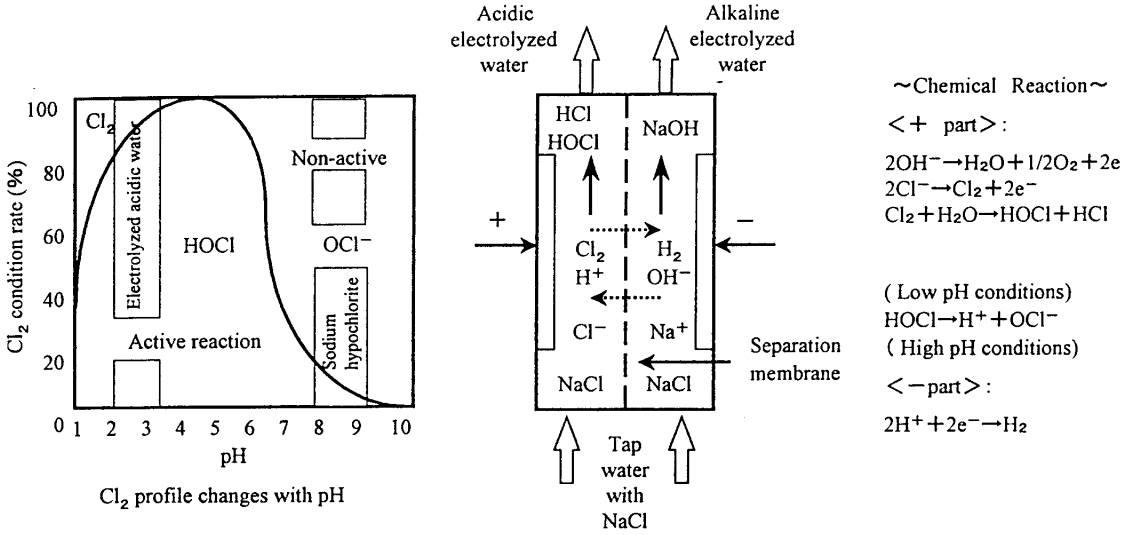

FIGURE 1. *Principle of EO water.*

and SE-2 (from bovine feces) were provided by the Laboratory of Zoonosis, National Institute of Animal Health, Tsukuba, Japan. *Salmonella* strain IFO-3313 (unknown) was obtained from the Institute for Fermentation, Osaka, Japan. *L. monocytogenes* strains ATCC 43256 (from Mexican-style cheese) and ATCC 49594 (derived from *L. monocytogenes* strain Scott A) (American Type Culture Collection, Manassas, Va.) and JCM 7676 (from roast beef), JCM 7672 (from salami sausage), and JCM 7671 (from lax ham) (Japan Collection of Microorganisms) were used in this study.

To minimize the growth of microorganisms naturally present on tomatoes, all test strains of *E. coli* O157:H7 and *Salmonella* were adapted for growth in tryptic soy broth (TSB, pH 7.3; Nissui Seiyaku, Tokyo, Japan) supplemented with nalidixic acid (50 μg/ml). Although some gram-positive microorganisms are less affected by nalidixic acid, *L. monocytogenes* strains were grown in tryptose phosphate broth (pH 7.0; Difco) containing 50 μg of nalidixic acid per ml before their use as an inoculum. Plating on media containing nalidixic acid greatly minimized interference with colony development by naturally occurring microorganisms, thus facilitating the detection of the test pathogen on recovery media.

EO water. Electrolyzed oxidizing (EO) water was generated with a model ROX-20TA EO water generator (Hoshizaki Electric Company Ltd., Toyoake, Aichi, Japan). The current passing through the EO water generator and the voltage between the electrodes were set at 19.8 A and 10 V, respectively. A 12% solution of sodium chloride (Sigma Chemical Co., St. Louis, Mo.) and deionized water from the laboratory supply line were simultaneously pumped into the equipment. The display indicator was activated and observed until the machine stabilized at a reading of 19.8 A. The EO water was collected from the appropriate outlet in sterile containers and was used within 2 to 3 h for the microbial study. Samples to be used for the determination of pH, ORP, and free-chlorine concentration were also collected at the same time. The pH of the tested solution was measured with a pH meter (D-22, Horiba, Kyoto, Japan). The ORP was measured with an ORP meter (HM-60V, TOA Electronics Ltd., Tokyo, Japan). The initial concentration of available chlorine in the test solution was quantified by spectrophotometric analysis at 530 nm with the use of *N,N*-diethyl-*p*-phenylen-diamine *(26)*.

The theoretical sequence of chemical reactions involved in the production of EO water is shown in Figure 1. During electrolysis, sodium chloride dissolved in deionized water in the electrolysis chamber dissociates into negatively charged chloride (Cl) and hydroxy (OH) ions and positively charged sodium (Na^+) and hydrogen (H^+) ions. The chloride and hydroxy ions are adsorbed to the anode, with each ion releasing an electron to become a radical. The chloric and hydroxy radicals combine, forming hypochlorous acid (HOCl), which separates from the anode. Two chloric radicals can also combine to produce chlorine gas. In the cathode section, each positively charged sodium ion receives an electron and becomes metallic sodium. The metallic sodium combines with water molecules, forming sodium hydroxide and hydrogen gas. A bipolar membrane separating the electrodes enhances the electrolysis of water to produce strong acidic and alkali waters from the anode and cathode, respectively. Electrolyzed acidic water at pH 2.7 contains available chlorine as a form of hypochlorous acid (HOCl), which is more effective in disinfection than hypochlorite (ClO^-) (Fig. 1).

Produce evaluation. Produce selected for evaluation consisted of red ripe tomatoes (90 ± 20 g each) to which no oil or wax had been applied. Tomatoes (*Lycopersicum esculentum* Mill.) var. Momotaro, used in each experiment, were purchased from a local supermarket and stored at room temperature (22 ± 2°C) for a maximum of 2 days before they were used in experiments.

Preparation of inocula. Each strain of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* was cultured in TSB (10 ml) supplemented with 50 μg of nalidixic acid per ml at 37°C. Cultures were transferred to TSB by loop at three successive 24-h intervals immediately before they were used as inocula. Cells of each strain were collected by centrifugation (3,000 × *g*, 10 min, 20°C) and resuspended in 5 ml of phosphate-buffered saline (PBS, pH 7.2) solution. The inoculum was maintained at 22 ± 2°C and applied to tomatoes within 1 h of preparation.

Procedure for inoculation. Tomatoes (22 ± 2°C) were placed stem-end-down in a biosafety cabinet. Within a 3-cm-diameter circle on the top of the tomato, 100 μl of a suspension of cells in PBS was applied with a micropipettor, with care taken to avoid placing inoculum on the blossom scar. To prevent the inoculum from running off the sides of the tomatoes and to facilitate drying, small, approximately equal numbers of cells were applied to 10 to 12 spots. The total numbers of *E. coli* O157: H7, *Salmonella*, and *L. monocytogenes* cells applied to the tomatoes were determined by surface plating the serially diluted inoculum in 0.1% peptone on appropriate enumeration media. Inoculated tomatoes were stored at 22 ± 2°C for 30 min before they were used for various treatments.

Survivability study. The survivability study involved the determination of numbers of pathogens that could be recovered from inoculated tomatoes after inoculation and drying at 22 ± 2°C for 30 min. *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were evaluated separately. Three replicate trials for each pathogen were performed, with three or four tomatoes being used for each trial. Thirty minutes postinoculation, each inoculated tomato in a biosafety cabinet was placed in a sealable Pyxon-20 (ELMEX Co. Ltd., Tokyo, Japan) bag containing 20 ml of EO water, 200-ppm chlorine water, or sterile distilled water and thoroughly rubbed by hand for 20 s. Sterile distilled water (200 ml) was then added to the bag, and the tomato was rinsed with vigorous agitation for 20 s (rinse step). The tomato was transferred to a clean bag, 20 ml of sterile 0.1% peptone water was added, and the tomato was thoroughly rubbed by hand for 40 s (residual wash step). Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in rinse water and peptone wash water were determined as described below. The tomato was removed from the bag, and the area of skin (ca. 10 g) originally inoculated with the pathogen was excised with a sterile scalpel. Care was taken to remove tomato tissue (pulp) no more than 0.1 cm below the skin surface. The excised tomato skin-pulp was then combined with 20 ml of sterile 0.1% peptone in a new quart bag and macerated between the fingers until the pulp was removed from the skin. The homogenate (macerate) was then analyzed to determine the level (CFU/ml) of the test pathogen. Excised tomato skin-pulp not macerated in sterile 0.1% peptone was also analyzed to determine populations of test pathogens.

Chlorine water treatment studies. The chlorine solution was prepared by adding sodium hypochlorite (Wako Chemical, Japan) solution to distilled water (vol/vol). The effectiveness of chlorine and electrolyzed acidic water in killing *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* separately applied to the surfaces of tomatoes was determined according to the basic protocol developed by Beuchat et al. *(3)*.

Microbiological analysis. Single- and mixed-strain suspensions of each pathogen in PBS were serially diluted in 0.1% sterile peptone water. Duplicate 0.1-ml quantities of appropriately diluted suspensions of *E. coli* O157:H7 and *Salmonella* were surface plated on tryptic soy agar (TSA) and TSA supplemented with 0.1% pyruvic acid and 50 μg of nalidixic acid per ml (TSAPN). In addition, diluted *E. coli* O157:H7 suspensions were surface plated onto sorbitol MacConkey agar (Nissui) supplemented with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter) (CT-selective supplement, Oxoid) (CT-SMAC) and CT-SMAC containing 50 μg of nalidixic acid per ml (CT-SMACN); samples containing *Salmonella* were plated on bismuth sulfite agar (BSA; Difco) and BSA supplemented with 50 μg of nalidixic acid per ml (BSAN). Diluted suspensions (0.1 ml) of *L. monocytogenes* were surface plated in duplicate on tryptose phosphate agar (TPA). TPA supplemented with 0.1% pyruvic acid and 50 μg of nalidixic acid per ml (TPAPN), and modified Oxford medium (Oxoid) supplemented with 50 μg of nalidixic acid per ml (MOXN). This medium contains 55.5 g of *Listeria* selective agar base (Oxoid CM 856, Unipath-Oxoid US) per liter of deionized water, 0.01 g of colistin methanesulfate (Sigma) per liter, 0.02 g of ceftazadime pentahydrate (Glaxo Group Research Ltd., Ware, Hertfordshire, UK) per liter, 50 μg of nalidixic acid per ml, and 5.0 g of agar (Difco) per liter. All ingredients except ceftazadime pentahydrate and nalidixic acid were combined and sterilized by heating at 121°C for 15 min. Ceftazadime solution and nalidixic acid were added to the molten agar before the medium was poured into petri plates.

Inoculated enumeration media were incubated at 37°C for 24 to 28 h before presumptive colonies of each pathogen were counted. At least five presumptive colonies of *E. coli* O157:H7 were confirmed with the *E. coli* O157 direct immunoassay test kit (Universal Health Watch, Columbia, Md.). *Salmonella* confirmation was carried out by testing reactions on triple sugar iron (Nissui) slants. Randomly picked presumptive colonies of *L. monocytogenes* were confirmed with API Listeria diagnostic kits.

Peptone wash water and phosphate-buffered macerate of skin-pulp from tomatoes prepared in the survivability study were analyzed for populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. Serially diluted peptone wash water (0.1 ml) from tomatoes inoculated with *E. coli* O157:H7 were surface plated in duplicate on TSAPN and CT-SMACN, samples from tomatoes inoculated with *Salmonella* were plated on TSAPN and BSAN, and samples inoculated with *L. monocytogenes* were plated on TPAPN and MOXN. Quadruplicate 0.25-ml samples of skin-pulp homogenate were also plated on appropriate recovery media. Plates were incubated at 37°C for 18 to 24 h before presumptive colonies of pathogens were counted and confirmed as described above.

Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in rinse water (200 ml) and peptone wash water (20 ml) after the rinsing or washing of tomatoes treated with chlorine (200 ppm), electrolyzed acidic water, or sterile water (control) were determined. Undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in sterile 0.1% peptone were surface plated on TSAPN and CT-SMACN for the recovery of *E. coli* O157:H7, on TSAPN or BSAN for the recovery of *Salmonella*, and on MOXN for the recovery of *L. monocytogenes*. Quantities (100 ml) of *E. coli* broth (Nissui), selenite cystine broth (Nissui), and *Listeria* enrichment broth (Oxoid), each supplemented with 50 μg of nalidixic acid per ml, were then combined with the 20-ml quantities of peptone wash solution in the bags containing the treated, rinsed, washed tomatoes that had been inoculated with *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively, and incubated for 24 to 26 h at 37°C. Cultures were streaked on appropriate recovery media for presumptive colonies, and confirmation of randomly selected colonies was carried out as described above.

Sensory evaluation. The quality of treated and untreated uninoculated tomatoes was evaluated by panelists (10 judges) selected from the National Food Research Institute who were experienced with sensory panels. The evaluation was based on a five-point hedonic scale (1, unacceptable; 2, limited quality for consumption; 3, medium; 4, good; 5, very good). The sensory characteristics of appearance, color, and taste were assessed for raw tomatoes after 6 h of treatment at room temperature. For each of the quality attributes, three of each treated and untreated to-

TABLE 1. *Physicochemical properties of tested solutions*[a]

Solution	pH	ORP (mV)	ACC (ppm)
Distilled water	7.1 ± 0.15	355 ± 7.0	—
AcEW	2.6 ± 0.1	1,140 ± 7.0	30.3 ± 3.1
NaOCl	9.3 ± 0.2	638 ± 18	198.5 ± 5.8

[a] Values are means ± standard deviations (n = 5). ORP, oxidation reduction potential; ACC, available chlorine concentration; AcEW, electrolyzed acidic water; NaOCl, sodium hypochlorite.

matoes were examined and/or tested by the judges. Rejection of a sample was based on a score of <2.5, which was the quality criterion used for both treated and untreated tomatoes.

Statistical analyses. All trials were replicated three times. Reported plate count data represented the mean values obtained for three individual trials, with each of these values being obtained from duplicate samples. Sensory evaluation tables represented mean values ± standard deviations obtained from three individual trails. Significant differences in plate count data were established by the least significant difference at the 5% level of significance.

RESULTS

The physicochemical properties of the tested solutions are shown in Table 1. The mean pHs of acidic electrolyzed water, chlorine water, and distilled water were 2.6, 9.3, and 7.1, respectively. The ORP values for acidic electrolyzed water, chlorine water, and distilled water were 1,140, 638, and 355 mV, respectively. The available chlorine concentrations of acidic electrolyzed water and chlorine water were 30.3 and 198.5 ppm, respectively. No free chlorine was detected in distilled water.

The results of studies undertaken to determine the efficacy of treatments with 200-ppm chlorine water and electrolyzed acidic water in killing *E. coli* O157:H7 on tomatoes are reported in Table 2. The numbers of *E. coli* O157:H7 cells applied, as calculated by plating of the inoculum on CT-SMACN and TSAPN, were 7.63 and 7.85 $\log_{10}$ CFU per tomato, respectively. The pHs of water rinses from tomatoes treated with chlorine and electrolyzed acidic water were 7.0 and 6.22, respectively, whereas the pHs of peptone washes were 7.2 and 6.8, respectively. Since the water rinse was diluted in 0.1% peptone before application to CT-SMACN and TSAPN, the residual effects of sanitizers or pH on *E. coli* O157:H7 would be minimal or nonexistent. Note that an additional 20 s elapsed from the time the tomato was rubbed to the time cells were plated or diluted in 0.1% peptone. Therefore, rubbing for 40 s represented approximately 1 min of exposure to sanitizers. The number of *E. coli* O157:H7 cells in rinse water represents the number removed from the control and treated tomatoes, and thus the number available to cross-contaminate other tomatoes or food preparation surfaces in food service or home use situations. The number of *E. coli* O157:H7 cells in peptone wash solutions represents the population remaining on the surfaces of control and treated tomatoes. The number of *E. coli* O157:H7 cells in macerated skin-pulp represents the population firmly attached to the skins of control and treated tomatoes. Overall, CT-SMACN and TSAPN were equally suitable for *E. coli* O157:H7 colony development regardless of the exposure of cells to sanitizers. Washing with water (control) resulted in a reduction of 2.10 $\log_{10}$ CFU per tomato; further significant reductions of 4.31 and 7.63 $\log_{10}$ CFU per tomato were achieved by treatment with 200-ppm chlorine water and electrolyzed acidic water, respectively, as detected on CT-SMACN. However, even with these large reductions, the pathogen was detected by enrichment of treated tomatoes.

Shown in Table 3 are the results of studies undertaken to determine the efficacy of 200-ppm chlorine water and electrolyzed acidic water in killing *Salmonella* on tomatoes. The numbers of *Salmonella* cells applied, as calculated by plating of the inoculum on BSAN and TSAPN, were 7.36 and 7.46 $\log_{10}$ CFU per tomato, respectively. TSAPN and BSAN were equally suitable for colony development, regardless of the exposure of cells to sanitizers. Washing with water (control) resulted in a reduction of 2.11 $\log_{10}$ CFU

TABLE 2. *Populations of* Escherichia coli *O157:H7 recovered from treated and untreated tomatoes*

Recovery medium	Treatment	Population (log CFU/tomato) recovered from[a]: Water rinse	Peptone wash	Skin-pulp homogenates	Reduction[b]	Enrichment[c]
CT-SMACN	None	ND	6.04	2.13	−0.54	9
	Water	3.27	1.23	1.03	2.10	9
	Chlorine	1.78	0.87	0.67	4.31	9
	EO water	<1.0[d]	<1.0[d]	<1.0[d]	7.63	2
TSAPN	None	ND	5.64	2.37	−0.16	
	Water	3.19	2.16	0.72	1.78	
	Chlorine	1.69	0.73	0.56	4.87	
	EO water	<1.0[d]	<1.0[d]	<1.0[d]	7.85	

[a] Mean value for three replicate experiments ($P \leq 0.05$). ND, not determined.
[b] Populations (log CFU per tomato) recovered from rinse water, peptone wash, and macerated skin-pulp combined and then subtracted from the population applied to the tomatoes. Populations applied to tomatoes were 7.63 and 7.85 $\log_{10}$ CFU per tomato as detected on CT-SMACN and TSAPN, respectively.
[c] Number of tomatoes (three tomatoes per replicate) on which *E. coli* O157:H7 was detected by enrichment.
[d] No colonies were observed during the incubation period.

TABLE 3. *Populations of* Salmonella *recovered from treated and untreated tomatoes*

Recovery medium	Treatment	Population (log CFU/tomato) recovered from[a]: Water rinse	Peptone wash	Skin-pulp homogenates	Reduction[b]	Enrichment[c]
BSAN	None	ND	4.95	2.79	−0.38	9
	Water	2.77	1.36	1.12	2.11	9
	Chlorine	1.63	0.76	0.43	4.54	9
	EO water	<1.0[d]	<1.0[d]	<1.0[d]	7.36	3
TSAPN	None	ND	5.12	2.23	−0.31	
	Water	2.96	1.57	1.03	1.90	
	Chlorine	1.37	0.83	0.57	4.69	
	EO water	<1.0[d]	<1.0[d]	<1.0[d]	7.46	

[a] Mean value for three replicate experiments ($P \leq 0.05$). ND, not detected.
[b] Populations (log CFU per tomato) recovered from rinse water, peptone wash, and macerated skin-pulp combined and then subtracted from the population applied to the tomatoes. Populations applied to tomatoes were 7.36 and 7.46 $\log_{10}$ CFU per tomato as detected on BSAN and TSAPN, respectively.
[c] Number of tomatoes (three tomatoes per replicate) on which *Salmonella* was detected by enrichment.
[d] No colonies were observed during the incubation period.

per tomato; further significant reductions of 4.54 and 7.36 $\log_{10}$ CFU per tomato were achieved by treatment with 200-ppm chlorine water and electrolyzed acidic water, respectively, as detected on BSAN. However, even with these large reductions, the pathogen was detected by enrichment of treated tomatoes.

Results of experiments undertaken to determine the efficacy of sanitizers in killing *L. monocytogenes* on the surfaces of tomatoes are summarized in Table 4. The numbers of *L. monocytogenes* cells applied, as calculated by plating of the inoculum on MOXN and TPAPN, were 7.54 and 7.59 $\log_{10}$ CFU per tomato, respectively. Overall, TPAPN was more suitable for *L. monocytogenes* colony development than MOXN was, regardless of the treatment of inoculated tomatoes with sanitizers. This finding indicates that some of the cells were injured and unable to resuscitate in the presence of selective chemicals in MOXN. This phenomenon was particularly evident when numbers of *L. monocytogenes* recovered from peptone wash samples on MOXN and TPAPN were compared. Treatment with water resulted in a reduction of 2.14 $\log_{10}$ CFU per tomato; treatment with 200-ppm chlorine water and electrolyzed acidic water significantly reduced populations by 4.76 and 7.54 $\log_{10}$ CFU per tomato, respectively.

DISCUSSION

Test strains of pathogens from different food sources were chosen for evaluation. The method evaluated involved four- or five-strain mixtures of each pathogen, which is consistent with currently accepted practices for use in studying the survival and growth of pathogens in food *(9, 10, 12, 28)* and with Scientific Advisory Panel recommendations *(11)*. The use of multiple-strain inocula likewise represents a conservative strategy, since the mixture may provide a more representative challenge than a single strain for the sanitization of products. Thus, data should more accurately

TABLE 4. *Populations of* Listeria monocytogenes *recovered from treated and untreated tomatoes*

Recovery medium	Treatment	Population (log CFU/tomato) recovered from[a]: Water rinse	Peptone wash	Skin-pulp homogenates	Reduction[b]	Enrichment[c]
MOXN	None	ND	5.34	2.35	−0.15	9
	Water	3.09	1.33	0.98	2.14	9
	Chlorine	1.49	0.93	0.36	4.76	9
	EO water	<1.0[d]	<1.0[d]	<1.0[d]	7.54	3
TPAPN	None	ND	5.41	2.53	−0.35	
	Water	3.23	1.49	1.12	1.75	
	Chlorine	1.54	1.07	0.47	4.51	
	EO water	<1.0[d]	<1.0[d]	<1.0[d]	7.59	

[a] Mean value for three replicate experiments ($P \leq 0.05$). ND, not detected.
[b] Populations (log CFU per tomato) recovered from rinse water, peptone wash, and macerated skin-pulp combined and then subtracted from the population applied to the tomatoes. Populations applied to tomatoes were 7.54 and 7.59 $\log_{10}$ CFU per tomato as detected on MOXN and TPAPN, respectively.
[c] Number of tomatoes (three tomatoes per replicate) on which *L. monocytogenes* was detected by enrichment.
[d] No colonies were observed during the incubation period.

predict physiological sensitivities of pathogenic strains occasionally present on produce and should also provide a conservative estimate of risk to public health. All strains examined were adapted to grow in the presence of 50 μg of nalidixic acid per ml, one of several markers used to evaluate the survival of bacterial pathogens in food products with potentially large numbers of interfering background microflora. Antibiotic-resistant markers have been widely used in studies to determine the fate of pathogens in nonsterile foods, including fresh produce *(22)*, meats *(7)*, and milk *(25)*. With the use of spot inoculation, a specified number of cells can be applied to the surface of produce, and the decrease in the number of viable cells during drying or under other pretreatment conditions can be more accurately assessed. Spot inoculation also facilitates the application of a large population of the test pathogen.

We used a short holding time in this experiment because a longer holding time at room temperature reduces populations to levels appropriate for use in sanitizer efficacy studies. For example, the population of *E. coli* O157:H7 decreased from 6.88 to 3.85 $\log_{10}$ CFU per tomato within 2 h at 22 ± 2°C *(3)*. Therefore, it may be difficult to use tomatoes containing large numbers of *E. coli* O157:H7 cells to test the efficacy of sanitizers. The existence of a large proportion of desiccation-stressed and injured cells throughout the ≥24-h drying period would likely be demonstrated as a decrease in tolerance to sanitizers, thereby potentially giving an overestimation of lethal activity. However, *Salmonella* and *L. monocytogenes* survived in adequate numbers for tests of susceptibility to the lethal activity of sanitizers.

The antagonistic effects of chlorine and low pH on microorganisms are well documented. Although organic acids (with low pHs) and hypochlorite solutions (with free chlorine) have been used widely in treatments for killing foodborne bacteria in the food industry, systems involving high ORP values (1,000 mV) have not commonly been used. The ORP of a solution is an indicator of its ability to oxidize or reduce, with positive and higher ORP values being correlated with greater oxidizing strengths *(15, 20, 21)*. An ORP of +200 to +800 mV is optimal for the growth of aerobic microorganisms, whereas an optimum range of 200 to 400 mV is favorable for the growth of anaerobic microorganisms *(15)*. Since the ORP of EO water in this study was >1,100 mV, ORP likely played an influential role (in combination with low pH and free chlorine) in killing microorganisms *(1)*. It is hypothesized that the low pH in EO water sanitizes the outer membranes of bacterial cells, thereby enabling hypochlorous acid to enter the bacterial cells more efficiently *(27)*. Moreover, Nakagawara et al. *(24)* showed that the microbicidal activity of electrolyzed acidic water depends primarily on the chemical equilibrium of Cl_2, HOCl, and ClO^-. These investigators also showed that the microbicidal activity of electrolyzed acidic water is quantitatively correlated with the concentration of hypochlorous acid that exists in the solution.

