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（出國類別：研究）

乳牛瘤胃模擬技術

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內容摘要: 瘤胃微生物主導乳牛飼糧在瘤胃的消化，也影響瘤胃溫室氣體甲烷的產生，但其族群數量與組成受飼料養分影響，因此增加對瘤胃微生物的瞭解，是營養與環保研究的重要工作。本次研習以學習瘤胃微生物研究方法爲主題，包括人工瘤胃發酵系統Rusitec (Rumen Simulation Technique) 的設立與運轉、瘤胃微生物培養基培養方法與計數方法及瘤胃微生物的phylogenetic分類方法。Rusitec發酵系統模擬乳牛瘤胃，可以進行較大範圍的飼糧處理研究，做爲事先篩選的測試，縮短研究時程同時也顧及動物福利。瘤胃微生物培養與計數，應用L-10培養基進行試管厭氧培養，可以瞭解各類微生物的種類與數量，評估飼糧利用效率。瘤胃微生物的phylogenetic分類是近年發展的方法，以共通性及專一性的細菌16S rDNA序列做爲分類方法，未來應可做爲微生物定性與定量的方法，目前分類樹及資料庫都在逐步擴展中。

本文電子檔已上傳至出國報告資訊網

摘　　要

瘤胃微生物主導乳牛飼糧在瘤胃的消化，也影響瘤胃溫室氣體甲烷的產生，但其族群數量與組成受飼料養分的影響，因此增加對瘤胃微生物的瞭解，是營養與環保研究的重要工作。本次研習以學習瘤胃微生物研究方法為主題，包括人工瘤胃發酵系統 Rusitec (Rumen Simulation Technique) 的設立與運轉、瘤胃微生物培養基培養方法與計數方法及瘤胃微生物的 phylogenetic 分類方法三方面。Rusitec 發酵系統模擬乳牛瘤胃，可以進行較大範圍的飼糧處理研究，做為事先篩選的試驗測試，縮短研究時程同時也顧及動物福利。瘤胃微生物培養與計數，應用 L-10 培養基進行試管厭氧培養，可以瞭解各類微生物的種類與數量，評估飼糧利用效率。瘤胃微生物的 phylogenetic 分類是近年發展的方法，以共通性及專一性的細菌 16S rDNA 序列做為分類方法，跳脫實際培養基培養方法，未來應可做為微生物定性與定量的方法，目前分類樹及資料庫都在逐步擴展中。

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一、目的

瘤胃是乳牛的主要消化器官之一，牛隻藉由其中無數共生的微生物(約 16.2 - 40.8 x 10^9 個細菌/mL) 分解飼糧中的纖維與其它組成後，產生微生物菌體蛋白質及揮發性脂肪酸，分別提供為牛隻主要的蛋白質與能量來源，進一步提供了人類重要的營養來源乳製品，因此增加對瘤胃微生物的瞭解，是研究瘤胃消化代謝的第一步，也是提高乳牛生產效率的重要基礎。

微生物在瘤胃發酵的結果，同時也產生了大量的發酵氣體，其中約75%為二氧化碳，20%為甲烷。牛隻無法利用甲烷，其產生約造成牛隻6%採食能量的損失，而且甲烷也是主要的溫室氣體之一，估計全球甲烷造成 15%的溫室效應，牛隻瘤胃甲烷的產生約佔了農業源的 35%，因此瘤胃甲烷減量研究不單可以提高牛隻的生產效率，同時也降低乳業對溫室效應的影響程度。

從更寬廣的角度看瘤胃生態，瘤胃微生物的數量相當多，分泌相當豐富的消化酵素，因此應可以做為尋覓特殊功能微生物的可能來源，瘤胃深具生技製品發展的潛力。藉由生產目標確立、厭氧微生物培養、菌種篩選、選殖功能性基因、穩定基因表現與擴大產量，瘤胃微生物將可用於經濟性產品的開發，目前纖維分解菌的分解機制研究，已有相當的進展。

瘤胃的消化、甲烷以及微生物的研究課題相當多，若以乳牛活體來進行將相當困難且進度緩慢，也容易影響動物健康與福利，因此大動物試驗處理的篩選方法多以試管 (*in vitro*) 方法進行，最終才以活體試驗確認。在人工瘤胃發酵模式中，屬於歐洲系統研發的瘤胃模擬技術 (Rumen Simulation Technique, Rusitec) 是一種非常受肯定的重要方法，英國 Rowett 研究院與加拿大農業 Lethbridge 研究中心，即以 RUSITEC 發表相當多探討瘤胃甲烷生成機制與尋找抑制劑的研究報告，同時這個方法也廣泛的使用於瘤胃消化代謝與微生物方面的研究。

本次研習前往加拿大農業 Lethbridge 研究中心，學習整套 Rusitec 系統的設立、發酵流程操作、設備維護、發酵樣品收集與分析、瘤胃微生

物培養與分類等實驗技術，同時研習活體瘤胃微生物的培養、DNA 萃取、PCR 放大、定性與定量等分生技術，希望推動未來國內相關研究工作：

1. 探討飼糧在瘤胃內的消化利用，以提高產乳效率及生乳品質。
2. 探討降低瘤胃甲烷產量的方法，以減緩全球暖化速度。
3. 建立瘤胃微生物的研究方法，將瘤胃微生物視為一特殊功能微生物源，以開發有經濟效益的微生物、酵素與基因。

二、內容

(一) 人工瘤胃 Rusitec 系統

瘤胃模擬技術 (Rumen simulation technique, Rusitec) 是研究瘤胃消化的人工瘤胃發酵系統之一，由英國蘇格蘭 the Hannah Research Institute 研究人員 Dr. J. W. Czerkawski 及 Dr. G. Breckenridge 於 1977 年發表，目前廣泛的被歐洲、加拿大與日本等國家的研究室採用，做為瘤胃營養及甲烷等研究工作的模型。Rusitec 發酵系統著重瘤胃多室的觀念，它採用上下混合方式，在發酵槽內裝入帶有兩份樣品的小罐，樣品裝在尼龍袋中培養 48 hr，樣品培養時間間隔一日，每日更換一袋，使發酵槽內一直有不同分解階段的樣品，以模擬瘤胃狀況。

Rusitec 的運轉概述

1. 微生物來源：以瘤胃開窗牛提供瘤胃固態與液態微生物，牛隻飼糧固定。試驗首日取適量瘤胃內容物，稍捏去水分後每個尼龍袋秤取 60 g，封口放入發酵瓶內罐，做為固態微生物接種。首日小罐內另放入一個飼料樣品。
2. 39℃水浴槽。
3. 發酵瓶：八組，一公升容積，總發酵液 800 - 900 mL，包括 50%人工唾液及 50%經攪拌機攪拌過濾後的瘤胃內容物濾液。發酵槽上蓋有四孔，分別為中心軸、人工唾液輸入孔、發酵液溢出孔及採樣孔。以水浴槽內底座及壓板固定於水浴槽內。中心軸底端接容積約 300 mL 的內罐，以放寬處理飼料樣品。
4. 攪拌系統：連續每分鐘八次上下方向攪拌混合、距離約 8-10 cm。

5. 試驗飼糧：以孔隙度約 53 μ 及大小約 8 x 10 cm^2 的尼龍袋，內秤入 10 – 15 g 樣品 (餵飼基，Wiley mill 的 1 mm 磨細)，車縫封口後放置在發酵瓶內罐，隨中心軸上下移動與微生物混合。樣品厭氧發酵培養 48 小時後取出，罐內二袋，保持發酵時間間隔 24 h。
6. 人工唾液 (1949 McDougall's buffer)：包括 9.8 g $NaHCO_3$、9.3 g $Na_2HPO_4.12H_2O$、0.47 g NaCl、0.57 g KCl、0.04 g $CaCl_2$ (預先配製 4%的 $CaCl_2$，CO_2 充氣時才加入，每公升加 1 mL) 及 0.065 g $MgCl_2$。以 CO_2 充氣 30 min，使 pH 近 7.0，並視需要調整 pH，使發酵液 pH 保持在 6.5 – 7.0 之間。人工唾液除啟始提供為 50%發酵液之外，培養全程以 3.5%/h 速度由蠕動馬達送入發酵槽，提供所需礦物質。
7. 發酵產物收集系統：每天以血清瓶收集約 750 – 800 mL (0.5 – 0.55 mL/min 送液速度) 溢出液。由血清瓶接氣體袋收集發酵氣體，扣除溢出液量後為產氣量。
8. 厭氧環境：發酵全程除每日換料開啟發酵槽外，儘可能保持厭氧狀態。瘤胃內容物取樣、攪拌機攪拌 (blending)、過濾及分送等過程，以 CO_2 送氣且儘速完成。每日發酵瓶開啟換料時，以 CO_2 送氣至液面，保持厭氧環境。整個發酵系統隨時保持密閉。
9. 發酵期間：適應期 10 – 14 天，正式採樣期 10 – 14 天。
10. 可測定項目：

(1) 適應期：每日溢出量、溢出量 pH、發酵液 pH、發酵氣體量。
(2) 採樣期：溢出量、溢出量 pH、發酵液 pH、發酵氣體量與組成 (CO_2 與 CH_4)、發酵液中的微生物數量與種類、微生物的酵素分泌與活性、發酵液 24-hr 的 pH、氨態氮 (NH_3-N) 與揮發性脂肪酸 (VFA) 變化 飼料組成消化率、溢出液中的微生物合成量等。

(二) 瘤胃微生物培養與計數--- Hungate tube 方法

雖然瘤胃微生物主導瘤胃內飼料的消化代謝，但其族群數量與組成也受飼料養分提供的影響。一個良好的平衡飼糧需要促進最高的微生物合成量與合成效率，以製造充分的 VFA 與菌體蛋白質，做為牛隻主要能量與胺基酸來源，因此瘤胃微生物的培養與計數，成為營養研究的重要測定項目。瘤胃是一個厭氧環境，要培養其微生物需要在厭氧環境下進行，厭氧環境多以 CO_2 來達成，可以在 CO_2 充氣的厭氧手套操作箱內進行，

或者是局部 CO_2 充氣環境的 Roll tube 內進行，本節敘述以後者為之。

表 1 列出常用來培養瘤胃微生物的培養基配方。若要培養不同消化能力細菌時，可以選擇配方中的碳水化合物部分做調整，如要培養澱粉分解菌，則可以將配方中的碳水化合物總量全由澱粉提供。

表 1. L-10 培養基與配製方法

項　　目	配製 100 mL 時	配製 8,000 mL 時
Tryptone	0.2 g	16.0 g
Yeast Extract	0.05 g	4.0 g
Glucose	0.1 g	8.0 g
Maltose	0.1 g	8.0 g
Cellobiose	0.1 g	8.0 g
Sodium Lactate 70% 或 Lactic Acid 85%	0.15 mL 0.1 mL	12.0 mL 8.0 mL
Starch (or Xylan)	0.1 g	8.0 g
Dithiothreitol	0.01 g	0.8 g
Mineral Solution I.[1]	3.75 mL	300.0 mL
Mineral Solution II.[2]	3.75 mL	300.0 mL
Resazurin 0.1%	0.1 mL	8.0 mL
VFA Solution [3] w/ prop. and buty. 或 w/o prop. and buty.	0.65 mL 0.45 mL	52.0 mL 36.0 mL
Vitamin Solution [4]	0.5 mL	40.0 mL
$FeSO_4.7H_2O$ 0.545% [5]	0.5 mL	40.0 mL

Hemin 0.05% [6]	0.2 mL	16.0 mL
Vitamin K 0.2% in EtOH	0.1 mL	8.0 mL
加水到 或當以下兩項為液體時	100.0 mL 93.0 mL	8,000.0 mL 7,440.0 mL
以 5 N NaOH 調整 pH 至 6.8		
Na_2CO_3, g 或 8% Na_2CO_3, mL	0.4 g 5.0 mL	32.0 g 400.0 mL
在 CO_2 充氣下煮沸		
L-cysteine HCl, g 或 2.5% L-cysteine HCl, mL	0.05 g 2.0 mL	4.0 g 160.0 mL
在 CO_2 充氣下煮沸		
以上為液態 L-10 製備。若要製備培養皿，則加入 agar (1.2% w/v)；若要製備 roll tube，則加入 agar (1.8% w/v)		
冷卻 5 分鐘，再次以 CO_2 充氣 5 –10 分鐘，儘速分裝至血清瓶，在 CO_2 充氣後，以橡皮塞及鋁瓶蓋封夾瓶口；或儘速分裝 5 mL 至 roll tube，在 CO_2 充氣後鎖緊 butyl 橡皮塞及中空螺紋蓋		
121℃滅菌 20 分鐘。存放實驗室內備用		

[1] Mineral Solution I: K_2HPO_4 0.6% w/v.

[2] Mineral Solution II: KH_2PO_4 0.6% w/v, $(NH_4)_2SO_4$ 1.2% w/v, NaCl 1.2% w/v, $MgSO_4$ 0.141% w/v, $CaCl_2$ 0.12% w/v, $CoCl_2.6H_2O$ 0.0027% w/v, $MnCl_2.4H_2O$ 0.03% w/v.

[3] Volatile Fatty Acid Solution: Acetic acid 36.0 mL, Isobutyric Acid 1.8 mL, n-Valeric Acid 2.0 mL, DL-2-Methylbutyric Acid 2.0 mL, Isovaleric Acid 2.0 mL, Propionic Acid 11.0 mL, n-Butyric Acid 9.0 mL.