There have been reports on the antimicrobial and antiviral activities of electrolyzed acidic water produced by the electrolysis of an aqueous sodium chloride solution with an instrument in which the anode and the cathode are separated by a membrane to form two compartments *(13, 17)*. The application of this technique to areas other than chlorine production, such as agriculture, water treatment, and food sanitation, is new. Recently, electrolyzed acidic water has been reported to be effective as a disinfectant for fruits and vegetables *(14, 18, 19)*.

TABLE 5. *Sensory evaluation of treated and untreated tomatoes at room temperature*

Parameter	Score for tomatoes[a]	
	Treated with EO water	Untreated
Taste	4.6 ± 0.25 A	4.6 ± 0.25 A
Color	4.8 ± 0.20 AB	4.8 ± 0.24 A
Appearance	4.5 ± 0.22 ABC	4.6 ± 0.20 AB

[a] Mean ± standard deviation (n = 10). Means with different letters in the same row are significantly different ($P \leq 0.05$). The values of the hedonic scale were as follows: 1, unacceptable; 2, limited quality for consumption; 3, medium; 4, good; 5, very good. The rejection criterion was a score of 2.5.

The effects of electrolyzed acidic water on the three pathogens were evaluated at ambient temperature in the interest of developing home use antibacterial treatments for unprocessed fresh produce. Although chlorine is highly effective in killing pathogenic microorganisms in simple aqueous systems, its antibacterial effects on microorganisms on foods are minimal, especially in the presence of organic materials that convert chlorine into inactive forms *(4)*.

No significant influence on the appearance, taste, or color of tomatoes was observed after treatment with electrolyzed acidic water (Table 5). These results, in combination with those of the efficacy studies, suggest that electrolyzed acidic water could be applied to control *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on the surfaces of tomatoes.

The results of this study reveal that electrolyzed acidic water is highly effective in killing *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* on the surfaces of tomatoes, indicating the potential for its application for the decontamination of fresh produce contact surfaces. An advantage of electrolyzed acidic water is that it can be produced with tap water, with no added chemicals other than sodium chloride. A large-scale series of experiments using this method must be carried out to determine the reproducibility of the results obtained in the studies reported here.

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Calcinated Calcium Killing of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on the Surface of Tomatoes

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ABSTRACT

This study was conducted to evaluate the efficacy of calcinated calcium, 200 ppm chlorine water (1% active chlorine), and sterile distilled water in killing *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on the surfaces of spot-inoculated tomatoes. Inoculated tomatoes were sprayed with calcinated calcium, chlorinated water, or sterile distilled water (control) and hand rubbed for 30 s. Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in the rinse water and in the residual (0.1% peptone) wash solution were determined. Treatment with 200 ppm chlorine and calcinated calcium resulted in 3.40- and 7.85-$\log_{10}$ reductions of *E. coli* O157:H7, respectively, and 2.07- and 7.36-$\log_{10}$ reductions of *Salmonella*, respectively. Treatment with 200 ppm chlorine and calcinated calcium reduced *L. monocytogenes* numbers by 2.27 and 7.59 $\log_{10}$ CFU per tomato, respectively. The findings of this study suggest that calcinated calcium could be useful in controlling pathogenic microorganisms in fresh produce.

Fresh fruits and vegetables are essential parts of the diets of people around the world. Nutritionists emphasize the importance of fruits and vegetables in a healthy diet, and researchers have recommended the consumption of at least five servings of fruits and vegetables per day *(14)*. Possibly as a result of these recommendations, over the past decade there has been an increase in the consumption of fresh fruits and vegetables, concurrent with increased global distribution, which has made more varieties of produce available year-round *(5)*. A larger number of microbial infections associated with the consumption of fresh fruits and vegetables have also been reported in recent years. Documented illnesses have been caused by bacteria, parasites, and viruses *(2, 16)* and transmitted via many types of fruits and vegetables, including tomatoes *(12, 18)*, lettuce, alfalfa sprouts, parsley, scallions, and cantaloupe, as well as unpasteurized apple and orange juices.

Although many sanitizers have been evaluated for their effectiveness in killing pathogenic microorganisms on different types of produce at a commercial level *(3)*, fewer options are available to consumers. Although the washing of produce with tap water may remove some soil and other debris, it cannot be relied upon to remove microorganisms completely *(1, 4, 6, 11)* and may result in cross-contamination of food preparation surfaces, utensils, and other food items *(1, 3, 4, 6)*. The washing of produce with chlorinated water is the method most commonly used to remove pathogens from fruit and vegetable surfaces, but the efficacy of such treatment has been reported to be inadequate for the elimination of pathogens. The treatment of raw produce with other disinfectants is partially effective in removing disease-causing microorganisms from the surfaces of raw fruits and vegetables *(3)*. Hence, there is a need for, and interest in, the development of effective antimicrobial treatments for the inactivation of pathogenic microorganisms on foods. The objective of this study was to evaluate the effectiveness of calcinated calcium in killing *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* in fresh produce with a view to its potential application to foods and food contact surfaces as an antimicrobial treatment.

MATERIALS AND METHODS

Test strains. The strains studied were enterohemorrhagic *E. coli* O157:H7 strains CR-3, MN-28, MY-29, and DT-66 (isolated from bovine feces); *Salmonella* Enteritidis SE-1, SE-3, and SE-4 (isolated from chicken feces); *Salmonella* Enteritidis SE-2 (isolated from bovine feces); and *Salmonella* Enteritidis IFO-3313. Dr. Nakazawa Muneo, of the Laboratory of Zoonosis, National Institute of Animal Health, Tsukuba, Japan, kindly provided all of the above-mentioned strains except IFO-3313, which was obtained from the Institute for Fermentation, Osaka, Japan. *L. monocytogenes* American Type Culture Collection (ATCC; Manassas, Va.) 43256, ATCC 49594, Japan Collection of Microorganisms (JCM) 7676, JCM 7672, and JCM 7671 were also used in this study. Dr. Akiko Nakama, of the Tokyo Metropolitan Laboratory, Japan, kindly provided all of these *Listeria* cultures.

To minimize the growth on enumeration media of microorganisms naturally present on tomatoes, all test strains of *E. coli* O157:H7 and *Salmonella* were adapted to grow in tryptic soy broth (pH 7.3; Nissiui Seiyaku, Tokyo, Japan) supplemented with nalidixic acid (50 μg/ml). Although some gram-positive microorganisms are less affected by nalidixic acid, the *L. monocyto-*

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genes strains were grown in tryptose phosphate broth (pH 7.0; Difco Laboratories, Detroit, Mich.) containing 50 μg of nalidixic acid per ml before they were used as an inoculum. Plating on media containing nalidixic acid greatly minimized the interference of colony development by naturally occurring microorganisms, thus facilitating the detection of the test pathogen on recovery media.

Produce evaluation. Produce selected for evaluation consisted of red ripe tomatoes (90 ± 20 g each) to which no oil or wax had been applied. Tomatoes were purchased from supermarkets and stored at room temperature (22 ± 2°C) for a maximum of 2 days before they were used in the experiments.

Preparation of inocula. Four- or five-strain mixtures of each pathogen were used as inocula. Each strain of *E. coli* O157:H7 and *Salmonella* was cultured at 37°C in tryptic soy broth supplemented with nalidixic acid (10 ml), and *L. monocytogenes* strains were cultured in tryptose phosphate broth containing 50 μg of nalidixic acid per ml at 37°C and transferred using loop inocula at three successive 24-h intervals immediately before they were used as inocula. Cells of each strain were collected by centrifugation (3,000 × *g*, 10 min, 20°C) and resuspended in 5 ml of phosphate-buffered saline (PBS, pH 7.2). Equal volumes of cell suspensions of five strains of each pathogen were combined to give approximately equal populations of each strain. The inoculum was maintained at 22 ± 2°C and applied to tomatoes within 1 h of preparation.

Procedure for inoculation. Tomatoes at 22 ± 2°C were placed stem-end-down in a biosafety cabinet. Within a 3-cm-diameter circle on the top of the tomato, 100 μl of a suspension of cells in PBS was applied with a micropipetter, with care being taken to avoid placing inoculum on the blossom scar. To prevent the inoculum from running off the side of the tomato and to facilitate drying, small, approximately equal numbers of cells were applied to 10 to 12 spots. The total numbers of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* inoculated onto the tomatoes (CFU per tomato) were calculated from the populations in the inocula (CFU/ml), which were determined by surface plating inoculum serially diluted in 0.1% peptone on appropriate enumeration media. Inoculated tomatoes were stored at 22 ± 2°C for 30 min and used for decontamination studies.

Survivability study. The survivability study involved the determination of the numbers of pathogens that could be recovered from inoculated tomatoes after inoculation and drying at 22 ± 2°C for 30 min. *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were evaluated separately. Two or three replicate trials for each pathogen were performed, and three or four tomatoes were used for each trial. Thirty minutes postinoculation, each inoculated tomato in a biosafety cabinet was sprayed with calcinated calcium, 200 ppm chlorine water, or sterile distilled water with a commercial 500-ml spray bottle with a nozzle held 10 to 15 cm from the tomato's surface. Care was taken to spray the entire surface, including the stem scar area. Thirty seconds after spraying, each tomato was placed in a sealable Pyxon-20 (ELMEX Co. Ltd., Tokyo, Japan) bag and thoroughly rubbed by hand for 30 s. Sterile distilled water (200 ml) was then added to the bag, and the tomato was rinsed by vigorous agitation for 30 s (rinse step). The tomato was transferred to a clean bag, 20 ml of sterile 0.1% peptone water was added, and the tomato was firmly rubbed by hand for 40 s (residual wash step). Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in rinse water and peptone wash water were determined as described below. The tomato was removed from the bag, and the area of skin originally inoculated with the pathogen was excised with a sterile scalpel. Care was taken to remove tomato tissue (pulp) no more than 0.1 cm below the skin surface. The excised tomato skin-pulp was then combined with 20 ml of sterile 0.85% saline water (NaCl, pH 7.0) in a new quart bag and macerated between the fingers until the pulp was removed from the skin. The homogenate (macerate) was then analyzed for the population (CFU/ml) of the test pathogen. Excised skin-pulp of tomatoes not macerated in sterile 0.1% peptone was also analyzed for populations of test pathogens.

Calcinated calcium and chlorine water treatment studies. Calcinated calcium (HYCEA-S, Kaiho Ltd., Tokyo, Japan) was dissolved in distilled water at a concentration of 0.5% (wt/vol) and filtered through double filter paper (150 mm; Advantec, 5C, Toyo Roshi Ltd., Japan), and the clear solution was used within 1 h of preparation. The chlorine solution was prepared by adding sodium hypochlorite (vol/vol; Wako Chemical Co., Tokyo, Japan) solution to distilled water. The effectiveness of calcinated calcium and chlorine water in killing *E. coli*, *Salmonella*, and *L. monocytogenes* separately and simultaneously applied to the surfaces of tomatoes was determined according to the basic protocol developed by Beuchat et al. *(4)*.

Microbiological analysis. Single- and mixed-strain suspensions of each pathogen in PBS were serially diluted in 0.1% sterile peptone water. Duplicate 0.1-ml quantities of appropriately diluted suspensions of *E. coli* O157:H7 and *Salmonella* were surface plated on trypto soya agar (TSA) and on TSA supplemented with 0.1% pyruvic acid and 0.5 μg of nalidixic acid per ml (TSAPN). In addition, diluted *E. coli* O157:H7 suspensions were surface plated onto sorbitol MacConkey agar (Nissui) supplemented with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter) (CT-selective supplement, Oxoid) (CT-SMAC) and on CT-SMAC containing 0.5 μg of nalidixic acid per ml (CT-SMACN). Samples containing *Salmonella* were plated on bismuth sulfite agar (BSA; Difco) and on BSA supplemented with 0.5 μg of nalidixic acid per ml (BSAN). Diluted suspensions of *L. monocytogenes* were surface plated (0.1 ml, in duplicate) on tryptose phosphate agar (TPA), on TPA supplemented with 0.1% pyruvic acid and 0.5 μg of nalidixic acid per ml (TPAPN), and on modified Oxford medium (Oxoid) supplemented with 0.5 μg of nalidixic acid per ml (MOXN). This medium contains 55.5 g of *Listeria*-selective agar base (Oxoid CM 856, Unipath-Oxoid US) per liter of deionized water, 0.01 g of colistin methanesulfate (Sigma Chemical Co., St. Louis, Mo.) per liter of deionized water, 0.02 g of ceftazadime pentahydrate (Glaxo Group Research Ltd., Ware, Hertfordshire, UK) per liter of deionized water, 0.5 μg of nalidixic acid per ml, and 5.0 g of agar (Difco) per liter of deionized water. All ingredients except ceftazadime pentahydrate and nalidixic acid were combined and sterilized by heating at 121°C for 15 min. Ceftazadime solution and nalidixic acid were added to the molten agar before the medium was poured into petri plates.

Inoculated enumeration media were incubated at 37°C for 24 to 28 h before presumptive colonies of each pathogen were counted. *E. coli* O157:H7 was confirmed with the *E. coli* O157 direct immunoassay test kit (Universal Health Watch, Columbia, Md.). *Salmonella* confirmation was carried out by testing reactions on triple sugar iron (Nissui) slants. Randomly picked presumptive colonies of *L. monocytogenes* were confirmed with API Listeria diagnostic kits.

Peptone wash water and phosphate-buffered macerate of skin-pulp from tomatoes prepared in the survivability study were analyzed for populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. Serially diluted peptone wash water from tomatoes inoculated with *E. coli* O157:H7 were surface plated (0.1 ml

TABLE 1. *Populations of* Escherichia coli *O157:H7 recovered from treated and untreated tomatoes*

Recovery medium	Treatment	Population recovered[a]				Reduction[b]	Enrichment[c]
		Water rinse		Peptone wash			
		CFU/ml	CFU/tomato	CFU/ml	CFU/tomato		
CT-SMACN	None	ND[d]	ND	6.93	7.90	−0.27	9
	Water	4.71	6.18	4.87	6.91	0.72	9
	Chlorine	2.59	4.56	2.63	4.71	2.92	9
	Calcinated calcium	<1.0[e]	<1.0[e]	<1.0[e]	<1.0[e]	7.63	2
TSAPN	None	ND	ND	6.54	7.34	0.51	
	Water	4.36	6.65	3.97	6.03	1.82	
	Chlorine	2.77	4.75	2.47	4.45	3.40	
	Calcinated calcium	<1.0[e]	<1.0[e]	<1.0[e]	<1.0[e]	7.85	

[a] Mean value for three replicate experiments.
[b] Number of tomatoes (three tomatoes per replicate) on which *E. coli* O157:H7 was detected by enrichment.
[c] Reduction (log CFU per tomato) compared with population on tomatoes not treated with water control, 200 ppm chlorine, or calcinated calcium and not rinsed with water. Initial populations (0 h) applied to tomatoes were 7.63 $\log_{10}$ CFU per tomato and 7.85 $\log_{10}$ CFU per tomato as detected on CT-SMACN and TSAPN, respectively.
[d] ND, not determined.
[e] No colonies were observed within the incubation period.

in duplicate) on TSAPN and CT-SMACN, samples from tomatoes inoculated with *Salmonella* were plated on TSAPN and BSAN, and samples inoculated with *L. monocytogenes* were plated on TPAPN and MOXN. Quadruplicate 0.25-ml samples of skin-pulp homogenate were also plated on appropriate recovery media. Plates were incubated at 37°C for 18 to 24 h before presumptive colonies of pathogens were counted and confirmed as described above.

Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in rinse water (200 ml) and peptone wash water (20 ml) were determined after the rinsing or washing of tomatoes treated with chlorine (200 ml), tomatoes treated with calcinated calcium, and tomatoes treated with sterile water (control). Undiluted samples (0.25 ml, in quadruplicate and 0.1 ml, in duplicate) and samples (0.1 ml, in duplicate) serially diluted in sterile 0.1% peptone were surface plated on TSAPN and CT-SMACN to recover *E. coli* O157:H7 or on TSAPN or BSAN to recover *Salmonella* or on MOXN to recover *L. monocytogenes*. One hundred milliliters of *E. coli* broth (Nissui), selenite cystine broth (Nissui), or *Listeria* enrichment broth (Oxoid) supplemented with 50 μg of nalidixic acid per ml was then combined with the 20 ml of peptone wash solution in the bag containing the treated, rinsed, washed tomato that had been inoculated with *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes*, respectively, and incubated for 24 to 26 h at 37°C. Cultures were streaked on appropriate recovery media for presumptive colonies, and confirmation of randomly selected colonies was carried out as described above. In studies involving the inoculation of tomatoes with *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, rinse water and peptone wash water diluted in 0.1% peptone were also surface plated (0.1 ml, in duplicate) on plate count agar (Nissui) to determine populations of aerobic mesophilic microorganisms. Plates were incubated at 30°C for 48 h before colonies were counted.

Sensory evaluation. The quality of treated and untreated uninoculated tomatoes was evaluated by 10 panelists selected from the National Food Research Institute who were experienced with sensory panels. The evaluation was based on a five-point hedonic scale: 1, unacceptable; 2, limited quality for consumption; 3, medium; 4, good; 5, very good. The sensory characteristics of appearance, color, and taste were assessed for raw tomatoes. For each of the quality attributes, three treated and three untreated tomatoes were examined and/or tested by the judges. The criterion for rejection for both treated and untreated tomatoes was a score of ≤2.5.

Statistical analyses. All trials were replicated three times. Reported plate count data are expressed as the mean value ± standard deviation obtained from three individual trials, with each of these values being obtained from duplicated samples. Significant differences in plate count data were established by a least significant difference test at the 5% level of significance.

RESULTS

The results of experiments to determine the efficacy of spray treatments with 200 ppm chlorine and calcinated calcium in killing *E. coli* O157:H7 on tomatoes are reported in Table 1. The sizes of the *E. coli* O157:H7 populations applied, as determined by the plating of the inoculum on CT-SMACN and TSAPN, were 7.63 and 7.85 $\log_{10}$ CFU per tomato, respectively. The pH values of water rinses used for tomatoes treated with chlorine and calcinated calcium were 7.0 and 9.18, respectively, whereas the pH values of peptone washes were 6.8 and 7.2. Since the water rinse was diluted in 0.1% peptone before its application to CT-SMACN and TSAPN, the residual effects of sanitizers or pH on *E. coli* O157:H7 would be minimal or nonexistent. Note that an additional 20 s elapsed from the time the tomato was rubbed to the time cells were plated or diluted in 0.1% peptone. Therefore, 40 s of rubbing represented approximately 1 min of exposure to the sanitizers. The numbers of *E. coli* O157:H7 cells in the rinse water are the numbers removed from the control and treated tomatoes and, thus, the numbers that would be available to cross-contaminate other tomatoes or food preparation surfaces in food service or home use situations. The numbers of *E. coli* O157:H7 cells in peptone wash solutions represent the population remaining on the surface of control and treated tomatoes. Overall, CT-SMACN supported colony develop-

TABLE 2. *Populations of* Salmonella *recovered from treated and untreated tomatoes*

Recovery medium	Treatment	Population recovered[a]				Reduction[b]	Enrichment[c]
		Water rinse		Peptone wash			
		CFU/ml	CFU/tomato	CFU/ml	CFU/tomato		
BSAN	None	ND[d]	ND	5.78	7.97	−0.61	9
	Water	4.17	6.21	3.92	6.19	1.17	9
	Chlorine	3.32	5.36	3.25	5.29	2.07	9
	Calcinated calcium	<1.0[e]	<1.0[e]	<1.0[e]	<1.0[e]	7.36	3
TSAPN	None	ND	ND	6.54	7.34	0.12	
	Water	4.28	6.55	4.23	6.13	1.33	
	Chlorine	3.50	5.54	3.46	5.50	1.96	
	Calcinated calcium	<1.0[e]	<1.0[e]	<1.0[e]	<1.0[e]	7.46	

[a] Mean value for three replicate experiments.
[b] Number of tomatoes (three tomatoes per replicate) on which *Salmonella* was detected by enrichment.
[c] Reduction (log CFU per tomato) compared with population on tomatoes not treated with water control, 200 ppm chlorine, or calcinated calcium and not rinsed with water. Initial populations (0 h) applied to tomatoes were 7.36 $\log_{10}$ CFU per tomato and 7.46 $\log_{10}$ CFU per tomato as detected on BSAN and TSAPN, respectively.
[d] ND, not determined.
[e] No colonies were observed within the incubation period.

ment by *E. coli* O157:H7 better than TSAPN did, regardless of whether cells were exposed to sanitizers. Washing with water (control) resulted in a reduction of 0.72 $\log_{10}$ CFU per tomato; further significant reductions of 2.92 and 7.63 $\log_{10}$ CFU per tomato were achieved by treatment with 200 ppm chlorine and calcinated calcium, respectively, as detected on CT-SMACN. However, even with these large reductions, the pathogen was detected with the enrichment of treated tomatoes.

Shown in Table 2 are results from experiments to determine the efficacy of 200 ppm chlorine and calcinated calcium in killing *Salmonella* on tomatoes. The sizes of *Salmonella* populations applied, as determined by the plating of the inoculum on BSAN and TSAPN, were 7.36 and 7.46 $\log_{10}$ CFU per tomato, respectively. TSAPN and BSAN were equally suitable for supporting colony development, regardless of whether cells were exposed to sanitizers. Washing with water (control) resulted in a reduction of 1.17 $\log_{10}$ CFU per tomato; further significant reductions of 2.07 and 7.36 $\log_{10}$ CFU per tomato were achieved by treatment with 200 ppm chlorine and calcinated calcium, respectively, as detected on BSAN. However, even with these large reductions, the pathogen was detected with the enrichment of treated tomatoes.

Results of experiments to determine the efficacy of sanitizers in killing *L. monocytogenes* on the surfaces of tomatoes are summarized in Table 3. The sizes of the *L. monocytogenes* populations applied, as determined by the plating of the inoculum on MOXN and TPAPN, were 7.54 and 7.59 $\log_{10}$ CFU per tomato, respectively. Overall, TPAPN supported colony development by *L. monocytogenes* better than MOXN did, regardless of whether inoculat-

TABLE 3. *Populations of* Listeria monocytogenes *recovered from treated and untreated tomatoes*

Recovery medium	Treatment	Population recovered[a]				Reduction[b]	Enrichment[c]
		Water rinse		Peptone wash			
		CFU/ml	CFU/tomato	CFU/ml	CFU/tomato		
MOXN	None	ND[d]	ND	6.19	7.32	0.22	9
	Water	5.15	7.23	4.29	6.19	1.35	9
	Chlorine	3.15	5.08	3.05	5.18	2.36	9
	Calcinated calcium	<1.0[e]	<1.0[e]	<1.0[e]	<1.0[e]	7.54	2
TPAPN	None	ND	ND	6.54	7.34	0.25	
	Water	5.19	7.43	5.23	6.32	1.27	
	Chlorine	3.64	5.72	3.26	5.32	2.27	
	Calcinated calcium	<1.0[e]	<1.0[e]	<1.0[e]	<1.0[e]	7.59	

[a] Mean value for three replicate experiments.
[b] Number of tomatoes (three tomatoes per replicate) on which *L. monocytogenes* was detected by enrichment.
[c] Reduction (log CFU per tomato) compared with population on tomatoes not treated with water control, 200 ppm chlorine, or calcinated calcium and not rinsed with water. Initial populations (0 h) applied to tomatoes were 7.54 $\log_{10}$ CFU per tomato and 7.59 $\log_{10}$ CFU per tomato as detected on MOXN and TPAPN, respectively.
[d] ND, not determined.
[e] No colonies were observed within the incubation period.

ed tomatoes had been treated with sanitizers. This finding indicates that some of the cells were injured and unable to resuscitate in the presence of selective chemicals in MOXN. This phenomenon was particularly evident when the size of the *L. monocytogenes* population recovered from peptone wash samples on MOXN was compared with the size of the population recovered on TSAPN. Treatment with water resulted in a reduction of 1.35 $\log_{10}$ CFU per tomato; treatment with 200 ppm chlorine and calcinated calcium resulted in significant reductions of 2.36 and 7.54 $\log_{10}$ CFU per tomato, respectively.

DISCUSSION

Test strains of pathogens from outbreaks of infections associated with foods of plant or animal origin were chosen for evaluation. The method used for the evaluation involved four- or five-strain mixtures of each pathogen, which is consistent with currently accepted practices for studying the survival and growth of pathogens in food *(8–10, 18)* and with Scientific Advisory Panel recommendations. The use of multiple-strain inocula represents a conservative strategy, since the mixture may provide a more representative challenge for sanitizing products than a single strain would. Thus, data should more accurately predict the physiological sensitivities of pathogenic strains occasionally present on produce and should also provide a conservative estimate of the risk to public health. All strains examined were adapted to grow in the presence of 50 μg of nalidixic acid per ml, one of several markers used to evaluate the survival of bacterial pathogens in food products with potentially high numbers of interfering background microflora. Antibiotic-resistant markers have been widely used in studies to determine the fate of pathogens in nonsterile foods, including fresh produce *(15)*, meats *(7)*, and milk *(17)*. With spot inoculation, a specified number of cells can be applied on produce surfaces, and the decrease in the numbers of viable cells during drying or under other pretreatment conditions can be more accurately assessed. Spot inoculation also facilitates the application of a large population of the test pathogen.