[4] Vitamin Solution: Thiamine HCl 200 mg, Ca-D-pantothenate 200 mg, Nicotinamide 200 mg, Riboflavin 200 mg, Pyridoxine HCl 200 mg, p-Aminobenzoate 10 mg, Biotin 5 mg, Folic Acid 5 mg, Vitamin B_{12} 0.2 mg, Thioctic Acid 5 mg. 以去離子水溶解成 500 mL 溶液，加入四滴 $CHCl_3$。

[5] $FeSO_4$ Solution 0.545%: 以去離子水溶解 $FeSO_4.7H_2O$ 2.725 g 近 500 mL 溶液，加入

15 滴濃 HCl 酸化，定量至 500 l。

[6] Hemin Solution 0.05%: 去離子水 125 mL 與 Ethanol (100%) 125 mL 混合，加入 2.5 g NaOH 溶解完成，加入 0.125 g Hemin。

Hungate tube 瘤胃微生物的厭氧培養與計數方法概述：

1. 在 CO_2 氣體輸出管路上，安裝去除氧氣及水分的 hungate column，加熱至 350℃ 以促進 CO_2 的還原。
2. 在通風櫥內，安裝 CO_2 氣體輸出管路末端數個出氣口，接上彎曲不銹鋼長針頭，使可掛在試管內，充氣 CO_2 造成厭氧小環境。
3. 計算試驗處理數、瘤胃內容物系列稀釋數量 ($1 - 10^{-8}$)、取三稀釋濃度、每濃度三重複 roll tube 等所需的 L-10 培養基量。配製並分裝至試管內，滅菌備用。
4. 採樣日準備 47℃ 水浴槽。Roll tubes 以 10 分鐘滅菌溶化 L-10 後放入，維持液化。內含 9 mL 0.1% pepton 溶液的稀釋用試管 39℃ 備用。
5. 自試驗處理的瘤胃開窗牛採取瘤胃內容物，經攪拌過濾後取得濾液做為微生物來源，在 CO_2 與 39℃ 熱水環境下送回實驗室，放置 39℃ 烘箱內備用。
6. 緩和搖勻第一頭牛的瘤胃濾液。先以 70 – 95%EtOH 在稀釋試管中蓋點火殺菌，以 1 mL 滅菌針筒取出 1 mL 濾液注入 9 mL pepton 試管中輕勻，得 10^{-1} 稀釋，重複 10 倍稀釋到約 10^{-8} 濃度。
7. 估計適當稀釋濃度，先以 70 – 95%EtOH 在 roll tube 中蓋點火殺菌，以 1 mL 滅菌針筒取出 0.5 – 1.0 mL 稀釋液注入 5 mL L-10 試管中輕勻，橫置入有冰塊堆積的 spinner 內搖動 15 – 25 秒鐘，使 L-10 均勻固化分佈在試管內壁上。舉例如要培養總菌數，取 10^{-6}、10^{-7} 與 10^{-8} 三種稀釋倍數，進行各三重複的接種；要培養纖維分解菌，取 10^{-3} 到 10^{-7} 五種稀釋倍數進行，纖維分解菌的 L-10 去除所有碳源及洋菜，以 1 x 3 cm^2 的濾紙為唯一碳源。

8. 在 39℃烘箱內培養兩天後計算總菌數、cellobiose 分解菌數、starch 分解菌數及 xylan 分解菌數，培養七天後計算 fungi 數、培養 14 天後計算纖維分解菌數。兩天與七天的菌數計數取其菌落數在 20 – 200 的培養管計算，可在低倍光學顯微鏡或者放大鏡下計算。纖維分解菌數計算採用 MPN (most probable number) 方法，可參考 AOAC (1998) 細菌分析手冊第八版敘述。
9. 依照菌落數、取量及稀釋倍數等，計算每 mL 瘤胃濾液的 CFU (colony forming unit) 數量。
10. 所有培養試管與培養基經完整滅菌後丟棄。

(三) 瘤胃微生物的 phylogenetic 分類

瘤胃微生物的種類與數量，是飼糧配方適當與否的重要指標。多數試驗以如上一節的培養方法，進行各種微生物的培養與計數，但常見的缺點是變異大。

近年來，有一群研究人員探討以 DNA sequencing 的分生方法，進行細菌種類與數量的分析，這個方法跳脫實際培養的工作，因為研究人員評估可以培養基培養方法養出的瘤胃微生物，不超過總數的 15%。目前這方面的研究報告中常見到細菌的分類樹狀圖，以其 DNA 序列的近似程度做遺傳分類，並已重新命名數種已知微生物的所屬，但 DNA 資料庫的建立仍然在初期階段，研究工作仍在逐步推動中，需要收集很多細菌種類、純化其 DNA、解讀其 DNA 序列、尋找個別細菌特殊的 DNA 序列、個別細菌特殊 DNA 序列的 primer 的設計等等。理論上，當此工作與資料庫完成後，將可以協助所有瘤胃試驗研究人員，很快的正確的瞭解其微生物的變化，更進一步若能將所有細菌特殊的 probe 放置在一個分類用 macroarray 上，則更可以省略大部分的分析與定序工作，但相對的經費付出也會增加。這是一個相當龐大的群體研究計畫，需要相當多的資金、儀器與人力的挹注。

細菌的 phylogenetic 分類的研究方法簡述於後：

1. 採集瘤胃內容物，分別以過濾方式取得液態內微生物，以 methylcellulose buffer 攪拌洗下貼附在固態的瘤胃微生物。
2. 以商業試劑組分離並純化瘤胃微生物 DNA。
3. 細菌屬於原核細胞 (Prokaryote)，細胞內有三種 rRNA，70S, 16S 及 5 S，因攜帶的遺傳資料數量較適中，因此研究工作多以 16S rRNA 為主。16S rRNA 上有一些片段是所有原核細胞都有的基因序列，稱為 conservative gene sequence，如 E. coli 的 400 – 1050 base。此段共通的 16S rRNA 有其對應的 16S rDNA 片段，因此研究人員設計適當的 universal primer (引子)，進行聚合酵素連鎖反應 (PCR) 放大所有細菌的此段 DNA 基因，因此 PCR 反應物總濃度測定後可以推算瘤胃內總細菌數量。
4. PCR 反應物經電泳分離後出現明確的 band，約在 1,500 鹼基位置。
5. 以試劑組純化膠片上的 band，可視為 insert，做為下一階段區別單一細菌的 DNA 來源。
6. 進行 random cloning，將這些 insert 插入載體 (vector)。
7. 將載體 transform 入 E. coli，進行培養，一個 E. coli 接受一個載體。
8. 以機器人將 E. coli 分散塗抹 (spread) 到大的培養盤 (約 30 x 30 cm^2)，以期得到單一菌落。
9. 以機器人感應個別單一菌落並送到個別 eppendorf 內培養。
10. 個別培養的單一菌液進行 DNA 分離、特殊 DNA 片段的 PCR 放大及 DNA 定序，與已有的細菌特殊 DNA 片段的資料庫比對，進行各種細菌的定性與定量。

三、心得

科學研究日新月異，研究人員需要隨時吸收各方面的資料來充實。Lethridge 研究中心是加拿大農業方面最大的研究單位，其涵括環境、永

續農業生產、食品安全與品質及生物技術生產等，工作人員近 600 人，因此其研究人力、設備與資源等都相當充分，只要有耐心與誠懇，就可以向各種領域的研究人員請益討論，並見到各種儀器設備與研究方法，是相當難能可貴的，這是此行很大的收獲。

四、建議

(一) 共同使用貴重儀器

如本次研習的 Real-time PCR、螢光濃度自動分析儀、機器人自動塗抹培養、機器人自動感應菌落與截取、自動 DNA 純化儀器等等，都十分昂貴，但該研究中心將這些儀器放置在公共實驗室，以利大家都能使用，是節省經費並提高研究水準的作法。

(二) 共通性實驗的專責管理

Lethbridge 研究中心有 Service Lab 的設置，如碳氮的分析即由專人負責，各實驗室完成樣品前處理後即可送至該實驗室，可以很迅速的完成分析，收取合理的費用，因此可以減少儀器的重複添購、污染的處理成本。另外一例如集中各實驗室的瘤胃微生物發酵系統，放置在同一間抽風設備良好的實驗室，以避免發酵氣體散佈在所有樓層，這也是不錯的觀念。

(三) 協助研究人員解決交通問題

建議加強對於出國研究人員的實質協助，如本次 Lethbridge 研究中心確實沒有公共交通工具可以到達，唯一的方法是租車，租車費加上更貴的保險費已超過日支費用的一半，但不能報銷，因此期望能對日後研究人員的狀況從寬認定，給與適當有彈性的協助。另外建議提醒研究人員在出國前要多瞭解當地習俗，如避免研究期間是當地的休假高峰期；同時先聯繫當地研究人員中的臺灣同鄉，以就近照顧。

五、附錄

(一) 1977 年 Dr. J. W. Czerkawski 及 Dr. G. Breckenridge 的 Rusitec 設計圖 (Design and development of a long-tern rumen simulation technique (Rusitec). British J. of Nutrition 38: 371-384)

Rumen simulation technique

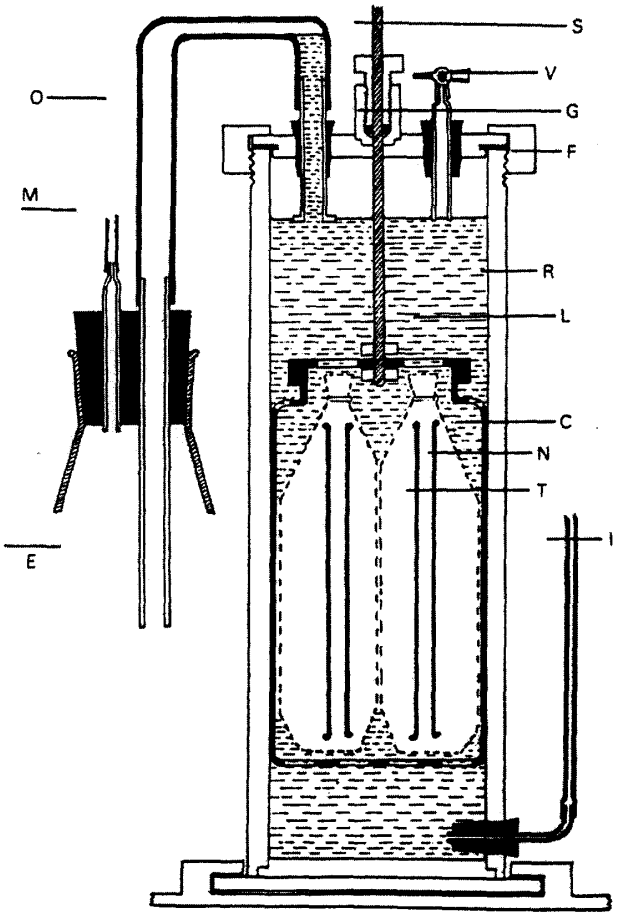
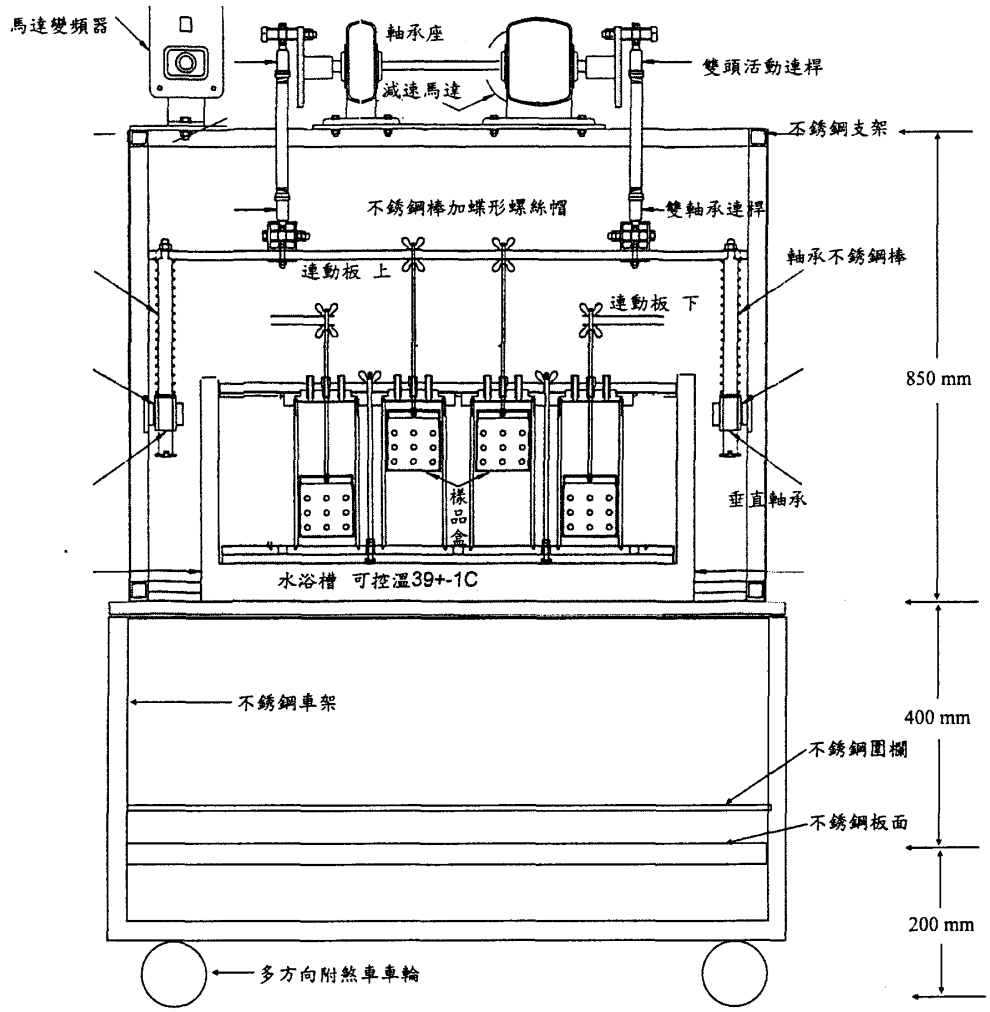


Fig. 1. Schematic diagram of one unit of the four-vessel long-term artificial rumen. (□), Made of perspex, (■), made of rubber or polyethylene. The driving shaft (S) was made of stainless steel. V, Sampling valve; G, gland (gas-tight); F, flange; R, main reaction vessel; L, rumen fluid; C, perforated food container; N, nylon gauze bag; T, rigid tube; I, inlet of artificial saliva; O, outlet through overflow; M, line to gas-collection bag; E, vessel for collection of effluent.

(二) 畜試所新竹分所依照加拿大農業 Lethbridge 研究中心資料所設計的 Rusitec 設計圖 (目前運作中)



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Appendix 2. Most Probable Number from Serial Dilutions

WALLACE E. GARTHRIGHT, Ph.D.

BACKGROUND

The most probable number (MPN) is particularly useful for low concentrations of organisms (<100/g), especially in milk and water, and for those foods whose particulate matter may interfere with accurate colony counts. The following background observations are adapted and extended from the article on MPN by James T. Peeler and Foster D. McClure in the Bacteriological Analytical Manual (BAM), 7th edition.

Only viable organisms are enumerated by the MPN determination. If, in the microbiologist's experience, the bacteria in the prepared sample in question can be found attached in chains that are not separated by the preparation and dilution, the MPN should be judged as an estimate of growth units (GUs) or colony-forming units (CFUs) instead of individual bacteria. For simplicity, however, this appendix will speak of these GUs or CFUs as individual bacteria.

The following assumptions are necessary to support the MPN method. The sample is prepared in such a way that the bacteria are distributed randomly within it. The bacteria are separate, not clustered together, and they do not repel each other. The growth medium and conditions of incubation have been chosen so that every inoculum that contains even one viable organism will produce detectable growth.

The essence of the MPN method is the dilution of a sample to such a degree that inocula will sometimes but not always contain viable organisms. The "outcome", i.e., the numbers of inocula producing growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions, incubating several tubes (or plates, etc.) at each dilution.

The first accurate estimation of the number of viable bacteria by the MPN method was published by McCrady (1915). Halvorson and Ziegler (1933), Eisenhart and Wilson (1943), and Cochran (1950) published articles on the statistical foundations of the MPN. Woodward (1957) recommended that MPN tables should omit those combinations of positive tubes (high for low concentrations and low for high concentrations) that are so improbable that they raise concerns about laboratory error or contamination. De Man (1983) published a confidence interval method that was modified to make the tables for this appendix.

CONFIDENCE INTERVALS

The 95 percent confidence intervals in the tables have the following meaning.

> Before the tubes are inoculated, the chance is at least 95 percent that the confidence interval associated with the eventual result will enclose the actual concentration.

It is possible to construct many different sets of intervals that satisfy this criterion. This manual uses a modification of the method of de Man (1983). De Man calculated his confidence limits iteratively from the smallest concentrations upward. Because this manual estimates concentrations of

pathogens, the intervals have been shifted slightly upward by iterating from the largest concentrations downward.

IMPROBABLE OUTCOMES

When excluding improbable outcomes, de Man's (1983) preferred degree of improbability was adopted. The included combinations of positive tubes are those that would be among the 99.985 percent most likely to result if their own MPNs were the actual bacterial concentrations. Therefore the entire set of results on any 10 different samples will be found in these tables at least 99 percent of the time.

PRECISION, BIAS, AND EXTREME RESULTS

The MPNs and confidence limits have been expressed to 2 significant digits. For example, the entry "400" has been rounded from a number between 395 and 405.