Calcinated calcium is a natural product of oyster shells. To obtain calcinated calcium, oyster shells are ground and their pearl layer alone is treated electrically with ohmic heating (220 V, 60 to 100 A, 10 to 60 min). Then the shells are crushed into powder (approximately 320 mesh) and used as a nutrient supplement in food in Japan *(13)*. In our previous study, calcinated calcium was dissolved in distilled water at the appropriate percentages (wt/vol), autoclaved at 121°C for 15 min, and stored as a stock solution; however, in the present study, we omitted the autoclaving step to make the process convenient for home or field use. The same effectiveness was obtained with the reuse of calcinated calcium. Just before calcinated calcium was used, filtration was carried out to obtain a clear solution with an alkaline pH (12.5 at 0.5%). If the filtered solution was kept for a long time at room temperature, it developed sediment. Therefore, calcinated calcium could be useful in controlling pathogens in fresh produce if a proper filtration procedure is developed.

TABLE 4. *Sensory evaluation of treated and untreated tomatoes at room temperature*[a]

Parameter	Score for samples treated with 0.5% calcinated calcium	Score for untreated samples
Taste	4.2 ± 0.25 A	4.5 ± 0.25 A
Color	4.8 ± 0.20 AB	4.8 ± 0.24 A
Appearance	4.3 ± 0.22 ABC	4.6 ± 0.20 AB

[a] Values are expressed as means ± standard deviations; $n = 10$. Means with the same letter in the same row are not significantly different ($P \leq 0.05$). The values of the hedonic scale were as follows: 1, unacceptable; 2, limited quality for consumption; 3, medium; 4, good; 5, very good. The rejection criterion was a score of ≤2.5.

No significant influence on the appearance, taste, or color of tomatoes was observed after treatment with calcinated calcium (Table 4). These results, in combination with those of the efficacy studies, suggest that calcinated calcium could be used to control *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on tomato surfaces.

In conclusion, results from survivability and sanitizer efficacy studies indicate that the method studied is effective in evaluating the antimicrobial activity of chemical sanitizers applied to tomatoes for the purpose of killing or removing *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. In the efficacy study, calcinated calcium was demonstrated to have antimicrobial activity that exceeded the antimicrobial activities of 200 ppm chlorine and distilled water. A large-scale series of experiments using this method must be carried out to determine the reproducibility of the results obtained in the experiments reported here.

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Original

Comparison of TaqMan™ *Salmonella* Amplification/Detection Kit with Standard Culture Procedure for Detection of *Salmonella* in Meat Samples

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We evaluated the TaqMan PCR *Salmonella* amplification/detection kit (PE Applied Biosystems) for rapid detection of *Salmonella* from a variety of meat samples. This system uses the 5′ nuclease activity of Taq DNA polymerase, which digests an internal fluorogenic probe to monitor the amplification of the target gene. The detection sensitivity of the kit, using 2 kinds of DNA extraction protocols, was compared with that obtained with 4 protocols of official culture methods. A total of 98 meat samples (16 raw beef, 31 pork and 51 chicken) were tested. The results of the TaqMan PCR method and the combined results of the 4 cultural protocols showed excellent agreement. However, no single culture protocol showed optimal recovery of *Salmonella* comparable to the PCR method. These results suggest that the TaqMan PCR method is a reliable and rapid method useful for detecting *Salmonella* in meat products.

Key words: TaqMan; PCR; *Salmonella*; detection

Introduction

Food-borne salmonellosis remains a major public health problem. Early detection of *Salmonella* is important for the limitation of outbreaks. There is a need for faster and simpler screening tests for the detection of *Salmonella* in foods, food sources, and food manufacturing plants. The conventional method for the detection of *Salmonella* requires multiple subculture steps followed by biochemical tests. They require at least 4 days in the case of noncontaminated products and 5 to 6 days for contaminated samples[1]. In recent years several rapid methods have been developed. These include rapid culture methods[2], various types of immunological methods[3,4] and DNA methods[5]. Although rapid methods have provided an improvement over traditional culture methods for *Salmonella* detection, their sensitivity is limited, and they still require both pre-enrichment and selective enrichment, which are time-consuming processes. Polymerase chain reaction (PCR) combines simplicity with a potential for high specificity and sensitivity in detection of foodborne, pathogenic bacteria. Accordingly, a number of PCR assays specific for different *Salmonella* genes such as the *invA* gene[6], 16S-rRNA gene[7], *phoP* gene[8], *phoE* gene[9] and *fimA* gene[10] have been reported. However, these PCR detection methods involve electrophoresis, which requires skill and is labor-intensive, time-consuming and difficult to automate. Moreover, the result of electrophoresis is subjective and documentation of results is difficult.

TaqMan *Salmonella* amplification/detection kit is a new PCR sequence detecting system that uses the 5′ nuclease assay of PCR products, employing fluorogenic probes[11]. Recently, the TaqMan PCR assay has been described as a unique system for the detection[12,13] or quantification of food pathogens[14]. We reported in an earlier study[15] that this system was very specific, recognizing all 68 strains of different serotypes of *Salmonella* tested, and not showing cross-reactivity with any of the non-*Salmonella* strains. In that study, the TaqMan *Salmonella* kit was also evaluated in a variety of naturally contaminated meat samples compared with the conventional culture method. However, only a single enrichment culture and selective agar medium were evaluated in that study. Thus, we might have underestimated the performance of the culture method.

A more thorough evaluation study is necessary before acceptance of this new PCR method for routine laboratory use. Accordingly, the present study was undertaken to compare the TaqMan PCR method with 4 different cultural protocols, recommended as ISO/CD standard methods and Food Hygiene Examination Guide methods[1].

Materials and Methods

Sample preparation

Ninety-eight samples of meats (chicken, beef and pork) were collected from 8 local stores; they comprised 16 raw beef, 31 raw pork and 51 raw chicken. These

samples were purchased on two occasions; first, on November 1997, 54 meat samples, second, another 44 samples (the same items from the same shops) one week after the first sampling occasion. The samples were immediately transported in insulated coolers to the laboratory for treatment and analysis on that day.

Culture method

The culture method followed the Food Hygiene Examination Guide[1]. Briefly, 25 g samples were aseptically measured, and stocked into sterile stomacher bags. These samples were added to 225 mL of sterile EEM (Eiken Chemical, Tokyo, Japan) or Trypticase Soy Broth (TSB; BBL, Cockeysville, MD, USA) and then pummeled for 2 min in a stomacher 400 Lab Blender (Seward, London, UK). Each sample mixture was incubated for 18 hr at 37℃, after which 1 mL of the mixture was transferred to 15 mL of SC broth (from EEM) (Eiken) or Hajna tetrationate Broth (from TSB) (Eiken). They were incubated for 20 hr at 43℃, then one loopful of the culture from each broth was streaked on DHL (Eiken) and MLCB (Nissui, Tokyo, Japan) agar plates. These plates were incubated at 37℃ overnight. Three to five suspicious colonies were submitted for biochemical screening (TSI and LIM) and seorological confirmation using *Salmonella* polyvalent O, O1 antisera (Denka Seiken, Tokyo, Japan). The sample inoculated in TSB was used not only for conventional culture methods, but also for DNA extraction. One mL of the TSB was transferred to a sterile Eppendorf tube twice. These tubes were used for DNA extraction.

DNA extraction

For *Salmonella* DNA extraction from meat samples, two extraction protocols, the Chelex method[16] and a method based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidine isothiocyanate (GuSCN)[17], were used as previously described[12]. Five microliters of the solution was used as the PCR template.

PCR assay condition

All amplification PCR reactions were performed in a total volume of 50 μL, with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Forster City, CA, USA). Thermal cycling conditions were 95℃ for 5 min; 40 cycles of 95℃ for 20 sec and 65℃ for 1 min. The PCR reaction employed 5 μL of template DNA; 34 μL of *Salmonella* Master Mix (Applied Biosystems); 11 μL of 25 mmol/L $MgCl_2$. Negative control (NAC; No Amplification Control, NTC; No Template Control) was used in each analysis according to the manufacturer's instructions.

Fluorescence reading and data analysis

The fluorescence of the PCR products was measured with an ABI PRISM 7700 Sequence Detector that included a plate reader accessory. Fluorescence was read prior to amplification (pre-read) and after thermal cycling (post-read). The pre-read data were used to correct for interference with fluorescence caused by the introduced food matrix. The fluorescence intensities of the fluorescent reporter dye (6-carboxyfluorescein; FAM; emission wavelength 518 nm and 6-carboxy-4,7,2′,7′-tetrachlorofluorescein; TET; emission wavelength 538 nm), the fluorescent quencher dye (6-carboxytetramethylrhodamine; TAMRA; emission wavelength 582 nm) and reference dye (6-carboxy-X-rhodamine; ROX; emission wavelength 602 nm; included in *Salmonella* Master Mix) were determined for each tube.

Data analysis and judgement were performed with SDS 1.6.3 Software (Applied Biosystems) according to the manufacturer's instructions. The data were judged with a 99.7% confidence level from negative control fluorescence data. In this system, an increase in the reporter fluorescence signal (FAM) corresponds to the amplification of target *Salmonella* DNA. To avoid false negative judgement, i.e., to discriminate between failure of the PCR reaction and the absence of *Salmonella*, the kit contained PCR amplification control DNA (PAC DNA). The detection of the reporter fluorescence (TET) from the PCR amplification of PAC DNA, which was amplified together with the target DNA in a reaction tube, served as a control for the effect of potential PCR inhibitors. Thus, the software judged either "Yes, +" which means that *Salmonella* exists in this sample; "No, −" which means that *Salmonella* does not exist in this sample; or "Retest, ?" which means that this sample needs to be reevaluated (PAC DNA signal was not obtained).

Results

Table 1 summarizes the results obtained by the conventional culture method and the TaqMan PCR method for the detection of Salmonellae in 98 food samples. Overall, the combined results of the 4 cultural protocols and the results of the TaqMan PCR method showed good agreement. A total of 37 samples were *Salmonella*-positive by the culture methods, while a total of 42 samples were *Salmonella*-positive by PCR. Among them, 34 samples were *Salmonella*-positive by both methods. A total of 53 samples were *Salmonella*-negative by the both methods. The combined results of the culture and PCR methods revealed that a total of 45 samples were *Salmonella*-positive. The 45 *Salmonella*-positive samples were composed of 35 raw chicken samples, 6 raw pork samples, and 4 raw beef samples. The rate of positive samples in each kind of meat was; chicken 68.6% (35/51), pork 19.4% (6/31), beef 25.0% (4/16) and total 45.9% (45/98). On the basis of these results, the sensitivity of the TaqMan PCR was 93.3% (42/45×100), compared with a sensitivity of 82.2% (37/45×100) for the conventional culture method. However, when *Salmonella* recovery by individual enrichment methods was compared with that by the TaqMan PCR method, the percentage of agreement was low. For example, the percentage agreement between the results of TSB-Hajna-DHL (Hajna tetrationate broth

Table 1. Number of *Salmonella*-positive Meat Samples for 4 Culture and 2 PCR Protocols

Protocol	Meat samples			Total
	Chicken	Pork	Beef	
No. of samples	51	31	16	98
Culture method[a]				
EEM-SC-DHL	14	1	0	15
EEM-SC-MLCB	21	3	2	26
TSB-Hajna-DHL	10	4	3	17
TSB-Hajna-MLCB	19	4	3	26
Culture combined	30	4	3	37
PCR method[b]				
Chelex extraction	26	5	4	35
GuSCN extraction	31	5	3	39
PCR combined	32	6	4	42
Positive by both methods	27	4	3	34
Negative by both methods	16	25	12	53
Two methods combined	35	6	4	45

[a] Abbreviations: EEM and TSB, enrichment in EEM or TSB broth, respectively; SC and Hajna; selective enrichment in SC or Hajna broth, respectively; DHL and MLCB; streaked on DHL or MLCB agar plate, respectively.

[b] DNA was extracted from the meat samples with 2 extraction protocols as described in the text.

after TSB enrichment followed by DHL plate) and the TaqMan PCR (GuSCN extraction) was 43.6% (17/39×100). Similarly, the percentage agreement between the results of EEM-SC-MLCB (SC broth after EEM enrichment followed by MLCB plate), which showed the best recovery among the cultural protocols, and the TaqMan PCR (GuSCN extraction) was 66.7% (26/39×100).

Table 2 shows the recovery of *Salmonella* from individual experiments (different shops, sampling occasions, and protocols). Eight samples (D-2, D-7, G-2, a-4, a-5, b-2, d-7 and d-8) which were negative by the conventional method were positive by the TaqMan method, while three samples (A-5, B-9 and b-9) positive by the former method was negative by the last. The data also indicate the variation in distribution of *Salmonella* from shop to shop and on different sampling occasions. While the absence of *Salmonella* was indicated in shops C and F, heavy contamination was noted in shops A and B. In the two successive samplings, the *Salmonella* contamination did not change in the heavily contaminated shops A and B, while the contamination rate decreased in shops G and H and increased in shops D and E.

Discussion

The analysis of natural meat samples in the present study revealed high recovery of *Salmonella* with both the conventional and the TaqMan PCR methods. However, combinations of 2 enrichment broths and 2 selective plates were necessary in culture methods to obtain as high *Salmonella* recovery as that of TaqMan PCR method (Table 1). No single culture method allowed optimal recovery and detection of *Salmonella* comparable to that of the PCR method. In our previous study[15], *Salmonella* recovery by only a single enrichment protocol (Hajna tetrationate broth followed by DHL plate) was compared with that by the TaqMan PCR method. In that study, the conventional and PCR methods showed good agreement (90.9%). However, the number of positive samples was limited in that study (11 positive samples). The results of present study are in agreement with the results reported by Perales and Audicana[18] and Aabo *et al.*[19], in that even the best combinations of enrichment and selective plating media for isolation of *Salmonella* spp. in naturally contaminated meats have a sensitivity of only 50–60%. Enrichment followed by PCR in these cases has a sensitivity of 99–100%[19].

At first glance, it would appear that the PCR gave false negative results in some samples (A-5, B-9 and b-9). However, it should be noted that the meat samples were divided into 2 for enrichment in this study. Accordingly, both methods may fail to detect *Salmonella* if the bacteria are present in low numbers and are heterogeneously distributed. In fact, if we look at the results carefully, the PCR did not fail to detect *Salmonella* in the same enrichment broth (TSB) from which a subsequent selective plate demonstrated *Salmonella* (A-9, G-4, H-4, a-2, a-3, a-7, a-10, b-6, d-6 and e-3).

In this study, *Salmonella* DNA was extracted by either the Chelex method or the GuSCN method from meat samples. Both of the extraction methods used are reliable with meat samples. This is in agreement with the results of our previous study[15]. In that study, we reported that the TaqMan PCR assay could detect 120 CFU/mL (3×10^3 CFU/25 g) of *Salmonella* in pure culture, being more sensitive than other methods for *Salmonella* detection, such as ELISA[20], biosensor[21], DNA-DNA hybridization[22] and immunofluorescence assay[23]. The same study also revealed that 8 hr of pre-enrichment was required to detect a single viable cell of *Salmonella* in 25 g of meat or shrimp samples by the PCR method. We conclude that the TaqMan PCR of DNA extracted by either of the above methods from an overnight culture of unselective enrichment (TSB) would be a practical protocol for detecting *Salmonella* from meat samples.

Epidemiologically, the contamination rate of raw chicken products observed in this study (68.6%) was higher than those observed in other studies in Tokyo (26.6%)[24], or in other countries: 43% in Ohio, USA[25], 55% in Portugal[26], 36.7% in Belgium[27]. Also, there was a great difference in *Salmonella* contamination rate from shop to shop, depending on sampling occasion. In the shops where meat samples were heavily contaminated, most meat samples were contaminated regardless of the parts and/or kinds of meats. Since chicken carcasses and meat blocks are cut up to individual parts in the backyard of the butcher's shops studied, cross-contamination between meats may occur. In the butcher's shops studied in this study, primal cuts and finished items are often processed by the same persons

Table 2. *Salmonella* Detection by the Culture and PCR Methods in Various Lots of Meat Samples from 8 Different Shops

1st purchase							2nd purchase							
Sample No.[a]	Culture[b]				PCR[b]		Sample No.	Culture				PCR		Sample
	EEM-SC		TSB-Hajna		Chelex	GuSCN		EEM-SC		TSB-Hajna		Chelex	GuSCN	
	DHL	MLCB	DHL	MLCB				DHL	MLCB	DHL	MLCB			
A- 1	+	+	−	+	+	+	a- 1	−	+	−	−	+	+	chicken minced
A- 2	+	+	+	+	+	+	a- 2	−	−	+	+	+	+	pork minced
A- 3	−	−	−	−	−	−	a- 3	−	−	+	+	+	+	beef minced
A- 4	−	−	−	−	−	−	a- 4	−	−	−	−	−	+	pork liver
A- 5	+	−	−	−	−	−	a- 5	−	−	−	−	−	+	chicken breast meat
A- 6	−	−	−	−	−	−	a- 6	−	+	+	+	+	+	pork guts
A- 7	+	+	+	+	+	+	a- 7	−	−	+	+	+	+	chicken gizzard
A- 8	−	−	−	−	−	−	a- 8	−	+	−	−	+	+	chicken liver
A- 9	−	−	+	+	+	+	a- 9	−	+	+	+	+	+	chicken thigh
A-10	−	+	−	+	+	+	a-10	−	−	+	+	+	+	chicken wing
A-11	−	−	−	−	−	−	a-11	−	+	+	+	+	+	pork frame
A-12	−	−	−	−	−	−	a-12	−	+	+	+	+	+	beef frame
B- 1	+	+	−	−	−	+	b- 1	−	+	−	+	+	+	chicken minced
B- 2	+	+	−	+	+	+	b- 2	−	−	−	−	+	+	chicken minced
B- 3	+	+	−	−	+	+	b- 3	NT[c]	NT	NT	NT	NT	NT	chicken minced
B- 4	+	+	+	+	+	+	b- 4	+	+	−	+	+	+	chicken breast meat
B- 5	+	+	−	−	+	+	b- 5	−	+	+	+	+	+	chicken frame
B- 6	+	+	−	−	−	+	b- 6	−	−	+	−	+	+	chicken thigh
B- 7	+	+	−	+	−	+	b- 7	+	+	−	+	+	+	chicken giblets
B- 8	+	+	+	+	+	+	b- 8	−	−	−	−	−	−	chicken gizzard
B- 9	+	+	−	−	−	−	b- 9	−	+	−	−	−	−	chicken liver
C- 1	−	−	−	−	−	−	c- 1	NT	NT	NT	NT	NT	NT	pork liver
C- 2	−	−	−	−	−	−	c- 2	NT	NT	NT	NT	NT	NT	chicken breast meat
C- 3	−	−	−	−	−	−	c- 3	NT	NT	NT	NT	NT	NT	pork minced
C- 4	−	−	−	−	−	−	c- 4	NT	NT	NT	NT	NT	NT	pork frame
C- 5	−	−	−	−	−	−	c- 5	NT	NT	NT	NT	NT	NT	chicken frame
C- 6	−	−	−	−	−	−	c- 6	NT	NT	NT	NT	NT	NT	chicken breast

[a] "A–H" and "a–h" represent the shops from which the meat samples were collected on the 1st (Nov., 1997) and the 2nd occasions (1 week after the 1st occasion), respectively. The numbers after the hyphen represent the sample numbers. The same number represents the same item of meat sample.

[b] Abbreviations are the same as described in Table 1.

[c] Not tested.

Table 2. (Continued)

1st purchase							2nd purchase						
Sample No.	Culture				PCR		Sample No.	Culture				PCR		Sample
	EEM-SC		TSB-Hajna		Chelex	GuSCN		EEM-SC		TSB-Hajna		Chelex	GuSCN	
	DHL	MLCB	DHL	MLCB				DHL	MLCB	DHL	MLCB			
D- 1	−	−	−	−	−	−	d- 1	−	−	−	−	−	−	pork minced
D- 2	−	−	−	−	−	+	d- 2	NT	NT	NT	NT	NT	NT	chicken minced
D- 3	−	−	−	−	−	−	d- 3	−	+	+	+	+	+	beef minced
D- 4	−	−	−	−	−	−	d- 4	−	−	−	−	−	−	pork frame
D- 5	−	−	−	−	−	−	d- 5	−	−	−	−	−	−	beef frame
D- 6	−	−	−	−	−	−	d- 6	−	−	−	+	+	+	chicken breast
D- 7	−	−	−	−	−	+	d- 7	−	−	−	−	+	−	chicken breast meat
D- 8	−	−	−	−	−	−	d- 8	−	−	−	−	+	−	beef minced
D- 9	−	−	−	−	−	−	d- 9	NT	NT	NT	NT	NT	NT	chicken wing
E- 1	−	−	−	−	−	−	e- 1	−	−	−	−	−	−	pork frame
E- 2	−	−	−	−	−	−	e- 2	−	−	−	−	−	−	pork minced
E- 3	−	−	−	−	−	−	e- 3	−	−	−	+	+	+	chicken breast meat
F- 1	−	−	−	−	−	−	f- 1	−	−	−	−	−	−	chicken breast meat
F- 2	−	−	−	−	−	−	f- 2	−	−	−	−	−	−	chicken minced
F- 3	−	−	−	−	−	−	f- 3	−	−	−	−	−	−	beef minced
F- 4	−	−	−	−	−	−	f- 4	−	−	−	−	−	−	pork minced
F- 5	−	−	−	−	−	−	f- 5	−	−	−	−	−	−	pork frame
G- 1	−	−	−	−	−	−	g- 1	−	−	−	−	−	−	chicken minced
G- 2	−	−	−	−	+	−	g- 2	−	−	−	−	−	−	pork minced
G- 3	−	−	−	−	−	−	g- 3	−	−	−	−	−	−	beef minced
G- 4	−	−	−	+	+	+	g- 4	−	−	−	−	−	−	chicken frame
G- 5	−	−	−	−	−	−	g- 5	−	−	−	−	−	−	pork frame
H- 1	−	+	+	−	+	+	h- 1	NT	NT	NT	NT	NT	NT	chicken minced
H- 2	−	−	−	−	−	−	h- 2	−	−	−	−	−	−	pork minced
H- 3	−	−	−	−	−	−	h- 3	−	−	−	−	−	−	beef minced
H- 4	−	−	−	+	+	+	h- 4	−	−	−	−	−	−	chicken breast meat
H- 5	−	−	−	−	−	−	h- 5	−	−	−	−	−	−	pork frame

with the same utensils. Also, raw meats of different animal species, including poultry and meat products, are often displayed on the same and/or directly adjacent counters. The hypothesis of cross contamination should be verified by studies of serotypes or genotypes of *Salmonella* isolated. These aspects were not investigated in this study. However, this investigation revealed which shops were more badly managed, i.e., poorly cleaned and disinfected. Variation in contamination rate from shop to shop depending on sampling occasion indicated that differences in operation of good manufacturing practice (GMP) depending on shops and occasions would greatly affect the *Salmonella* contamination rate. The TaqMan PCR method, which is easy to perform and allows handling of as many as 96 samples at one time, would be a robust tool for performing these evaluations.

The main conclusion from this study is that the TaqMan PCR method is superior to the conventional culture method for routine detection of *Salmonella* from meat samples. The main advantage in utilization of the method for food analysis is that it reduces both labor and cost. Moreover, the total time needed to accomplish the analysis would be reduced from 4 to 6 days to 24 hr.

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Rapid, Quantitative PCR Monitoring of Growth of *Clostridium botulinum* Type E in Modified-Atmosphere-Packaged Fish

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A rapid, quantitative PCR assay (TaqMan assay) which quantifies *Clostridium botulinum* type E by amplifying a 280-bp sequence from the botulinum neurotoxin type E (BoNT/E) gene is described. With this method, which uses the hydrolysis of an internal fluoregenic probe and monitors in real time the increase in the intensity of fluorescence during PCR by using the ABI Prism 7700 sequence detection system, it was possible to perform accurate and reproducible quantification of the *C. botulinum* type E toxin gene. The sensitivity and specificity of the assay were verified by using 6 strains of *C. botulinum* type E and 18 genera of 42 non-*C. botulinum* type E strains, including strains of *C. botulinum* types A, B, C, D, F, and G. In both pure cultures and modified-atmosphere-packaged fish samples (jack mackerel), the increase in amounts of *C. botulinum* DNA could be monitored (the quantifiable range was 10^2 to 10^8 CFU/ml or g) much earlier than toxin could be detected by mouse assay. The method was applied to a variety of seafood samples with a DNA extraction protocol using guanidine isothiocyanate. Overall, an efficient recovery of *C. botulinum* cells was obtained from all of the samples tested. These results suggested that quantification of BoNT/E DNA by the rapid, quantitative PCR method was a good method for the sensitive assessment of botulinal risk in the seafood samples tested.

Botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* is responsible for food-borne botulism, and the nonproteolytic *C. botulinum* (group II) has been reported to grow and produce toxin at temperatures as low as 3.3 to 4°C (12, 39), although a recent report (29) indicated that toxin was produced at an even lower temperature (2°C). Accordingly, there is a great concern over the growth and toxin production of these organisms in low-temperature-storage food such as vacuum- or modified-atmosphere (MA)-packaged seafood (35) or refrigerated processed foods of extended durability (REPFED) (33). Currently, mouse assay is the only universally acknowledged method for the detection of *C. botulinum*. It is highly sensitive and specific, but costly, time-consuming, laborious, and requires handling of laboratory animals; thus, only a limited number of samples can be analyzed at one time. If a more rapid and convenient risk assessment could be established, the development of new packaged food products such as REPFED, which is at present potentially discouraged because of concerns over *C. botulinum*, would be stimulated. To date, several alternative in vitro assays have been developed (11, 47), and among them, enzyme-linked immunosorbent assay (ELISA) has been most widely used for food analysis (36). However, ELISA has several deficiencies, including sensitivity, complexity in handling, and accuracy. Recently, a novel in vitro bioassay (47) has been developed for the detection of BoNT type B (BoNT/B) which seems to have solved these deficiencies. Multiple alternative methods should be developed and evaluated for their performance, cost, and adaptability to automation before they are fully integrated into the food industry.