Numerous articles have noted a bias toward over-estimation of microbial concentrations by the MPN. Garthright (1993) has shown, however, that there is no appreciable bias when the concentrations are expressed as logarithms, the customary units used for regressions and for combining results. Therefore, these MPNs have not been adjusted for bias.

The tables below show the MPN for the (0, 0, 0) outcome as less than the MPN of the (1, 0, 0) outcome. (Note: although there are outcomes with lower MPNs [e.g., (0, 0, 1)], these are in no sense intermediates between the (1, 0, 0) and (0, 0, 0) outcomes.)

Since no particular density is indicated for an outcome of (0.0.0), a density must be assigned arbitrarily (and stated explicitly in the report) in order to calculate statistics. For the logarithm of the density, log[0.5*MPN(1,0,0)] is a reasonable choice. For statistics using the (non-logarithmic) density itself, calculate once with a density of 0.0 and once with a density of 0.5*MPN(1,0,0). Either report both statistics or report one statistic accompanied by a comment on the difference between that statistic and the other one.

SELECTING THREE DILUTIONS FOR TABLE REFERENCE

An MPN can be computed for any numbers of tubes at any numbers of dilutions. MPN values based on 3 decimal dilutions, however, are very close approximations to those based on 4 or more dilutions. When more than three dilutions are used in a decimal series of dilutions, refer to the 3 dilution table according to the following two cases, illustrated by the table of examples below (with 5 tubes at each dilution).

Case 1. One or more dilutions show all tubes positive. Select the highest dilution that gives positive results in all tubes (even if a lower dilution gives negative results) and the next two higher dilutions (ex. a and b); if positive results occur in higher unselected dilutions, shift each selection to the next higher dilution (ex. c). If there are still positive results in higher unselected dilutions, add those higher-dilution positive results to the results for the highest selected dilution (ex. d). If there were not enough higher dilutions tested to select three dilutions, then select the next lower dilutions (ex. e).

Case 2. No dilutions show all tubes positive. Select the 3 lowest dilutions (ex. f). If there are positive results in higher unselected dilutions, add those higher-dilution positive results to the results for the highest selected dilution (ex. g).

Example	1 g	0.1 g	0.01 g	0.001 g	0.0001 g	Combination of Positives	MPN/g
a	5	5	1	0	0	5-1-0	33
b	4	5	1	0	0	5-1-0	33
c	5	4	4	1	0	4-4-1	40
d	5	4	4	0	1	4-4-1	40
e	5	5	5	5	2	5-5-2	5400
f	0	0	1	0	0	0-0-1	0.20
g	4	4	1	1	0	4-4-2	4.7

Other compendia of methods require that no excluded lower dilutions may have any negative tubes. This manual differs when the highest dilution that makes all tubes positive follows a lower dilution that has one or more negative tubes. Example b above would be read according to other compendia as (4, 5, 1, 0, 0) with MPN 4.8/g. The BAM reading, 33/g, is 7 times larger. The BAM selection method is based on FDA experience that for some organisms in some food matrices such outcomes as (2, 5, 1, 0, 0) and (0, 3, 1, 0, 0) occur too often to be random occurrences. In these cases, it appears that some factor (a competing organism or adverse set of compounds) is present at the lowest dilutions in such concentrations that it can reduce the detection of the target microbes.

Until further research clarifies this situation, analysts should continue to exclude dilutions lower than the highest dilution with all tubes positive. The findings should, however, report the extent to which such lower, partially-negative dilutions have been excluded. Analysts working with materials with known limited complexity in research settings will want to use their professional judgement to read outcomes such as (4, 5, 1, 0, 0) as (4, 5, 1, 0, 0). They may also read outcomes such as (3, 5, 1, 0, 0) as too improbable to record, because they are not included in the tables.

CONVERSION OF UNITS

The tables below apply directly to inocula 0.1, 0.01, and 0.001 g. When different inocula are selected for table reference, multiply the MPN/g and confidence limits by whatever number is required to make the inocula match the table inocula. For example, if the inocula were 0.01, 0.001, and 0.0001 for 3 tubes each, multiplying by 10 would make these inocula match the table inocula. If the positive results from this 3 tube series were (3, 1, 0), one would multiply the Table 1 MPN/g estimate, 43/g, by 10 to arrive at 430/g.

APPROXIMATIONS FOR AN UNUSUAL SERIES OF DILUTIONS

The MPNs for a series of dilutions not addressed by any tables (e.g., resulting from accidental loss of some tubes) may be computed by iteration or may be estimated as follows. First, select the lowest dilution that doesn't have all positive results. Second, select the highest dilution with at least one positive result. Finally, select all the dilutions between them. Use only the selected dilutions in the following formula of Thomas (1942):

$$MPN/g \approx \frac{P}{(N*T)^{1/2}}$$

where $(\ )^{1/2}$ means square root,
P is the number of positive results,
T is the total grams of sample in the selected dilutions, and
N is the grams of sample in the selected negative tubes.

The following examples will illustrate the application of Thomas's formula. We assume that the dilutions are 1.0, 0.1, 0.01, 0.001, and 0.0001 g.

Example (1). Dilution results are (5/5, 10/10, 4/10, 2/10, 0/5). We use only(--,--, 4/10, 2/10,--); so T= 10*0.01 + 10*0.001 = 0.11. There are 6 negative tubes at 0.01 and 8 negative tubes at 0.001, so N = 6*0.01 + 8*0.001 = 0.068. There are 6 positive tubes, so

$$MPN/g \approx \frac{6}{(0.068 \times 0.11)^{1/2}} = \frac{6}{0.086} = 70/g.$$

Example (2). Dilution results are (5/5, 10/10, 10/10, 0/10, 0/5). We use only(--,--, 10/10, 0/10,--), so by Thomas's formula,

$$MPN/g \approx \frac{10}{(0.01 \times 0.11)^{1/2}} = \frac{10}{.0332} = 300/g.$$

These two approximated MPNs compare well with the MPNs for (10, 4, 2) and (10,10,0) (i.e., 70/g and 240/g, respectively).

Example (2) above is a special case for which an exact solution for the two selected dilutions can be calculated directly, as follows. When all the results at the highest dilutions are negative, all the results at the remaining dilutions are positive, and when V is an individual inoculum at the highest dilution with all positive tubes, then

$$MPN/g = (\frac{1}{V})\ [2.303\ \log_{10}(\frac{T}{N})]$$

where T and N are defined as for Thomas's formula. For the second example above, the third dilution is the highest with positive portions, so V = 0.01. The MPN for the third and fourth dilution would be exactly

$$MPN/g = (\frac{1}{0.01}) \times [2.303\ \log_{10}(\frac{0.11}{0.01})] = 240/g.$$

Approximate confidence limits can be calculated for the MPN by using the method of Cochran as follows. Let "a" denote the dilution ratio (e.g., a = 10 for the tables in this appendix). If there are n tubes per dilution, the standard error of $\log_{10}$(MPN) is

$$s.e. = c\ [\frac{(\log_{10}a)}{n}]^{1/2}$$

where c = 0.58 for dilution ratios of 10 or more and c = 0.55 for dilution ratios less than 10. If the dilutions used for the MPN determination are not all the same, Peeler et al. (1992) recommend using for n the harmonic mean number of tubes in the k dilutions:

$$n_H = \frac{k}{(1/n_1 + \ldots + 1/n_k)}$$

SPECIAL REQUIREMENTS AND TABLES INCLUDED

Requests for special computations and different designs will be honored as resources permit. Designs may be requested with more or less than 3 dilutions, uneven numbers of tubes, different confidence levels, etc. (Telephone or write the Division of Mathematics, FDA/CFSAN, 200 C St., SW, Washington, DC 20204.) The most-published designs, three 10-fold dilutions with 3, 5, or 10 tubes at each dilution, are presented here.

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Table 1. For 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Pos. tubes				Conf. lim.		Pos. tubes				Conf. lim.	
0.10	0.01	0.001	MPN/g	Low	High	0.10	0.01	0.001	MPN/g	Low	High
0	0	0	<3.6	--	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	--

Table 2. For 5 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf.lim.	
0.1	0.01	0.001		Low	High	0.1	0.01	0.001		Low	High
0	0	0	<2.0	--	6.8	3	3	2	24	9.8	70
0	0	1	1.8	0.09	6.8	3	4	0	21	6.8	40
0	1	0	1.8	0.09	6.9	3	4	1	24	9.8	70
0	1	1	3.6	0.7	10	3	5	0	25	9.8	70
0	2	0	3.7	0.7	10	4	0	0	13	4.1	35
0	2	1	5.5	1.8	15	4	0	1	17	5.9	36
0	3	0	5.6	1.8	15	4	0	2	21	6.8	40
1	0	0	2.0	0.1	10	4	0	3	25	9.8	70
1	0	1	4.0	0.7	10	4	1	0	17	6.0	40
1	0	2	6.0	1.8	15	4	1	1	21	6.8	42
1	1	0	4.0	0.7	12	4	1	2	26	9.8	70
1	1	1	6.1	1.8	15	4	1	3	31	10	70
1	1	2	8.1	3.4	22	4	2	0	22	6.8	50
1	2	0	6.1	1.8	15	4	2	1	26	9.8	70
1	2	1	8.2	3.4	22	4	2	2	32	10	70
1	3	0	8.3	3.4	22	4	2	3	38	14	100
1	3	1	10	3.5	22	4	3	0	27	9.9	70
1	4	0	11	3.5	22	4	3	1	33	10	70
2	0	0	4.5	0.79	15	4	3	2	39	14	100
2	0	1	6.8	1.8	15	4	4	0	34	14	100
2	0	2	9.1	3.4	22	4	4	1	40	14	100
2	1	0	6.8	1.8	17	4	4	2	47	15	120
2	1	1	9.2	3.4	22	4	5	0	41	14	100
2	1	2	12	4.1	26	4	5	1	48	15	120
2	2	0	9.3	3.4	22	5	0	0	23	6.80	70
2	2	1	12	4.1	26	5	0	1	31	10	70
2	2	2	14	5.9	36	5	0	2	43	14	100
2	3	0	12	4.1	26	5	0	3	58	22	150
2	3	1	14	5.9	36	5	1	0	33	10	100
2	4	0	15	5.9	36	5	1	1	46	14	120
3	0	0	7.8	2.1	22	5	1	2	63	22	150
3	0	1	11	3.5	23	5	1	3	84	34	220
3	0	2	13	5.6	35	5	2	0	49	15	150
3	1	0	11	3.5	26	5	2	1	70	22	170
3	1	1	14	5.6	36	5	2	2	94	34	230
3	1	2	17	6.0	36	5	2	3	120	36	250
3	2	0	14	5.7	36	5	2	4	150	58	400
3	2	1	17	6.8	40	5	3	0	79	22	220
3	2	2	20	6.8	40	5	3	1	110	34	250
3	3	0	17	6.8	40	5	3	2	140	52	400
3	3	1	21	6.8	40	5	3	3	180	70	400
5	3	4	210	70	400	5	5	0	240	70	710
5	4	0	130	36	400	5	5	1	350	100	1100
5	4	1	170	58	400	5	5	2	540	150	1700
5	4	2	220	70	440	5	5	3	920	220	2600
5	4	3	280	100	710	5	5	4	1600	400	4600
5	4	4	350	100	710	5	5	5	>1600	700.00	--
5	4	5	430	150	1,100						

Table 3. For 10 tubes at each of 1, 0.1, and 0.01 g inocula, the MPNs and 95 percent confidence intervals.

Pos. tubes				Conf.. lim.		Pos. tubes				Conf. lim.	
.1	.01	.001	MPN/g	Low	High	.1	.01	.001	MPN/g	Low	High
0	0	0	<.94	--	3.1	3	5	0	8.8	3.6	17
0	0	1	.90	.040	3.1	4	0	0	4.5	1.6	11
0	0	2	1.8	.33	5.1	4	0	1	5.6	2.2	12
0	1	0	.90	.040	3.6	4	0	2	6.8	3.0	14
0	1	1	1.8	.33	5.1	4	1	0	5.6	2.2	12
0	2	0	1.8	.33	5.1	4	1	1	6.8	3.0	14
0	2	1	2.7	.80	7.2	4	1	2	8.0	3.6	17
0	3	0	2.7	.80	7.2	4	2	0	6.8	3.0	15
1	0	0	.94	.050	5.1	4	2	1	8.0	3.6	17
1	0	1	1.9	.33	5.1	4	2	2	9.2	3.7	17
1	0	2	2.8	.80	7.2	4	3	0	8.1	3.6	17
1	1	0	1.9	.33	5.7	4	3	1	9.3	4.5	18
1	1	1	2.9	.80	7.2	4	3	2	10	5.0	20
1	1	2	3.8	1.4	9.0	4	4	0	9.3	4.5	18
1	2	0	2.9	.80	7.2	4	4	1	11	5.0	20
1	2	1	3.8	1.4	9.0	4	5	0	11	5.0	20
1	3	0	3.8	1.4	9.0	4	5	1	12	5.6	22
1	3	1	4.8	2.1	11	4	6	0	12	5.6	22
1	4	0	4.8	2.1	11	5	0	0	6.0	2.5	14
2	0	0	2.0	.37	7.2	5	0	1	7.2	3.1	15
2	0	1	3.0	.81	7.3	5	0	2	8.5	3.6	17
2	0	2	4.0	1.4	9.0	5	0	3	9.8	4.5	18
2	1	0	3.0	.82	7.8	5	1	0	7.3	3.1	15
2	1	1	4.0	1.4	9.0	5	1	1	8.5	3.6	17
2	1	2	5.0	2.1	11	5	1	2	9.8	4.5	18
2	2	0	4.0	1.4	9.1	5	1	3	11	5.0	21
2	2	1	5.0	2.1	11	5	2	0	8.6	3.6	17
2	2	2	6.1	3.0	14	5	2	1	9.9	4.5	18
2	3	0	5.1	2.1	11	5	2	2	11	5.0	21
2	3	1	6.1	3.0	14	5	3	0	10	4.5	18
2	4	0	6.1	3.0	14	5	3	1	11	5.0	21
2	4	1	7.2	3.1	15	5	3	2	13	5.6	23
2	5	0	7.2	3.1	15	5	4	0	11	5.0	21
3	0	0	3.2	.90	9.0	5	4	1	13	5.6	23
3	0	1	4.2	1.4	9.1	5	4	2	14	7.0	26
3	0	2	5.3	2.1	11	5	5	0	13	6.3	25
3	1	0	4.2	1.4	10	5	5	1	14	7.0	26
3	1	1	5.3	2.1	11	5	6	0	14	7.0	26
3	1	2	6.4	3.0	14	6	0	0	7.8	3.1	17