Because of their high specificity and sensitivity, PCR-based assays have advantages for rapid, accurate identification of pathogenic bacteria. However, PCR assays with electrophoresis are primarily qualitative techniques and not appropriate for accurate quantification of a target sequence. Quantification of target gene numbers by PCR has been attempted with most probable number PCR (MPN PCR) (18), competitive PCR (21, 24), and PCR-ELISA (16, 41). These methods require the multiple handling of culture tubes (MPN PCR), an internal standard with identical PCR efficiency which must be analyzed during the log phase of the reaction (competitive PCR), and post-PCR treatment (PCR-ELISA). The approach to botulinal risk assessment would be greatly simplified and advantageous, if quantitative PCR could be accomplished in an automated system.

Recently 5′ nuclease assays (TaqMan assay) have been described as a unique detection system for PCR products (20, 26). The 5′ nuclease assay exploits the 5′→3′ nuclease activity of *Taq* DNA polymerase, which digests an internal probe labeled with a quencher dye and a reporter dye. The probe is designed to hybridize to an internal region of the amplified sequence. For the intact probe, the fluorescence from the reporter dye is suppressed efficiently by the quencher dye due to its spatial proximity to the reporter. As the PCR amplification proceeds, the 5′ nuclease activity of *Taq* DNA polymerase cleaves the probe, separating the two dyes and thus resulting in an increase in reporter fluorescence signal that can be detected on a fluorescence spectrometer. Therefore, the increase in the reporter dye fluorescence is a direct consequence of target amplification. The method has been applied to detect *Listeria monocytogenesis* (2), Shiga-like toxin-producing *Escherichia coli* (48), *Salmonella* (6, 22), and *E. coli* O157:H7 (31). These

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studies have mainly addressed "yes-or-no" detection rather than the quantification of pathogens, which relies on end point data collection with a microplate reader format (TaqMan LS-50B PCR detection system).

A truly automated, quantitative PCR of target genes in a large number of samples has been successfully realized with a rapid, quantitative PCR assay with the ABI PRISM 7700 sequence detection system (SDS) employing a 5′ nuclease assay. This method has been shown to be rapid and sensitive for the quantification of PCR or reverse transcription-PCR products (7, 15). This system, a combination of a thermal cycler, laser, and a detection-software system, allows for the quantification of PCR products in real time as revealed by the fluorescence increase produced by 5′ nuclease activity during the amplification process. When the threshold cycle, C_t, for each standard is plotted on the y axis and the starting quantity for each standard is plotted on the x axis, a standard curve can be obtained. This standard curve can be used to quantify the template in unknown samples. This system has been applied for quantification of *Mycobacterium tuberculosis* (9), *Yersinia pestis* (19), and *Salmonella* (30).

In this paper, we describe a rapid, quantitative PCR assay employing the ABI PRISM 7700 SDS for the estimation of *C. botulinum* type E populations. The complete nucleotide sequences of the neurotoxin genes for type E were published (34), allowing the design of PCR primers for detection (5, 14, 40, 42, 43). However, the reported primers cannot be applied to the rapid, quantitative PCR method, because the method requires designing an internal probe region that must have a higher annealing temperature than the primers. In this study, we have designed a new primer set and a probe suitable for the rapid, quantitative PCR method and applied the assay to the quantification of *C. botulinum* type E in MA-packaged fish.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Clostridium* strains and non-*Clostridium* strains tested in this study are listed in Table 1. The *Clostridium* cultures were grown at 30°C overnight in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) anaerobically (BBL anaerobic jar; BBL Microbiology Systems, Cockeysville, Md.). Non-*Clostridium* strains were grown at 37 to 25°C overnight in Trypticase soy broth (TSB) (BBL) under aerobic conditions, with NaCl (2.5%) added when necessary.

***C. botulinum* type E spore preparations.** Spore suspensions of *C. botulinum* were prepared as described previously (25). The spores were suspended in a small volume of cold sterile distilled water and stored at or below 4°C. The number of spores per milliliter was determined on a poured medium of Clostridia count agar (Nissui Pharmaceutical Co, Ltd., Tokyo, Japan) consisting of peptone (15.0 g), soy peptone (8.0 g), yeast extract (8.0 g), meat extract (7.5 g), L-cysteine hydrochloride (0.75 g), ferric ammonium citrate (1.0 g), sodium metabisulfite (1.0 g), and agar (30 g) dissolved in 1 liter of distilled water (final medium pH of 7.6). Ten milliliters of the 10-fold dilutions of samples and 15 ml of the medium (heated at 50°C) were mixed into a P. T. Pouch (Sakami Medical Instruments, Tokyo, Japan) and sealed, preventing contamination with air bubbles. After incubation at 30°C for 24 h, black colonies formed by sulfite-reducing clostridia were counted. Based on spore counts in each stock suspension, appropriate volumes of suspensions of the same type were mixed and diluted with water to make the desired spore concentration with equal numbers of spores of each strain. Spore suspensions were heat shocked (60°C, 10 min) before they were inoculated on the fish fillets. A spore mixture of four strains was used for both growth and toxin production experiments in fish fillets. A single strain (Iwanai) was used in pure culture experiments in preference to a mixture of strains to allow the growth curve to be determined more accurately, because in a mixed suspension, growth can be dominated by the strain best able to grow under each condition, making it difficult to obtain reproducible data on growth.

For DNA extraction from clean spores, clean spore suspensions without vegetative cells and sporangia were prepared by the enzymatic method as described previously (1). After confirmation that vegetative cells and sporangia were completely digested, the spores were washed 10 times by centrifugation with sterile distilled water and used for DNA extraction experiments.

Seafood samples. All of the seafood samples used in this study were purchased from a local retail shop. They were transported to the laboratory on ice, processed immediately in the laboratory, and frozen at −20°C until used. Before the experiments, three fillets were randomly selected for the initial presence of *C. botulinum* type E by both mouse assay and the PCR method after enrichment in Trypticase-peptone-glucose-yeast extract (TPGY) broth (Trypticase from BBL Microbiology Systems Cockeysville, Md.; all others from Difco Products, Detroit, Mich.). The absence of *C. botulinum* type E was confirmed.

PCR primers and fluorogenic probe. PCR primers and a fluorogenic probe based on the nucleotide sequence data of the BoNT type E (BoNT/E) gene were designed by using Primer Express, version 1.0 (Applied Biosystems) from the GenBank database (34) (accession no. X62089). Selection of primers and a probe allowed for adjustment of the melting temperatures to those optimal for the ABI 7700 SDS. The TaqMan probe consists of an oligonucleotide with a 5′-reporter dye and a downstream, 3′-quencher dye. The fluorescent reporter dye 6-carboxy-fluorescein (FAM) is covalently linked to the 5′ end of the oligonucleotide. This reporter dye is quenched by 6-carboxy-tetramethyl rhodamine (TAMRA) located at the 3′ end. A 280-bp region was selected for specific real-time PCR amplification, after the nucleotide sequences of BoNT/A, -B, -C, -D, and -E (3, 17, 34, 44, 46) were aligned by using the Genetyx-Mac computer program (Software Development Co., Tokyo, Japan). Information regarding the primers and probe sequences is given in Table 2. The specificity and efficiency of the PCR primers and probe was determined by PCR with purified DNA templates from *C. botulinum* type E and other bacteria strains. Chromosomal DNA of cultures grown overnight was purified by standard methods (37). Approximately 5 ng of DNA from each strain of bacteria was amplified by using these primers.

Rapid, quantitative PCR assays. All amplification reactions were performed in a total volume of 50 μl. Thermal cycling was performed with a two-step PCR protocol: 50°C for 2 min, 95°C for 10 min, and 60 cycles of 95°C for 15 s and 65°C for 1 min. Reaction volumes (50 μl) for the PCR consisted of 5 μl of template DNA; 5.0 mM $MgCl_2$; 5 μl of 10×TaqMan buffer A; 200 nM each primer; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems); 0.5 U of uracil-*N*-glycosidase (Amp Erase UNG; Applied Biosystems); and 100 nM TaqMan probe. Amplification and detection were performed with the ABI PRISM 7700 sequence detector (Applied Biosystems). The use of the sequence detector allows measurement of the fluorescent spectra of all 96 wells of the thermal cycler. During amplification, the fluorescence intensities of the fluorescent reporter dye (FAM; emission wavelength at 518 nm), the fluorescent quencher dye (TAMRA; emission wavelength at 582 nm), and the reference dye (6-carboxy-X-rhodamine; ROX; emission wavelength at 602 nm; included in TaqMan buffer A) were determined in real time for each well. The fluorescence signal was normalized by dividing the emission intensity of the reporter dye (FAM) by the emission intensity of a reference dye (ROX) to obtain a ratio defined as R_n (normalized reporter) for a given reaction tube. The ABI 7700 employs a computer algorithm to calculate a value termed ΔR_n as follows: $\Delta R_n = R_n^+ - R_n^-$, where R_n^+ is R_n at any given time during the reaction, and R_n^- is the baseline R_n during cycles 3 to 15. Data for individual reactions are graphically displayed as ΔR_n on the y axis, and the cycle number is on the x axis. The threshold ΔR_n is set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15. A reaction is considered positive if its ΔR_n curve exceeds the threshold at the completion of 60 cycles. For quantification of PCR products, a fluorescent threshold was manually set across all samples in the experiment such that it bisected the exponential phase of the fluorescent signal increase. The cycle threshold (C_t) was defined as the cycle number at which a sample's ΔR_n fluorescence crossed the threshold. Data collection and multicomponent analysis were performed with the Sequence Detection Software version 1.6.3 (Applied Biosystems) programs on a personal computer. Target DNA content in 96 samples, including the linear standard samples, was measured simultaneously in one assay run according to the manufacturer's protocol. The PCR product was verified with ethidium bromide-stained agarose gels by procedures described elsewhere.

Standard curve and amplification efficiency. Standard curves of C_t versus $\log_{10}$ of the copy number were used to estimate the copy number of DNA targets in unknown samples. For a comparison of PCR amplification efficiencies among different strains of *C. botulinum* type E, slopes of the standard curve lines constructed for each strain were calculated by performing a linear regression

TABLE 1. Strains tested by the rapid, quantitative PCR method

Species	Type	Strain	Source[a]	TaqMan assay result
Clostridium botulinum	A	62A	SCNIH	−
		97	SCNIH	−
		Renkon-1	SCHU	−
		Hall	SCHU	−
		Kyoto	SCHU	−
	B	9B	SCNIH	−
		Okra	SCNIH	−
		QC	SCHU	−
		Karashi	SCHU	−
	C	003-9	SCHU	−
	D	Karugamo	SCHU	−
	E	Iwanai	SCNIH	+
		Tenno 2	SCNIH	+
		5545	SCNIH	+
		164-1	SCNIH	+
		35396	SCHU	+
		Biwako	SCHU	+
	F	Langeland	SCHU	−
		Yaeyama	SCHU	−
		9H-01F	SCHU	−
		Cardella	SCHU	−
		4257	SCHU	−
	G	G2734	SCHU	−
		G2741	SCHU	−
Clostridium perfringens		BBC 2401	BBC	−
Clostridium sporogenes		IFO 13950	IFO	−
Alteromonas macleodii		NCIMB 1963	NCIMB	−
Bacillus cereus		IFO 13494	IFO	−
Escherichia coli		IFO 3806	IFO	−
Morganella morganii			Fish	−
Photobacterium phosphoreum		ATCC 11040	ATCC	−
Pseudomonas fluorescene		IAM 12022	IAM	−
Pseudomonas putida			Fish	−
Staphylococcus aureus		IFO 3761	IFO	−
Salmonella enterica serovar Typhimurium		IFO13245	IFO	−
Vibrio parahaemolyticus		IFO 12711	IFO	−
Vibrio alginolyticus			Kyushu University (Japan)	−

Continued on following page

TABLE 1—*Continued*

Species	Type	Strain	Source[a]	TaqMan assay result
Vibrio hollisae		JCM 1283	JCM	–
Klebsiella pneumoniae			Roast chicken	–
Hafnia alvei			Meat products	–
Serratia marcescens		IAM 12142	IAM	–
Proteus vulgaris		IAM 12542	IAM	–
Enterobacter cloacae			Meat products	–
Listonella anguillarum		NCIMB 2286	NCIMB	–
Photobacterium angustum		NCIMB 1895	NCIMB	–
Photobacterium damsela		ATCC 33539	ATCC	–
Photobacterium leiognathi		NCIMB 2193	NCIMB	–
Salinivibrio costicola		NCIMB 701	NCIMB	–

[a] SCNIH, G. Sakaguchi's Collection at National Institute of Health, Japan (given by S. Igimi); SCHU, G. Sakaguchi's Collection at Hiroshima University, Hiroshima, Japan; ATCC, American Type Culture Collection, T Manassas, Va.; NCIMB, National Collection of Industrial Bacteria; IFO; Institute for Fermentation, Osaka, Japan; BBC, Japanese Association of Veterinary Biologics; JCM, Japan Collection of Microorganisms, Saitama, Japan.

analysis with the computer software Microsoft Excel 98 (Microsoft, Redmond, Wash.). From this slope, the amplification efficiency (e) was estimated by the formula $e = 10^{-1/s} - 1$, where s is the slope. The e value can also be defined as $X_n = X_o \times (1 + e)^n$, where X_n is the number of target molecules at cycle n, X_o is the initial number of target molecules, and n is the number of cycles. Thus, when the amplification efficiency is 100%, the e value becomes 1.0.

Sequence of the amplicon region. DNA segments of about 570 bp (positions 1350 to 1920, corresponding to the nucleotide numbers downstream from the ATG start codon of the BoNT/E gene) including the upstream and downstream regions of the amplicon were sequenced for various strains of *C. botulinum* type E. These regions were amplified with primers BE1350F (5′-TCCTAAAGAAA TTGACGATACAGTAAC-3′) and BE1920R (5′-AAGCTCGGGTTCAAATT CTAATA-3′). The amplified products were cloned in pCR II vector plasmids by using the TA cloning kit (Invitrogen). These plasmids were used to transform competent *E. coli* JM109 cells. The plasmid DNAs were purified by the standard method (37). The purified plasmid DNAs were sequenced with the Thermo Sequence II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

DNA extraction procedures. For *C. botulinum* DNA extraction from pure culture and food samples, two extraction protocols, the Chelex method (45) and a method based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidine isothiocyanate (GuSCN) (8), were used as previously described (22) with a slight modification. Briefly, with protocol 1 (Chelex extraction method), 1 ml of bacterial culture fluid or food slurry was placed into a microcentrifuge tube and centrifuged for 5 min at 15,000 × *g*. The resulting pellet was resuspended with 200 μl of 5% Chelex 100 (Bio-Rad Laboratories, Hercules, Calif.) solution. After vigorous mixing, the bacterial suspension was boiled for 10 min and then placed on ice and centrifuged for 5 min at 15,000 × *g* to pellet the particulate matter. Five microliters of the supernatant was used as PCR template. With protocol 2 (GuSCN method), 1 ml of culture fluid or food slurry was centrifuged for 5 min at 15,000 × *g*. After the supernatant was discarded, the

TABLE 2. Primers and fluorogenic probe specific for the amplification of the BoNT/E gene

Primer or probe	Sequence (5′→3′)	Denaturation temp (°C)[a]	Location within the BoNT/E gene[b]
Primers			
BE1430F	5′-GTGAATCAGCACCTGGACTTTCAG-3′	66.5	1430–1453
BE1709R	5′-GCTGCTTGCACAGGTTTATTGA-3′	64.7	1709–1688
Probe			
BE1571FP	5′-R-ATGCACAGAAAGTGCCCGAAGGTGA-Q-p-3′[c]	73.6	1571–1595

[a] Calculated by nearest-neighbor algorithm.
[b] BoNT/E gene as described in GenBank accession no. X62089. Positions correspond to the nucleotide numbers downstream from the ATG start codon of the BoNT/E gene.
[c] R, 6-FAM; Q, TAMRA; p, phosphate cap.

pellet was resuspended in 500 μl of 4 M guanidine isothiocyanate solution (Gibco BRL, Gaithersburg, Md.) added with Tween 20 (2% [wt/vol]) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After vigorous mixing, the bacterial suspension was boiled for 10 min and then placed on ice and centrifuged for 10 min at 15,000 × *g* to pellet the particulate matter. A portion (400 μl) of the supernatant was transferred to a new tube containing 400 μl of 100% isopropanol. After vigorous mixing, the mixture was centrifuged for 10 min at 15,000 × *g*, the supernatant was discarded, and the pellet was rinsed with 75% isopropanol. The pellet was then dissolved in 160 μl of distilled water by heating for 3 min at 70°C. Prior to being used, the template DNA solution was centrifuged for 5 min at 15,000 × *g* to further remove water-insoluble impurities according to Chen et al. (6). Five microliters of the solution was used as a PCR template.

Recovery of BoNT/E DNA from various samples spiked with *C. botulinum* type E. The ability to recover BoNT/E DNA in fish fillets was evaluated by spiking fish samples with different numbers of *C. botulinum* type E cells, recovering the DNA from each spiked sample, and subjecting it to the rapid, quantitative PCR assay. Subsamples (25 g) of jack mackerel fillet, either fresh or spoiled (after 9 days of storage at 10°C) were combined with 75 ml of phosphate-buffered saline (PBS) in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender (Seward, London, United Kingdom). After stomaching, the slurries were poured into sterile microcentrifuge tubes and immediately inoculated with 100 μl of 10-fold serial dilutions of *C. botulinum* type E (Iwanai) culture grown anaerobically in GAM broth (30°C, 18 h). Subsamples inoculated with 100 μl of sterile buffer served as a negative control. Inoculated food slurries were mixed vigorously for 1 min on a gyratory shaker to assure an even distribution of cells. For determination of inoculum populations, 1-ml 10-fold serial dilutions of the undiluted, original cultures were taken for *C. botulinum* type E enumeration (CFU per milliliter) by the anaerobic pouch method as described above and incubated at 30°C for 48 h.

Experiments with recovery of *C. botulinum* type E cells from a variety of seafood samples were performed as described above, except that the spoiled samples used were those stored at 10°C for 7 days. In these experiments, the recovery of two concentrations (8.5×10^4 and 8.5×10^1 CFU per PCR tube) of *C. botulinum* type E cells deposited into the food slurries were calculated with a universal standard curve produced from a pure culture. Thus, when a delay in the C_t was observed in the food samples, the interpolation from the C_t value resulted in a lower cell number calculated.

Growth curve of *C. botulinum* type E measured by the rapid, quantitative PCR method in pure culture. Erlenmeyer flasks containing 800 ml of anaerobic GAM broth were inoculated with 2.9×10^1 spores of *C. botulinum* type E (Iwanai) and incubated at 30°C for 24 h. The number of *C. botulinum* cells during growth was determined both by culture and the rapid, quantitative PCR method. Enumeration by culture was carried out on poured medium of Clostridia count agar as described above.

Calibration of the copy numbers of BoNT/E DNA in fish samples. For quantification of the copy number of BoNT/E DNA in fish samples, a calibration was made by depositing a known amount of *C. botulinum* type E cells (10^6 CFU) into 1 ml of control fish slurries. This calibration was made for each sample. The fish fillets used for this purpose were those which had not been inoculated with *C. botulinum* at the beginning of storage and had been stored under the same conditions for the same periods as the samples. For preparation of the cells for calibration, cell culture in the exponential growth phase (10^7 CFU/ml) in TPGY broth at 30°C was diluted 10-fold with PBS, and portions (1 ml) of the suspension were transferred to 1.5-ml Eppendorf tubes. The cells in the tubes were harvested by centrifugation and stored at −30°C until they were used. For each analysis, these tubes containing the cell pellets of 10^6 CFU were thawed and poured with 1 ml of control fish slurry. After vigorous mixing, the slurries were extracted in the same way as the samples. A standard curve was produced for every 96-well analysis. The cells used for this purpose were prepared in the same way as the cells used for calibration, except that the thawed cell pellets of the tube containing 10^6 CFU were extracted directly with extraction reagents. Four serial dilutions of of BoNT/E DNA solution (i.e., corresponding to 10^6, 10^5, 10^4, or 10^3 CFU/ml) were made after the extraction. These four serial dilutions of BoNT/E DNA were analyzed in triplicate for every 96-well analysis to make a standard curve. After PCR amplification, the calibrated number of BoNT/E DNA per milliliter of fish slurry, X, was determined by the formula $X = X' \times I/I'$ where X' is the amount of BoNT/E DNA in samples given by C_t values on a standard curve (Fig. 2A), I is the known amount of *C. botulinum* type E cells (10^6 CFU/g) deposited into the control fish slurries in each reaction, and I' is the recovered amount of cells in the control fish slurries determined by interpolation from the C_t values on the standard curve.

Growth curve of *C. botulinum* type E measured by the rapid, quantitative PCR method in MA-packaged fish. Fish fillets, each 25 g, placed on sterile petri dishes were either uninoculated or inoculated with the spore suspensions (100 μl) of *C. botulinum* type E (a mixture of the four strains Iwanai, Tenno 2, 5545, and 164-1). The spore suspensions were inoculated and spread on the fish fillets with sterile glass spreaders (2.0×10^1 spores per g of sample). Immediately after inoculation, petri dishes containing one fillet were packed in gas-impenetrable laminate film bags (300 by 200 mm; Mitsubishi Chemical Industry Co. Ltd., Japan), flushed with N_2 at 100% after evacuation. The O_2 transmission rate of the film was 8.7 $cm^3/m^2/24$ h/atm. The details of the packaging methods and gas analyses have been described previously (23). Packaged samples were incubated at 10°C. Total aerobic counts, toxin levels, and BoNT/E DNA were analyzed daily. Fillets sampled once used were not used again in the investigation. After opening the bags, inoculated and uninoculated samples were asceptically put into stomacher bags with 75 ml of sterile salt (0.85% NaCl) water. The suspensions were then pummeled for 2 min in a stomacher 400 Lab Blender (Seward, London, United Kingdom). After the necessary serial dilutions were made with the sterile salt water, total aerobic counts were determined with a standard agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) plate (50% artificial seawater–tap water). The plates were incubated aerobically at 25°C for 48 h. Extraction and quantitative analysis of BoNT/E DNA were performed as described above. To verify whether the amplification product was a target gene, the amplified PCR product of one sample was subjected to direct sequencing as described previously (38).

Toxin detection. The procedure to assay *C. botulinum* toxin in fish samples followed the FDA protocol (13), with a slight modification. Briefly, fish samples (25 g) were defrosted and then extracted with an equivalent volume of gelatin phosphate buffer, pH 6.2. The mixture was centrifuged at 15,000 × *g* for 10 min. The pH of the supernatant liquid was adjusted to 6.0 to 6.2 with 1 N HCl. A portion (0.2 ml) of a 10% trypsin solution was added to 1.8 ml of the supernatant liquid, and this mixture was incubated at 37°C for 1 h Trypsinization was done for all samples. A pair of mice were infected with 0.5 ml of the supernatant liquid. All mice were observed for 96 h for symptoms of botulism.

Nucleotide sequence accession number. The sequences determined in this study have been deposited in the DDBJ nucleotide sequence database under the following accession numbers: *bont/E* (Iwanai), AB040123; *bont/E* (Tenno2), AB040124; *bont/E* (Biwako), AB040125, *bont/E* (5545), AB040126; *bont/E* (35396), AB040127; and *bont/E* (164-1), AB040128.

RESULTS

Designing *C. botulinum* type E-specific primers and fluorogenic probe. The BoNT/E DNA is highly AT rich (GC ratio, 25%), and it is generally difficult to design PCR primers for it. Several specific PCR primers have been reported (5, 14, 40, 42, 43). However, the annealing temperature for these primers is low, resulting in low PCR efficiency, and they could not be adapted to the rapid, quantitative PCR assay. Also, we could not find a proper region for TaqMan probe design in the internal region between the primers previously reported. Therefore, we have decided to design our own specific primers and a fluorogenic TaqMan probe. The software primer express (version 1.0) (ABI) was used following the recommended guidelines for designing fluorogenic probes for rapid, quantitative PCR assays. Briefly, PCR quantification needs high PCR efficiency and specificity; thus, primers should have a high melting temperature (T_m) (above 65°C) in the BoNT/E gene. The PCR amplicon should not exceed 350 bp in length to increase PCR efficiency. The fluorogenic probe should have a T_m higher than that of the primers. Consequently, only one region (1430 to 1709, nucleotide numbering downstream from the ATG start codon of BoNT/E gene) was proposed as a possible region for the primer and probe design. From eight combinations of primers and probes recommended by the software, which differed slightly in sequence, but were located in the same region, we have designed two forward and reverse primers each and one probe in the BoNT/E gene considering

the sequence specificity for the BoNT/E DNA. We tested the PCR efficiency with various combinations of the primers and probes and chose the combination most efficient in PCR amplification (Table 2).