Pos. tubes				Conf.. lim.		Pos. tubes				Conf. lim.	
.1	.01	.001	MPN/g	Low	High	.1	.01	.001	MPN/g	Low	High
3	2	0	5.3	2.1	12	6	0	1	9.2	3.6	17
3	2	1	6.4	3.0	14	6	0	2	11	5.0	20
3	2	2	7.5	3.1	15	6	0	3	12	5.6	22
3	3	0	6.5	3.0	14	6	1	0	9.2	3.7	18
3	3	1	7.6	3.1	15	6	1	1	11	5.0	21
3	3	2	8.7	3.6	17	6	1	2	12	5.6	22
3	4	0	7.6	3.1	15	6	1	3	14	7.0	26
3	4	1	8.7	3.6	17	6	2	0	11	5.0	21
6	2	1	12	5.6	22	8	1	2	19	9.0	34
6	2	2	14	7.0	26	8	1	3	21	10	39
6	2	3	15	7.4	30	8	2	0	17	7.7	34
6	3	0	12	5.6	23	8	2	1	19	9.0	34
6	3	1	14	7.0	26	8	2	2	21	10	39
6	3	2	15	7.4	30	8	2	3	23	11	44
6	4	0	14	7.0	26	8	3	0	19	9.0	34
6	4	1	15	7.4	30	8	3	1	21	10	39
6	4	2	17	9.0	34	8	3	2	24	11	44
6	5	0	16	7.4	30	8	3	3	26	12	50
6	5	1	17	9.0	34	8	4	0	22	10	39
6	5	2	19	9.0	34	8	4	1	24	11	44
6	6	0	17	9.0	34	8	4	2	26	12	50
6	6	1	19	9.0	34	8	4	3	29	14	58
6	7	0	19	9.0	34	8	5	0	24	11	44
7	0	0	10	4.5	20	8	5	1	27	12	50
7	0	1	12	5.0	21	8	5	2	29	14	58
7	0	2	13	6.3	25	8	5	3	32	15	62
7	0	3	15	7.2	28	8	6	0	27	12	50
7	1	0	12	5.0	22	8	6	1	30	14	58
7	1	1	13	6.3	25	8	6	2	33	15	62
7	1	2	15	7.2	28	8	7	0	30	14	58
7	1	3	17	7.7	31	8	7	1	33	17	73
7	2	0	13	6.4	26	8	7	2	36	17	74
7	2	1	15	7.2	28	8	8	0	34	17	73
7	2	2	17	7.7	31	8	8	1	37	17	74
7	2	3	19	9.0	34	9	0	0	17	7.5	31
7	3	0	15	7.2	30	9	0	1	19	9.0	34
7	3	1	17	9.0	34	9	0	2	22	10	39
7	3	2	19	9.0	34	9	0	3	24	11	44
7	3	3	21	10	39	9	1	0	19	9.0	39
7	4	0	17	9.0	34	9	1	1	22	10	40

Pos. tubes				Conf.. lim.		Pos. tubes				Conf. lim.	
.1	.01	.001	MPN/g	Low	High	.1	.01	.001	MPN/g	Low	High
7	4	1	19	9.0	34	9	1	2	25	11	44
7	4	2	21	10	39	9	1	3	28	14	58
7	4	3	23	11	44	9	1	4	31	14	58
7	5	0	19	9.0	34	9	2	0	22	10	44
7	5	1	21	10	39	9	2	1	25	11	46
7	5	2	23	11	44	9	2	2	28	14	58
7	6	0	21	10	39	9	2	3	32	14	58
7	6	1	23	11	44	9	2	4	35	17	73
7	6	2	25	12	46	9	3	0	25	12	50
7	7	0	23	11	44	9	3	1	29	14	58
7	7	1	26	12	50	9	3	2	32	15	62
8	0	0	13	5.6	25	9	3	3	36	17	74
8	0	1	15	7.0	26	9	3	4	40	20	91
8	0	2	17	7.5	30	9	4	0	29	14	58
8	0	3	19	9.0	34	9	4	1	33	15	62
8	1	0	15	7.1	28	9	4	2	37	17	74
8	1	1	17	7.7	31	9	4	3	41	20	91
9	4	4	45	20	91	10	5	3	100	44	180
9	5	0	33	17	73	10	5	4	110	50	210
9	5	1	37	17	74	10	5	5	130	57	220
9	5	2	42	20	91	10	5	6	140	70	280
9	5	3	46	20	91	10	6	0	79	34	180
9	5	4	51	25	120	10	6	1	94	39	180
9	6	0	38	17	74	10	6	2	110	50	210
9	6	1	43	20	91	10	6	3	120	57	220
9	6	2	47	21	100	10	6	4	140	70	280
9	6	3	53	25	120	10	6	5	160	74	280
9	7	0	44	20	91	10	6	6	180	91	350
9	7	1	49	21	100	10	7	0	100	44	210
9	7	2	54	25	120	10	7	1	120	50	220
9	7	3	60	26	120	10	7	2	140	61	280
9	8	0	50	25	120	10	7	3	150	73	280
9	8	1	55	25	120	10	7	4	170	91	350
9	8	2	61	26	120	10	7	5	190	91	350
9	8	3	68	30	140	10	7	6	220	100	380
9	9	0	57	25	120	10	7	7	240	110	480
9	9	1	63	30	140	10	8	0	130	60	250
9	9	2	70	30	140	10	8	1	150	70	280
10	0	0	23	11	44	10	8	2	170	80	350
10	0	1	27	12	50	10	8	3	200	90	350

Pos. tubes				Conf.. lim.		Pos. tubes				Conf. lim.	
.1	.01	.001	MPN/g	Low	High	.1	.01	.001	MPN/g	Low	High
10	0	2	31	14	58	10	8	4	220	100	380
10	0	3	37	17	73	10	8	5	250	120	480
10	1	0	27	12	57	10	8	6	280	120	480
10	1	1	32	14	61	10	8	7	310	150	620
10	1	2	38	17	74	10	8	8	350	150	620
10	1	3	44	20	91	10	9	0	170	74	310
10	1	4	52	25	120	10	9	1	200	91	380
10	2	0	33	15	73	10	9	2	230	100	480
10	2	1	39	17	79	10	9	3	260	120	480
10	2	2	46	20	91	10	9	4	300	140	620
10	2	3	54	25	120	10	9	5	350	150	630
10	2	4	63	30	140	10	9	6	400	180	820
10	3	0	40	17	91	10	9	7	460	210	970
10	3	1	47	20	100	10	9	8	530	210	970
10	3	2	56	25	120	10	9	9	610	280	1300
10	3	3	66	30	140	10	10	0	240	110	480
10	3	4	77	34	150	10	10	1	290	120	620
10	3	5	89	39	180	10	10	2	350	150	820
10	4	0	49	21	120	10	10	3	430	180	970
10	4	1	59	25	120	10	10	4	540	210	1300
10	4	2	70	30	150	10	10	5	700	280	1500
10	4	3	82	38	180	10	10	6	920	350	1900
10	4	4	94	44	180	10	10	7	1200	480	2400
10	4	5	110	50	210	10	10	8	1600	620	3400
10	5	0	62	26	140	10	10	9	2300	810	5300
10	5	1	74	30	150	10	10	10	>2300	1300	--
10	5	2	87	38	180						

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SURVEY AND SUMMARY

Real-time PCR in virology

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ABSTRACT

The use of the polymerase chain reaction (PCR) in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory. Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. There are currently five main chemistries used for the detection of PCR product during real-time PCR. These are the DNA binding fluorophores, the 5′ endonuclease, adjacent linear and hairpin oligoprobes and the self-fluorescing amplicons, which are described in detail. We also discuss factors that have restricted the development of multiplex real-time PCR as well as the role of real-time PCR in quantitating nucleic acids. Both amplification hardware and the fluorogenic detection chemistries have evolved rapidly as the understanding of real-time PCR has developed and this review aims to update the scientist on the current state of the art. We describe the background, advantages and limitations of real-time PCR and we review the literature as it applies to virus detection in the routine and research laboratory in order to focus on one of the many areas in which the application of real-time PCR has provided significant methodological benefits and improved patient outcomes. However, the technology discussed has been applied to other areas of microbiology as well as studies of gene expression and genetic disease.

BACKGROUND

The polymerase chain reaction (PCR) (1,2) has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties, including virology. The method utilises a pair of synthetic oligonucleotides or primers, each hybridising to one strand of a double-stranded DNA (dsDNA) target, with the pair spanning a region that will be exponentially reproduced. The hybridised primer acts as a substrate for a DNA polymerase (most commonly derived from the thermophilic bacterium *Thermus aquaticus* and called *Taq*), which creates a complementary strand via sequential addition of deoxynucleotides. The process can be summarised in three steps: (i) dsDNA separation at temperatures >90°C, (ii) primer annealing at 50–75°C, and (iii) optimal extension at 72–78°C (Fig. 1A). The rate of temperature change or ramp rate, the length of the incubation at each temperature and the number of times each set of temperatures (or cycle) is repeated are controlled by a programmable thermal cycler. Current technologies have significantly shortened the ramp times using electronically controlled heating blocks or fan-forced heated air flows to moderate the reaction temperature. Consequently, PCR is displacing some of the gold standard cell culture, antigenaemia and serological assays (3). Existing combinations of PCR and detection assays (called 'conventional PCR' here) have been used to obtain quantitative data with promising results. However, these approaches have suffered from the laborious post-PCR handling steps required to evaluate the amplicon (4).

Traditional detection of amplified DNA relies upon electrophoresis of the nucleic acids in the presence of ethidium bromide and visual or densitometric analysis of the resulting bands after irradiation by ultraviolet light (5). Southern blot detection of amplicon using hybridisation with a labelled oligonucleotide probe is also time consuming and requires multiple PCR product handling steps, further risking a spread of amplicon throughout the laboratory (6). Alternatively, PCR–ELISA may be used to capture amplicon onto a solid phase using biotin or digoxigenin-labelled primers, oligonucleotide probes (oligoprobes) or directly after incorporation of the digoxigenin into the amplicon (7–12). Once captured, the amplicon can be detected using an enzyme-labelled avidin or anti-digoxigenin reporter molecule similar to a standard ELISA format.

The possibility that, in contrast to conventional assays, the detection of amplicon could be visualised as the amplification progressed was a welcome one (13). This approach has provided a great deal of insight into the kinetics of the reaction

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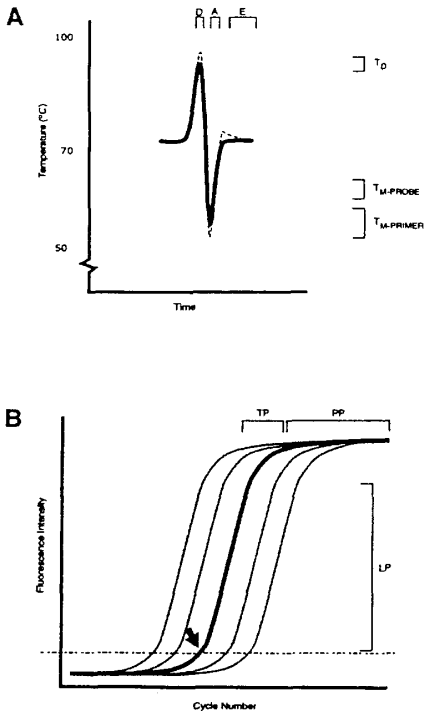

Figure 1. Kinetic amplification. (**A**) An idealised plot of temperature versus time during a single PCR cycle comprised of the denaturation (D), primer and probe annealing (A) and primer extension (E) steps. At the indicated optimal temperature ranges, dsDNA is denatured (T_D), oligoprobes anneal ($T_{M\text{-}PROBE}$) and finally the primers anneal as a precursor to their extension ($T_{M\text{-}PRIMER}$). The actual temperature, shown as a dashed line, may overshoot the desired temperature to varying degrees, depending on the quality of the thermocycler employed. (**B**) The ideal amplification curve of a real-time PCR (bold), when plotted as fluorescence intensity against the cycle number, is a typical sigmoidal growth curve. Early amplification cannot be viewed because the detection signal is indistinguishable from the background. However, when enough amplicon is present, the assay's exponential progress can be monitored as the rate of amplification enters a log-linear phase (LP). Under ideal conditions, the amount of amplicon increases at a rate of approximately one $\log_{10}$ every three cycles. As primers and enzyme become limiting and products inhibitory to the PCR accumulate, the reaction slows, entering a transition phase (TP), eventually reaching the plateau phase (PP) where there is little or no further increase in product yield. The point at which the fluorescence passes from insignificant levels to clearly detectable is called the threshold cycle (C_T; indicated by an arrow), and this value is used in the calculation of template quantity during quantitative real-time PCR. Also shown are curves representing an optimal titration of template (grey), consisting of higher and lower starting template concentrations, which produce lower or higher C_T values, respectively. Data for the construction of a standard curve are taken from the LP, which subsequently allows the concentration of unknown samples to be determined.

and it is the foundation of kinetic or 'real-time' PCR (Fig. 1B) (6,14–17). Real-time PCR has already proven itself valuable in laboratories around the globe, building on the enormous amount of data generated by conventional PCR assays.

The monitoring of accumulating amplicon in real time has been made possible by the labelling of primers, probes or amplicon with fluorogenic molecules, This chemistry has clear benefits over radiogenic oligoprobes that include an avoidance of radioactive emissions, ease of disposal and an extended shelf life (18).

The increased speed of real-time PCR is largely due to reduced cycle times, removal of post-PCR detection procedures and the use of fluorogenic labels and sensitive methods of detecting their emissions (19,20). The reduction in amplicon size generally recommended by the creators of commercial real-time assays may also play a role in this speed, however we have shown that decreased product size does not necessarily improve PCR efficiency (21).

The disadvantages of using real-time PCR in comparison with conventional PCR include the inability to monitor amplicon size without opening the system, the incompatibility of some platforms with some fluorogenic chemistries, and the relatively restricted multiplex capabilities of current applications. Also, the start-up expense of real-time PCR may be prohibitive when used in low-throughput laboratories. These shortcomings are mostly due to limitations in the system hardware or the available fluorogenic dyes or 'fluorophores', both of which will be discussed in more detail.

Because most of the popular real-time PCR chemistries depend upon the hybridisation of an oligoprobe to its complementary sequence on one of the strands of the amplicon, the use of more of the primer that creates this strand is beneficial to the generation of an increased fluorescent signal (22). Asymmetric PCR, as this is known, has been shown to produce improved fluorescence from a hairpin oligoprobe PCR (23) and we have found it directly applicable to other oligoprobe-hybridisation assays.

The most commonly used fluorogenic oligoprobes rely upon fluorescence resonance energy transfer (FRET; Fig. 2) between fluorogenic labels or between one fluorophore and a dark or 'black-hole' non-fluorescent quencher (NFQ), which disperses energy as heat rather than fluorescence. FRET is a spectroscopic process by which energy is passed between molecules separated by 10–100 Å that have overlapping emission and absorption spectra (24,25). Förster primarily developed the theory behind this process: the mechanism is a non-radiative induced-dipole interaction (26). The efficiency of energy transfer is proportional to the inverse sixth power of the distance (R) between the donor and acceptor ($1/R^6$) fluorophores (27,28).

Post-amplification manipulation of the amplicon is not required for real-time PCR, therefore these assays are described as 'closed' or homogeneous systems. The advantages of homogeneous systems include a reduced result turnaround, minimisation of the potential for carry-over contamination and the ability to closely scrutinise the assay's performance (29). In addition, real-time PCR has proven cost effective when implemented in a high throughput laboratory (30), particularly when replacing conventional, culture-based approaches to virus detection.

In the remainder of this article, the theory behind real-time PCR will be reviewed and its rapidly increasing use in the fields of human virology will be used as an illustration.

AMPLICON DETECTION

In the following section we will focus on the detection processes that discriminate real-time PCR from conventional

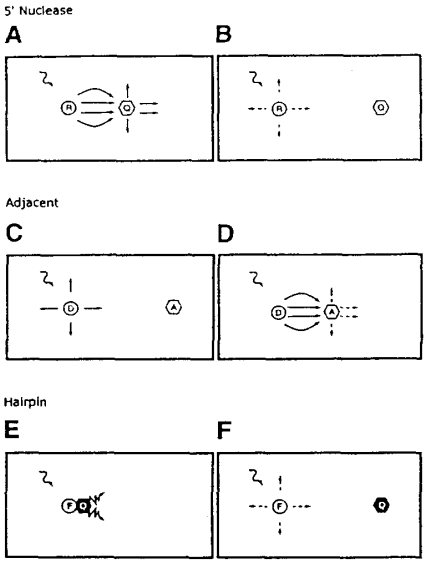

Figure 2. Fluorogenic mechanisms. When a 5′ nuclease probe's reporter (R) and quencher (Q, open) are in close proximity as in (**A**), the quencher 'hijacks' the emissions that have resulted from excitation of the reporter by the instrument's light source. The quencher then emits this energy (solid arrows). When the fluorophores are separated beyond a certain distance, as occurs upon hydrolysis as depicted in (**B**), the quencher no longer exerts any influence and the reporter emits at a distinctive wavelength (dashed arrows) which is recorded by the instrument. In the reverse process as depicted in (**C**) using adjacent oligoprobes, the fluorophores begin as separated entities. A signal from the acceptor (A) can only be generated when the donor (D) comes into close proximity as shown in (**D**). This occurs as the result of adjacent hybridisation of the oligoprobes to the target amplicon. In (**E**) another form of quenching is shown, caused by the intimate contact of labels attached to hairpin oligoprobes (molecular beacon, sunrise or scorpion). The fluorophore (F) and a NFQ (Q, closed) interact more by collision than FRET, disrupting each other's electronic structure and directly passing on the excitation energy which is dissipated as heat (jagged, dashed arrows). When the labels are separated by disruption of the hairpin, as is the case in (**F**), the fluorophore is free to fluoresce (dashed arrows).

PCR assays. There are five major chemistries currently in use, and they can be classified into amplicon sequence specific or non-specific methods of real-time PCR detection (31). Each of the chemistries has an associated nomenclature to describe the fluorescent labels; however, for general discussion, fluorophore will continue to be used to describe these moieties. Although this review focuses on the use of these chemistries in real-time applications, they can also be used as a label for end-point amplicon detection.

DNA-binding fluorophores

The basis of the sequence non-specific detection methods is the DNA-binding fluorogenic molecule. Included in this group are the earliest and simplest approaches to real-time PCR. Ethidium bromide (32), YO-PRO-1 (33,34) and SYBR® green I (35) all fluoresce when associated with dsDNA which is exposed to a suitable wavelength of light. This approach requires less specialist knowledge than the design of fluorogenic oligoprobes, is less expensive and does not suffer when the template sequence varies, which may abrogate hybridisation of an oligoprobe (36). Formation of primer-dimer (37) is common and, together with the formation of specific products, is strongly associated with entry of the PCR into the plateau phase (Fig. 1B) (38,39). Association of a DNA-binding fluorophore with primer-dimer or other non-specific amplification products can confuse interpretation of the results. Adding a short, higher temperature incubation after the extension step in which fluorescence data are acquired minimises the contribution of these products to the fluorescence signal (40). The problem of primer-dimer can also be addressed using software capable of fluorescent melting curve analysis. This method makes use of the temperature at which the dsDNA amplicon is denatured (T_D; Fig. 1A). The shorter primer-dimer can be discriminated by its reduced T_D compared with the full-length amplicon. Analysis of the melting curves of amplicon in the presence of SYBR green I has demonstrated that the practical sensitivity of DNA-binding fluorophores is limited by non-specific amplification at low initial template concentrations.

DNA binding fluorophores also increase the T_D and broaden the melting transition, requiring substantial sequence change to produce a shift in the T_D. Oligoprobes are able to discriminate single point mutations using the temperature at which 50% of oligoprobe-target duplexes separate (41). This temperature is called the melting temperature (T_M) and it is dependent upon the concentration of the dsDNA, its length, nucleotide sequence and the solvent composition, and is often confused with T_D (Fig. 1A) (42).

Linear oligoprobes

The use of a pair of adjacent, fluorogenic hybridisation oligoprobes was first described in the late 1980s (43,44) and, now known as 'HybProbes', they have become the method of choice for the LightCycler™ (Roche Molecular Biochemicals, Germany), a capillary-based, microvolume fluorimeter and thermocycler with rapid temperature control (20,45). The upstream oligoprobe is labelled with a 3′ donor fluorophore (FITC) and the downstream probe is commonly labelled with either a LightCycler Red 640 or Red 705 acceptor fluorophore at the 5′ terminus so that when both oligoprobes are hybridised, the two fluorophores are located within 10 nt of each other, sometimes attracting the name 'kissing' probes (Figs 2C, D and 3C). The plastic and glass composite capillaries are optically clear and act as cuvettes for fluorescence analysis, as well as facilitating rapid heat transfer. Capillaries are rotated past a blue light-emitting diode and fluorescence is monitored by three photodetection diodes with different wavelength filters. The temperature is varied by rapidly heating and cooling air using a heating element and fan which produce ramp rates of 20°C/s, prolonging polymerase survival (46). Additionally, because the oligoprobes are not significantly hydrolysed during amplification (47) and the LightCycler is able to monitor the changes in fluorescence emission during denaturation of the adjacent oligoprobes from their amplicon, this system can perform single tube genotyping. This capability, which makes use of fluorescent melting curve analysis, provides significant information about the sequence to which the oligoprobes are binding. Mutation(s) under one or both oligoprobes can be determined by the decrease in melting

p.29

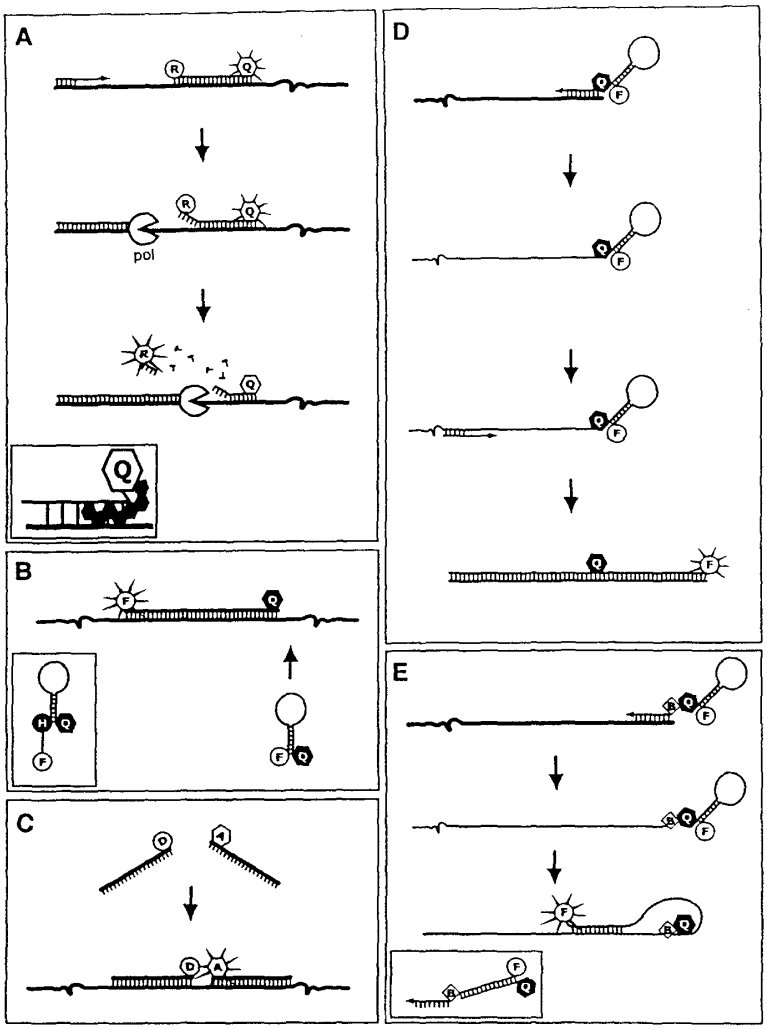

Figure 3. Oligoprobe chemistries. (**A**) 5′ Nuclease oligoprobes. As the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyses the oligoprobe via its 5′→3′ endonuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q, open), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher. Inset shows the NFQ and MGB molecule that make up the improved MGB nuclease-oligoprobes. (**B**) Hairpin oligoprobes. Hybridisation of the oligoprobe to the target separates the fluorophore (F) and non-fluorescent quencher (Q, closed) sufficiently to allow emission from the excited fluorophore, which is monitored. Inset shows a wavelength-shifting hairpin oligoprobe incorporating a harvester molecule. (**C**) Adjacent oligoprobes. Adjacent hybridisation results in a FRET signal due to interaction between the donor (D) and acceptor (A) fluorophores. This bimolecular system acquires its data from the acceptor's emissions in an opposite manner to the function of nuclease oligoprobe chemistry. (**D**) Sunrise primers. The opposite strand is duplicated so that the primer's hairpin structure can be disrupted. This separates the labels, eliminating the quenching in a similar manner to the hairpin oligoprobe. (**E**) Scorpion primers. The primer does not require extension of the complementary strand; in fact it blocks extension to ensure that the hairpin in the probe is only disrupted by specific hybridisation with a complementary sequence designed to occur downstream of its own, nascent strand. Inset shows a duplex scorpion that exchanges the stem–loop structure for a primer element terminally labelled with the fluorophore and a separate complementary oligonucleotide labelled with a quencher at the 5′ terminus.

temperature that they incur due to destabilisation of the oligoprobe/target duplex (Fig. 4). This has imparted significant improvements in speed upon the diagnosis of genetic disease as well as a growing number of multiplex PCR approaches for the detection of related viral pathogens. Despite the fact that the hybridisation does not reach equilibrium using these ramp rates, the apparent T_M values are both reproducible and characteristic of a given probe/target duplex (48). However,

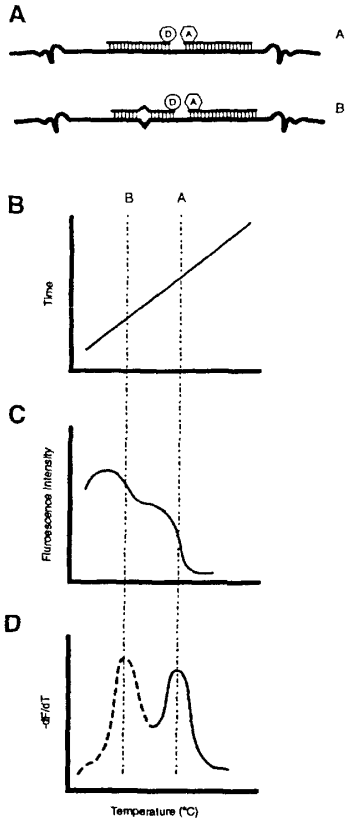

Figure 4. Fluorescent melting curve analysis. At the completion of a real-time PCR using a pair of adjacent oligoprobes, the reaction can be cooled to a temperature below the expected T_M of the oligoprobes then heated above 85°C at a fraction of a degree per second (**B**). During heating, the emissions of the acceptor can be constantly acquired (**C**). Software calculates the negative derivative of the fluorescence over time, producing clear peaks that indicate the T_M of the oligoprobe-target melting transition (**D**). When one or more mutations are present under one or both oligoprobes (**A**), this T_M is shifted and this shift can be used diagnostically to discriminate single nucleotide polymorphisms in the template. Ideally, one of the oligoprobes, the anchor, is designed to bind to a stable sequence region, whereas the other, sensor, will span the mismatch. Mismatches near the centre of the probe and flanked by G:C pairs are more destabilising than mismatches near the ends of the oligoprobe flanked by A:T pairs.

the capillaries are fragile and require some experience to handle (49).

When comparing signals from the different chemistries, the destruction of nuclease oligoprobes continues despite a plateau in product accumulation whereas SYBR green I fluorescence in the no template control generally increases non-specifically during later cycles. Adjacent oligoprobe fluorescence begins to decrease as the rate of collision between the growing numbers of complementary amplicon strands increases favouring the formation of dsDNA over the hybridisation of oligoprobe to its target DNA strand. Additionally, there is the possibility that some oligoprobe is consumed by sequence-related endonuclease activity (50,51). All three oligoprobe chemistries (SYBR Green I, nuclease and adjacent oligoprobes) seem capable of detecting amplified product with approximately the same sensitivity (20).

Combinations of the above approaches are now appearing as more users of the instrumentation become familiar with the concepts behind real-time PCR and contribute to the literature. If a sequence-specific, fluorophore-labelled linear oligoprobe is added to a SYBR green I mix, currently called the Bi-probe system, FRET will occur and an additional layer of specificity can be obtained (44,52,53). An assay using a BODIPY®FL-labelled oligoprobe was adapted to run in the LightCycler using a β-globin target sequence (54). The probe was designed so that the fluorophore was located on a terminal cytosine and was quenched by proximity with a complementary guanine. The assay demonstrated that quenching varies linearly with the concentration of template across a defined concentration range. The commonly used fluorophore FITC is inherently quenched by deoxyguanosine nucleotides. The level of quenching can be increased if more guanines are present or a single guanine is located in the first overhang position, 1 nt beyond the fluorophore-labelled terminus of the probe. This approach to amplicon detection is easier to design than fluorogenic oligoprobes, simpler to synthesise and use in real-time PCR and does not require a DNA polymerase with nuclease activity (55).

The light-up probe is a peptide nucleic acid to which the asymmetric cyanine fluorophore thiazole orange is attached (56). When hybridised with a nucleic acid target, either as a duplex or triplex, depending on the oligoprobe's sequence, the fluorophore becomes strongly fluorescent. These probes do not interfere with the PCR, do not require conformational change, are sensitive to single nucleotide mismatches allowing fluorescence melting analysis, and because a single reporter is used, a direct measurement of fluorescence can be made instead of the measurement of a change in fluorescence between two fluorophores (56,57). However, non-specific fluorescence has been reported during later cycles using these probes (58).

5′ Nuclease oligoprobes

In the late 1980s homogeneous assays were few and far between, but rapid advances in thermocycler instrumentation and the chemistry of nucleic acid manipulation have since made these assays commonplace. The success of these assays revolves around a signal changing in some rapid and measurable way upon hybridisation of a probe to its target (59). By using an excess, the time required for hybridisation of an oligoprobe to its target, especially when the amount of that target has been increased by PCR or some other amplifying process, is significantly reduced (41,59).

In 1991, Holland *et al.* (6) described a technique that was to form the foundation for homogeneous PCR using fluorogenic oligoprobes. Amplicon was detected by monitoring the effect of *Taq* DNA polymerase's 5′→3′ endonuclease activity on specific oligoprobe/target DNA duplexes. The radiolabelled products were examined using thin layer chromatography and the presence or absence of hydrolysis was used as an indicator of duplex formation. These oligoprobes contained a 3′ phosphate moiety, which blocked their extension by the

polymerase, but otherwise had no affect on the amplicon's yield.

The desirable criteria for an oligoprobe label are (i) easy attachment of the label to DNA, (ii) detectability at low concentrations, (iii) detectability using simple instrumentation, (iv) production of an altered signal upon specific hybridisation, (v) biological safety, (vi) stability at elevated temperatures and (vii) an absence of interference with the activity of the polymerase (6,18).