Specificity of PCR primers and a TaqMan probe in the detection of *C. botulinum* type E. The specificity of the assay was tested against a panel of bacterial templates from 6 strains of *C. botulinum* type E and 18 genera of 42 non-*C. botulinum* type E strains, including strains of *C. botulinum* types A, B, C, D, F, and G (Table 1). Only *C. botulinum* type E (strains Iwanai, 5545, Tennno 2, 35396, Biwako, and 164-1) reacted with the probe. These samples gave C_t values of <25, which we designated as the upper limit of positivity. All of the other strains tested, including *C. botulinum* types A, B, C, D, F, and G were essentially nonreactive to the probe (Table 1), with C_t values of >60 cycles, confirming the species-specific nature of the assay.

PCR amplification pattern. Quantification of the PCR-amplified product from serial dilutions of *C. botulinum* type E (Iwanai) DNA revealed sigmoid-shaped curves typical of PCR samples (Fig. 1A). A comparison of the fluorescence intensities of the samples at any given cycle indicated that the intensity was greater for samples containing higher DNA templates than for those containing lower DNA templates. The PCR product concentration from samples containing 0.5 pg of genomic DNA could be determined after 45 cycles. The number of cycles at which the fluorescence intensity rose above the threshold levels ranged from 22 (5 ng/PCR tube) to 45 (0.5 pg/PCR tube). With 0.05 pg of genomic DNA per PCR tube, no multiplication of fluorescence occurred. Agarose gel electrophoresis of PCR products amplified from 5 ng of template DNA per PCR tube is also shown in Fig. 1B. The gel showed that only the toxin gene (280 bp) was being amplified regardless of PCR cycles.

PCR amplification efficiency of various strains of *C. botulinum* type E. Amplification efficiency was measured by comparing the standard curves constructed with six strains of *C. botulinum* type E (Iwanai, 5545, Tenno 2, 35396, Biwako, and 164-1) by rapid, quantitative PCR. The C_t of each concentration of BoNT/E DNA was determined. Then, the C_t versus log DNA was plotted to produce a standard curve. The slope of the standard curves allowed us to calculate the average amplification efficiency (Table 3). Our comparison did not reveal any significantly different amplification kinetics among these six strains (Table 3). These results were further confirmed by sequencing the amplicon region of these strains. The sequences in the primer and probe regions were identical in all of the strains tested (data not shown; DDBJ nucleotide sequence database accession no. AB040123 to AB040128). Therefore, a single standard curve generated with DNA of *C. botulinum* type E (Iwanai) was used for quantification for mixed population analysis.

Recovery of BoNT/E DNA from various samples. The sensitivity of the assay was evaluated with either a pure culture (Fig. 2A), fish (jack mackerel) slurry (Fig. 2A), or a clear spore suspension (without vegetative cells) (Fig. 2B) of *C. botulinum* type E (Iwanai) by two DNA extraction methods (Chelex and GuSCN). Recovery of BoNT/E DNA from these samples was evaluated from 10-fold serial dilutions of *C. botulinum* cell or spore suspensions of pure culture or fish slurry spiked before DNA extraction. For pure culture, a good reverse correlation between cell number and threshold cycle was obtained by both extraction methods (for the GuSCN method, see Fig. 2A) (data not shown for the Chelex method). However, with inoculated fish slurries, only the GuSCN method worked well (Fig.

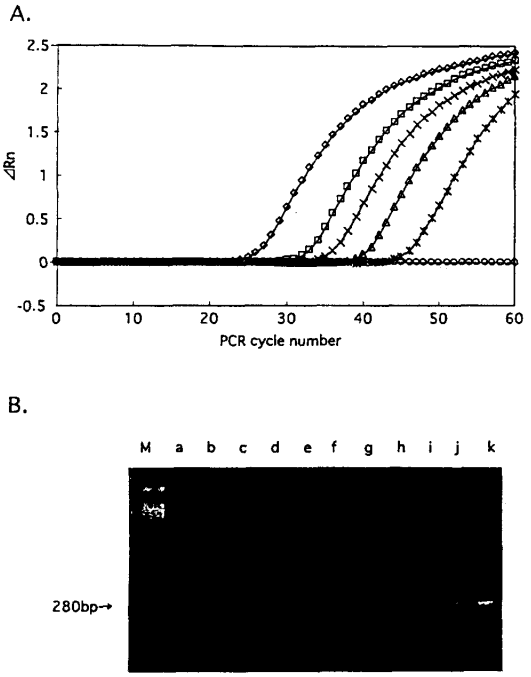

FIG. 1. (A) Real-time PCR amplification plots for reactions with a starting DNA template concentration of *C. botulinum* type E (Iwanai) ranging from 50 fg to 5 ng per PCR tube. Cycle number is plotted versus change in normalized reporter signal (ΔR_n) defined as described in Materials and Methods. Two replicate plots were made for each standard sample, but the data for only one are shown here. ◇, 5 ng; □, 500 pg; ×, 50 pg; △, 5 pg; *, 0.5 pg; ○, 0.05 pg of DNA per PCR tube, respectively. (B) Agarose gel electrophoresis of PCR products amplified from 5 ng of template DNA per PCR tube with different cycles. Lanes: M, 100-bp DNA ladder; a, 10 cycles; b, 15 cycles; c, 20 cycles; d, 25 cycles; e, 30 cycles; f, 35 cycles; g, 40 cycles; h, 45 cycles; i, 50 cycles; j, 55 cycles; k, 60 cycles.

TABLE 3. Amplification parameters of six different strains of *C. botulinum* type E

Strain	Slope[a]	Amplification efficiency[b]
Iwanai	−3.633	0.885
Tenno 2	−3.669	0.873
5545	−3.881	0.810
164-1	−3.869	0.813
35396	−3.744	0.850
Biwako	−3.778	0.840

[a] The slope of standard curve line (C_t values versus $\log_{10}$ copy number) constructed for each strain.
[b] Efficiencies of PCR amplification calculated from the slope by the formula described in Materials and Methods.

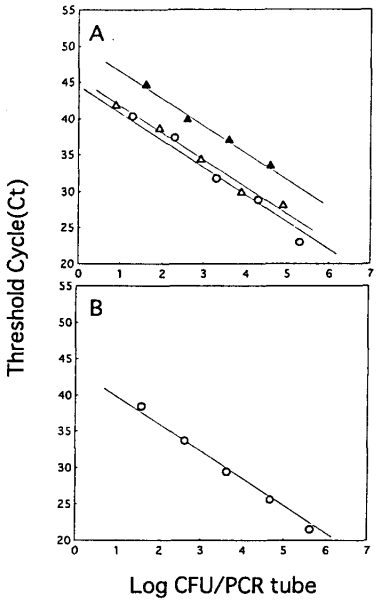

FIG. 2. Relationship between C_t values and numbers of *C. botulinum* type E (Iwanai) vegetative cells (A) or spores (B) in the rapid, quantitative PCR assay. DNA was extracted by the GuSCN method from vegetative cells or spores from pure culture (○), from a post spiked fresh fish fillet slurry (△), and from a postspiked spoiled (after 9 days of storage at 10°C) fish fillet slurry (▲).

2A). Extraction was equally successful from clean spores by the GuSCN method (Fig. 2B). By the Chelex method, the presence of food components attenuated PCR signals severely, and a very low correlation was obtained between cell number and fluorescence intensity (data not shown). Therefore, we decided to use the GuSCN method in the subsequent experiments. By the GuSCN method, the fluorescence intensity (PCR product concentrations) in fish samples, both fresh and spoiled, and pure cultures containing comparable bacterial loads was linear over 5 orders of magnitude, with the R^2 values of the lines being greater than 0.97 in each case (Fig. 2A). However, the y axis was higher in spoiled fish fillets than fresh fillets and pure cultures (Fig. 2A).

Similar experiments performed with a variety of seafood samples revealed linear relationships between the fluorescence intensity and *C. botulinum* cell numbers, although in some cases, the C_t values were 1 to 3 cycles higher than those obtained with the pure culture (data not shown). Table 4 shows the results of the recovery of two different concentrations of *C. botulinum* type E (Iwanai) cells measured by the rapid, quantitative PCR method from a variety of seafood samples. Overall, recovery was good (ranging from 19.1% to 173.5%), and there was no serious difference in the order of the bacterial number between the seafood samples (Table 4). However, in some samples (spoiled samples of salmon and common mackerel), BoNT/E DNA was not recovered unless the supernatant after GuSCN precipitation was extracted with phenol-chloroform.

Comparison of growth curves with the PCR assay and culture method. To determine if the rapid, quantitative PCR method could be used for quantifying *C. botulinum* type E instead of a conventional culture method, the growth curves constructed with the data obtained by the rapid, quantitative PCR method (translated to cell number) and the data obtained by the culture method were compared (Fig. 3). Similar growth curves were obtained by both methods, indicating that the rapid, quantitative PCR method could be used instead of the culture method for monitoring *C. botulinum* growth. Although the 10-CFU/PCR sample was the most diluted spiked sample used for drawing the standard curve (Fig. 2B), the rapid, quantitative PCR method was able to detect *C. botulinum* type E (Iwanai) in this experiment at as little as 0.1 CFU/PCR (C_t = 48.3, translated to 4 CFU/ml of culture) in an 8-h incubation, when the culture count obtained at the same time was 10 CFU/ml (Fig. 3). The results of similar experiments with pure culture showed that *C. botulinum* type E at ≦1 CFU/tube could be detected occasionally, while that at 1 to ~5 CFU/PCR (10 to ~200 CFU/ml of culture) could be detected constantly (data not shown). The presence of *C. botulinum* type E toxin was also tested in 1-ml cultures at each sampling point. The mouse assay required an incubation time of 18 h for toxin detection (corresponding to a cultural count of ca. 10^9). It was apparent that the rapid, quantitative PCR method was much more sen-

TABLE 4. Quantification of *C. botulinum* type E cell number (per PCR tube) recovered from a variety of fresh and spoiled (stored at 10°C for 7 days) seafood samples via rapid, quantitative PCR assay

Seafood sample	No. of bacteria recovered (% recovery)[a]			
	High inoculation (8.5×10^4)[b]		Low inoculation (8.5×10^1)[b]	
	Fresh	Spoiled	Fresh	Spoiled
Flathead	3.5×10^4 (41.2)	3.3×10^4 (38.2)	4.3×10^1 (50.0)	6.5×10^1 (76.5)
Sea bream	1.5×10^5 (173.5)	2.4×10^4 (28.5)	3.5×10^1 (41.2)	4.3×10^1 (50.0)
Salmon	3.8×10^4 (44.1)	1.6×10^4 (19.1)[c]	8.0×10^1 (94.1)	8.5×10^1 (100.0)[c]
Common mackerel	5.3×10^4 (61.8)	1.7×10^4 (19.7)[c]	9.0×10^1 (105.9)	7.0×10^1 (82.4)[c]
Shrimp	6.0×10^4 (70.6)	1.9×10^4 (22.1)	3.0×10^1 (35.3)	4.0×10^1 (47.1)
Crab	6.0×10^4 (70.6)	5.3×10^4 (61.8)	1.0×10^2 (120.6)	9.3×10^1 (108.8)

[a] Determined by a universal standard curve made with a pure culture of *C. botulinum* type E (Iwanai) as described in the Materials and Methods.
[b] The number of *C. botulinum* cells (determined by the culture method) actually deposited into the food slurries.
[c] A phenol-chloroform extraction step was required for recovery.

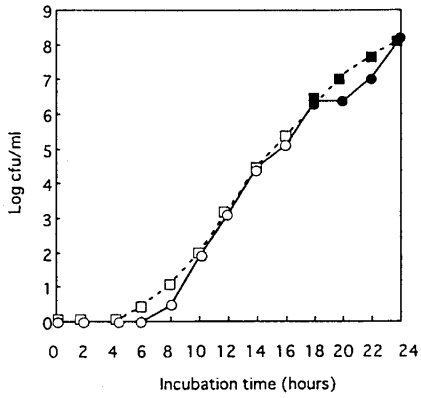

FIG. 3. Growth curves constructed by culture methods (□) and the rapid, quantitative PCR assay (○) with a pure culture of *C. botulinum* type E (Iwanai). The culture (in TSB) was incubated at 30°C. Solid symbols denote the samples detected with BoNT by mouse assay.

sitive than the mouse assay, making it possible to monitor the early log growth.

Time course monitoring of *C. botulinum* levels in MA-packaged fish. Using the rapid, quantitative PCR method, the time course of *C. botulinum* type E in fish fillet (jack mackerel) packaged with a modified gas atmosphere (N_2 at 100%) was examined (Fig. 4). Spore mixtures of four strains of *C. botulinum* type E (Iwanai, Tenno 2, 5545, and 164-1) were inoculated to jack mackerel fillets, which were then gas packaged and stored at 10°C. The presence of *C. botulinum* type E was quantified by the rapid, quantitative PCR method. We have calibrated the amount of BoNT/E DNA per gram of fish fillets by depositing *C. botulinum* type E (Iwanai) cells into control samples (uninoculated with *C. botulinum* type E) prior to DNA extraction. By doing so, it was possible to determine the amount of BoNT/E DNA in fish samples, eliminating the effects of both DNA extraction loss and the possible PCR inhibitors in food samples.

Given the initial spore number of 2.0×10^1 CFU/g, *C. botulinum* in fish fillets revealed no fluorescence signal by 2 days of storage (Fig. 4B). The signal was first detected at 3 days of storage (calculated as 7.9×10^1 CFU/g), and enhanced signals were obtained thereafter (Fig. 4B). At day 7, the *C. botulinum* count calculated by the rapid, quantitative PCR method reached 1.6×10^6 CFU/g. No signal was obtained from uninoculated fish fillets, either fresh or spoiled, throughout the experiment (Fig. 4A), confirming that our primers and probe for *C. botulinum* type E do not cross-react with the natural background bacterial flora of fish. Also, representative PCR products from the inoculated samples were sequenced and confirmed to have the BoNT/E DNA sequence. The mouse toxin assay was done at the same time. Toxin was first detected by mouse assay on day 7 (corresponding to the 1.6×10^6 CFU of *C. botulinum* per g as measured by the rapid, quantitative PCR method), which was 5 days after the first signals were detected by the rapid, quantitative PCR method.

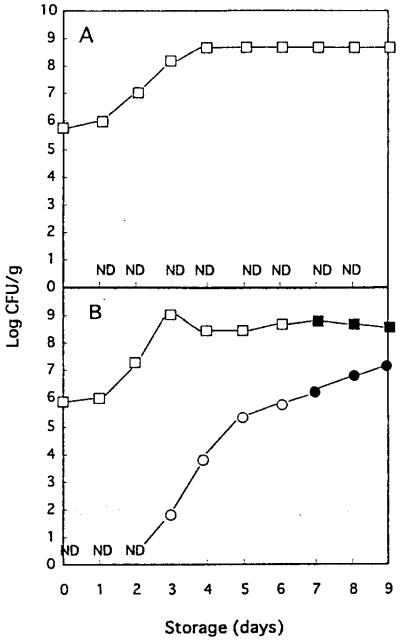

FIG. 4. Time course change in the cell number of *C. botulinum* type E (a mixture of four strains: Iwanai, Tenno 2, 5545, and 164-1) estimated by the rapid, quantitative PCR assay (○) and viable counts of aerobic spoilage bacteria (□) in MA-packaged fish fillets uninoculated (A) or inoculated (B) with *C. botulinum* spore mixtures (four strains). The packaged fillets were incubated at 10°C for 9 days. Solid symbols denote the samples detected with BoNT by mouse assay. ND, no BoNT/E DNA detected.

DISCUSSION

The ability to quantify the BoNT gene from diverse kinds of samples by using an automated procedure could be beneficial to food testing laboratories. The detection of the BoNT gene by PCR has proven to be a sensitive and rapid alternative to the mouse assay (5, 14, 40, 42, 43). However, these methods using PCR are qualitative, not quantitative. In this report, we have developed and evaluated a rapid, quantitative PCR (TaqMan; ABI 7700 system) for automatically quantifying the BoNT/E gene. The method, based on real-time analysis of PCR amplification (7, 15), has several advantages. (i) Unlike PCR-ELISA and MPN PCR, it is a simple and rapid method because it is a real-time assay in a closed tube without a need for any post-PCR manipulations. (ii) Unlike competitive PCR, real-time PCR makes DNA quantification much more precise and reproducible, since it is based on C_t values rather than an end-point measurement of the amount of accumulated PCR product. Consequently, the system allows for a large dynamic range, and we have achieved linearity over 6 log units of input DNA. (iii) Unlike competitive PCR, real-time PCR does not require an internal standard, the design and validation of which are laborious.

The present results suggest that our primers and probe could

be used for quantification of various strains of *C. botulinum* type E. The adaptation of the 5′nuclease quantitative PCR to previously reported PCR primers (5, 14, 40, 42, 43) is not possible due to the requirement of regions with T_m at least 5°C higher than those of the primer regions. Accordingly, we have designed an original primer and probe region (Table 2). Beside the primer and probe design, primer concentrations, and temperature cycling profile were found to be important parameters as reported for the TaqMan PCR assay (2). Also, since the T_m of the TaqMan probe does not increase during PCR amplification because of its inability to be extended by the *Taq* DNA polymerase, we have chosen a two-step PCR to ensure the hybridization of the TaqMan probe during extension, in which the annealing and extension steps were combined and carried out at 65°C. The results of the study revealed that the primers and probe were highly specific for the *C. botulinum* type E strains tested (Table 1). Moreover, PCR efficiency was almost the same between the different strains of *C. botulinum* type E tested in this study (Table 3). Sequencing the amplicon region of these strains revealed that they were identical in the primer and probe regions (data not shown). These results suggest that our primers and probe could be used for quantification of various strains of *C. botulinum* type E in food and environmental samples, although these aspects should be studied more carefully with additional strains of *C. botulinum* type E.

The most important advantage of the rapid, quantitative PCR method is the ability to monitor the growth of *C. botulinum* type E in food where natural microbial competitors are present. At present, laborious and time-consuming studies with the mouse assay are necessary in challenge tests of *C. botulinum* for risk assessment of food products. The quantitative monitoring of the growth of *C. botulinum* by conventional culture methods is, if not impossible, time-consuming and erroneous, because isolation agar media such as egg yolk agar would not effectively inhibit certain other members of the genus *Clostridium* with similar morphologic characteristics of colonies (13). A selective agar medium has been reported (10) and used for monitoring *C. botulinum* type E in a recent study (27) and our laboratory (B. Kimura et al., unpublished data). However, the selectivity of the medium is still limited, and a confirmation test for representative colonies is necessary. This is laborious and time-consuming as well as erroneous because of the difficulty in discriminating typical colonies. This is true particularly when a large number of background bacteria are present. An MPN technique coupled with PCR has been applied for monitoring *C. botulinum* type E in fish and the environment (18). However, a large number of dilutions and replicates is required. This makes the rapid analysis of multiple samples difficult by this method. Also, it should be noted that the MPN counts are a compromise, because the equation presumes an even distribution of cells among samples. With the rapid, quantitative PCR method, a more accurate, rapid, and automated monitoring of the copy number of BoNT/E DNA in as many as 96 samples at one time is possible. Thus, a large number of packaged food samples (different components, lots, and storage conditions) could be examined. Also, the data indicated that the rapid, quantitative PCR assay is capable of quantifying *C. botulinum* type E in fish if $\geqq 10^2$ to 10^3 CFU/g is present. This means that growth could be monitored much earlier than toxin could be detected by the mouse assay (Fig. 3 and 4).

One limitation of this method is that it is for quantifying the BoNT/E DNA copy number, not the toxin itself. At present, there is only a little information (32) available on the conditions for toxin production by *C. botulinum* at the cellular level. *C. botulinum* toxin is generally detected in the late exponential phase in pure culture (4). During the preparation of this manuscript, McGrath et al. (28) reported on the quantification of mRNA of the BoNT/E gene of *C. botulinum* type E by competitive reverse transcription-PCR. They have demonstrated that gene expression was 100 times stronger in the stationary phase than in the mid-log phase. Also, they found different levels of gene expression in two different types of broth, indicating that culture medium affects toxin production. These results suggest that quantification of mRNA, rather than DNA, would be more appropriate for risk assessment of *C. botulinum*. However, from a practical point of view, in food testing laboratories, quantification of DNA is simpler to perform. Also, there is no reason to reject the idea that an increase in BoNT/E DNA reflects a potential risk of toxin production. Thus, the sensitive and rapid detection of the increase of BoNT/E DNA in food could serve as an early warning system. Recently, a novel in vitro bioassay for direct detection of BoNT/B, amplifying the enzymatic activity of the neurotoxin light chain, has been described (47). This method seems more sensitive and less expensive than the mouse assay or ELISA method (11). An advantage of the rapid, quantitative PCR method over the in vitro bioassay would be a much higher sensitivity and quantitative accuracy, while an obvious disadvantage is that it is not a method for direct detection of BoNT. It would be interesting to evaluate these methods all at one time in the future. Unfortunately, however, the in vitro bioassay method has not been established for BoNT/E yet. In practical terms, it would be most effective to use the rapid, quantitative PCR methods in combination with other methods for directly detecting BoNT (11, 47) or the mouse assay (when necessary). In such cases, samples demonstrating growth potential would be tested by either of the latter assays for confirmation. By doing so, a much more rapid and convenient risk assessment of *C. botulinum* would be realized.

When DNA was extracted either by the Chelex method or the GuSCN method from fish samples, a linear relationship between the initial number of template copies and C_t values was obtained only with the GuSCN method. The Chelex method worked well with DNA isolated from pure cultures (data not shown), but only a very low correlation could be obtained between the initial number of template copies and C_t values when DNA was isolated from fish slurries (data not shown). This is likely due to the inhibition of PCR amplification by the large amounts of food components, which could not be eliminated from the PCR tube. In our previous study (22), the Chelex method worked well for "yes-or-no" detection of *Salmonella invA* from raw meat and shrimp. In that study, subsamples (25 g) were diluted with 225 ml (10× dilution) of Trypticase soy broth (TSB) or Universal preenrichment broth (UB; Difco Laboratories, Detroit, Mich.). In the present study, subsamples (25 g) were diluted with 75 ml of phosphate buffer in order to increase the detection sensitivity. Thus, it appears that the Chelex method does not work well when samples are

not diluted enough to eliminate the inhibitory effect of food components. Since the Chelex extract protocol is a one-tube reaction, this seems inevitable. With the GuSCN method, isopropanol precipitation is performed, and the influence of the PCR inhibitor may be less than that for the Chelex extract protocol. A similar phenomenon was described by Chen et al. (6). From these results, it was suggested that the GuSCN method was more appropriate for extraction of DNA from fish samples for quantification.

The application of the rapid, quantitative PCR method to other foods could be limited unless the performance of PCR is carefully evaluated food by food. The present results showed that the GuSCN DNA extraction method was efficient for the quantification of BoNT/E DNA in a variety of fish samples, although in some samples, a phenol-chloroform extraction step was required (Table 4). However, higher C_t values (1 to 3 cycles) than those obtained with the pure culture were obtained in some cases (data not shown), resulting in the variation in recovery (Table 4). The variation may be due to either the extraction efficiency of DNA, the inhibition of PCR amplification by PCR inhibitors, or a large amount of DNA from background spoilage bacteria. Thus, a universal standard curve cannot be used for accurate quantification of BoNT/E DNA in a variety of fish samples (based on fish species and duration of storage). Accordingly, in this study, an accurate quantification of BoNT/E DNA in jack mackerel samples was achieved by calibrating the standard curve by depositing a known amount of *C. botulinum* type E cells into the fish slurry (uninoculated with *C. botulinum*) for each analysis before extraction. By doing so, it was possible to determine the copy numbers of BoNT/E DNA, eliminating the effect of both the loss of DNA during extraction and the potential PCR inhibitors (Fig. 4). However, in practical use, it would be more convenient to apply the universal standard curve, since the variation in recovery was not significant enough to make a difference in the order of the bacterial number (Table 4). The merit of using a universal standard curve is that it is simple and saves money, although there are some limitations to the interpretation of the data. The possible variations should be determined before routine analysis of various food samples, and the most practical and time/money-saving calibration method should be selected.

In conclusion, although DNA extraction protocols may need to be optimized for food by food evaluation, the use of the rapid, quantitative PCR assay could provide a rapid, quantitative assay for *C. botulinum* type E in seafood samples. At present, one of the most serious drawbacks of conventional techniques in food microbiology is the difficulty of determining the growth of a target microorganism in a complex mixture of bacteria by plate count techniques. This method, enabling us to elucidate how bacteria grow in the real environment, where they are typically associated with surfaces and are in dynamic competition with a heterogeneous microflora, could be applicable to other food pathogens or certain target microbes in the environment.

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生食用野菜・果物の自主衛生管理へのMicro Foss法の導入

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Evaluation of MicroFoss Method in Raw-vegetable and Fruits for Quality Control

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The MicroFoss methods for estimating viable count of aerobic bacteria and coliforms from various vegetables and fruits were evaluated. Total 130 of samples were tested using conventional methods such as plate count agar and desoxycholate-agar plate count (coliform count). Each samples were tasted using the MicroFoss total viable count (TVC) and Coliform medium. The correlation coefficients of standard curves were given in 0.7～0.9. And, the MicroFoss method has widely range of quantification that it was unnecessary for serial dilution of sample solution. The MicroFoss method can detect the larger the initial bacteria concentration, the shorter the detection time (D_t). In theologically, it was estimated to detect for 1 cell less than 18 hours by these standard curves. Therefore, the MicroFoss method may be extremely useful for quality control in food industries. The other side, we try to detect *E. coli* from cultivation water of sprout by the MicroFoss *E. coli* medium. In inoculation area, the MicroFoss method jointly with Durham's tubes method could detect *E. coli* for 1 day of cultivation water, though FDA was recommended strongly to test 3 days of cultivation water for sprout.