An innovative approach used nick-translation PCR in combination with dual-fluorophore labelled oligoprobes (14). In the first truly homogenous assay of its kind, one fluorophore was added to the 5′ terminus and one to the middle of a sequence specific oligonucleotide probe. When in such close proximity, the 5′ reporter fluorophore (6-carboxy-fluoroscein) transferred laser-induced excitation energy by FRET to the 3′ quencher fluorophore (6-carboxy-tetramethyl-rhodamine; TAMRA), which reduced the lifetime of the reporter's excited state by taking its excess energy and emitting it as a fluorescent signal of its own (Fig. 2A and B). TAMRA emitted the new energy at a wavelength that was monitored but not utilised in the presentation of data. However, when the oligoprobe hybridised to its template, the fluorophores were released due to hydrolysis of the oligoprobe component of the probe/target duplex. Once the labels were separated, the reporter's emissions were no longer quenched and the instrument monitored the resulting fluorescence. These oligoprobes have been called 5′ nuclease, hydrolysis or TaqMan® oligoprobes (Fig. 3A). Nuclease oligoprobes have design requirements that are applicable to the other linear oligoprobe chemistries, including (i) a length of 20–40 nt, (ii) a GC content of 40–60%, (iii) no runs of a single nucleotide, particularly G, (iv) no repeated sequence motifs, (v) an absence of hybridisation or overlap with the forward or reverse primers and (vi) a T_M at least 5°C higher than that of the primers, to ensure the oligoprobe has bound to the template before extension of the primers can occur (60).

This technology, however, required the development of a platform to excite and detect fluorescence as well as perform thermal cycling. A charge-coupled device had been described in 1992 for the quantification of conventional reverse transcription (RT)–PCR products (61). In 1993 this approach was combined with a thermal cycler resulting in the first real-time PCR fluorescence excitation and detection platform (29). To date, the ABI Prism® 7700 sequence detection system (Perkin Elmer Corporation/Applied Biosystems, USA) has been the main instrument used for 5′ nuclease oligoprobes. Non-PCR related fluorescence fluctuations have been normalised using a non-participating or 'passive' internal reference fluorophore (6-carboxy-*N,N,N′,N′*-tetramethylrhodamine; ROX). The corrected values, obtained from a ratio of the emission intensity of the reporter signal and ROX, are called RQ^+. To further control amplification fluctuations, the fluorescence from a 'no-template' control reaction (RQ^-) is subtracted from RQ^+ resulting in the ΔRQ value that indicates the magnitude of the signal generated for the given PCR (62).

The fractional cycle number at which the real-time fluorescence signal mirrors progression of the reaction above the background noise was used as an indicator of successful target amplification (63). This threshold cycle (C_T) is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating amplicon exceeds 10 standard deviations of the mean baseline fluorescence, using data taken from cycles 3 to 15 (Fig. 1B) (64). The C_T is proportional to the number of target copies present in the sample (17).

A recent improvement to the nuclease oligoprobe has resulted in the minor groove binding (MGB) oligoprobes (Fig. 3A, inset). This chemistry replaces the standard TAMRA quencher with an NFQ and incorporates a molecule that stabilises the oligoprobe-target duplex by folding into the minor groove of the dsDNA (65). This allows the use of very short (14 nt) oligoprobes, which are ideal for detecting single nucleotide polymorphisms (SNPs). A related use of dual labelled oligonucleotide sequences has been to provide the signal-generating portion of the DzyNA–PCR system (66). Here, the reporter and quencher are separated after cleavage of the probe by a DNAzyme, which is created during PCR as the complement of an antisense DNAzyme sequence included in the 5′ tail of one of the primers. Upon cleavage, the dual labelled substrate releases the fluorophores and generates a signal in an analogous manner to the 5′ nuclease probe.

Hairpin oligoprobes

Molecular beacons were the first hairpin oligoprobes and are a variation of the dual-labelled nuclease oligoprobe (Fig. 3B). The hairpin oligoprobe's fluorogenic labels are called fluorophore and quencher, and they are positioned at the termini of the oligoprobe. The labels are held in close proximity by distal stem regions of homologous base pairing deliberately designed to create a hairpin structure which results in quenching either by FRET or a direct energy transfer by a collisional mechanism due to the intimate proximity of the labels (Fig. 2E and F) (67). In the presence of a complementary sequence, designed to occur within the bounds of the primer binding sites, the oligoprobe will hybridise, shifting into an open configuration. The fluorophore is now spatially removed from the quencher's influence and fluorescent emissions are monitored during each cycle (68). The occurrence of a mismatch between a hairpin oligoprobe and its target has a greater destabilising effect on the duplex than the introduction of an equivalent mismatch between the target and a linear oligoprobe. This is because the hairpin structure provides a highly stable alternate conformation. Therefore, hairpin oligoprobes have been shown to be more specific than the more common linear oligoprobes making them ideal candidates for detecting SNPs (67). The quencher, 4-(4′-dimethylamino-phenylazo)-benzene (DABCYL), differs from that described for the nuclease oligoprobes because it is an NFQ.

The wavelength-shifting hairpin probe is a recent improvement to this chemistry which makes use of a second, harvesting fluorophore. The harvester passes excitation energy acquired from a blue light source and releases it as fluorescent energy in the far-red wavelengths. The energy can then be used by a receptive 'emitter' fluorophore that produces light at characteristic wavelengths (Fig. 3B, inset). This offers the potential for improved multiplex real-time PCR and SNP analysis (Fig. 4), using currently available instruments (69). Because the function of these oligoprobes depends upon correct hybridisation of the stem, accurate design is crucial to their function (47).

Self-fluorescing amplicon

The self-priming amplicon is similar in concept to the hairpin oligoprobe, except that the label becomes irreversibly incorporated into the PCR product (Fig. 3D and E). Two approaches have been described: sunrise primers (now commercially called Amplifluor™ hairpin primers) and scorpion primers (31,70). The sunrise primer consists of a 5′ fluorophore and a DABCYL NFQ. The labels are separated by complementary stretches of sequence that create a stem when the sunrise primer is closed. At the 3′ terminus is a target-specific primer sequence. The sunrise primer's sequence is intended to be duplicated by the nascent complementary strand and, in this way, the stem is destabilised, the two fluorophores are held ~20 nt (70 Å) apart and the fluorophore is free to emit its excitation energy for monitoring (70). This system could suffer from non-specific fluorescence due to duplication of the sunrise primer sequence during the formation of primer-dimer.

The scorpion primer is almost identical in design except for an adjacent hexethylene glycol molecule that blocks duplication of the signalling portion of the scorpion. In addition to the difference in structure, the function of scorpion primers differs slightly in that the 5′ region of the oligonucleotide is designed to hybridise to a complementary region within the amplicon. This hybridisation forces the labels apart disrupting the hairpin and permitting emission in the same way as hairpin probes (31).

VIRAL QUANTITATION

The majority of diagnostic PCR assays reported to date have been used in a qualitative, or 'yes/no' format. The development of real-time PCR has brought true quantitation of target nucleic acids out of the pure research laboratory and into the diagnostic laboratory.

Determining the amount of template by PCR can be performed in two ways: as relative quantitation and as absolute quantitation. Relative quantitation describes changes in the amount of a target sequence compared with its level in a related matrix. Absolute quantitation states the exact number of nucleic acid targets present in the sample in relation to a specific unit (71). Generally, relative quantitation provides sufficient information and is simpler to develop. However, when monitoring the progress of an infection, absolute quantitation is useful in order to express the results in units that are common to both scientists and clinicians and across different platforms. Absolute quantitation may also be necessary when there is a lack of sequential specimens to demonstrate changes in virus levels, no suitably standardised reference reagent or when the viral load is used to differentiate active versus persistent infection.

A very accurate approach to absolute quantitation by PCR is the use of competitive co-amplification of an internal control nucleic acid of known concentration and a wild-type target nucleic acid of unknown concentration, with the former designed or chosen to amplify with an equal efficiency to the latter (72–76). However, while conventional competitive PCR is relatively inexpensive, real-time PCR is far more convenient, reliable and better suited to quick decision making in a clinical situation (77,78). This is because conventional, quantitative, competitive PCR (qcPCR) requires significant development and optimisation to ensure reproducible performance and a predetermined dynamic range for both the amplification and detection components (79).

Although a comparison of absolute standard curves, relative standard curves and C_T values produces similar final values (80), the general belief remains that an internal control in combination with replicates of each sample are essential for reliable quantitation by PCR (38,39). Unfortunately, real-time PCR software with the ability to calculate the concentration of an unknown by comparing signals generated by an amplified target and internal control is only beginning to emerge. This issue will hopefully be addressed in upcoming commercial releases (81). Therefore, the next best approach to quantitation by PCR is the use of an external standard curve. This approach relies upon titration of an identically amplified template, in a related sample matrix, within the same experimental run. While the external standard curve is the more commonly described approach, it suffers from uncontrolled and unmonitored inter-tube variations. Because of this omission, such experiments should be described as semi-quantitative. Despite this sub-optimal approach, fluorescence data is generally collected from PCR cycles that span the linear amplification portion of the reaction where the fluorescent signal and the accumulating DNA are proportional. Because the emissions from fluorescent chemistries are temperature dependent, data is generally acquired only once per cycle at the same temperature in order to monitor amplicon yield (45). The C_T of the sample at a specific fluorescence value can then be compared with similar data collected from a series of standards by the calculation of a standard curve. The determination of the C_T depends upon the sensitivity and ability of the instrument to discriminate specific fluorescence from background noise, the concentration and nature of the fluorescence-generating component and the amount of template initially present.

Real-time PCR offers significant improvements to the quantitation of viral load because of its enormous dynamic range that can accommodate at least eight $\log_{10}$ copies of nucleic acid template (33,52,77,82–89). This is made possible because the data are chosen from the linear phase (LP; Fig. 1B) of amplification where conditions are optimal, rather than the end-point where the final amount of amplicon present may have been affected by inhibitors, poorly optimised reaction conditions or saturation by inhibitory PCR by-products and double-stranded amplicon. The result of taking data from the end-point is that there may not be a relationship between the initial template and final amplicon concentrations.

Real-time PCR is also an attractive alternative to conventional PCR for the study of viral load because of its low inter-assay and intra-assay variability (77,87,90) and its equivalent or greater analytical sensitivity in comparison with traditional viral culture, or conventional single-round, and nested PCR (77,85,91–96). Real-time PCR has been reported to be at least as sensitive as Southern blot (92). However, these reports could be an over-estimate due to the choice of smaller targets, which amplify more efficiently, or due to the use of different or improved primers for the real-time assays because the use of software to design optimised primers and oligoprobes is more common.

When this increased sensitivity and broad dynamic range are combined, it is possible to quantitate template from samples containing a large range of concentrations, as is often the case in patient samples. This avoids the need for dilution of the

amplicon prior to conventional detection or repeat of the assay using a diluted sample because the first test result falls outside the limits of the assay. These are problems encountered when using some conventional qcPCR assay kits, which cannot encompass high viral loads whilst maintaining suitable sensitivity (52,97–99). The flexibility of real-time PCR is also demonstrated by its ability to detect one target in the presence of a vast excess of another target in duplexed assays (84).

Viral load is also a useful indicator of the extent of active infection, virus–host interactions and the response to antiviral therapy, all of which can play a role in the treatment regimen selected (100,101). Conventional quantitative PCR has already proven the benefits of applying nucleic acid amplification to the monitoring of viral load as a useful marker of disease progression and as a component of studies into the efficacy of antiviral compounds (74,100,102–104). The severity of some diseases has been shown to correlate with the viral load making real-time PCR quantitation useful to study not simply the presence of a virus but the role of viral reactivation or persistence in the progression of disease (78,82,91,105–112).

An example of the benefits which real-time PCR has brought to the quantitative detection of human cytomegalovirus (CMV) is seen in patients who are immunosuppressed following solid organ or bone marrow transplantation. Although qualitative detection of CMV DNA by PCR has been used as an indicator for the success of antiviral therapy, quantitative assays are preferred in order to monitor patient's therapeutic responses. Moreover, since it has been postulated that the monitoring of viral replication over time is a more reliable indicator of a developing viral disease than the determination of absolute viral amounts at a single point of time, several quantitative assays have been established and evaluated to increase diagnostic accuracy. Quantitative competitive assays based on end-point analysis have displayed detection limits of 5×10^1 genome equivalents (ge) per assay and a dynamic range of 5×10^1–5×10^4 ge/assay (113). Hybridisation-based assays covered approximately the same dynamic range of four orders of magnitude with detection limits of 20 ge/assay (114,115). Although these assays possess dynamic ranges that may be sufficient for most clinical applications, they display a high inter- and intra-assay variability, up to 40% (115).

In contrast, one of the first published real-time PCR assays for the detection of CMV DNA could be performed in <90 min, spanned a dynamic range of six to seven orders of magnitude with a detection limit of at least 10 ge/assay and an inter-assay and intra-assay variability of <10% and <5%, respectively, using plasma samples from bone marrow transplant patients (116).

MULTIPLEX REAL-TIME PCR

Multiplexing (using multiple primers to allow amplification of multiple templates within a single reaction) is a useful application of conventional PCR (117). However, its transfer to real-time PCR has confused its traditional terminology. The term multiplex real-time PCR is more commonly used to describe the use of multiple fluorogenic oligoprobes for the discrimination of multiple amplicons. The transfer of this technique has proven problematic because of the limited number of fluorophores available (14) and the common use of a monochromatic energising light source. Although excitation by a single wavelength produces bright emissions from a suitably selected fluorophore, this restricts the number of fluorophores that can be included (69). Recent improvements to the design of the hairpin primers, and hairpin and nuclease oligoprobes as well as novel combinations of fluorophores such as in the bi-probe and light-up probe systems, have promised the ability to discriminate an increasing number of targets.

The discovery and application of the non-fluorescent quenchers has liberated some wavelengths that were previously occupied by the emissions from the early quenchers themselves. This breakthrough has permitted the inclusion of a greater number of spectrally discernable oligoprobes per reaction, and highlighted the need for a single non-fluorescent quencher, which can quench a broad range of emission wavelengths (e.g. 400–600 nm). Early real-time PCR systems contained optimised filters to minimise overlap of the emission spectra from the fluorophores. Despite this, the number of fluorophores that could be combined and clearly distinguished was limited when compared with the discriminatory abilities of conventional multiplex PCR. More recent real-time PCR platforms have incorporated either multiple light-emitting diodes to span the entire visible spectrum, or a tungsten light source, which emits light over a broad range of wavelengths. When these platforms incorporate high quality optical filters it is possible to apply any current real-time PCR detection chemistries on the one machine. Nonetheless, these improvements generally allow only four-colour oligoprobe multiplexing, of which one colour is ideally set aside for an internal control to monitor inhibition and perhaps even act as a co-amplified competitor. Some real-time PCR designs have made use of single or multiple nucleotide changes between similar templates to allow their differentiation by T_M (Fig. 4) thus avoiding the need for multiple fluorophores (49,91,118–122). This approach has been used far more commonly in the detection of human genetic diseases where as many as 27 possible nucleotide substitutions have been detected using only one or two fluorophores (48,123–128).

To date, there have only been a handful of truly multiplexed real-time PCR assays described in the literature and few of these have been applied to the diagnosis of infectious disease. Some of these approaches cannot, technically, be considered real-time, homogenous assays because they require interruption of the procedure to transfer template, their fluorescence is detected by end-point analysis or the assays are not performed within the same tube. One of the best viral, multiplex, real-time PCR protocols can discriminate between four retroviral target sequences (129), however, conventional multiplex PCR using end-point detection can easily discriminate more than five different amplified sequences, indicating a greater flexibility when compared with real-time PCR (130–133).