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1996年の腸管出血性大腸菌O157：H7の大規模食中毒の発生[1]を境にして，食中毒事例の発生率は増加の傾向にある．このため，食品企業では自主衛生検査の必要性が自覚され，さらに消費者から問われるようになってきており，より実用的な食品衛生技術の開発が求められている[2]．

食品中の細菌検査は，食品衛生検査指針に従って寒天培養法や最確数法（MPN法）を用いて，汚染指標である一般細菌数・大腸菌群数・大腸菌数を測定することで行なわれている[3]．しかし，本指針による食品検査法の操作は煩雑で多大な労力を必要とする．また，寒天培養法での測定法はコロニー形成を必要とするため，少なくとも2日以上，病原菌であれば4～6日以上の培養時間を必要とする[4]．このため実際の食品製造等の現場における微生物検査の導入は難しく，必要十分量の検査を行うことすらできないのが現状である．また仮に検査結果を得られたとしても，もはや製品は出荷・流通し消費者に渡っている場合もあり，食中毒発生の未然防止には役立てられない．このように食品衛生検査指針による検査法は，食中毒事例発生後の原因究明には極めて確実で有効な検査法であるが，食品現場で必要とされているであろう食中毒発生の事前警告システムとすることは困難である．

このような背景から，細菌検査において様々な簡易迅速測定手法が開発されつつある．例えば，スパイラル・プレーターによる自動塗抹法[5]は1枚の平板培地にて10^2～10^5CFU/mlの生菌数の測定が可能である．また，ペトリフィルム[6]のようなフイルム型培地も操作が簡便かつ生菌数の測定が可能である．しかし，微生物のコロニー形成数を測定するために培養の過程を必要とするため，労力の省力化にはなりうるが，計測時間の短縮には至らない．また，ATP量を測定することにより細菌の発

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育増殖をモニタリングする[7]手法も出現しており簡易化されているが，微生物以外の生物由来の ATP も同時に測定されるため，洗浄度検査というニーズには適合するが，汚染指標細菌を選択的に測定することは難しく，食品最終製品の細菌測定に適用できる検体も限られている．

このように汚染指標となりうる細菌を初心者でも正確かつ簡易迅速に測定でき，大量検体の処理が可能で，さらに HACCP に基づく衛生管理等に必要な結果を数値化して記録に残せるシステムを構築しているものは少ない．また，多種多様な自動検査システムが存在するものの，実際の自主衛生管理へ導入するための評価や実験例は少なく，特に生野菜・芽物野菜・果物についてはその知見が乏しい．上記の点が必要とされる状況のなか，我々は微生物迅速測定機器 Micro Foss を導入した自主衛生管理の可能性について注目した．

本稿では，一般生菌数と大腸菌群数の Micro Foss による迅速測定結果と従来法で得られた結果との検量線作成を試み，衛生管理の高度化が模索されている[8][9]，生鮮野菜・果物における定量評価を行った．さらに，カイワレ大根の栽培をモデルとして糞便由来大腸菌（fecal-coliform）汚染の迅速検出への応用についても検討し，食品製造流通現場などにおける自主衛生管理のための警告システム構築の可能性について検討を行った．これらの結果を報告する．

実 験 方 法

1. 供試菌及び実験試料

供試菌は *Escherichia coli* JCM 1649（理化学研究所より入手）を用いた．供試菌を普通ブイヨン（栄研化学製）にて 35℃ 18 時間培養後，リン酸生理食塩緩衝溶液（PBS）にて 10 段階希釈し，生菌数を測定すると同時に実験に供した．実験に用いたサンプルは，茨城県内の食品小売店舗にて購入した，野菜・果物（カイワレ大根・モヤシ・レタス・キャベツ・キュウリ・パプリカ・メロン・イチゴ）計 8 種類を使用した．また，カイワレ大根の栽培についてはアメリカ産とオーストラリア産の 2 種類の種子を用いて実験を行った．

2. 従来法による試験法

比較対照として，従来法（食品衛生検査指針[3]）による一般生菌数及び大腸菌群数計測を平行して行った．小売店舗より購入した検体は即座に 25 g に小分けし測定するまで冷蔵した．これに 225 m*l* のリン酸生理食塩緩衝溶液を加え 60 秒間ストマッカーに供した．この乳液 1 m*l* をリン酸生理食塩緩衝溶液により段階希釈し，混釈法にて一般生菌数及び大腸菌群数を測定した．測定用培地には，一般生菌数測定には標準寒天培地（日水製薬製），大腸菌群数測定にはデソキシコーレート培地（栄研化学製）を用いて行った．

3. Micro Foss を用いた測定

実験には，Micro Foss model 32（Foss 社製）を使用した．従来法による試験法で生菌数測定用に作成した乳液を，Micro Foss 専用バイアルである TVC medium（一般生菌数測定用）[10] と Coliform medium（大腸菌群測定用）[11] にそれぞれ 2 m*l*, 5 m*l* を供した．これらの測定用バイアルは Micro Foss model 32 とその付属ソフトウェアにて，その増殖によるバイアル内の培地の色調変化と色調変化を検出するのに要した時間（Dt）を測定した．測定は 585 nm の吸光度で 6 分毎に行った．測定条件は 35℃, 18 時間の培養条件にて行った．

サンプル乳液の pH が 5.5 以下の検体については 0.05 N の NaOH 水溶液を用いて pH の調整を行った．pH メーターを用いて，サンプルの乳液 10m*l* に 0.05 N の NaOH 水溶液で pH が 6.7～6.9 になるために必要な滴定量を求めた．この滴定量の 1/10 量をあらかじめ Micro Foss の検出用バイアルに加え pH を事前調整し，これにサンプル溶液を加え，検出時間（Dt）を測定した．

4. カイワレ種子への大腸菌汚染法とカイワレ大根の栽培法

初めにカイワレ種子への吸水を目的として，カイワレ大根種子約 4 g（約 100 個相当）を無菌シャーレ中に置き，無菌 D.W. を 20 m*l* 加えた．この時，別途に培養しておいた *E. coli* を 0.1～100 CFU/m*l* になるよう段階希釈し，この吸水時の無菌 D.W. に 1 m*l* 加え，感染カイワレ種子を作成した．20 分室温放置し吸水させた．次に水耕栽培するために，ポリスチレン・カップを用意し底面に約 3 cm の切れ目を十字に入れ，カップ内に脱脂綿を敷いた．脱脂綿に十分な無菌 D.W. を含ませた後，吸水させた感染種子とシャーレ上にある水をカップ内の脱脂綿上に蒔いた．散水は，1 日 1 回カップ内の種子に無菌 D.W. を加えることで行った．カイワレ種子の生育は 25℃ 遮光条件で 3 日間行った．

5. カイワレ種子生育液からの大腸菌回収実験

4. での散水時にポリスチレン・カップ底面の切れ目から流れ出る成育液 25m*l* を採取し，この流れ出た栽培排水 5m*l* を Micro Foss の *E. coli* medium（大腸菌測定用）[12][13] に加え，3. と同様に検出時間（Dt）を求めた．さらに，*E. coli* medium の液層にダーラム管を無菌的に加え，ガ

ス産生能についても観察を行った．測定条件は 44.5℃，12 時間の培養条件にて行った．同時に成育液 1 m*l* を 9 m*l* の EC 培地（日水製薬製）に加え 44.5℃で 24 時間培養し，従来法との比較も行った．上記の実験は 3 日間，1 日毎に行った．

実験結果

1. 様々な生野菜における検量線の作成

生カット野菜・果物を用いて，従来法にて測定した一般生菌数と Micro Foss で得られた検出時間（Dt）との相関を図 1 に示した．一般生菌数について，相関係数は 0.86，生野菜全般の検量線として，y＝－0.5264x＋9.4612（r＝0.86）と得られた．この時の各種サンプル毎の検量線の傾き及び切片を表 1 に示した．どの検体においても相関は 0.80 以上と得られ，検量線から Log±2 以内の誤差内で全て収まる結果となった．傾きと切片においては，－0.43～－0.87，8.2～11.1 と検体によるバラツキが認められた．しかし，検体毎（作物毎）に検量線を作成した場合，各々において相関係数が 0.80 に近い，もしくは越える，高い相関を得ることができた．

一方，大腸菌群について同様に従来法と本法との相関を求めたところ，相関係数は 0.78，生野菜全般の検量線として，y＝－0.531x＋8.7119（r＝0.83）と得られた（図 2，表 1）．大腸菌群においても一般生菌数の場合と同様，サンプル間において傾き及び切片にバラツキがあるものの，サンプル個別の検量線を作成すると高い相関（R_2＝0.70～0.99）が得られた．

2. 大腸菌（fecal-coliform）検出実験

カイワレ種子へ低レベルの大腸菌を接種した際の回収実験の結果を表 2 に示した．大腸菌を 10^0 cells 以上接種した種子の栽培水では，1 日目においても本法による検出が可能であった．この時に必要な検出時間は 7.5 時間以内であった．しかし，栽培 3 日目においては大腸菌を接種していないサンプルについても，色調変化による陽性反応が得られた．この擬陽性を解決するため，Micro Foss 検出用バイアルの液体層にダーラム管を無菌的に加え再び実験したところ，大腸菌非接種区においてのガス発生は認められなかった．さらに，EC 培地にて確認

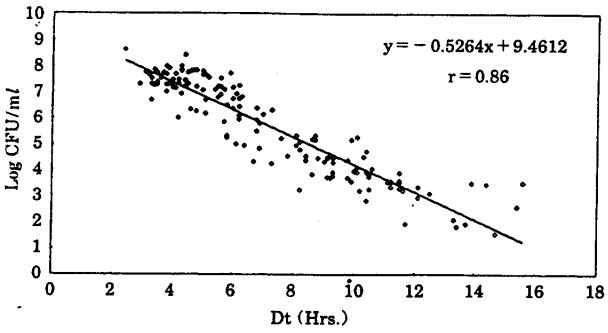

図 1　一般生菌数測定用バイアル（TVC medium）による検量線作成結果

表 1　一般生菌数及び大腸菌群数検量線の検体毎での比較

検体名	一般生菌数			大腸菌群数傾き		
	傾き	Y 切片	相関係数	傾き	Y 切片	相関係数
カイワレ大根	－0.716	11.072	0.821	－0.711	9.994	0.893
モヤシ	－0.739	10.141	0.784	－0.873	10.384	0.746
レタス	－0.427	8.703	0.835	－0.525	8.984	0.883
キャベツ	－0.539	9.321	0.791	－0.499	8.213	0.701
メロン	－0.631	9.588	0.991	－0.429	8.308	0.815

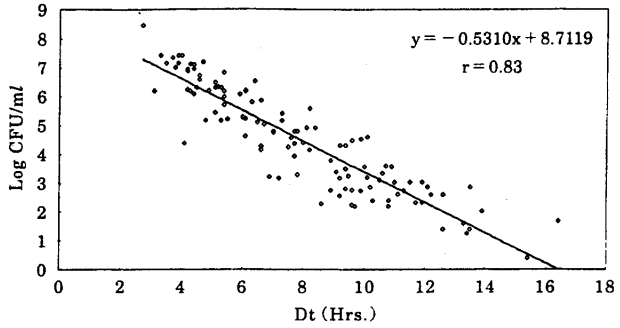

図 2　大腸菌群測定用バイアル（Coliform medium）による検量線作成結果

表 2　大腸菌汚染させたカイワレ大根種子からの回収実験結果

栽培日数	汚染菌数（CFU/種子 4 g）				
	非接種	4.5×10^{-1}	4.5×10^{0}	4.5×10^{1}	4.5×10^{2}
0	0/4	0/4	0/4	0/4	0/4
1	0/4	0/4	4/4	4/4	4/4
2	0/4	0/4	4/4	4/4	4/4
3	0/4	0/4	4/4	4/4	4/4

したところ，EC 培地での反応は陰性であった．このように，測定用バイアルにダーラム管を組み合わせることで，EC 培地との結果が一致して得られた．

考　　察

迅速微生物検査法には，インピーダンス測定法[14]・溶存酸素測定法[15]・メンブランフィルター法[16]・蛍光染色法[17]・フローサイトメトリー法[18]など様々な手法がこれまで報告され，その一部は実用化されている．このように様々な迅速検出法が存在しているが，食品検体は多種多様であり測定原理によって検出に向き不向きが存在する．今回，我々の実験系では，野菜や芽物野菜について実験を行った．1996 年の大規模食中毒事例以来，野菜の安全性の確保が必要とされており，特に芽物野菜についての安全性確保については海外においても重要な問題となっている．このような野菜や芽物野菜といった検体からの微生物測定には，検体からの食品残渣が多く含まれるため，メンブランフィルターやフローサイトメトリーを用いた手法では計測が困難と思われる．

しかし本測定装置は，微生物の代謝産物による培地中の pH 指示薬の色調変化をリアルタイムで検知することで菌数を推定するシステムである．培養セルの中には，微生物増殖に必要な液体培養液が入っており，これに検体希釈液を加えて培養する．培養セルの色調変化検出部には，検体中からの食品残渣による影響を受けることがないように寒天層が底部に埋め込まれており，この部位の色調変化を測定することで安定した検出結果を得ることができる（図 3）．微生物の代謝は寒天平板でのコロニー形成よりも短時間で検出できるため，より迅速な測定が可能である[19]．微生物の代謝産物による培地内の pH の変化は，検体に存在する菌量が多ければ多いほど，早期に変化する．このため，pH の変化を検出するのに必要な時間と初発菌数との関係は逆相関の関係にあり，このことから菌数を推定できる．この pH の変化は，生菌数がおよそ 10^{6} CFU/l に達した際，著しく変化することが Shelef ら[20]により報告されている．Shelef らは，培地中に加えた pH 指示薬（BCP）の変化をモニタリングすることで牛挽肉からの汚染レベルの推定を試みた．また，Russell[10] は増殖基礎培地と CM 培地（bio-Merieux Vitek, Inc. 製），2% dextrose 加 CM 培地を用

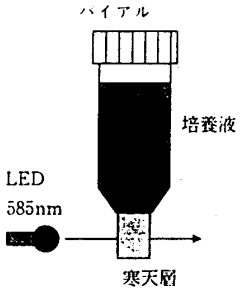

図 3　Micro Foss による微生物検出の原理
pH 指示薬の色調変化を経時的に測定し，色調の急激な変化が生じた時を検出時間（Dt）とする．図は Russell らを改変．

いて，豚挽肉中からの一般生菌数・大腸菌群，大腸菌の迅速定量を試みている．このように，近年，畜肉においては上記の迅速定量実験の試み[1]がなされてはいるが，野菜などをはじめその他の食品についての応用例や実用についての情報は未だ少ない．

Micro Foss 微生物迅速測定器については Russell[10]が畜肉を用いて，一般生菌数・大腸菌群・大腸菌の迅速定量の評価を行っている．今回の我々の実験では，検体を生野菜に絞って実験を行った．その理由として，

1)　Micro Foss の検出原理は微生物の代謝産物を pH 指示薬の色調変化に置き換え，その変化をリアルタイムに計測しカイネティクス解析することで菌数計測を行っているため，pH により測定結果に影響が顕著に現れると考えられる．特に野菜について pH は多様であるため，実用可能であるか検討が必要であること，

2)　生野菜には多数・未知の一般生菌と大腸菌群が付着しており，その微生物フローラによっては競合による増殖阻害のため，測定結果に影響があるかの検討が必要であること，が挙げられる．

今回，計 130 検体の野菜・果物において従来法にて測定した生菌数と本法における検出時間（Dt）との相関を求めたところ，全体で相関係数 0.80 前後の相関が得られており，生鮮野菜の自然汚染の迅速菌数測定に十分実用可能なものと考えられる．検出感度について検量線から推定すると，1 CFU/m*l* のサンプルを検出するには，一般生菌数の場合 18.0 時間，大腸菌群の場合 16.4 時間と理論上考えられた．実際に上記の感度が保証されるかどうかは疑問であるが，少なくとも食品衛生法と同等の感度（30～300 CFU/m*l* において検出可能）を持つと考えられた．本法にて一般生菌数を測定するには，従来では 2 日必要であるところを 15 時間以内に短縮でき，食品現場での汚染の指標や目標値の設定に使用できると考えられる．特に，初発菌量が多ければ多いほど，シグナル変化は早く起こる．このシグナル変化を警告として，現場へフィードバックすることも可能であった．上記の結果は，これまで微生物迅速自動測定機器として報告されているインピーダンス法や溶存酸素濃度測定法における測定結果と同等であった．具体的には，インピーダンス測定法を用いた例では 1 CFU/m*l* の大腸菌群を 16.1 時間で検出している報告があり，[22] 本法の結果と同等の検出時間として得られている．また溶存酸素濃度測定法においても，1 CFU/m*l* の一般生菌数を検出するには理論上 19.2 時間と検量線から求められており，この結果も本法と同等レベルの検出時間[15]であり，pH 指示薬による変化という単純明快な検出原理であるにもかかわらず，十分な定量性が得られていた．また，簡易な測定原理であるため，微生物増殖による最終的な変化を肉眼で確認することも可能である．このように最終確認が複数の手段で行える微生物検査システムは実際の食品現場において重要である．

本法による一般生菌数の検出実験では，各々の細菌やサンプルに付着している細菌相により多少の相違が現れる可能性が予想された．今回の我々の実験において，それぞれのサンプルの種類毎にて検量線を作成したところ，その傾きと切片に多少の違いが認められた．従って，今後検体毎や製造ルーチン毎に検量線を作成する必要があると考えられる．しかし，全てのサンプルを用いて作成した検量線は，相関係数が 0.80 程度の相関を得ており，その誤差は±Log 2 以内で収まっていることから，この検量線でおおまかな定量の用途に十分用いることが可能と考えられた．相関係数について，インピーダンス法での過去の様々な報告[23]では 0.68～0.94 程度と報告されており，本法の結果においてもこの範囲内に収まるものとなった（表 1）．

実験の結果，定量に必要な検量線の作成については，実際への応用面について多少問題点が存在した．例えば，カイワレ・モヤシといった水耕栽培野菜については，初発菌数が 10^6 CFU/m*l* 以上と高く，低濃度汚染レベルにおいてのプロットを取ることができない．また，逆にレタス・キャベツ（特にイチゴ）などにおいては，通常 10^3 CFU/m*l* 程度の汚染であり，高濃度汚染のプロットを得ることができない．このため，野菜個別にお

いての相関は低くなってしまう傾向にある．これを解消するには，汚染の危害モデルを作成（例えば，温度不備の危害をモデルに，常温にサンプルを一定時間おいて初発菌数を増やしてから測定する，加熱不備をモデルに緩やかな加熱処理を施す，など）し，実験を行うことで広範囲の検量線を確保できると考えられる．一般生菌や大腸菌群は食品製造現場に広く分布して存在しているため直接病原菌汚染の指標とは結びつかないが，一般生菌数は最も一般的な微生物学的評価であり，大腸菌群数の定量についても加熱処理の適正評価に重要な意味を持つ．本法はこれらの定量を迅速かつ簡便に行えるため，細菌汚染レベルのルーチン検査の用途だけでなく，加熱処理条件の検討や賞味期限の設定に威力を発揮するものと考えられる．

低 pH である果物などを実験に供した場合では（ex. イチゴ）擬陽性が生じた．特に pH5.5 以下のサンプルをバイアルに供した際，実験前に明らかな指示薬の変化が認められた．このため，明らかに低 pH のサンプルはあらかじめ NaOH を事前に適当量（実際には，イチゴの pH3.6 に対して 190 μl の 0.5 N NaOH）加え pH 調整した後，サンプルを加えることで，擬陽性を防止できた．pH3.2 のリンゴジュースについても既知量の大腸菌を人工接種して同様に試験したところ，これについても定量が可能であった（未発表）．BCP の pH 変域領域は 6.8～5.2 であり，pH を 6.8 前後に調整すれば検討可能になると考えられた．

大腸菌検査への応用実験については，44.5℃の温度条件にも関わらず，色調変化による擬陽性が生じた．このため，ガス発生の検出を補うことにした．12 時間の観察において，色調が変化してガス発生が認められるものは大腸菌，と判定したところ，この結果は EC 培地の結果と一致した．これにより糞便系大腸菌群（facal-coliform）の迅速スクリーニングも可能になると考えられ，色調変化とガス検知による 2 重のチェックが簡便に行えることで，より正確性を与えるものと考えられた．カイワレ大根をモデル栽培し，その栽培水からの検出実験を行った結果，大腸菌を接種したサンプルは 1 日目の栽培水より検出可能であった．米国 FDA[24] ではモヤシなど水耕栽培の栽培水は 3 日目の水を検討せよと強く推奨しているが，これよりも早期に大腸菌が検出可能で，水耕栽培の早期チェックに有効であると考えられた．しかし，今回の実験系はあくまでも栽培モデルでの実験系であり，水耕栽培現場への適用が可能か，さらなる検討が必要である．

また，*Salmonella* について実験を行ったところ，陽性反応が現れた．我々の実験では糞便系大腸菌としてのスクリーニングを検討したため *Salmonella* が検出されても当然の結果と考えられる．このように糞便系大腸菌群を汚染指標にすることで，大腸菌だけでなく *Salmonella* を含んだ幅広い警告システムを確立できると考えられる．大腸菌群では，野菜を標的とした場合には，そもそも初めから多数の大腸菌群が存在しているため汚染指標にはなりえない．しかし，本法の糞便系大腸菌の有無・菌量の測定により，野菜に対して十分な汚染指標を作成できるのではないかと考えられる．その他の微生物の選択性については，インピータンス法では培養時の培地を様々な選択培地に変更可能なため，選択的な検出に汎用性が高い．本法においては現在，一般生菌数・大腸菌群数・大腸菌数測定用バイアルが販売されているが，今後さらなる培地の改良により，その他の細菌や食中毒菌・酵母などを選択的に測定できる可能性があると考えられる．

さらに，測定終了後には培養済みバイアルが残るため，その培養液から大腸菌・病原性微生物の分離・同定試験が可能であり，より詳細な衛生管理への対応や事後確認も可能であると考えられる．これらの結果から本法は，自主衛生管理手法としての有用性が認められた．

要　約

細菌検査を簡易迅速化することを目的に開発された微生物迅速測定器 Micro Foss を用いて，生食用野菜・果物における一般生菌数・大腸菌群の迅速菌数推定法の評価を行った．

(1) 生食用野菜・果物（カット野菜・フルーツ）計 130 検体を購入し，各検体の一般生菌数と大腸菌群を従来法にて測定した．同時に Micro Foss 専用の一般生菌数・大腸菌群測定用バイアルに供し，pH 指示薬の変化の検出に必要な時間（Dt）を求めた．

(2) 従来法にて求めた菌数と Dt とのグラフを作成し相関を求めることで，本法の有用性を評価した．また芽物野菜としてカイワレ大根を用いて大腸菌の検出実験への応用についても評価した．

(3) 本法は広い定量幅を持っており従来法では必要な段階希釈が不要であった．測定時間は初発菌数が多い程短く，3～18 時間で菌数推定結果が得られた．

(4) 大腸菌の検出も，ダーラム管と併用することで可能となった．

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Menadione-Catalyzed Luminol Chemiluminescent Assay for the Viability of *Escherichia coli* ATCC 25922

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Abstract: ***Escherichia coli* ATCC 25922 produced O_2^- in the presence of menadione, and O_2^--dependent luminol chemiluminescence intensity was proportional to colony-forming unit (CFU) in the exponential phase. CFU was determined by using a 96-well plate at a range of 3×10^3 to 8×10^7 CFU /well (0.1 ml) after a 10-min incubation with menadione, followed by chemiluminescent assay for 5 s. After a 4-hr incubation of *E. coli* (10^5 CFU/0.1 ml) with menadione and an antimicrobial agent inhibiting the synthesis of peptidoglycan, protein, and DNA, the inhibitory concentration (IC) of the antimicrobial agent determined by menadione-catalyzed luminol chemiluminescent assay was in good agreement with minimal inhibitory concentration (MIC) of the NCCLS (National Committee for Clinical Laboratory Standard) method requiring 18 hr. Menadione-catalyzed luminol chemiluminescent assay is expected to be useful for the rapid determination of cell viability under the conditions of various cell growths and stresses.**

Key words: **Menadione, Chemiluminescence, Antibiotics, Viability**

Dilution (6) and disk antimicrobial susceptibility tests (8) require more than 18 hr to obtain results. The minimal inhibitory concentration (MIC) is used to quantitatively measure the *in vitro* activity of an antimicrobial agent against a bacterial isolate, and it is determined by observing the lowest concentration of antimicrobial agent that will inhibit visible growth of the bacterium after overnight incubation.

In order to reduce the testing time, various modified tests have been proposed. For example, the rapid measurements of specific enzyme activities and ATP concentration in bacteria have been expected to be performed with fluorogenic or chromogenic substances (4, 5) and by bioluminescent assay (1, 6), respectively. However, the measurement of bacterial enzyme activities are not useful in many laboratories because of the unstable methods affected by pH, temperature, ion concentration, and enzyme inhibitors. The bioluminescent assay of bacterial ATP is also affected by the extracellular ATP in medium and by the treatment of cell lysis (2).

On the other hand, menadione-catalyzed luminol chemiluminescent assay is expected to be simple and rapid. As shown in Fig. 1, the extracellular generation of active oxygen species (O_2^- and H_2O_2) catalyzed by exogenous menadione (redox catalyst) has been proposed to depend on the cell activity to keep the intracellular NAD(P)H concentration and the activity of NAD(P)H:menadione oxidoreductase, which is sensitive to the change in pH and ion concentration (10, 11). Solid and dashed lines in Fig. 1 show the production of O_2^- from the oxidation of unstable semiquinone radicals and of H_2O_2 from the autoxidation of menadiol after a reduction of menadione by NAD(P)H:menadione reductase.

Recently, O_2^- was found to be main product in the reaction between menadione and *E. coli* ATCC 25922 (solid line in Fig. 1), and the viability was related to menadione-catalyzed luminol chemiluminescence, depending on the generation of O_2^- (12). This luminol chemiluminescent assay requires a few minutes for the incubation of cells with menadione, then a few seconds for the measurement of luminol chemiluminescence intensity after an injection of luminol solution without the

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Abbreviations: ATCC, American Type Culture Collection; ATP, adenosine triphosphate; CAMHB, cation-adjusted Mueller-Hinton broth; CFU, colony-forming unit; H_2O_2, hydrogen peroxide; LCI, luminol chemiluminescence intensity; MIC, minimal inhibitory concentration; NAD(P), nicotinamide adenine dinucleotide (phosphate); O_2^-, superoxide anion; NCCLS, National Committee for Clinical Laboratory Standards.

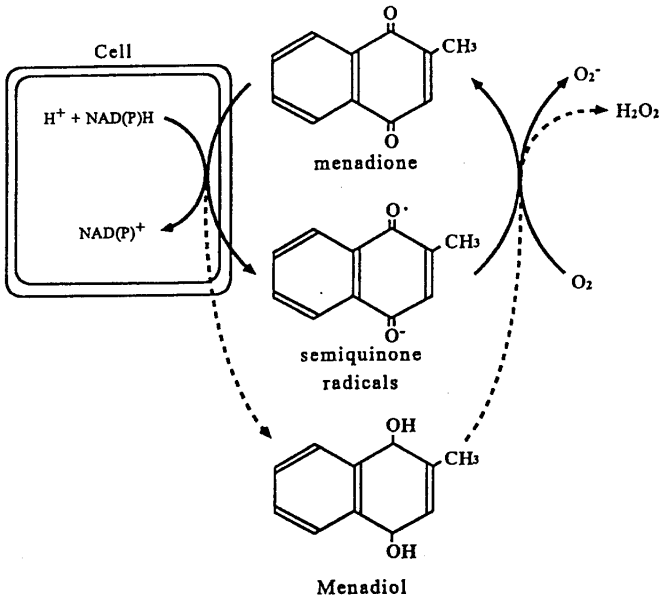

Fig. 1. Proposed mechanism for menadione-catalyzed production of active oxygen species by viable cells.

treatment of cell lysis. Thus the menadione-catalyzed luminol chemiluminescent assay is expected to be useful for the rapid determination of cell viability under the conditions of the various stresses.

Because *E. coli* ATCC 25922 is one of the quality control strains for broth microdilution MIC testing of NCCLS, the inhibitory effect of antimicrobial agent on menadione-catalyzed luminol chemiluminescence by this strain was tested to study the sensitivity of menadione-catalyzed luminol chemiluminescent assay to the antimicrobial agents inhibiting the synthesis of peptidoglycan, protein, and DNA. This paper suggests that menadione-catalyzed luminol chemiluminescence by *E. coli* ATCC 25922 shows the rapid response to the change in cell viability and physiology under the conditions of various cell growth and stresses.

Materials and Methods

Organisms and growth conditions. Escherichia coli (ATCC 25922) was cultured in Mueller-Hinton broth containing 25 mg of Ca^{2+} and 12.5 mg of Mg^{2+} per liter (cation-adjusted Mueller-Hinton broth, CAMHB) at 35 C overnight. The absorbance at 600 nm of the cell suspension was adjusted to 0.1 with CAMHB, and the suspension was incubated at 35 C for about 2 hr. After incubation, the absorbance of each cell suspension was adjusted to the appointed absorbance at 600 nm with CAMHB on the basis of correlation between absorbance and CFU of the bacterium, and the diluted cell suspension was used for the chemiluminescent assay.

Chemiluminescent assay. The above diluted cell suspension (50 µl) was placed in each well in a white 96-well microplate, and 50 µl of CAMHB containing menadione (10 µg/ml) was added. When the appointed incubation time was finished, luminol chemiluminescence intensity was determined after an injection of 100 µl of luminol-horseradish peroxidase solution containing 5 mg of luminol, 5 units of horseradish peroxidase (Type I), and 50 mg of bovine serum albumin (Fraction V) in 50 ml of 0.5 M boric acid-Na (pH 9.5). The chemiluminescence intensity after this injection was automatically determined by using luminescencer AB-2000 (ATTO, Tokyo).

Determination of colony-forming unit (CFU). A series of tenfold dilutions of bacterial cell suspension was prepared in CAMHB. A 0.1 ml portion of the dilution was spread on a Mueller-Hinton agar (Becton Dickinson) plate in duplicate. The colonies on each plate were counted after 18- to 24-hr incubation at 35 C, and the number of viable bacteria per milliliter of each original suspension was calculated as CFU.

Determination of inhibitory concentration (IC) by chemiluminescent assay. Fifty microliters of cell suspension containing approximately 2×10^6 CFU/ml and menadione (10 µg/ml) were mixed with 50 µl of

CAMHB containing an antimicrobial agent in each well of a white 96-well microplate. Antimicrobial agents used in this study are listed in Table 1 and are prepared by serial twofold dilutions described in NCCLS (6). After incubation at 35 C for 4 hr, the IC was determined as the concentration of antimicrobial agent decreasing the luminol chemiluminescence intensity (LCI) from less than 20% of antimicrobial agent-free LCI.

Assay of intracellular NAD(P)H concentration. The extraction and estimation of NAD(P)H of *E. coli* were performed according to the method of Lilius et al (3).

Chemicals. All reagents were analytical grade.

Results and Discussion

LCI increased after the addition of menadione to *E. coli* cell suspension and reached the maximum LCI a few minutes later. Superoxide dismutase diminished the menadione-catalyzed luminol chemiluminescence more effectively than catalase, indicating the generation of O_2^- in the interaction between menadione and *E. coli* (12). The desirable concentration of menadione required for the chemiluminescent assay was 5 μg/ml because menadione inhibited cell growth at above 20 μg/ml and caused poor chemiluminescence intensity at below 2 μg/ml (data not shown).

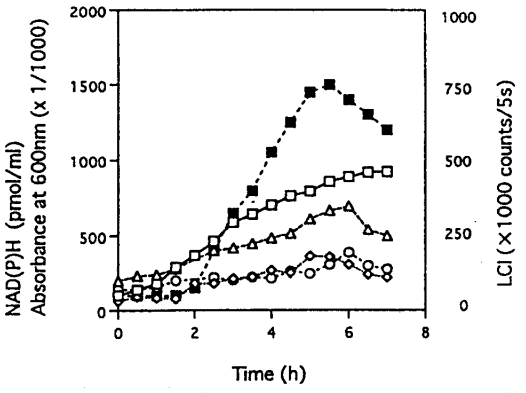

Fig. 2. Correlationships between menadione-catalyzed LCI and intracellular NAD(P)H concentration during growth of *E. coli*. The absorbance of cell suspension was adjusted to 0.1 at 600 nm with CAMHB and was incubated at 35 C. The total volume of cell suspension was 50 ml, and 0.5 ml of cell suspension was used for the determination of NAD(P)H concentration. LCI and intracellular NAD(P)H concentration were determined by the methods described in "Materials and Methods." ■, LCI; ◇, NADH concentration; ○, NADPH concentration; △, NAD(P)H concentration; □, absorbance at 600 nm.

Figure 2 shows the relationships among menadione-catalyzed LCI, intracellular NAD(P)H concentration, and cell growth monitored by measuring the turbidity at 600 nm. Menadione-catalyzed LCI was not correlated with the turbidity of *E. coli* cells, but with the intracellular NAD(P)H concentration during cell growth, because the peak of LCI overlapped with that of intracellular NAD(P)H concentration in the late exponential phase. These results suggest that menadione-catalyzed LCI depends on intracellular NAD(P)H concentration required for the activity of NAD(P)H:menadione reductase as described in the previous paper (12) and that the chemiluminescent assay is applicable to the determination of bacterial viability.

When the viable cell number of *E. coli* was determined in the late exponential phase, CFU were proportional to LCI as shown in Fig. 3. CFU from 2×10^3 to 2×10^6/well (0.1 ml) could be determined 10 min after the incubation of *E. coli* and menadione (data not shown). The detectable CFU by the chemiluminescent assay was similar to that by the bioluminescent assay of ATP (7). Menadione-catalyzed luminol chemiluminescent assay is expected to be simple and rapid compared to the bioluminescent assay of ATP, requiring both the enzymatic decomposition of extracellular ATP and the extraction of intracellular ATP.

As menadione-catalyzed luminol chemiluminescence was so strongly dependent on colony-forming units, the application of the chemiluminescent assay to an antimicrobial susceptibility test was examined. Figure 4 shows

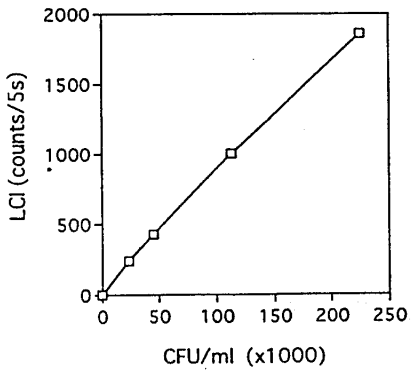

Fig. 3. Correlation between CFU and LCI in the late exponential phase. Growth conditions and the chemiluminescent assay are described in "Materials and Methods." *E. coli* was harvested in the late exponential phase, and the cell density was diluted from 0 to 23×10^4 CFU/ml with CAMHB. LCI was determined 10 min after the incubation of *E. coli* and menadione at 35 C and was shown as the mean of four determinations.

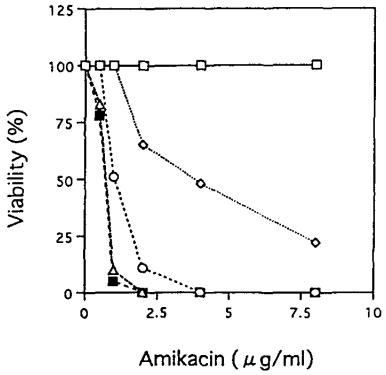

Fig. 4. Dose- and time-dependent effects of amikacin on menadione-catalyzed LCI. The assay conditions are the same as those to determine IC in "Materials and Methods." The viability of *E. coli* cells exposed to amikacin was determined as the percentage based on the LCI of intact cells. Each symbol shows the following incubation time: □, 1 hr; ◇, 2 hr; ○, 3 hr; △, 4 hr; ■, 6 hr.

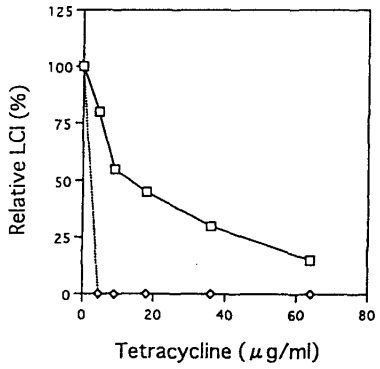

Fig. 5. Direct inhibition of menadione-catalyzed LCI by tetracycline. After the cell suspension, which contained approximately 10^6 CFU/ml and 5 μg/ml of menadione, was incubated at 35 C for 4 hr, tetracycline was added to it. LCI was determined after 1-min and 60-min incubations with tetracycline (□, 1-min incubation; ◇, 60 min incubation).

Table 1. MIC determined by menadione-catalyzed luminol chemiluminescent assay after 4-hr incubation

Antimicrobial agent	MIC (μg/ml) Chemiluminescent assay	NCCLS
Penicillins		
ampicillin	8	2−8
piperacillin	4	1−4
Monobactams		
aztreonam	0.125	0.06−0.25
Carbapenems		
imipenem	0.5	0.06−0.25
Quinolones		
norfloxacin	0.06	0.03−0.12
ofloxacin	0.03	0.015−0.12
Chloramphenicols		
chloramphenicol	2	2−8
Aminoglycosides		
kanamycin	2	1−4
amikacin	1	0.5−4
gentamicin	0.5	0.25−1
Cephems		
cefazolin	1	1−4
ceftazidime	0.5	0.06−0.5
cefmetazole	1	0.25−2
cefotetan	0.25	0.06−0.25
Tetracyclines		
tetracycline	0.5	0.5−2
minocycline	0.5	0.25−1
Others		
fosfomycin	2	0.5−2
sulfamethoxazole-trimethoprim	0.06/1.2	≦0.5/9.5

that amikacin reduces the LCI in time-dependent and dose-dependent manners and that the change in antimicrobial action of amikacin stops at 2 μg/ml after a 4- or 6-hr incubation time. Cefazolin, ampicillin (inhibitor of peptidoglycan synthesis), tetracycline (inhibitor of protein synthesis), and norfloxacin (inhibitor of DNA synthesis) also showed the antimicrobial action in time-dependent and dose-dependent manners (data not shown), and IC could be determined 4 hr after the incubation of *E. coli* with menadione and an antimicrobial agent.

Table 1 shows that the MICs determined by the chemiluminescent assay are in good agreement with the acceptable quality control limit ranges in NCCLS (6), because IC of imipenem alone was greater than the limit ranges when 18 antimicrobial agents were tested. Menadione-catalyzed luminol chemiluminescence was sensitive to the antimicrobial agents inhibiting the synthesis of peptidoglycan, protein, and DNA (9). On the other hand, all MIC of the antimicrobial agents listed in Table 1 was in the acceptable quality control limit ranges of NCCLS (data not shown) when the MIC of antimicrobial agents was determined according to the dilution antimicrobial susceptibility test described in NCCLS (7).

Minocycline and tetracycline inhibited menadione-catalyzed luminol chemiluminescence immediately after the addition of these antimicrobial agents into the mixture of menadione and *E. coli* that generates enough O_2^-. Figure 5 shows the inhibitory effect of tetracycline on menadione-catalyzed LCI 1 min and 60 min after the mixing of *E. coli* and tetracycline. The inhibitory effect observed after 1 min of incubation is considered to be the direct inhibition of luminol chemiluminescence by tetra-

cycline, because menadione-catalyzed lucigenin chemiluminescence was not inhibited by tetracycline (data not shown here). The inhibitory effect observed after 60 min of incubation was greater than after 1 min and was therefore considered to depend on combination of antimicrobial action and direct inhibition. As shown in Fig. 5, the 50% inhibition concentration of tetracycline on LCI was about 10 μg/ml, and that of minocycline was about 1 μg/ml (data not shown). In the case of *E. coli*, the IC of tetracycline could be estimated because it was much lower than the 50% inhibition concentration on LCI.

As mentioned in "Results and Discussion," the menadione-catalyzed luminol chemiluminescent assay is expected to be useful for the rapid determination of the viability of *E. coli* under various growth conditions.

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8-Deoxy-trichothecin production by *Spicellum roseum* isolated from a cultivated mushroom in Japan

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8-Deoxy-trichothecin production by *Spicellum roseum* isolated from a cultivated mushroom in Japan

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Summary

Ex-type culture of *Spicellum roseum* isolated from silage in France has been known to produce some trichothecenes. As the causative agent of the pink mold damping-off disease, we isolated some strains of *S. roseum* from a cultivated mushroom *Flammulina velutipes* at Nagano, Japan. To examine whether the Japanese strains of *S. roseum* produce trichothecenes or not, the isolates were cultivated on rice medium and extraction was made for mycotoxin analysis. 8-Deoxy-trichothecin was accordingly detected and confirmed with GC/MS by means of EI and CI mode. However, deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X and nivalenol were not detected in the extract, all of which are well known to be detected from scabby wheat.

Key words: 8-deoxy-trichothecin, *Flammulina velutipes*, mycotoxins, *Spicellum roseum*, trichothecene

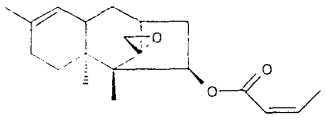
(Received, February 26, 2001, Revised & Accepted May 23, 2001)

Introduction

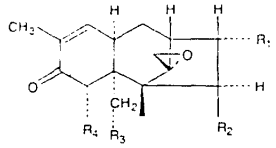
Nakamura *et al.*[1] reported that *Sporothrix* sp. was the causative agent of the pink mold damping-off disease of *Flammulina velutipes* (Curt. : Fr.) Sing., a cultivated mushroom in Japan. The hyphomycete was later reidentified as *Spicellum roseum* Nicot & Roquebert based on morphology and rDNA sequence analysis

Okada *et al.*[2]).

Spicellum roseum was first isolated from silage in France (Nicot and Roquebert[3]). Other strains were isolated from mushroom compost in USA, paper bags in India, and leather in UK (Seifert *et al.*[1]). Seifert *et al.*[4] recently reported that ex-type culture of *S. roseum* produced trichothecene mycotoxins: i.e., 12,13-trichothec-9-ene, 8-deoxy-trichothecin, trichodermol and trichodermin. The structures of some trichothec mycotoxins were shown in Fig. 1. In this paper, we examined new isolates of *S. roseum* from cultivated *F. velutipes* in Japan to determine if these strains produce trichothecenes or not.



8-deoxy-trichothecin



	R_1	R_2	R_3	R_4
Deoxynivalenol	OH	H	OH	OH
3-Acetyl-deoxynivalenol	OAc	H	OH	OH
Fusarenon-X	OH	OAc	OH	OH
Nivalenol	OH	OH	OH	OH

Fig. 1 Structures of some trichothecenes.

Materials and Methods

Some strains of *S. roseum* were isolated from cultivated *F. velutipes* at Nagano, Japan, which was infected by the pink mold damping-off disease. The isolates were deposited in Japan Collection of Microorganisms (JCM; http://www.jcm.riken.go.jp/). Two strains of *S. roseum* JCM 8964 and 8965 were used for the productivity test of trichothecenes.

Culture of *S. roseum* was done as follows. Thirty gram of 4 % polished rice was weighed in conical flask and 15 ml of water was added. It was maintained for 3 hours in room temperature and then autoclaved. For pre-culturing, *S. roseum* was inoculated by a needle at the center of potato dextrose agar (PDA) plates in 9 cm diam Petri dish. The pre-culture was kept at 25 ℃ for 7 days. Inoculation of *S. roseum* to the rice medium was made by adding three pieces of 8 mm diam disks cut from the pre-culture plate by a cork bowler. The inoculated rice medium was kept at 25 ℃ for 14 days.

Extraction and clean-up procedures for mycotoxins were as listed in Figs. 2 and 3, respectively.

TMS derivatization of extract from *S. roseum* cultures was as follows. TMS derivatizing reagent was

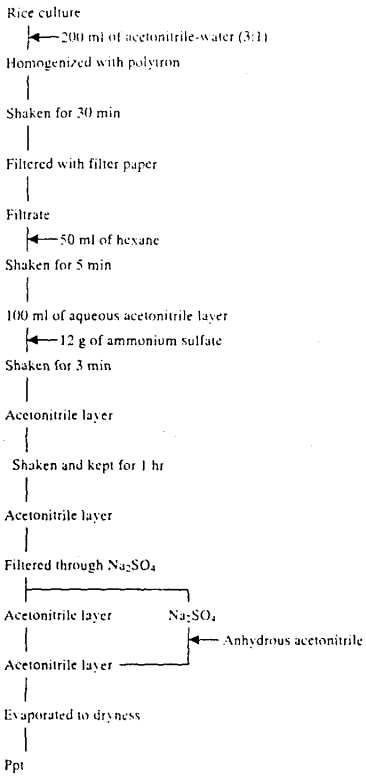


Fig. 2 Extraction of trichotheceses.

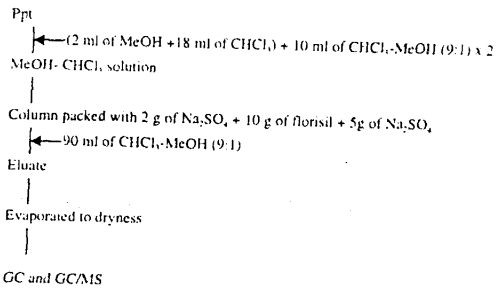


Fig. 3 Clean-up method of trichothecenes.

composed of 1.0 ml of N-trimethylsilylimidazole, 0.2 ml of trimethylchlorosilane and 9.0 ml of ethyl acetate. Zero point one ml of TMS derivatizing reagent was added to the extract after clean-up and kept at room temperature for 15 min. Then 3.9 ml of ethyl acetate was added to this reacting solution. This solution was filtered with filter disk of Ekikurodisk R 13CR with 0.2 μ m PTFE (Gelman Japan). The filtrate was analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS).

The GC, Shimadzu GC-15A (Shimadzu Co., Kyoto, Japan) equipped with ECD detector, was used. The conditions of GC were listed as follows. DB-1 megabore column (J & W Scientific, Folsom, CA, USA), 10 m x 0.53 mm i.d. and 1.5 μ m in film thickness, was used. The column temperature was held at 185℃ for 50 min. It was increased by 10℃/min to 240℃ and maintained for 15 min. Injection port was 280℃. N2 gas for column connection purge was 22 ml/min and that for cell purge was 60 ml/min. Flow rate of He gas was 15 ml/min. Detection was made by ECD.

GC/MS conditions of EI mode by Shimadzu GCMS-QP5000 were as follows. Shimadzu GC-17A was

used as a gas chromatograph. A 30 m x 0.25 mm i.d. DB-1 column (J & W Scientific, Folsom, CA, USA) with film thickness of 0.25μm was used. The column temperature, initial at 190℃, was increased by 1.5℃ /min to 217℃ and then by 6.0℃/min to 280℃ and maintained at 280℃ for 5 min. Injection port temperature was 250℃ and interface temperature was 300℃. Flow rate of He gas was 1.8 ml/min. Detection voltage was 2.50 kV.

The cleaned-up extract was trimethylsilylated and injected into GC. The TMS derivatives were also analyzed by GC/MS, Shimadzu QP-5000 (Shimadzu Co., Japan), both in the electron ionization (EI) and chemical ionization (CI) modes. For CI, methane gas was used as the reagent gas. ThermoQuest Finnigan Trace GC/MS instrument was used to compare authentic 8-deoxy-trichothecin with the trichothecin in order to confirm the presence of 8-deoxy-trichothecin. Spectra were recorded in the EI and CI modes. For CI, both methane and isobutane (which had been used by Plattner et. al.[5]) were used as reagent gases.

Results and Discussion

Deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-Ac-DON), fusarenon-X (Fus. -X) and nivalenol (NIV) are well known trichothecenes often found in scabby wheat and have some toxicity against animals and human. These mycotoxins were analyzed using gas chromatography that showed the detection limit as 0.2 μ g/g for DON, 3-Ac-DON, Fus.-X and NIV. Gas chromatogram and mass spectra obtained showed that these trichothecenes were not present in the culture extract of *S. roseum* isolated from affected *F. velutipes* in Japan, although the extraction is suitable for these trichothecenes. Therefore, we conclude that these four mycotoxins were not produced by *S. roseum* JCM 8964 and JCM 8965.

One peak of trichothecenes was obtained in gas chromatogram, so trimethylsililated sample was injected into QP-5000 type of GC/MS. In GC/MS analysis mass spectra similar to that reported for 8-deoxy-trichothecin were obtained from the extract of *S. roseum* JCM 8965 by means of EI and CI mode from QP-5000 GC/MS. The molecular weight of 8-deoxy-trichothecin is 318.

To confirm this result TMS derivatives of the *S. roseum* were analyzed on the TRACE GC/MS along with authentic 8-deoxy-trichothecin[5]. In the chromatogram of the extract of JCM 8965, components were observed which had identical spectra with the EI and isobutane CI spectra previously reported for the TMS derivative of 8-deoxy-trichothecin. The larger of these two components, 70% of the total, eluted at 7.14 min, while the latter eluted at 7.22 min. By contrast, the chromatogram of the TMS derivative of authentic 8-deoxy-trichothecin revealed only a single component that eluted with the same retention time (7.14min). Thus the component eluting at 7.14 min is confirmed to be 8-deoxy-trichothecin. The EI mass spectrum and the isobutane CI spectrum are identical with the ones reported previously by *Plattner et al.*[5]. From these results, it becomes clear that *S. roseum* JCM 8965 produced 8-deoxy-trichothecin. The exact structure of the minor component in the extract of JCM 8965 has presently not been completely characterized but is presumably a stereoisomer of 8-deoxy-trichothecin. The total ion chromatograms and EI mass spectra of 8-deoxy-trichothecin and the extract of JCM 8965 are shown in Fig. 4 and Fig. 5, respectively. The total ion chromatograms, the single ion monitor chromatograms of 319 (M+1) and CI (isobutane) mass spectra of 8-deoxy-trichothecin and the extract of JCM 8965 are shown in Fig. 6 and Fig. 7, respectively. In the case of *S. roseum* JCM 8964, the production of 8-deoxy-trichothecin was obscure.