Despite these limitations, a number of assays have been applied to the detection of several viral genomes at once, including the use of non-specific label, SYBR green 1 to detect herpes simplex viruses (HSV), varicella zoster virus (VZV) or CMV, in separate tubes (134) or, by adaptation of a conventional multiplex PCR, to identify HSV-1 and HSV-2, VZV and enteroviruses within a single capillary by applying fluorescent melting curve analysis (121). Another single-tube multiplexed nuclease oligoprobe RT–PCR was capable of simultaneously detecting Influenza A and B in patient's respiratory samples

with improved sensitivity compared with conventional or shell-viral culture (95).

Future developments of novel chemistries such as combinatorial fluorescence energy transfer tags (135), and improvements to the design of real-time instrumentation and software will greatly enhance the future of multiplex real-time PCR.

APPLICATIONS TO VIROLOGY

Real-time PCR has been extremely useful for studying viral agents of infectious disease and helping to clarify disputed infectious disease processes. Most of the assays presented in the literature allow an increased frequency of virus detection compared with conventional techniques, which makes the implementation of real-time PCR attractive to many areas of virology.

Of course, real-time PCR has proven increasingly valuable for general virological studies, although increasingly, these applications are difficult to review due to the nature of their use as a tool rather than the focus of published study. Such studies have investigated the role of viruses in a range of diseases by simply confirming the presence or absence of the virus (136,137) or, in the future, by monitoring the levels of specific gene activity (138) as a result of growth under manipulated conditions. Altered viral entry or replication, caused by the modification of target tissues, can also be followed using real-time PCR as can links between virus replication and the expression of cellular genes (139–141).

Real-time PCR has enhanced the speed and scope of measuring viral strain and titre differences in patients displaying different syndromes due to varieties of the same virus (106). Also, epidemiological studies have been improved in speed and scope through the use of real-time PCR because it can reliably measure the amount of two nucleic acid targets within a single reaction (91,142). New chemistries have allowed better discrimination of multiple viral genotypes within a single reaction vessel (143) and provided an alternative to methods of virus detection based on morbidity and mortality assays.

The use of real-time PCR has provided insight into the role(s) some compounds have on PCR inhibition as well as shedding light on the efficiency of different nucleic acid extraction methods, from a diverse range of sample types (144–146). This ability to utilise template from a range of sample types fulfils a requirement for an ideal detection system, which is the ability to apply a single technology across many fields. This flexibility is highlighted by the detection of viral nucleic acids derived, in different ways, from plants (147–149), animals (86,89,94,150–152), urban sludge (85,153), tissue culture (23,77,96,154–160), various solid tissues (108,118,161–166), cerebrospinal fluid (49,167,168), peripheral blood mononuclear cells (82,93,105,112,169–171), plasma (81,88,90,172–176), serum (30,33,36,87,97,99,109,177–181), swabs (182,183), saliva (106) and urine (78,122,184,185). Also, chronic conditions such as sarcoma (186–189), carcinoma (92,111), cervical intraepithelial neoplasia (190–192), and lymphoproliferative disorders (193,194) can be relatively easily studied to investigate direct or indirect links with viral infection. These studies have targeted viruses which include the flaviviruses (33,36,96,109,195), hepadnaviridae (52,97), herpesviruses (21,77,78,82–84,91,92,98,105,106,108, 111,112,144,167,171), orthomyxoviruses (95), parvoviruses (88), papovaviruses (122,143,145), paramyxoviruses (94), picornaviruses (85,86), retroviruses (90,93,156,196,197) and TT virus (137). Viral load monitoring by real-time PCR has also proven beneficial for studying patients following organ transplant (21,83,107,198–201).

This technology has now become an essential tool in the thorough assessment of viral gene therapy vectors prior to their use in clinical trials. Nuclease oligoprobes have been most commonly reported for these studies, which assess the biodistribution, function and purity of these drug preparations (164,197,202–205).

Additionally, the study of emerging viruses has been complimented by the use of real-time PCR as a tool to demonstrate links between unique viral sequences and patient clinical signs and symptoms (94,96,150,160,206,207).

The speed and flexibility of real-time PCR has also proven useful to commercial interests for the screening of microbial contamination of large-scale reagent preparations produced from eukaryotic expression systems (208,209).

CONCLUSIONS AND SUMMARY

Advances in the development of fluorophores, nucleotide labelling chemistries, and the novel applications of oligoprobe hybridisation have provided real-time PCR technology with a broad enough base to ensure its acceptance. Recently, instrumentation has appeared that is capable of incredibly short cycling times combined with the ability to detect and differentiate multiple amplicons. New instruments are also flexible enough to allow the use of any of the chemistries described in this review making real-time nucleic acid amplification an increasingly attractive and viable proposition for the routine diagnostic laboratory. In many cases these laboratories perform tissue culture to isolate virus and serological methods to confirm the identity of the isolate, which may take a considerable, and clinically relevant, amount of time.

The familiarity that leads to comfortable routine use of a technology is now apparent in the inclusion of the fluorogenic oligoprobe chemistries in many laboratories. According to the literature, the most widely used format is the 5′ endonuclease oligoprobe although that is most likely due to its commercial maturity. The more recently developed oligoprobe chemistries have been used in a number of innovative applications and it is apparent from the rate and content of real-time PCR-related publications that they are becoming more widely accepted.

Recent developments in multiplex real-time PCR have suggested a future in which easy identification, genotyping and quantitation of viral targets in single, rapid reactions will be commonplace. Of course, this technology is by no means restricted to virology, as significant achievements have appeared in the area of mutation detection, applying all the benefits described above to enhance the detection of genetic disease and, where applicable, allow quantitation of the extent of such genetic changes.

Micro- and macro-array technology will surely play some chimeric role in the real-time PCR of future research, but for now there is significant potential for routine, research and commercial interests to redesign existing systems for greater sophistication, flexibility and the ability to generate high quality quantitative data. The development of assays capable

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of real-time PCR that can discriminate as many targets as conventional multiplex PCR assays, whilst producing quantitative data at a greatly increased speed, will consolidate fluorogenic nucleic acid amplification as a routine tool for the laboratory of tomorrow.

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Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen

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Abstract

We reported the first attempt to describe mucosa-associated bacterial populations in the chicken ceca by molecular analysis of 16S rRNA genes. Bacteria in the mucosa were highly diverse but mainly Gram-positive with low G+C. *Fusobacterium prausnitzii* and butyrate-producing bacteria comprised the largest groups among 116 cloned sequences. Twenty five percent of the clones had less than 95% homology to database sequences. Many sequences were related to those of uncultured bacteria identified in human feces or the bovine rumen. Terminal restriction fragment length polymorphism (T-RFLP) analysis revealed some differences between bacterial populations present in the mucosa and lumen of ceca. Greater resolution of bacterial population was obtained using a culture-independent approach rather than a culture-based approach.

Keywords: Chicken; Cecal bacterium; 16S rRNA; Phylogeny; Diversity

1. Introduction

The normal gut microflora plays an important role in the health and well-being of host animals. In poultry, the absence of healthy microflora in the ceca has been considered to be a major factor in the susceptibility of chicks to bacterial infection [1]. To prevent chicks from bacterial infection, poultry producers in North America currently rely on the prophylactic use of antibiotics. There is public concern about the development and spread of antibiotic resistance in bacteria, which has led to greater interest in the use of probiotics in commercial practice to control bacterial infection and reduce the reliance on antibiotics. Clearly, a better understanding of microbial ecology of the chicken gut is required for the development of probiotics and their most effective use.

Chicken gut microflora have been studied previously by culture-based methods (reviewed in [1]). Because of the selectivity of the culture approach for readily cultivated bacteria, it may introduce a biased view of microbial diversity. Increasingly, molecular approaches are being used to examine the diversity of gut microflora independent of culturing [2–4]. While molecular approaches based on PCR may introduce bias of a different kind [5–7], they provide powerful tools to investigate the phylogenetic diversity of microbes in gut samples [2–4].

This report is the first study to our knowledge, to use molecular analysis of 16S rRNA genes to examine the phylogenetic diversity of bacterial communities in mucosa of chicken ceca, to compare bacterial populations in the mucosa and lumen, and to compare the results of culture-dependent and -independent methods to analyze mucosal bacteria.

2. Materials and methods

2.1. Chicken maintenance and sample collection

Broiler chickens (Ross/Ross) were reared under controlled management similar to commercial practice. Management and experimental procedures were according the

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welfare guideline of the Animal Care Committee, University of Guelph (AUP 98R161). The birds were fed with corn-soy broiler diets [8] containing 18–22% crude protein and 3073–3195 kcal ME per kg and no antibiotics. Gut samples were collected from the ceca of 10 6-week-old broiler chickens. All gut samples were kept on ice and processed immediately after dissection. Bacterial samples from digesta of ceca were prepared essentially by the method of Apajalahti et al. [9]. To prepare cecal wall-associated bacterial samples, ceca were opened longitudinally and briefly washed three times in saline to remove unattached or loosely attached bacteria from the wall. Bacterial cells were then released from the cecal wall by two washes in saline containing 0.1% Tween 80 with vigorous hand shaking for 30 s per wash followed by centrifugation (27 000 × *g*, 20 min) at 4°C to pellet the cells. This fraction of bacterial cells was referred to as mucosal bacteria in our investigation. The procedure described above was shown to release about 95% bacterial cells from the cecal wall, which was as efficient as scraping mucosa to prepare the bacterial samples. Samples for DNA extraction were frozen in liquid nitrogen and stored at −70°C, while samples for bacterial growth and storage were collected anaerobically and stored in 15% glycerol at −70°C.

2.2. Bacterial growth

Bacteria were grown at 41°C on brain–heart-infusion agar (BHI, Difco) supplemented with hemin (5 mg l^{-1}), yeast extract (5 g l^{-1}), and L-cysteine (0.5 g l^{-1}) in an anaerobic atmosphere (80% N_2, 10% CO_2, and 10% H_2).

2.3. Cell lysis and DNA extraction

Bacterial samples were subjected to five freeze–thaw cycles, alternating between liquid nitrogen and 65°C for 5 min in the presence of β-mercaptoethanol (5 μl ml^{-1}), followed by bead-beating as described previously [2] to lyse cells. DNA was extracted from cell lysates using the method of phenol/chloroform extraction and ethanol precipitation as described [2].

2.4. Random cloning of 16S rRNA genes

16S rRNA genes were amplified by PCR from genomic DNA of mucosal bacteria using eubacterial primers F8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-GGTTACCTTGTTACGACTT-3′). PCR reaction mixtures were the same as described previously [2]. The amplification program was 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C for 25 cycles followed by 10 min at 72°C. PCR products were cloned into the vector, pCR®4-TOPO®, using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions, and partially sequenced with an ABI PRISM® 377 Automated DNA sequencer.

2.5. Sequence and phylogenetic analysis

Partial 16S rDNA sequences corresponding to *Escherichia coli* 16S rRNA bases 400–1050 were compared directly with the GenBank, EMBL, and DBJI non-redundant nucleotide databases using BLAST. Sequence alignment and phylogenetic analysis were conducted as described previously [2]. Briefly, cloned 16S rDNA sequences and closely related reference sequences were manually aligned using the program SeqPup (Don Gilbert, Biocomputign Office, Biology Department, Indiana University, Bloomington, IN, USA). Phylogenetic trees were generated using a neighbor-joining method [10], in the PHYLO_WIN package [11], with pair-wise gap removal and Jukes–Cantor correction [12]. In order to validate the tree, statistical bootstrapping [13] was carried out with data resampled 1000 times. Sequences were also compared by generating similarity matrices. Putative chimeric sequences were identified using the program Check Chimera [14].

2.6. T-RFLP (terminal restriction fragment length polymorphism) analysis

Two pairs of eubacterial primers, F8 and R1492, F8 and R926 (5′-CCGTCAATTCCTTTRAGTTT-3′) [15] were used for T-RFLP. F8 was labeled with 6-FAM (6-carboxyfluorescein, Applied Biosystems) while the reverse primers were labeled with NED (Applied Biosystems). PCR conditions were the same as those for random cloning. Aliquots of amplified rDNA products were separately digested with *Alu*I, *Hha*I, and *Msp*I (New England Biolabs) according to the manufacturer's instructions. The lengths of T-RFs were determined by comparison with Rox-labeled internal standards using an ABI PRISM® 377 Automated DNA sequencer and GeneScan® Analysis Software (Applied Biosystems).

3. Results

3.1. Diversity and phylogenetic analysis

Microscope-counts of bacterial cells were 10^{10}–10^{11} cells g^{-1} cecal digesta and about 10^{11} cells in total recovered from the cecal mucosa of one bird. More than 90% of bacterial cells stained Gram-positive.

Partial sequences of 116 random 16S rDNA clones from bacteria present in the cecal mucosa were analyzed. In addition to *E. coli*, at least 48 other molecular species were obtained among cloned sequences. The presumptive relationships of these sequences were obtained from database comparison. As shown in Table 1, 75% of cloned sequences exhibited a 95% or higher identity to database sequences, and were assigned to the closest genus. There were 29 cloned sequences (25%) with less than 95% of

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Table 1
Bacteria found in the mucosa of chicken ceca

Cloned rDNA sequence	Homology to database sequence[a]	
	≥95%	<95%
Bacilli	1	2
Butyrate-producing bacteria	27	2
Clostridia	5	1
Enterococcus cecorum	7	0
E. coli	1	0
Eubacteria	2	2
F. prausnitzii	7	0
Holdemania filiformis	1	1
Lactobacilli	4	0
Ruminococci	11	4
Streptococcus alactolyticus	2	0
Uncultured bacteria from human feces[b]	14	9
Uncultured rumen bacteria[b]	5	6
Uncultured bacteria from mouse gut[b]	0	1
Others	0	1
Total number of clones	87	29[c]

[a]Based on a BLAST analysis of partial 16S rDNA sequences corresponding to *E. coli* 16S rRNA bases 400–1050.
[b]Sequences showing a similarity in the databases to those identified in human feces, bovine rumen, or mouse gut.
[c]GenBank accession numbers: AF429354–AF429382.

relatedness to database sequences. We also analyzed full-length 16S rRNA gene sequences from the seven predominating cloned sequences to confirm the homology determined by partial sequence analysis. Only two clones exhibited 1% of variation in the sequence homology, suggesting that partial sequences used in this study can represent full-length sequences for the analysis.

Phylogenetic analysis revealed that the cloned sequences were mainly those of low G+C, Gram-positive bacteria (Fig. 1). This group was highly diverse, with many sequences being related to sequences identified in human feces [16] or in the bovine rumen [2]. There were 11 groups comprising four or more closely related sequences. The largest group comprised sequences (34) more or less related to *Fusobacterium prausnitzii*. Of the 116 clones, more than 25% were related to butyrate-producing bacteria.

3.2. T-RFLP analysis of bacterial populations present in the mucosa and lumen

T-RFLP analysis was used to compare bacterial populations in the mucosa and lumen. Fig. 2 shows the T-RFLP profiles generated by *Hha*I and *Msp*I digestions. While *Msp*I and *Alu*I were unable to differentiate the two bacterial populations significantly, *Hha*I generated several major T-RFs with a preference in the mucosa or lumen. For instance, one T-RF (197 bp) was mainly located in the mucosa, which was hardly detected in the lumen (Fig. 2A).

3.3. Comparison of culture-dependent and -independent mucosal bacteria

Two random 16S rDNA clone pools from the mucosal samples were compared. One was the pool (culture-independent) used for the phylogenetic analysis, the other was generated from the mucosal bacteria grown on BHI agar for 2 days. As shown in Table 2, the BHI-cultured sample had a less diverse bacterial population and a less complicated community structure than the uncultured sample.