Though the toxicity of 8-deoxy-trichothecin has not been known yet, the toxicity test of the extract of *S.*

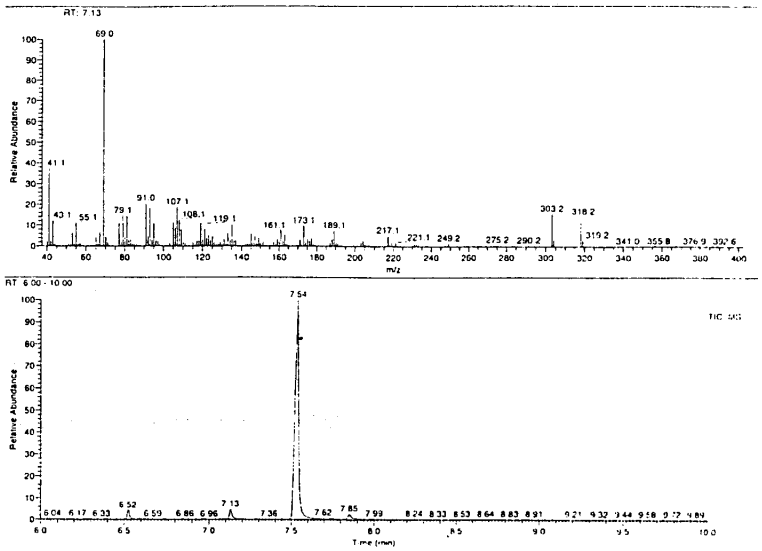


Fig. 4 EI mass spectrum of 8-deoxy-trichothecin and the total ion chromatogram.

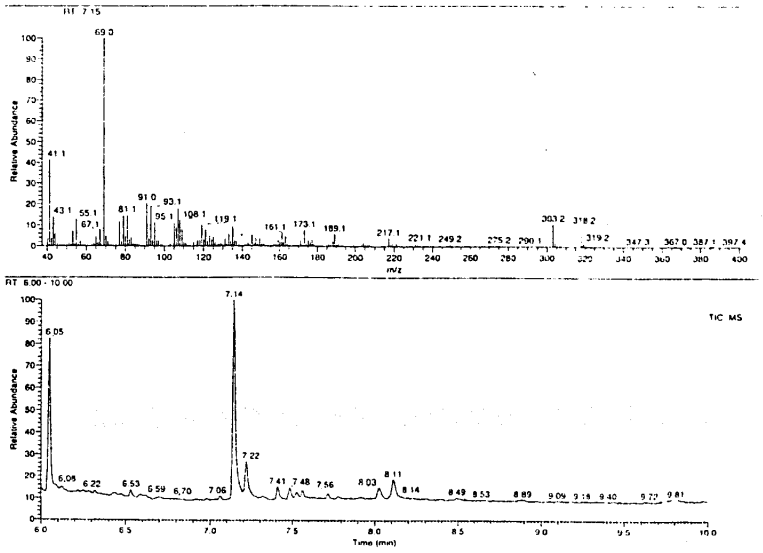


Fig. 5 EI mass spectrum of the extract of JCM 8965 and the total ion chromatogram.

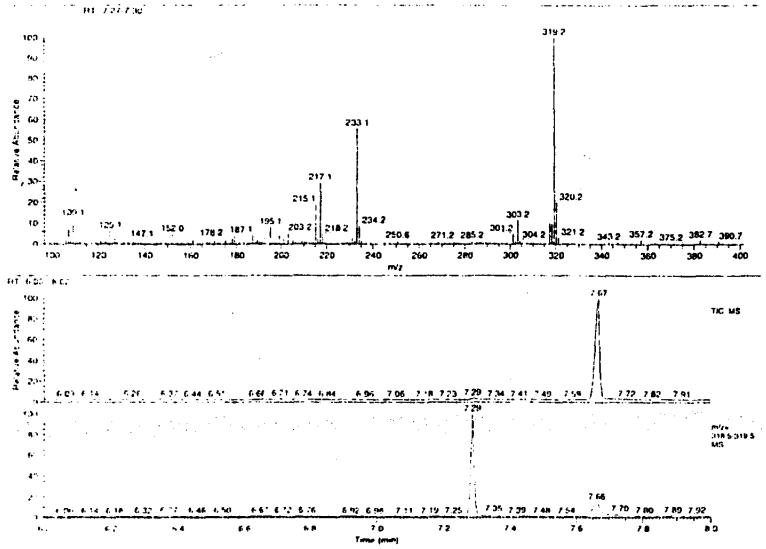


Fig. 6 CI(isobutane)mass spectrum of 8-deoxy-trichothecin, the total ion chromatogram, and the single ion monitor chromatogram of 319(M+1

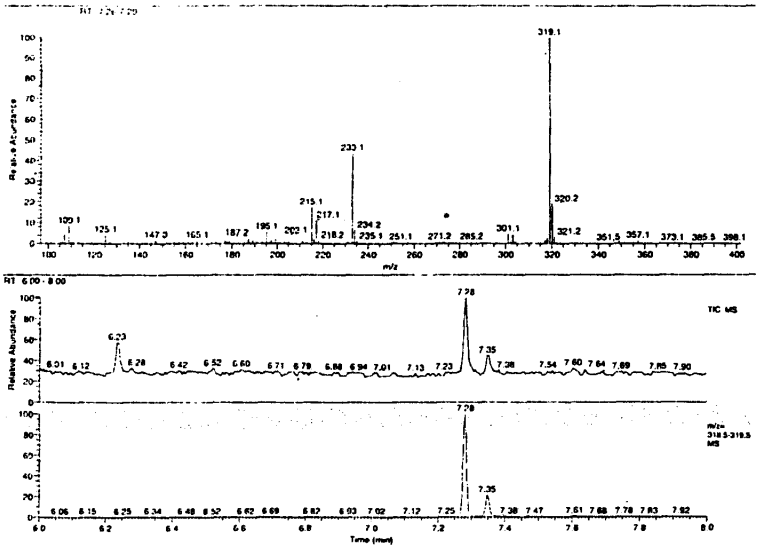


Fig. 7 CI(isobutane)mass spectrum of the extract of JCM 8965, the total ion chromatogram and the single ion monitor chromatogram of 319(M+1).

roseum cultures should be carried out for more complete safety on mushroom cultivation.

We thank Mr. K. Nakamura and Mr. S. Tsunoda, Nagano Vegetable and Ornamental Crops Experiment Station, and Dr. K. Yamanaka, Mushroom Research Laboratory, Hokuto Corp. (Kyoto Mycological Institute, at present), who gave us the valuable instruction on the pink mold damping-off disease of *F. velutipes*.

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日本の栽培キノコから分離した*Spicellum roseum*による8－デオキシートリコテシンの産生

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フランスの堆肥から分離された不完全菌*Spicellum roseum*のタイプ由来株を培養すると，トリコテセン系マイコトキシンを産生することが知られている．桃色かび立枯病を引き起こす原因菌として，我々は長野県のエノキタケ(*Flammulina velutipes*)から数株の*S. roseum*を分離した．これら日本産の*S. roseum*がトリコテセン系マイコトキシンを産生するか否かを調べるために，分離菌株を米培地で培養し，マイコトキシンの分析を行った．EIモード及びCIモードのガスクロマトグラフ質量分析計で8－デオキシートリコテシンを検出し，確認した．しかしながら，赤かび病罹病のムギから検出されるトリコテセン系マイコトキシンとして有名なデオキシニバレノール，3－アセチルーデオキシニバレノール，フザレノン-X及びニバレノールは検出されなかった．

キーワード：8－デオキシートリコテシン，*Flammulina velutipes*，マイコトキシン，
Spicellum roseum，トリコテセン

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Traditional Japanese Fermented Foods Free from Mycotoxin Contamination

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Traditional Japanese Fermented Foods Free from Mycotoxin Contamination

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Abstract

Miso (soybean paste), shoyu (soy sauce), sake (rice wine) and katsuo-bushi (dried bonito), which are traditional Japanese fermented foods, have been consumed for a long period of time and are considered to be the safest foods. However, fermented foods may be contaminated with mycotoxins. Fungi used for the fermentation of miso, shoyu, and sake consist of *Aspergillus oryzae* and *A. sojae*. Although *A. oryzae* and *A. sojae* belong to the *A. flavus* group, which is known to produce aflatoxins, none of the strains examined actually produced aflatoxins. Aflatoxin contamination of rice, miso, shoyu, sake and katsuo-bushi could not be detected. Aflatoxigenic fungi do not occur in areas with a mean temperature below 16°C. Since the mean temperature in most areas of Japan is lower than 16°C, it appears that food contamination with aflatoxigenic strains does not occur in most parts of Japan. Some koji molds are known to produce kojic acid (KA) and cyclopiazonic acid (CA). Production of KA and CA was examined and the producing strains were eliminated from the commercial fermented foods. The fate of KA and CA in shoyu fermentation was also examined and it became clear that the contents of KA and CA decreased during shoyu fermentation. Contamination of katsuo-bushi with aflatoxins, sterigmatocystin and ochratoxin A could not be detected. As mentioned previously, mycotoxin contamination, which is presently very low, should be completely eliminated in future.

Discipline: Food / Postharvest technology
Additional key words: aflatoxins, kojic acid, cyclopiazonic acid, sterigmatocystin, ochratoxin A

Aflatoxins

Miso (soybean paste), shoyu (soy sauce) and sake (rice wine) which are traditional Japanese fermented foods have been consumed for a long period of time, and are considered to be the safest foods. However, the discovery of aflatoxins became a cause for concern for the producers of traditional Japanese fermented foods. *Aspergillus flavus* and *A. parasiticus*, which are known to produce aflatoxins, belong to the *A. flavus* group. Koji mold is considered to be composed of *A. oryzae* and *A. sojae*. Koji mold involved in the first step of fermentation is also classified into this group. Aflatoxins exhibit a strong acute toxicity and the strongest carcinogenicity found in the natural world to animals. Koji mold first and then the other microorganisms are involved in the fermentation process. In order to examine the production of aflatoxins by koji mold strains practically used in the Japanese fermentation companies, we collected commercially used koji mold. A total of 212 strains of koji mold were tested for their aflatoxin production. Though substances similar to aflatoxins were observed on the TLC (thin layer chromatography) plate under UV light, it was found that none of them produced aflatoxins, as shown in Table 1 based on the analysis of the UV spectra.

Rice is a staple food in Japan and also a raw material of traditional Japanese fermented foods. The possibility

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This paper is based primarily on a presentation at the International Cooperative Research Works Symposium on Food Safety held in 1999[9].

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Table 1. Production of aflatoxin-like substances by koji molds used for various purposes

Strains	Production of aflatoxins	Production of aflatoxin-like substances						Number of strains
		+		±		−		
For miso	0	6	(18%)	4	(12%)	23	(70%)	33
For shoyu	0	14	(29%)	7	(15%)	27	(56%)	48
For sake	0	4	(22%)	0	(0%)	14	(78%)	18
For tamari shoyu	0	3	(50%)	0	(0%)	3	(50%)	6
For shochu	0	1	(50%)	0	(0%)	1	(50%)	2
For hon-koji	0	0	(0%)	0	(0%)	1	(100%)	1
Others	0	21	(20%)	11	(11%)	72	(69%)	104
Total	0	49		22		141		212
%	0	23		10		67		100

Aflatoxins were analyzed by TLC first. Then aflatoxin-like substances that were observed under UV light could not be identified as aflatoxins based on UV spectra.

of contamination with aflatoxins was examined. Ninety-eight rice samples produced in Japan in 1966 and 1967 were analyzed at a rate of 2 samples from each prefecture. Two rice samples from Burma (Myanmar), 1 rice sample from Thailand, 3 samples from USA, 3 rice samples from the People's Republic of China, 1 rice sample from Taiwan, and 1 rice sample from Spain imported between 1966 and 1967 were also analyzed. Aflatoxins were not detected from any of these samples.

One hundred and eight commercial miso samples, 33 home-made miso samples and 28 koji (molded rice inoculated with koji mold) samples were also analyzed. Aflatoxins were not detected from any of these samples. Therefore, it appears that miso is free from aflatoxin contamination. The decomposition of aflatoxins in miso was studied by spiking aflatoxins before fermentation. Although aflatoxins B_1 and G_1 were decomposed at the primary stage of fermentation, the contents of most of aflatoxins B_2 and G_2 did not decrease. These results indicate that, in order to prevent aflatoxin contamination of miso, it is necessary to use aflatoxin-free raw materials and to prevent the koji mold from becoming contaminated with aflatoxin-producing fungi.

Aflatoxin contamination of shoyu was also examined[4]. Thirty-nine shoyu samples were collected from all parts of the country and analyzed. No aflatoxins were detected from these samples. Therefore, it was concluded that shoyu samples were not contaminated with aflatoxins.

The geographical distribution of the fungi that produce aflatoxins in soil in Southeast Asia and Japan was also studied[2,3]. It appeared that the frequency of isolation of aflatoxin-producing fungi in soil increased from the subtropical zone to the tropical zone as shown in Fig. 1. No aflatoxigenic strains were isolated from areas where the mean temperature was lower than 16°C. Therefore, it was concluded that in most parts of the Japanese islands *A. flavus* and *A. parasiticus* do not occur.

Kojic acid

Kojic acid is known to be produced by *A. oryzae*. Kojic acid does not display a strong toxicity, but shows a weak mutagenicity in the Rec assay and Ames test. Production of kojic acid by *A. oryzae* strains was examined[6,7] and is shown in Fig. 2. Most of the strains used for the production of sake and shoyu did not produce detectable amounts of kojic acid. Twelve out of 17 strains used for miso fermentation produced kojic acid. These strains were supplied by the Japan Association of Koji-starter. Based on these results, we informed the Japan Association of Koji-starter that the strains with a high production of kojic acid should not be used for koji-starter. As Sakaguchi's medium is considered to be suitable for the production of kojic acid, Sakaguchi's medium was used for time course studies of kojic acid production by koji molds at 30°C. The production of kojic acid which was negligible after 2 or 3 days of incubation, increased rapidly during the 3 to 14-day incubation period. After 2 weeks of incubation, the amount of kojic acid in the culture medium decreased. As rice is used for koji (molded rice) production, rice was used for the time course studies of kojic acid production by koji molds at 30°C. The results were almost similar to those obtained in Sakaguchi's medium. However, the amount of kojic acid in the rice culture medium did not decrease after 2 weeks. In

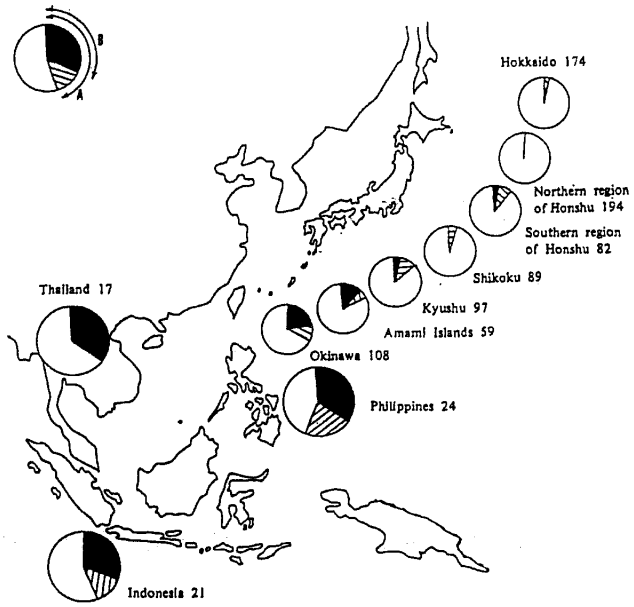

Fig. 1. Detection ratio of aflatoxin-producing fungi in soil samples collected from several countries of Southeast Asia and Japan
A: Ratio of samples where *A. flavus* and *A. parasiticus* were detected.
B: Ratio of samples where aflatoxin-producing fungi were detected.
Number: number of examined samples.

the Japanese fermented food industries, the incubation period required for making koji to produce sake, shoyu and miso is usually within 2 days. It was observed that kojic acid in koji after 2 days of incubation could not be detected or that the amount was very low, even when strains with a high production of kojic acid were used. We expect that kojic acid-producing strains will be eliminated from the koji molds used in the Japanese fermented food industries in the near future. The decomposition of kojic acid during shoyu fermentation was examined. When kojic acid was spiked during shoyu fermentation, kojic acid could not be detected after 150 days. Presently, the usual shoyu fermentation period is about 180 days. Therefore, even if kojic acid were to be produced during the koji-making period, it might be decomposed during shoyu fermentation and the probability that kojic acid remains in the final shoyu product would be very low.

Cyclopiazonic acid

It was reported that several strains of *A. oryzae* produce cyclopiazonic acid (CA). Since CA is also a kind of mycotoxin and shows a comparatively strong acute toxicity, the production of this toxin by several *Aspergillus* strains was examined[1]. The results are shown in Table 2. Thirty-six strains of *A. oryzae*, which were used in traditional Japanese fermented foods, were examined. As a

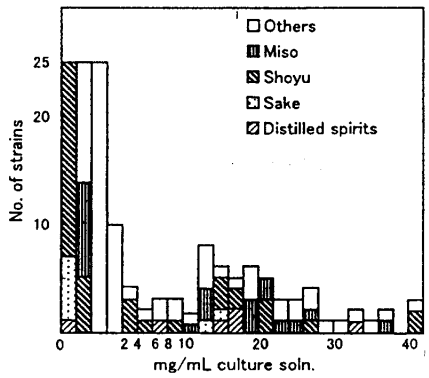

Fig. 2. Production ability of kojic acid by strains of koji molds

Table 2. Production of cyclopiazonic acid by several *Aspergillus* strains

Aspergillus species	Strains No.	Cyclopiazonic acid	Characteristics
Aspergillus oryzae	NFRMCA01	N.D.	
	NFRMCA02	N.D.	
	NFRMCA03	N.D.	
	NFRMCA04	N.D.	
	NFRMCA05	N.D.	
	NFRMCA06	N.D.	
	NFRMCA07	N.D.	
	NFRMCA08	N.D.	
	NFRMCA09	N.D.	
	NFRMCA10	N.D.	
	NFRMCA11	N.D.	
	NFRMCA12	N.D.	
	NFRMCA13	19.6 μg/g	
	NFRMCA14	N.D.	
	NFRMCA15	N.D.	for shoyu
	NFRMCA16	N.D.	for shoyu
	NFRMCA17	N.D.	for shoyu
	NFRMCA18	N.D.	for shoyu
	NFRMCA19	N.D.	
	NFRMCA20	N.D.	
	NFRMCA21	N.D.	
	NFRMCA22	N.D.	
	NFRMCA23	N.D.	
	NFRMCA24	N.D.	
	NFRMCA25	24.4 μg/g	
	NFRMCA26	N.D.	
	NFRMCA27	5.5 μg/g	
	NFRMCA28	N.D.	
	NFRMCA29	N.D.	
	NFRMCA30	N.D.	
	NFRMCA31	N.D.	
	NFRMCA32	N.D.	
	NFRMCA33	N.D.	
	NFRMCA34	N.D.	
	NFRMCA35	N.D.	
	IFO4278	1.9 μg/g	
Aspergillus sojae	MFR-S-52	N.D.	for shoyu
	NFR-NO9	N.D.	for shoyu
Aspergillus flavus	TH-1	16.0 μg/g	aflatoxigenic
	US-F-2	5.2 μg/g	aflatoxigenic
Aspergillus parasiticus	NRRL2999	N.D.	aflatoxigenic
	NRRL3145	N.D.	aflatoxigenic
	ATCC15517	N.D.	aflatoxigenic
Aspergillus niger		N.D.	

N.D.: Not detected.

result, the 4 strains were found to produce CA. When CA production by *A. flavus* and *A. parasiticus* was compared, all of the *A. flavus* strains produced CA while none of the *A. parasiticus* strains produced CA. Four strains of *A. oryzae* used for shoyu fermentation and 2 strains of *A. sojae* also used for shoyu fermentation did not produce CA. Three strains of *A. oryzae*, which showed comparatively high levels of production of CA, were used in order to determine the time course of production of CA using liquid media. These experiments showed that none of the strains produced detectable amounts of CA until 40 h after inoculation. Koji mold, which is used for the production of traditional fermented foods, only proliferates for about 40 h at most after inoculation. CA was not produced in detectable amounts until 40 h, even when strains with a high CA production ability were inoculated to the media. Most of the *A. oryzae* strains did not produce any detectable amount of CA. These results suggested that the possibility of CA contamination of Japanese fermented foods was extremely low, but that it is preferable not to use CA-producing strains for the production of foods. Therefore, these CA-producing strains were eliminated from commercial koji mold. Decomposition of CA was also examined[8]. When CA was spiked in shoyu before fermentation, about 70% of the amount of CA decreased in the first 40-day period. This decrease was induced mainly by yeast, especially *Zygosaccharomyces rouxii*, by decomposition or assimilation.

Katsuo-bushi

Katsuo-bushi (dried bonito) is a kind of traditional fermented food used for the seasoning of soup base. For the production of dried bonito, fungi belonging to the *A. glaucus* group have been used. *Aspergillus* strains are inoculated to boiled, smoked and half-dried bonito. The inoculation had been naturally performed in a special room where *Aspergillus* strains had been grown over a long period of time. Twenty-six samples of katsuo-bushi were collected from all of the producing areas of Japan. As most of the mold growing on katsuo-bushi belonged to *Aspergillus*, the possibility of contamination with aflatoxins, ochratoxin A and sterigmatocystin was examined[5]. The results showing the biological effects of the extracts from dried bonito are presented in Table 3. Toxins were not detected from any of the samples examined. Most of the isolated strains from katsuo-bushi belonged to the *A. glaucus* group, *A. ochraceus* group, and *A. versicolor* group. Twelve representative strains were selected and the production ability of mycotoxins such as aflatoxins, ochratoxin A and sterigmatocystin was examined. Eleven strains did not produce any of the

mycotoxins listed above. Only one strain of *A. flavus*, which seemed to be attached to the surface of katsuo-bushi, and was isolated from the washing solution of katsuo-bushi, produced aflatoxin B_1 at the level of 1.1 ppm on rice medium. Inoculation was also performed using 3 mycotoxin-producing strains. These 3 strains grew well on the katsuo-bushi at 28°C. From a sample of katsuo-bushi inoculated with *A. flavus* and cultured, 10 ppm of aflatoxin B_1 was detected, and from one sample inoculated with *A. ochraceus* and cultured, 5 ppm of ochratoxin A was detected. Sterigmatocystin was not detected from samples of katsuo-bushi inoculated and cultured with *A. versicolor*. However, strains with a stronger toxicity may produce sterigmatocystin, if they infect katsuo-bushi. Also, if the starter of katsuo-bushi becomes infected with mycotoxin-producing strains, mycotoxin contamination of katsuo-bushi may occur. Therefore, strains that do not produce mycotoxins were selected and have been used for katsuo-bushi fermentation.

As mentioned above, in the present study, several Japanese fermented foods were found to be free from contamination with mycotoxins such as aflatoxins, sterigmatocystin, kojic acid and cyclopiazonic acid.

Further studies should be carried out to ensure that other traditional Japanese fermented foods are also safe.

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Table 3. Chemical analysis of mycotoxins in katsuo-bushi (dried bonito) and biological test for the extracts from dried bonito

Sample No.	Source	Aflatoxins	Ochratoxin A	Sterigmatocystin	Chick embryo test[a]		Brine shrimp test[a]	
					24 h	48 h	24 h	48 h
1	Chikura, Chiba Prefect.	–	–	–	2/15	2/15	1/49	3/49
2		–	–	–	0/15	0/15	2/35	3/25
3		–	–	–	2/15	3/15	1/45	8/45
4	Nishiizu, Shizuoka Prefect.	–	–	–	0/14	2/14	1/48	5/48
5	Gozensaki, Shizuoka Prefect.	–	–	–	0/15	2/15	1/71	4/71
6	Yaidzu, Shizuoka Prefect.	–	–	–	1/15	3/15	0/55	3/55
7		–	–	–	0/15	0/15	2/38	4/38
8		–	–	–	0/15	0/15	0/61	2/61
9		–	–	–	2/15	3/15	1/42	2/42
10		–	–	–	2/15	2/15	0/37	2/37
11	Daioh, Mie Prefect.	–	–	–	2/13	2/13	0/39	4/39
12		–	–	–	0/15	1/15	1/48	3/48
13	Tosashimidzu, Kochi Prefect.	–	–	–	3/15	3/15	1/50	3/50
14		–	–	–	0/15	0/15	1/82	2/82
15		–	–	–	0/15	0/15	3/56	4/56
16		–	–	–	0/15	0/15	1/41	2/41
17		–	–	–	2/15	4/15	1/79	4/79
18	Yamakawa, Kagoshima Prefect.	–	–	–	0/15	0/15	2/39	3/39
19		–	–	–	2/15	3/15	2/29	5/29
20		–	–	–	2/14	2/14	1/36	5/36
21	Makurazaki, Kagoshima Prefect.	–	–	–	0/15	0/15	1/99	14/99
22		–	–	–	0/15	0/15	0/68	4/68
23		–	–	–	0/15	0/15	4/61	10/61
24		–	–	–	0/14	0/14	0/110	5/110
25		–	–	–	0/15	0/15	0/116	6/116
26	Miyako Island, Okinawa Prefect.	–	–	–	2/15	0/15	0/70	5/70

a): Denominator denotes the numbers of tested samples and numerator denotes the numbers of dead individuals.
–: No production of corresponding mycotoxin.

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