4. Discussion

Previous studies using culture-based methods have defined cultivable microflora in the chicken gut (reviewed in [1]). The present study was to investigate the diversity and phylogenetic relationships of mucosa-associated cecal bacteria, both cultivable and non-cultivable, by molecular analysis of 16S rRNA genes. 16S rDNA analysis has shown greater diversity of the bacterial population in the chicken ceca than had previously been achieved by the culture approach. Twenty nine out of 116 cloned sequences showed less than 95% homology to database sequences, which may represent new species [17] previously unidentified in the chicken gut. Some sequences were related to uncultured bacteria reported from human feces [16] or from the bovine rumen [2]. While the ecological and physiological role of these bacteria remains to be determined, it is likely that previous estimates by cultures may have overestimated certain groups of bacteria, such as lactobacilli and clostridia, in the cecal microflora. Gut-surface-associated bacterial population has long been of a research interest because of their importance in pathogen control, immune modulation, and their effects upon nutrient absorption by their hosts. Our observations reported here may, therefore, have significant implications for the health and nutrition of the host, and are particularly relevant for the development of probiotics and their effective use.

T-RFLP analysis has become a useful tool for the studies of microbial ecology, as a generated T-RFLP profile can serve as a 'community fingerprint' to characterize a particular microbial community [15]. In this study, the use of *Hha*I was able to differentiate bacterial populations present in the mucosa and lumen, as indicated by the seven major polymorphic bands which were located differently in the mucosa or lumen (Fig. 2A). It is unclear at present that which bacterial groups contribute to the polymorphism. However, our sequence analysis of full-length 16S rRNA genes from the seven predominating cloned sequences revealed that one group butyrate-producing bacteria would generate a 5′-T-RF upon digestion with *Hha*I which has a similar size to one of the seven polymorphic bands (197 bp). In addition, our recent cloning and sequence analysis suggested that the butyrate-producing bacteria were present in the mucosa, but not in the

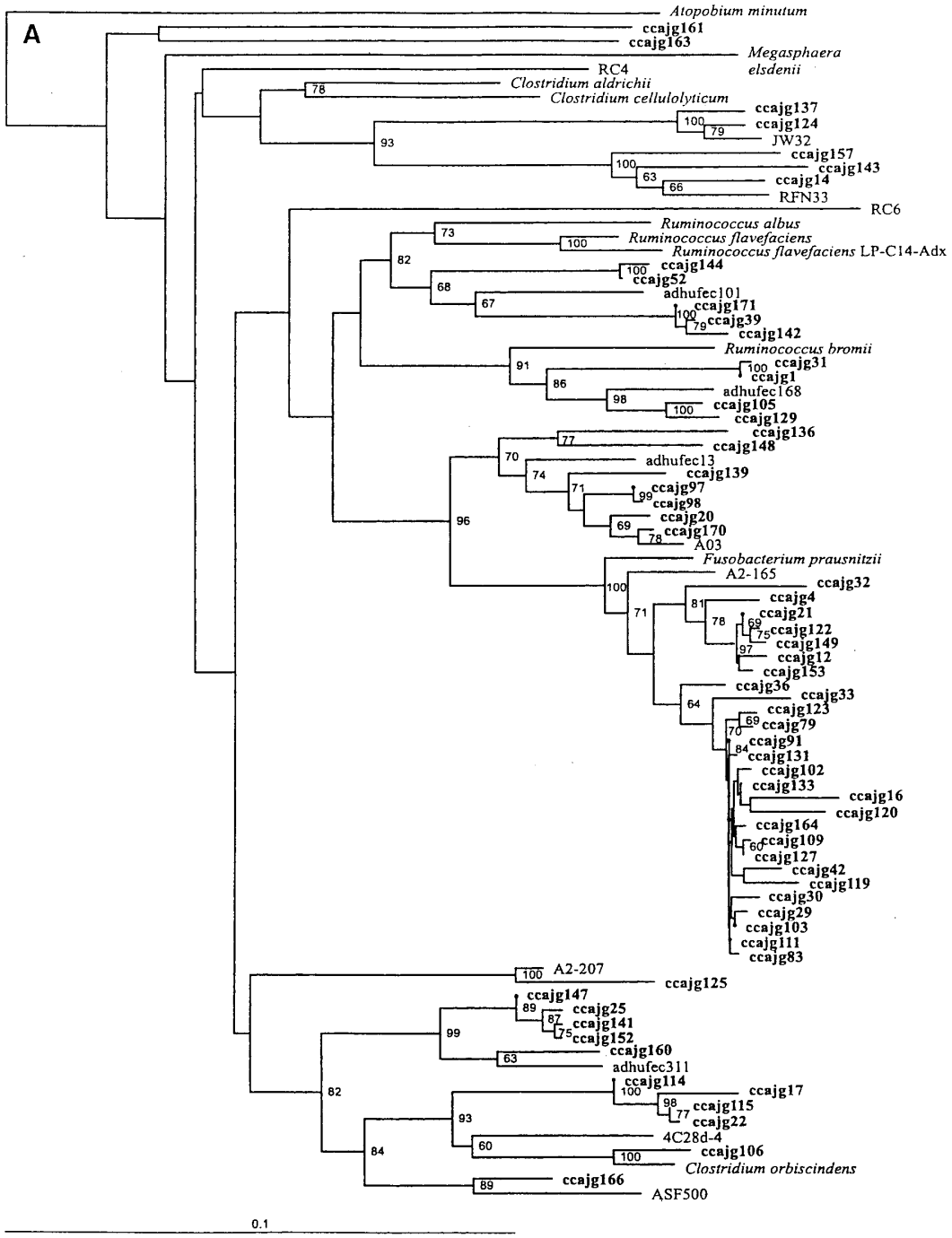

Fig. 1. A,B: Unrooted phylogenetic tree of mucosal bacteria in the chicken ceca constructed using a neighbor-joining method. Our cloned sequences are shown in bold numbers. Bootstrap values for 1000 trees are shown at branch points. Only values of 60% or above are shown. The bar represents a sequence divergence of 0.1%.

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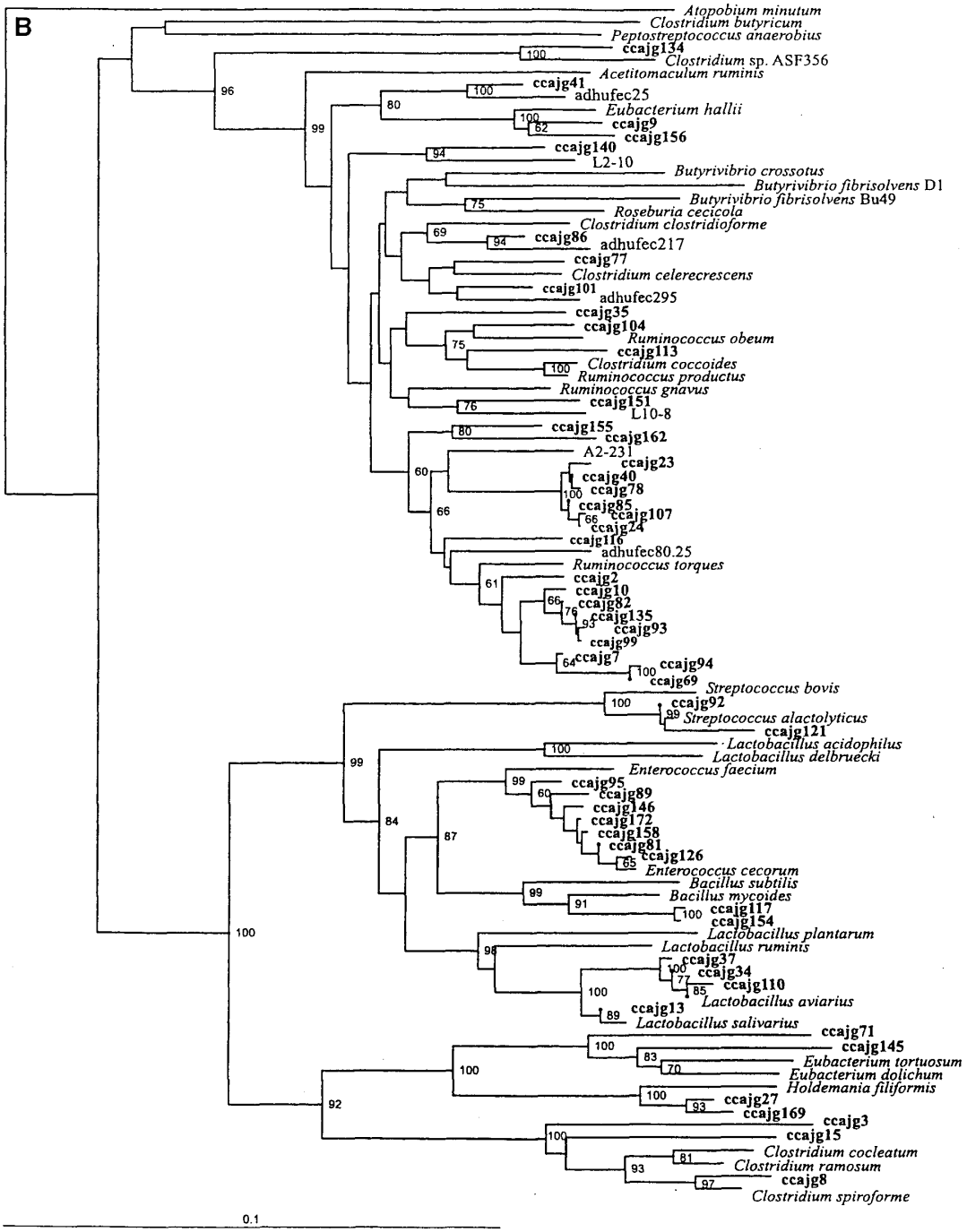

Fig. 1. (*continued*)

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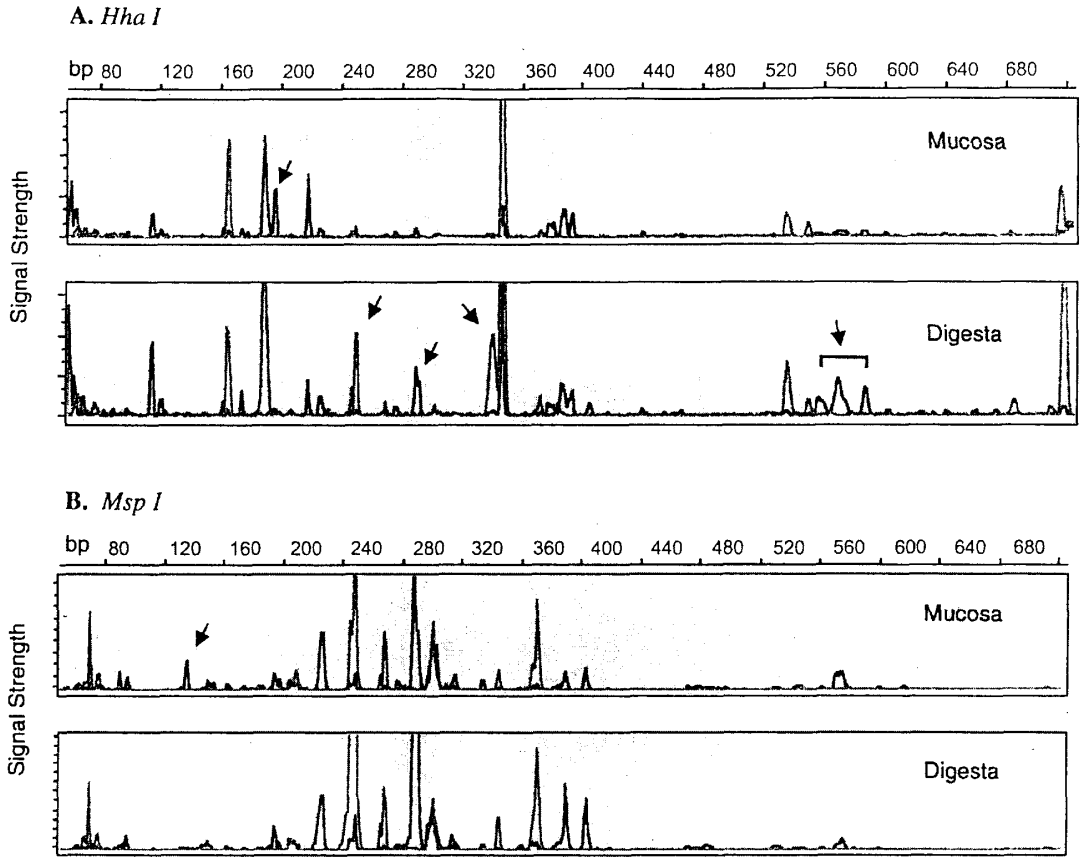

Fig. 2. TRFLP analysis of 16S rDNA amplified by primers F8 and R926 from bacteria present in the mucosa and lumen of chicken ceca. PCR products were digested with *Hha*I (A) or *Msp*I (B). F8 and R926 were labeled with 6-FAM (blue) and NED (orange), respectively. The major T-RF bands with a different location are indicated by arrows.

Table 2
Culture-dependent and -independent bacteria in the mucosa of chicken ceca[a]

	Culture-independent		Culture-dependent[b]	
Cloned sequence	No. of clones	Percent	Number of clones	Percent
Butyrate-producing bacteria	29	25	0	0
Clostridia	6	5	4	7
E. cecorum	7	6	26	44
E. coli	1	1	21	36
F. prausnitzii	7	6	0	0
Lactobacilli	4	4	4	7
Ruminococci	15	13	1	2
Sequences identified in human feces[c]	23	20	2	3
Sequences identified in bovine rumen[c]	11	10	0	0
Sequences identified in mouse gut[c]	1	1	0	0
Others	12	10	1	2
Total	116	101	59	101

[a]Based on a BLAST analysis. Cloned sequences were assigned to the closest database sequences.
[b]Culture-dependent bacteria were grown on BHI agar and analyzed by random cloning and sequence analysis of 16S rRNA genes. Culture-independent bacteria were analyzed by direct DNA extraction from cecal samples, random cloning and sequence analysis of 16S rRNA genes.
[c]Sequences showing a similarity in the databases to those identified in human feces, bovine rumen, or mouse gut.

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lumen of ceca (data not shown). These data imply that the major T-RF (197 bp) may represent one group butyrate-producing bacteria.

In our comparison of culture-dependent and -independent bacterial populations from chicken ceca, the bacteria population revealed by the culture-dependent approach exhibited a lower diversity and a less complicated community structure. This may reflect the limitation of the culture method since some bacteria may not be cultivable. Furthermore, any medium used is more or less selective for certain groups of cultivable bacteria. In our comparison, we have presented groups of sequences as percentages of the total number of cloned sequences. This is an approximation since the exact proportion was not necessarily conserved in the PCR step. Alternative molecular methods, such as Real Time PCR, dot blot or in situ hybridization will be needed to confirm these results.

One significant observation in the present study was the predominance of butyrate-producing bacteria in the chicken ceca. These bacteria were more or less closely related to *F. prausnitzii*, or related to unidentified butyrate-producing bacteria isolated from human feces [16]. Butyrate plays an important role in animal health by influencing the regulation, metabolism and development of colonic epithelial cells [18,19]. The ecological and physiological significance of this numerically important group of bacteria in the chicken ceca remains to be elucidated.

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